Overweight and obesity status in pregnant women are related to intestinal microbiota and serum metabolic and inflammatory profiles

Noora Houttu[a,*], Kati Mokkala[a], Kirsi Laitinen[a]

[a]Institute of Biomedicine, University of Turku, Turku, Finland

[*]Correspondence: Noora Houttu, University of Turku, Institute of Biomedicine, Kiinamyllynkatu 10, 20520 Turku, FINLAND, tel.: +358 2 333 6063, e-mail: nhmhou@utu.fi

ABSTRACT

Background: Overweight and obesity may predispose women to clinical complications during their pregnancy. We hypothesize that a higher degree of overweight status is related to a range of aberrations in biomarkers already in early pregnancy. Our objective was to investigate whether intestinal microbiota, serum metabolic and inflammatory profiles differ in relation to the degree of overweight status in pregnant women.

Methods: This study investigated 52 overweight and 47 obese pregnant women in early pregnancy. Fecal samples were analyzed for intestinal microbiota composition by 16S ribosomal RNA gene sequencing and Qiime pipeline. Circulating serum metabolites, including lipids, amino acids and GlycA, a marker of low-grade inflammation, were analyzed by NMR metabolomics and hsCRP was quantified by immunoassay. Serum zonulin levels were analyzed to depict intestinal permeability by Zonulin ELISA kit and LPS activity for endotoxemia by Limulus amebocyte lysate assay. The analyses were adjusted for multiple comparisons using Benjamini-Hochberg procedure for false discovery rate controlling.

Results: The relative abundance of bacterial family *Prevotellaceae* (adjusted P=0.19) and markers of low-grade inflammation, hsCRP (P=0.0015) and GlycA (P<0.001) and three branched chain amino acids (isoleucine, adjusted P=0.024; leucine, adjusted P=0.026; valine, adjusted P=0.10) and one aromatic amino acid (phenylalanine, adjusted P=0.050) and concentrations of several VLDL particles and lipid measures in several VLDL particles were higher in obese pregnant women compared to their overweight pregnant counterparts (adjusted P<0.12). In contrast, lipid measures in a few HDL particles and many fatty acids were lower in obese compared to overweight pregnant women (adjusted P<0.12).

Conclusions: The detected alterations in intestinal microbiota and metabolic and inflammatory profiles related to obesity status may offer new alternative tools to supplement standard clinical measures to predict the risk for metabolic alterations during the early phase of pregnancy.

Keywords: Intestinal microbiota, metabolic profile, low grade inflammation, obesity, overweight, pregnancy

Abbreviations: BMI, body mass index; GlycA, glycoprotein acetyls, mainly α1-acid glycoprotein; HbA1c, glycated haemoglobin; hsCRP, high-sensitive C-reactive protein; HOMA2-IR, homeostatic model assessment-method; LPS, lipopolysaccharide; PCA, principal component analysis

INTRODUCTION

Several metabolic changes take place during pregnancy to meet the high demands posed by fetal growth and development[1]. Furthermore, changes have been observed in the intestinal microbiota composition[2] and inflammatory profile[1]. These physiological changes are stringently regulated by several hormones from thyroid gland, ovaries and placenta. If the woman is either overweight (body mass index, BMI, 25-30) or obese (BMI < 30), this may impose an additional burden during pregnancy, as manifested in metabolic disturbances such as insulin resistance[3]. Indeed, overweight and obesity have been shown to associate with an increased risk of adverse pregnancy outcomes, these including gestational diabetes[4] and even miscarriage[5]. In the offspring, the risks include excessive fetal growth[6], and also metabolic diseases in later life mediated through early programming mechanisms[7].

However, the extent to which intestinal microbiota composition and metabolic and inflammatory profiles i.e. markers potentially related to adverse events during pregnancy,

differ according to the women's overweight and obesity status is poorly known. Some previous studies have indicated that an increasing maternal weight is related to lowered numbers of Bacteroidetes and *Bifidobacterium*[8] and a higher number of *Staphylococcus* as assessed by targeted microbiota analyses[9]. Further, some metabolic markers, i.e. decreased urinary concentrations of hippurate and phenylalanine and increased levels of creatine, lactate, lysine, citrate and acetate[10] and increased concentrations of serum inflammatory markers, including C-reactive protein, monocyte chemoattractant protein-1, interleukin-6 and interleukin-1 receptor antagonist[11] were detected in pregnant women with increasing weight. Currently the potential adverse consequences of overweight and obesity during pregnancy is regulated by limiting the pregnancy weight gain defined according to the prepregnancy BMI[12]. We would argue that an early identification of additional modifiable risk factors in this at-risk group of pregnant women would be likely to provide motivational strategy for health counselling of pregnant women, and thus be helpful in lowering the risk of adverse clinical manifestations in both mother and child.

The objective of this study was to investigate whether intestinal microbiota composition and serum metabolic and inflammatory profiles differ and are interrelated in overweight and obese women during early pregnancy.

SUBJECTS AND METHODS

Subjects

The study investigated 99 overweight and obese pregnant women at early pregnancy who participated in the on-going mother-infant dietary intervention trial (ClinicalTrials.gov, NCT01922791). Mothers were taken to the study for the analysis of intestinal microbiota and serum markers at baseline in order of enrollment in the trial. A total of 52 mothers were overweight (BMI 25-30) with the remaining 47 classified as obese (BMI $\geq$ 30). The inclusion

criteria into the trial were overweight (prepregnancy BMI ≥ 25) and early pregnancy (< 17 weeks of gestation). The exclusion criteria were gestational diabetes diagnosed before enrollment, multifetal pregnancy, presence of metabolic or inflammatory chronic disease, including type 1 and 2 diabetes, coeliac disease and inflammatory bowel disease. Written informed consent was obtained from all participants. The study was conducted according to the Declaration of Helsinki and all procedures were approved by the Ethics Committee of the Hospital District of Southwest Finland (permission 115/180/2012).

Clinical procedures and sampling

Blood and fecal samples and clinical measurements were obtained in early pregnancy (a mean of 13.2 ± 4.4 weeks of gestation). Height was measured by a wall stadiometer to the nearest 0.1 cm, fat percent by air displacement plethysmography (Bod Pod system, COSMED, Inc., Concord, CA, USA), blood pressure by digital sphygmomanometer, prepregnancy weight was self-reported and was obtained from the maternal welfare clinic records. Prepregnancy BMI was calculated as weight in kilograms/(height in meters squared). Overweight was defined a BMI equal to or greater than 25 kg/m^2, whereas the woman was obese if their BMI was equal to or greater than 30 kg/m^2 (ref. 13). Values of 120 mmg Hg for systolic and 80 mm Hg for diastolic blood pressure or below were considered normotensive.

Diet and physical activity

Three-day food diaries were gathered in the week prior to study visit. The subjects were instructed to record their food intake and to guarantee the completeness and accuracy diaries were checked with a portion picture booklet. Mean daily intakes of energy, energy yielding nutrients and fiber were calculated by using computerized software (Aivo diet 2.0.2.3, Aivo, Turku, Finland). Women completed a physical activity questionnaire containing questions regarding the physical activity at occupation, commuting and from work and during leisure

time. An index number (MET index, h/wk) was calculated from three questions describing the leisure time activity level[14].

Glucose metabolism

The glucose concentration was measured using an enzymatic method utilizing hexokinase (Cobas 8000 automatic c702-analyzer, Roche Diagnostics, Mannheim, Germany), the insulin concentration with an immunoelectrochemiluminometric assay (a modular E170 automatic analyzer, Roche Diagnostics, Mannheim, Germany), glycated haemoglobin (HbA1c) by ion-exchange HPLC (Bio-Rad Variant II Haemoglobin A1c Program, Bio-Rad Laboratories, Marnes-la-Coquette, France) and insulin resistance was estimated with the homeostatic model assessment-method (HOMA2-IR) using HOMA calculator (http://www.dtu.ox.ac.uk/)[15]. Glucose concentration below value of 5.3 mmol/l, insulin values below 26 mU/I and HAbc1 levels under 6.5 % were considered within reference limits.

Low-grade inflammation, LPS and zonulin

High-sensitivity C-reactive protein (hsCRP) was determined to assess low-grade inflammation by using an automated colorimetric immunoassay on the Dade Behring Dimension RXL autoanalyzer (Siemens Healthcare, Camberly, Surrey, UK) and lipopolysaccharide (LPS) activity for endotoxemia by Limulus amebocyte lysate assay coupled with a chromogenic substrate (HyCult Biochemistry, Uden, theNetherlands). For hsCRP, the lower limit of detection was 0.1 mg/l and for LPS, the interassay coefficient of variation was 5.9%. Serum zonulin levels were was analyzed for intestinal permeability by the Zonulin ELISA kit (Immundiagnostik, Bernsheim, Germany); the zonulin interassay variation assay was 10.6%.

Metabolomics

A high-throughput proton NMR metabolomics platform (Brainshake, Helsinki, Finland) was used to analyze serum metabolic profile as described earlier[16]. The analysis platform encompasses 223 variables (213 lipids, 9 amino acids and Glycoprotein acetyls, mainly α1-acid glycoprotein (GlycA), a novel marker of low-grade inflammation). GlycA consists of a complex of heterogeneous nuclear magnetic resonance signal containing N-acetyl sugar groups originating from multiple acute phase circulating glycoproteins; α1-acid glycoprotein, haptoglobin, α1-antitypsin, α1-antichymotrypsin and transferrin[17].

Intestinal microbiota composition, richness and diversity

Intestinal microbiota composition was analyzed using 16S RNA gene sequencing and Qiime pipeline as previously described[18]. Based on the sequences, a total of 731 operating taxonomic units (OTUs) were detected, and the relative abundance was determined using these OTUs. The bacteria with relative abundance > 1% were considered to be reliable and were taken for further analysis. To evaluate richness and diversity, we analyzed Chao1, observed species, phylogenetic diversity (PD) and Shannon index[19].

Statistical analysis

SPSS Statistics 24.0 (IBM, Chicago, IL, USA) for Windows was used for statistical analyses. The normality distributions of the data was checked through visual inspection of histograms and with Kolmogorov-Smirnov test. The independent samples t-test was used for comparing normally distributed data and the Mann-Whitney *U* test for comparing the non-parametric data (insulin, HbA1c, HOMA2-IR, hsCRP, observed species, PD, Shannon index, intestinal microbiota and NMR metabolites) between overweight and obese pregnant women. Chi-square-test was used to compare categorical data. To further test whether specific metabolic patterns could be detected, a principal component analysis (PCA) of serum metabolites was performed. The correlations between prepregnancy BMI, diet, microbiota, metabolites and

low-grade inflammation markers were evaluated with a Spearman rank order test. To examine the interrelations among microbiota, metabolic and low-grade inflammatory markers a stepwise linear regression was conducted. Two linear regression models with either HOMA2-IR or GlycA (mmol/l) as outcome variable with multiple variables as predictors were used. These factors are known risk factors for the development of metabolic diseases including type 2 diabetes[20,21], gestational diabetes[22] and cardiovascular diseases[23] (serum triglycerides, mmol/l; very large VLDL particles, mol/l; cholesterol in large HDL, mmol/l; ratio of omega-3, omega-6 and polyunsaturated fatty acids to total fatty acids, % from lipid metabolites; MET index, h/wk; intake of energy and energy yielding nutrients as grams and as percentages. Further, the variables from microbiota (*Prevotellaceae*) and amino acids (leucine, isoleucine, valine and phenylalanine, mmol/l) variables that remained significant, after correction for multiple analyses, in comparison between overweight and obese women and after correction for multiple analysed were included in the model. These variables have also been associated with BMI[24].

The results are shown as mean ± standard deviation (SD), median and interquartile range (IQR) or median difference and confidence interval (95 % CI), percentage difference of median or correlation coefficient (rho) or regression coefficient (β) and 95% CI. Differences were considered significant with P-value below 0.05. The statistical analysis of metabolites (lipids and amino acids separately) and intestinal microbiota (at each taxonomic level) was adjusted for multiple comparisons using adaptive Benjamini-Hochberg procedure for the false discovery rate controlling. Adjusted P-values < 0.20 concerning microbiota (number of tested variables 57) and adjusted P-values < 0.12 concerning metabolites (number of tested variables 222) and adjusted P-values < 0.05 considering Spearman rank order test between pregestational BMI with microbiota (number of tested variables 57), lipids (number of tested variables 213) or amino acids (number of tested variables 9) and Spearman rank order test between low-grade

inflammation and lipids (number of tested variables 213) or amino acids (number of tested variables 9) and Spearman rank order test between diet and metabolites (number of tested variables 222) were considered significant.

RESULTS

Clinical characteristics

Clinical characteristics of the women are shown in Table 1. The groups of overweight and obese women were clearly distinguishable according to their prepregnancy BMI, weight and body fat percent at early pregnancy. Half of the pregnant women were highly educated with college or university degrees. Both groups were normotensive and normoglycemic according to their mean systolic and diastolic blood pressure and fasting glucose concentration. Even though, mean glucose and insulin concentrations as well as HOMA2-IR were within reference limit in both groups, obese pregnant women had significantly higher values than the overweight pregnant women. Five women had thyroxine related disease and thyroxine medication, nine women had lung disorders with four of them being administered corticosteroid medication, fifteen women had allergies of which one was taking antihistamines and two women had psoriasis, with one of them receiving corticosteroid therapy. Mean daily intakes of energy, energy yielding nutrients and fiber or MET index describing physical activity did not differ between overweight and obese pregnant women. Neither, serum zonulin levels, a marker of intestinal permeability, nor LPS activity, a marker of endotoxemia, differ between the two groups.

Metabolic profile

In the evaluation of lipid profile, 157 of 213 lipid metabolites differed statistically significantly between overweight and obese pregnant women (Figure 1; Exact values are presented in

Supplementary Table 1a). After adjusting the P-values (Benjamini-Hochberg procedure) 84 lipid metabolites remained statistically significantly different between the two groups. The majority of these (59/84) were higher in obese compared to overweight pregnant women. Specifically, concentrations of several VLDL subclasses and several lipid measures in many VLDL subclasses were higher in obese than overweight pregnant women. In contrast, lipids in certain HDL subclasses and omega-6 fatty acid, 18:2 linoleic acid plus the ratio of polyunsaturated fatty acids to total fatty acids, as well as the estimated degree of unsaturation of fatty acids were lower in obese (Figure 1).

Further, we evaluated the association of prepregnancy BMI as continuous variable with lipid metabolites and found a correlation with 92/213 lipid metabolites. After adjusting the P-values (Benjamini-Hochberg procedure), 65/213 remained statistically significant (Supplementary Table 1b), these including mainly measures related to VLDL and HDL particles as well as fatty acids.

The concentrations of three branched chain amino acids and one aromatic amino acid were statistically significantly different between overweight and obese pregnant women. After adjusting the P-values (Benjamini-Hochberg procedure), these four amino acids, namely isoleucine (median 0.05 (IQR 0.04-0.06) vs median 0.04 (IQR 0.04-0.05) mmol/l, P = 0.024), leucine (median 0.07 (IQR 0.06-0.08) vs median 0.06 (IQR 0.06-0.07) mmol/l, P = 0.026), valine (median 0.2 (IQR 0.1-0.2) vs median 0.1 (IQR 0.1-0.2) mmol/l, P = 0.10) and phenylalanine (median 0.08 (IQR 0.08-0.09) vs median 0.08 (IQR 0.07-0.08) mmol/l, P = 0.050) remained statistically significantly higher in the obese pregnant women. Exact values of the analyzed metabolites in overweight and obese pregnant women are presented in Supplementary Table 1a. PCA was conducted to evaluate whether overweight and obese pregnant women differed in their amino acid metabolic pattern. Glycine, alanine, phenylalanine, isoleucine, leucine, tyrosine and valine clustered with obese pregnant women

while glutamine and histidine clustered with overweight pregnant women (Figure 2a & b). When analyzing the association between prepregnancy BMI and the nine amino acids and after adjusting for multiple testing (Benjamini-Hochberg procedure), branched chain and aromatic amino acids remained statistically significant (isoleucine adjusted P-value < 0.001, leucine adjusted P-value = 0.01, valine adjusted P-value = 0.01 and phenylalanine adjusted P-value = 0.04) while glycine did not (adjusted P-value = 0.07) (see Supplementary Table 1b for unadjusted and adjusted correlation coefficient values).

Intestinal microbiota profile, richness and diversity

In both groups of pregnant women the main dominating phyla were Bacteroidetes and Firmicutes which did not differ between overweight and obese women (Table 2). The relative abundances of four bacteria belonging to the phylum Bacteroidetes, including a bacterial family *Prevotellaceae* (mean 3.69 ± SD 9.03 vs mean 2.50 ± SD 6.82, P = 0.019), genus *Prevotella* (mean 3.69 ± SD 9.03 vs mean 2.50 ± SD 6.82, P = 0.019) and species *copri* (mean 3.14 ± SD 8.47 vs mean 2.18 ± SD 6.84, P = 0.033) were higher in obese compared to overweight pregnant women whilst species *uniformis* (mean 3.62 ± SD 3.09 vs mean 6.00 ± SD 5.06, P = 0.012) was lower in the obese pregnant women. After adjusting for multiple variables (Benjamini-Hochberg procedure), the bacterial family *Prevotellaceae* was found to be higher in obese compared to overweight pregnant women (p = 0.19). No statistically significant differences were detected between obese and overweight pregnant women in the richness index Chao1 (mean ± SD: 376.4 ± 58.7 vs 387.5 ± 56.6, P = 0.36), observed species (median (IQR): 336.2 (296.2-372.1) vs 348.6 (296.2-372.1), P = 0.38), PD (median (IQR): 36.5 (31.7-39.8) vs 36.93 (31.5-41.8), P = 0.53), Shannon index (median (IQR): 5.4 (5.0-5.9) vs 5.48 (5.3-5.8), P = 0.64) and Firmicutes to Bacteroidetes ratio (median 0.89 (IQR 0.61-1.22) vs 0.92 (0.62-1.23), P=0.59). Prepregnancy BMI correlated statistically significantly with species *uniformis* (rho =

-0.22, P = 0.036), although this did not remain significant after adjusting for multiple variables (Benjamini-Hochberg adjusted P-value = 0.76; Supplementary Table 1b).

Interrelations of low-garde inflammation, microbiota, metabolic markers and diet

Low-grade inflammatory markers, GlycA and hsCRP, were statistically significantly elevated in obese compared to overweight pregnant women (Table 1). Moreover, prepregnancy BMI correlated with hsCRP and GlycA (rho = 0.38, P < 0.001 and rho = 0.46, P < 0.001, respectively) (Supplementary Table 1b).

Next, we evaluated the relation of GlycA and hsCRP with lipid and amino acid profiles separately as continuous variables. After adjusting the P-values (Benjamini-Hochberg procedure) of the Spearman rank order test, 171 of 213 lipids correlated statistically significantly with GlycA while 59 of 213 lipids correlated with hsCRP. The correlation coefficients were also higher between GlycA and lipids than between hsCRP and lipids (Supplementary Table 1c).

With respect to the amino acids, both GlycA and hsCRP correlated with the following concentrations; isoleucine (r = 0.64, P > 0.001 and r = 0.27, P = 0.007, respectively, Spearman rank order test), leucine (r = 0.44, P < 0.001 and r = 0.23, P = 0.023) and phenylalanine (r = 0.50, P < 0.001 and r = 0.40, P < 0.001). GlycA also correlated with alanine (ρ = 0.36, P < 0.001) (Supplementary Table 1c).

We evaluated the interrelations of inflammation, amino acids and other metabolic risk markers by conducting a PCA correlation plot (Figure 2c). GlycA and four amino acids, these including isoleucine, leucine, phenylalanine and valine, were found to cluster with HOMA2-IR and insulin.

In the stepwise linear regression models the best explanatory factors for HOMA2-IR were prepregnancy BMI (β 0.072; 95% CI 0.046, 0.099; P < 0.001), very large VLDL particle (β 9.58, 95% CI 5.65, 13.51, P < 0.001) and valine (β 6.22; 95% CI 1.63, 10.80; P = 0.008) and for GlycA very large VLDL particle (β 3.15, 95% CI 2.15, 4.14, P < 0.001), phenylalanine (β 5.96; 95% CI 4.10, 7.82; P < 0.001), leucine (β -8.42; 95% CI -12.75, -4.09; P < 0.001) and isoleucine (β 9.65; 95% CI 3.74, 15.57; P = 0.002) (Table 3). Physical activity and diet were found not to explain HOMA2-IR or GlycA. Instead, dietary intake correlated with several lipid metabolites (Supplementary Table 1d). After adjusting for multiple comparison, correlation between fiber and lipid metabolites remained significant.

DISCUSSION

We found that the intestinal microbiota, as well as serum metabolic and inflammatory profiles differ according to the degree of overweight status in women with no clinical manifestations of pregnancy related complications, with the aberrations being more pronounced in obese women. As far as we are aware, this is the first study which has investigated jointly these profiles in relation to the overweight and obesity status of the pregnant women. These results may be of significance considering the elevated risk for both short and long-term health risks associated with higher degree of obesity[13].

We demonstrated that lipid values in several VLDL subclasses were found to be higher whereas there was a reduction in the lipid measures in HDL particles and several fatty acid related measures in obese compared to overweight pregnant women. These obesity status related alterations in lipid profile have also been detected previously in both pregnant[25,26] and non-pregnant subjects[27]. The new finding in our study relates to the detailed examination of several lipid measures in VLDL, LDL and HDL lipoprotein subclasses utilizing metabolomics analysis in obese and overweight pregnant women. In general, by utilizing more traditional measures,

dyslipidemia in obesity has been characterized by elevated triglycerides, total cholesterol and LDL cholesterol, as well as decreased HDL cholesterol concentrations[28] which can further contribute to pregnancy related adverse clinical manifestations[1,29,30,31]. In addition to prepregnancy BMI, we found that diet, but not exercise, associated with lipid metabolites, particularly the relation of dietary fibre with serum polyunsaturated fatty acids was detected. This correlation may be mediated by intestinal microbiota as we have recently shown in pregnant women that fiber enhances gut microbiota richness[32], which again has been linked to a healthier metabolic phenotype[33,34].

We propose that the key denominator for the adverse clinical manifestations is likely related to the interaction of aberrant metabolism and low-grade inflammation as we found that the levels of two markers of low-grade inflammation, hsCRP and GlycA, were higher in obese compared to overweight pregnant women. In particular, the novel marker, GlycA[19], has been shown to correlate with obesity and insulin resistance[29], which may be of particular importance during pregnancy considering the heightened risk of gestational diabetes in overweight and obese pregnant women[4]. Furthermore, to confirm the interaction aspect we showed that several lipids and amino acids correlated with GlycA and hsCRP. Interestingly, we found that the correlation with GlycA was more pronounced than with hsCRP. Our finding is in line with previous studies in which GlycA has been shown to correlate with higher concentrations of triglycerides and other lipid levels, such as LDL cholesterol, in non-pregnant subjects[27] and obese and overweight pregnant women[35]. In addition to lipids, the level of GlycA has been shown to correlate with the amounts of branched chain amino acids in non-pregnant subjects[27]. This is the first time that a correlation between GlycA and branched chain and aromatic amino acids has been detected in pregnant women. Interestingly, in stepwise linear regression modelling the relation of branched chain amino acids to GlycA was even stronger than that of prepregnancy BMI. Furthermore, we found that the levels of GlycA, branched chain and

aromatic amino acids associated with HOMA2-IR and insulin. In contrast, in the stepwise linear regression modelling the best explanatory predictor of HOMA2-IR was prepregnancy BMI.

In previous studies, the concentrations of branched chain amino acids along with aromatic amino acids, have been related to obesity and insulin resistance[36,37], as well as to an increased risk of type 2 diabetes[38] in non-pregnant individuals. In the evaluation of amino acid profile, we found that in comparison to overweight pregnant women, the obese women had higher serum concentrations of four amino acids, these including three branched chain amino acids and one aromatic amino acid, which is in line with the previous studies demonstrating an association of maternal BMI with concentration of circulating branched chain amino acids[39]. Moreover, the detected correlations between prepregnancy BMI and branched chain and aromatic amino acids in our study strengthen the finding. These results may be of significance regarding maternal health as serum concentrations of amino acids, particularly those of arginine and glycine, in the targeted mass-spectrometry analysis were associated with an increased risk of gestational diabetes[40]. In our study, the elevated concentration of branched chain amino acids was coincidental with higher insulin and glucose concentrations already during early pregnancy.

The mechanism to explain how increased levels of branched chain amino acids induce insulin resistance has been postulated to involve uncoupling of insulin receptor from insulin receptor substrate-1 by activation of mammalian target rapamycin complex 1[41]. Possible factors influencing the branched chain amino acid levels in the serum include dietary intake and catabolism of branched chain amino acids[41]. In our study, we did not detect differences in protein intake between obese and overweight pregnant women or correlation between protein intake and amino acid metabolites, suggesting that the diet was not a contributing factor in our study. Although, it is possible that instead of single nutrients, it could be useful to evaluate the

355 associations of dietary patterns with serum metabolites[42]. The second mechanism relates to the
356 catabolism of the branched chain amino acids through down-regulation of mitochondrial
357 activity, as observed in a study conducted in twins[43], with subsequent elevated blood
358 concentrations. Recently, intestinal microbiota composition has been suggested as a contributor
359 to these changes. We detected a higher relative abundance of *Prevotella copri* in obese
360 compared to overweight pregnant women, although after adjusting for multiple variable
361 comparison, this was not statistically significant. Nevertheless, this finding is of interest[44] as
362 *Prevotella copri* has been linked to the biosynthesis of branched chain amino acids and
363 subsequent development of insulin resistance. Microbiota composition and the metabolic
364 activity of the microbiota may also explain our findings on the more enhanced levels of serum
365 glucose and cholesterol in obese compared to overweight pregnant women. Indeed, the
366 detected higher relative abundance of family *Prevotellaceae* in obese compared to overweight
367 pregnant women may contribute to glucose metabolism through the produced metabolites. It
368 has been shown previously that the family *Prevotellaceae* that belong to phylum Bacteroidetes,
369 produce propionate and acetate that take part in gluconeogenesis and synthesis of cholesterol
370 and de novo synthesis of lipids, respectively[45]. Furthermore, one previous study showed that
371 women with a history of metabolic disorder, have a *Prevotellaceae*-dominated intestinal
372 microbiome and lower abundance of the phylum Firmicutes compared to women with no
373 history of metabolic disorder[46]. In contrast, there was a lower abundance of species *uniformis*
374 in obese compared to overweight pregnant women in our study. However, after adjusting for
375 multiple comparisons, the change in the abundance of *uniformis* did not remain statistically
376 significant. Nevertheless, administration of species *uniformis* has been shown to decrease
377 metabolic and immune dysfunction by affecting macrophage and dendritic cell function and
378 intestinal dysbiosis in obese mice consuming a high fat diet[47]. Of note is that the gut microbiota
379 composition may alter during pregnancy[2], but further studies are needed to establish which

bacteria and in which state of pregnancy could be considered as predictive markers of metabolic disturbances.

The strength of our study lies in its detailed analysis of a large number of circulating metabolites and intestinal microbiota data from well characterized overweight and obese pregnant women who were otherwise healthy. We also used robust statistical methods and corrected for multiple comparisons. One possible limitation relates to the lack of normal weight pregnant women as a comparative groups. Although our focus was in a group of pregnant women at risk for clinical complications, normal weight pregnant women as a comparative group would have allowed generalization of the results to a wider population.

In conclusion, this study highlights the impact of overweight and obesity status on maternal intestinal microbiota, metabolic and inflammatory profiles during early pregnancy. The observations of early alterations in these markers could provide new predictors to supplement standard clinical markers, particularly as costs associated with microbiota and particularly metabolomics analytics are becoming reasonable, providing information about a large number of metabolites while at the same time providing comparable results to the traditional assays of biomarkers.

ACKNOWLEDGEMENTS

We thank biostatistician Tero Vahlberg for advice on statistical analyses and Päivi Isaksson for contacting the study participants.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

Funding was received from the Academy of Finland (#258606) and State research funding for university-level health research of the Turku University Hospital Expert Responsibility Area. Personal support to KM was received from the Finnish Cultural Foundation (Varsinais-Suomi Regional Fund) and to NH from Orion Research Foundation sr.

AUTHOR CONTRIBUTIONS

KL and KM designed the research. KL organised the data collection. NH analyzed the data. NH wrote the first draft, and all authors wrote, read, commented and approved the final manuscript.

APPENDIX

Supplementary Data.

REFERENCES

[1] Wang Q, Würtz P, Auro K, Mäkinen VP, Kangas AJ, Soininen P *et al*. Metabolic profiling of pregnancy: cross-sectional and longitudinal evidence. BMC Med 2016; 14: 205.

[2] Koren O, Goodrich JK, Cullender TC, Spor A, Laitinen K, Bäckhed HK *et al.* Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell 2012; 150: 470-480.

[3] Lain KY, Catalano PM. Metabolic changes in pregnancy. Clin Obstet Gynecol 2007; 50: 938–48.

[4] Pantham P, Aye IL, Powell TL. Inflammation in maternal obesity and gestational diabetes mellitus. Placenta 2015; e-pub ahead of print 28 April 2015; doi: 10.1016/j.placenta.2015.04.006.

[5] Agenor A, Bhattacharya S. Infertility and miscarriage: common pathways in manifestation and management. Womens Health (Lond) 2015; e-pub ahead of print 4 August 2015; doi: 10.2217/whe.15.19.

[6] Heerwagen MJ, Miller MR, Barbour LA, Friedman JE. Maternal obesity and fetal metabolic programming: a fertile epigenetic soil. Am J Physiol Regul Integr Comp Physiol 2010; e-pub ahead of print 14 July; doi: 10.1152/ajpregu.00310.2010

[7] Koletzko B, Beyer J, Brands B, Demmelmair H, Grote V, Haile G *et al*. Early influences of nutrition on postnatal growth. Nestle Nutr Inst Workshop Ser 2013; e-pub ahead of print 22 January 2013; doi: 10.1159/000342533

[8] Santacruz A, Collado MC, García-Valdés L, Segura MT, Martín-Lagos JA, Anjos T *et al.* Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women. Br J Nutr 2010; e-pub ahead of print 8 March 2010; doi: 10.1017/S0007114510000176

[9] Collado MC, Isolauri E, Laitinen K, Salminen S. Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women. Am J Clin Nutr 2008; 88: 894-9.

[10] Desert R, Canlet C, Costet N, Cordier S, Bonvallot N. Impact of maternal obesity on the metabolic profiles of pregnant women and their offspring at birth. Metabolomics 2015; 11: 1896–1907.

[11] Friis CM, Paasche Roland MC, Godang K, Ueland T, Tanbo T, Bollerslev J *et al.* Adiposity-related inflammation: effects of pregnancy. Obesity (Silver Spring) 2013; 21:E124-30

[12] Rasmussen KM, Yaktine AL. Institute of Medicine and National Research Council Committee to Reexamine IOM Pregnancy Weight Guidelines. Weight gain during pregnancy: reexamining the guidelines. National Academies Press, 2009

[13] World Health Organization. Obesity: Preventing and Managing the Global Epidemic. Report on a WHO Consultation on Obesity. World Health Organization: Geneva, 2007. WHO/NUT/NCD/98.1.

[14] Pahkala K, Heinonen OJ, Lagström H, Hakala P, Simell O, Viikari JS *et al.* Vascular endothelial function and leisure-time physical activity in adolescents. Circulation 2008; 118: 2353-9.0.

[15] The Oxford Centre for Diabetes. Endocrinology & metabolism. Diabetes trial unit. HOMA calculator. Available from: http://www.dtu.ox.ac.uk/. [Accessed April 2016]

[16] Soininen P, Kangas AJ, Würtz P, Suna T, Ala-Korpela M. Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics. Circ Cardiovasc Genet 2015; 8: 192–206.

[17] Otvos JD, Shalaurova I, Wolak-Dinsmore J, Connelly MA, Mackey RH, Stein JH *et al.* GlycA: A Composite Nuclear Magnetic Resonance Biomarker of Systemic Inflammation. Clin Chem 2015; e-pub ahead of print 16 March 2015; doi: 10.1373/clinchem.2014.232918.

[18] Mokkala K, Röytiö H, Munukka E, Pietilä S, Ekblad U, Rönnemaa T *et al*. 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; e-pub ahead of print 27 July 2016; doi: 10.3945/jn.116.235358

[19] Lozupone CA, Knight R. Species divergence and the measurement of microbial diversity. FEMS Microbiol Rev 2008; e-pub ahead of print 22 April; doi: 10.1111/j.1574-6976.2008.00111.x.

[20] German JB,. Smilowitz JT, and Zivkovi AM. Lipoproteins: When size really matters. Curr Opin Colloid Interface Sci 2006; 11(2-3): 171-183

[21] Mora S, Otvos JD, Rosenson RS, Pradhan A, Buring JE et al. Lipoprotein Particle Size and Concentration by Nuclear Magnetic Resonance and Incident Type 2 Diabetes in Women. Diabetes 2010: e-pub ahead of print 25 February 2010; doi: 10.2337/db09-1114.

[22] Enquobahrie DA, Williams MA, Qiu C, Luthy DA. Early pregnancy lipid concentrations and the risk of gestational diabetes mellitus. Diabetes Res Clin Pract 2005; e-pub ahead of print 8 November 2005; doi: 10.1016/j.diabres.2005.03.022.

[23] Klop B, Elte JW, Cabezas MC. Dyslipidemia in obesity: mechanisms and potential targets. Nutrients 2013; 5:1218–1240.

[24] Sandler V, Reisetter AC, Bain JR, Muehlbauer MJ, Nodzenski M, Stevens RD et al. Associations of maternal BMI and insulin resistance with the maternal metabolome and newborn outcomes. Diabetologia 2017; e-pub ahead of print 16 December 2016; doi: 10.1007/s00125-016-4182-2

[25] Hellmuth C, Lindsay KL, Uhl O, Buss C, Wadhwa PD, Koletzko B *et al*. Association of maternal prepregnancy BMI with metabolomic profile across gestation. Int J Obes (Lond) 2017; e-pub ahead of print 27 September; doi: 10.1038/ijo.2016.153

[26] Sandler V, Reisetter AC, Bain JR, Muehlbauer MJ, Nodzenski M, Stevens RD *et al*. Associations of maternal BMI and insulin resistance with the maternal metabolome and newborn outcomes. Diabetologia 2016; e-pub ahead of print 16 December 2016; doi: 10.1007/s00125-016-4182-2

[27] Bogl LH, Kaye SM, Rämö JT, Kangas AJ, Soininen P, Hakkarainen A *et al*. Abdominal obesity and circulating metabolites: A twin study approach. Metabolism 2016; e-pub ahead of print 31 October 2015; doi: 10.1016/j.metabol.2015.10.027

[28] Klop B, Elte JW, Cabezas MC. Dyslipidemia in obesity: mechanisms and potential targets. Nutrients 2013; 5:1218–1240.

[29] Bao W, Dar S, Zhu Y, Wu J, Rawal S, Li S *et al*. Plasma concentrations of lipids during pregnancy and the risk of gestational diabetes mellitus: A longitudinal study. J Diabetes 2017; e-pub ahead of print 24 April 2017; doi: 10.1111/1753-0407.12563.

[30] Enquobahrie DA, Williams MA, Qiu C, Luthy DA. Early pregnancy lipid concentrations and the risk of gestational diabetes mellitus. Diabetes Res Clin Pract 2005; e-pub ahead of print 8 November 2005; doi: 10.1016/j.diabres.2005.03.022.

[31] Li G, Kong L, Zhang L, Fan L, Su Y, Rose JC *et al*. Early Pregnancy Maternal Lipid Profiles and the Risk of Gestational Diabetes Mellitus Stratified for Body Mass Index. Reprod Sci. 2015; e-pub ahead of print 12 November 2014; doi: 10.1177/1933719114557896.

[32] Röytiö H, Mokkala K, Vahlberg T, Laitinen K. Dietary intake of fat and fibre according to reference values relates to highergut microbiota richness in overweight pregnant women. Br J Nutr 2017; 118(5):343-352.

[33] Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G et al. Richness of human gut microbiome correlates with metabolic markers. Nature 2013; 500:541-546.

[34] Cotillard A, Kennedy SP, Kong LC, Prifti E, Pons N, Le Chatelier E et al. GlycAm a maerker of acure phase glycoproteins, and the risk of incident type 2 diabetes mellitus: PREVEND study. Clin Chim Acta 2016; e-pub ahead of print 5 November 2015; doi: 10.1016/j.cca.2015.11.001.

[35] 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; e-pub ahead of print 4 January 2017; doi: 10.1016/j.metabol.2016.12.015

519 [36] Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF *et al*. A branched-
520 chain amino acid-related metabolic signature that differentiates obese and lean humans and
521 contributes to insulin resistance. Cell Metab 2009; 9: 311-26.
522 [37] Zhang S, Zeng X, Ren M, Mao X, Qiao S. Novel metabolic and physiological functions
523 of branched chain amino acids: a review. J Anim Sci Biotechnol 2017; 8: 10.
524 [38] Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E *et al*. Metabolite
525 profiles and the risk of developing diabetes. Nat Med 2011; e-pub ahead of print 20 March
526 2011; doi: 10.1038/nm.2307
527 [39] Sandler V, Reisetter AC, Bain JR, Muehlbauer MJ, Nodzenski M, Stevens RD *et al*.
528 Associations of maternal BMI and insulin resistance with the maternal metabolome and
529 newborn outcomes. Diabetologia 2017; e-pub ahead of print 16 December 2016; doi:
530 10.1007/s00125-016-4182-2
531 [40] Nevalainen J, Sairanen M, Appelblom H, Gissler M, Timonen S, Ryynänen M. First-
532 Trimester Maternal Serum Amino Acids and Acylcarnitines Are Significant Predictors of
533 Gestational Diabetes. Rev Diabet Stud 2016; e-pub ahead of print 10 Februart 2017; doi:
534 10.1900/RDS.2016.13.236.
535 [41] Yoon MS. The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance
536 and Metabolism. Nutrients 2016; 8: E405.
537 [42] Playdon MC, Moore SC, Derkach A, Reedy J, Subar AF, Sampson JN, Albanes D, Gu
538 F, Kontto J, Lassale C, Liao LM, Männistö S, Mondul AM, Weinstein SJ, Irwin ML7,
539 Mayne ST, Stolzenberg-Solomon R. Identifying biomarkers of dietary patterns by using
540 metabolomics. Am J Clin Nutr 2017: e-pub ahead of prin 28 December 2016; doi:
541 10.3945/ajcn.116.144501.

[43] Pietiläinen KH, Naukkarinen J, Rissanen A, Saharinen J, Ellonen P, Keränen H *et al.* Global transcript profiles of fat in monozygotic twins discordant for BMI: pathways behind acquired obesity. PLoS Med 2008; 5: e51.

[44] Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyötylainen T, Nielsen T, Jensen BA *et al*. Human gut microbes impact host serum metabolome and insulin sensitivity. Nature 2016; e-pub ahead of print 13 July 2016; doi: 10.1038/nature18646

[45] Chakraborti CK. New-found link between microbiota and obesity. World J Gastrointest Pathophysiol 2015: 6: 110–119.

[46] Fugmann M, Breier M, Rottenkolber M, Banning F, Ferrari U, Sacco V *et al.* The stool microbiota of insulin resistant women with recent gestational diabetes, a high risk group for type 2 diabetes. Sci Rep 2015; 5: 13212.

[47] Gauffin Cano P, Santacruz A, Moya Á, Sanz Y. Bacteroides uniformis CECT 7771 Ameliorates Metabolic and Immunological Dysfunction in Mice with High-Fat-Diet Induced Obesity. PLoS One 2012; e-pub ahead of print 26 July 2012; doi: 10.1371/journal.pone.0041079

TABLES

Table 1. Clinical characteristics and intake of energy yielding nutrients and fiber of overweight and obese pregnant women at early pregnancy.

	Overweight pregnant women, n = 52	Obese pregnant women, n = 48	P-value
Characteristics:			
Age (years)	30 ± 5	30 ± 5	0.78
Education (college or university)	0.519	0.511	0.88
Gestational age (weeks)	13 ± 3	13 ± 3	0.82
Prepegnancy BMI (kg/m2)	27 ± 2	34 ± 4	< 0.001
Weight (kg)	77.3 ± 8.6	94.5 ± 14.0	< 0.001
Fat percent (%)	39.8 ± 4.6	48.3 ± 3.8	< 0.001

Systolic blood pressure (mmHg)	117 ± 14	119 ± 10	0.37
Diastolic blood pressure (mmHg)	76 ± 9	79 ± 9	0.12
Intake of energy, energy yielding nutrients & fiber:			
Energy (kJ)	8090 ± 1503	8194 ± 2203	0.79
Carbohydrates			
(g)	215 ± 56	229 ± 75	0.31
(%)	45 ± 7	47 ± 6	0.090
Protein			
(g)	85 ± 22	80 ± 19	0.29
(%)	18 ± 5	17 ± 3	0.24
Fat			
(g)	76 ± 22	75 ± 25	0.73
(%)	35 ± 7	34 ± 6	0.38
Fiber			
(g)	19 ± 6	20 ± 7	0.58
Physical activity:			
MET index (h/wk)	7.5 (3.0-12.0)	3.9 (1.2-12.0)	0.27
Serum glucose markers:			
Insulin (mU/l)	9.0 (7.0-11.0)	14.0 (9.0-17.0)	< 0.001
Glucose (mmol/l)	4.7 ± 0.3	4.9 ± 0.3	0.015
HbA1c (%)	4.98 ± 0.22	4.9 ± 0.3	0.36
HOMA2-IR	1.2 (0.9-1.4)	1.7 (1.1-2.2)	< 0.001
Low-grade inflammation markers:			
hsCRP (mg/l)	4.0 (1.8-6.9)	6.1 (4.0-10.0)	0.0015
GlycA (mmol/l)	1.45 ± 0.11	1.57 ± 0.19	< 0.001
Intestinal permeability marker:			
Zonulin (ng/ml)	44.6 ± 8.7	48.9 ± 13.1	0.052
Metabolic endotoxemia marker:			
LPS (EU/ml)	0.37 ± 0.06	0.37 ± 0.08	0.80

560 Values are mean ± SD for normally distributed, median (interquartile range) for non-distributed
561 variables or percent (%) of total. Independent samples t-test, Mann-Whitney U test or chi-
562 square-test. P value < 0.05 is considered significant.

563

564 Table 2. Relative abundance (percentage of total bacteria) of intestinal microbiota in overweight and obese pregnant women.

	Overweight pregnant women, relative abundance of (%) of total bacteria, n = 48		Obese pregnant women, relative abundance of (%) of total bacteria, n = 43			
Bacteria	mean ± SD	median (IQR)	mean ± SD	median (IQR)	P-value	Benjamini-Hochberg P-value
k__Bacteria;p__Actinobacteria	1.22 ± 1.16	0.79 (0.34-1.23)	1.12 ± 1.59	0.65 (0.34-1.23)	0.36	0.58
k__Bacteria;p__Bacteroidetes	48.89 ± 9.95	48.86 (42.01-58.38)	50.98 ± 12.76	51.19 (42.18-58.38)	0.44	0.58
c__Bacteroidia	48.89 ± 9.95	48.86 (42.01-58.38)	50.98 ± 12.76	51.19 (42.18-58.38)	0.44	0.65
o__Bacteroidales	48.89 ± 9.95	48.86 (42.01-58.38)	50.98 ± 12.76	51.19 (42.18-58.38)	0.44	0.65
f__Bacteroidaceae	33.04 ± 11.77	32.77 (23.75-38.57)	32.62 ± 13.85	30.69 (23.18-38.57)	0.61	0.99
g__Bacteroides	33.04 ± 11.77	32.77 (23.75-38.57)	32.62 ± 13.85	30.69 (23.18-38.57)	0.61	0.98
s__non identified	20.43 ± 9.04	19.16 (13.25-28.24)	22.17 ± 13.52	20.17 (13.38-28.24)	0.74	0.98
s__caccae	2.01 ± 3.82	0.83 (0.30-1.77)	1.36 ± 2.00	0.93 (0.08-1.77)	0.43	0.98
s__eggerthii	0.55 ± 1.53	0.00 (0.00-0.01)	1.03 ± 2.51	0.00 (0.00-0.01)	0.51	0.98
s__fragilis	0.90 ± 1.70	0.33 (0.03-1.33)	1.54 ± 3.64	0.44 (0.00-1.33)	0.60	0.98
s__ovatus	3.08 ± 4.68	1.76 (0.84-2.89)	2.73 ± 4.27	1.43 (0.75-2.89)	0.44	0.98

s__uniformis	6.00 ± 5.06	4.66 (2.53-6.36)	3.62 ± 3.09	3.38 (0.90-6.36)	0.01	0.26
f__Porphyromonadaceae	3.87 ± 2.61	3.28 (2.11-5.08)	3.82 ± 2.63	3.34 (2.40-5.08)	0.99	0.99
g__Parabacteroides	3.87 ± 2.61	3.27 (2.11-4.83)	3.79 ± 2.62	3.34 (2.40-4.83)	0.98	0.98
s__non identified	2.44 ± 2.41	1.82 (0.56-3.58)	2.38 ± 2.68	1.87 (0.05-3.58)	0.69	0.98
s__distasonis	1.36 ± 1.11	1.17 (0.59-2.05)	1.36 ± 1.54	0.90 (0.16-2.05)	0.44	0.98
f__Prevotellaceae	2.50 ± 6.82	0.01 (0.00-0.01)	3.69 ± 9.03	0.00 (0.00-0.01)	0.02	0.19
g__Prevotella	2.50 ± 6.82	0.01 (0.00-0.01)	3.69 ± 9.03	0.00 (0.00-0.01)	0.02	0.29
s__copri	2.18 ± 6.84	0.01 (0.00-0.01)	3.14 ± 8.47	0.00 (0.00-0.01)	0.03	0.35
f__Rikenellaceae	6.19 ± 3.99	6.09 (3.62-9.28)	6.70 ± 5.02	5.36 (3.29-9.28)	0.99	0.99
g__non identified	6.19 ± 3.99	6.09 (3.62-9.25)	6.69 ± 5.02	5.36 (3.29-9.25)	0.98	0.98
s__non identified	6.19 ± 3.99	6.09 (3.62-9.25)	6.69 ± 5.02	5.36 (3.29-9.25)	0.98	0.98
f__[Barnesiellaceae]	1.78 ± 2.11	1.21 (0.00-3.00)	1.88 ± 2.00	1.60 (0.00-3.00)	0.91	0.99
g__non identified	1.78 ± 2.11	1.21 (0.00-3.00)	1.88 ± 2.00	1.60 (0.00-3.00)	0.91	0.98
s__non identified	1.78 ± 2.11	1.21 (0.00-3.00)	1.88 ± 2.00	1.60 (0.00-3.00)	0.91	0.98
k__Bacteria;p__Firmicutes	45.10 ± 10.13	45.13 (36.65-53.29)	43.60 ± 13.10	45.06 (33.73-53.29)	0.79	0.79
c__Clostridia	43.93 ± 9.25	44.41 (36.11-52.19)	42.92 ± 12.88	43.82 (33.35-52.19)	0.89	0.89
o__Clostridiales	43.90 ± 9.24	44.21 (36.11-52.17)	42.88 ± 12.86	43.73 (33.34-52.17)	0.88	0.88

f__non identified	4.55 ± 3.57	3.37 (1.94-6.32)	4.39 ± 3.14	3.94 (1.78-6.32)	0.91	0.99
g__non identified	4.55 ± 3.57	3.37 (1.94-6.32)	4.39 ± 3.14	3.94 (1.78-6.32)	0.91	0.98
s__non identified	4.55 ± 3.57	3.37 (1.94-6.32)	4.39 ± 3.14	3.94 (1.78-6.32)	0.91	0.98
f__Lachnospiraceae	16.97 ± 7.27	17.00 (12.15-19.89)	15.90 ± 5.08	15.45 (11.18-19.89)	0.58	0.99
g__non identified	9.32 ± 5.40	8.12 (5.41-10.38)	8.24 ± 3.15	7.89 (5.90-10.38)	0.76	0.98
s__non identified	9.32 ± 5.40	8.12 (5.41-10.38)	8.24 ± 3.15	7.89 (5.90-10.38)	0.76	0.98
g__Blautia	2.28 ± 2.04	1.91 (0.97-2.26)	1.99 ± 1.56	1.49 (1.22-2.26)	0.61	0.98
s__non identified	2.25 ± 1.99	1.91 (0.96-2.26)	1.98 ± 1.56	1.49 (1.22-2.26)	0.60	0.98
g__Coprococcus	1.45 ± 1.38	1.03 (0.50-2.08)	1.58 ± 1.48	0.94 (0.61-2.08)	0.59	0.98
s__non identified	0.97 ± 0.99	0.62 (0.39-1.54)	1.14 ± 1.18	0.69 (0.48-1.54)	0.34	0.98
g__Lachnospira	2.01 ± 1.55	1.73 (0.61-3.56)	2.11 ± 2.05	1.24 (0.55-3.56)	0.81	0.98
s__non identified	2.01 ± 1.55	1.73 (0.61-3.56)	2.11 ± 2.05	1.24 (0.55-3.56)	0.81	0.98
f__Ruminococcaceae	19.40 ± 6.14	19.42 (14.21-25.50)	19.90 ± 9.94	19.92 (14.27-25.50)	0.70	0.99
g__non identified	9.54 ± 5.43	8.30 (6.20-12.97)	9.85 ± 6.64	10.18 (4.84-12.97)	0.84	0.98
s__non identified	9.54 ± 5.43	8.30 (6.20-12.97)	9.85 ± 6.64	10.18 (4.84-12.97)	0.84	0.98
g__Faecalibacterium	5.55 ± 3.04	4.85 (3.50-7.03)	5.34 ± 3.20	5.39 (2.94-7.03)	0.83	0.98
s__prausnitzii	5.55 ± 3.04	4.85 (3.50-7.03)	5.34 ± 3.20	5.39 (2.94-7.03)	0.83	0.98

g__Oscillospira	1.26 ± 0.80	1.00 (0.85-1.51)	1.33 ± 0.94	1.13 (0.76-1.51)	0.95	0.98
s__non identified	1.26 ± 0.80	1.00 (0.85-1.51)	1.33 ± 0.94	1.14 (0.76-1.51)	0.95	0.98
g__Ruminococcus	3.04 ± 2.59	2.54 (0.81-5.21)	3.37 ± 2.97	2.61 (1.15-5.21)	0.77	0.98
s__non identified	3.04 ± 2.59	2.54 (0.81-5.21)	3.37 ± 2.97	2.61 (1.15-5.21)	0.77	0.98
f__Veillonellaceae	1.62 ± 1.39	1.13 (0.65-2.31)	1.55 ± 1.29	1.16 (0.59-2.31)	0.93	0.99
k__Bacteria;p__Proteobacteria	2.99 ± 1.94	2.55 (1.49-4.00)	2.89 ± 2.97	1.99 (1.21-4.00)	0.34	0.58
c__Betaproteobacteria	1.76 ± 1.44	1.56 (0.845-2.21)	1.87 ± 2.52	0.98 (0.53-2.21)	0.31	0.65
o__Burkholderiales	1.76 ± 1.44	1.56 (0.84-2.21)	1.87 ± 2.52	0.98 (0.53-2.21)	0.31	0.65
f__Alcaligenaceae	1.76 ± 1.44	1.56 (0.84-2.21)	1.87 ± 2.52	0.98 (0.53-2.21)	0.31	0.99
g__Sutterella	1.75 ± 1.44	1.55 (0.84-2.21)	1.82 ± 2.54	0.87 (0.47-2.21)	0.20	0.98
s__non identified	1.75 ± 1.44	1.55 (0.84-2.21)	1.82 ± 2.54	0.87 (0.47-2.21)	0.20	0.98

565 Values are mean ± SD and median (interquartile range). Mann-Whitney U test. P value < 0.05 and Benjamini-Hochberg P-value < 0.20 are

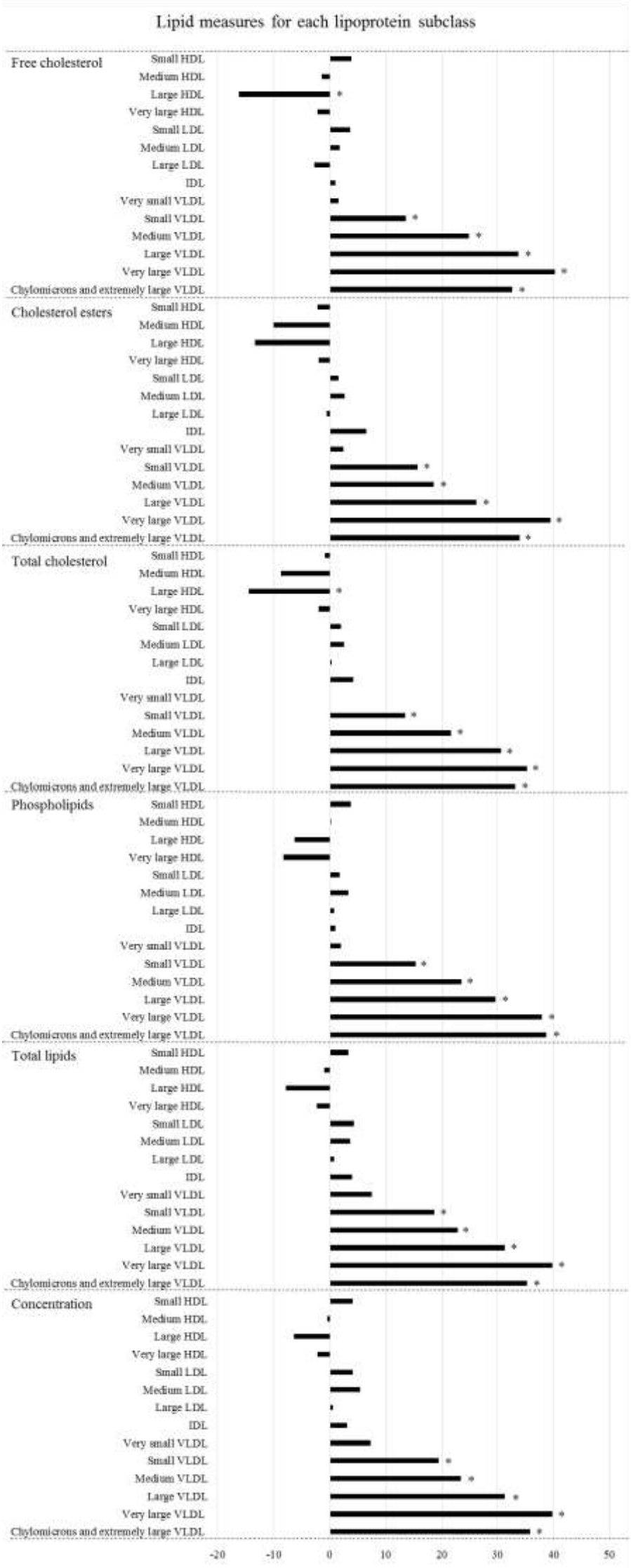
566 considered significant. p, Phylum; c, class; o, order; f, family; g, genus; s, species.

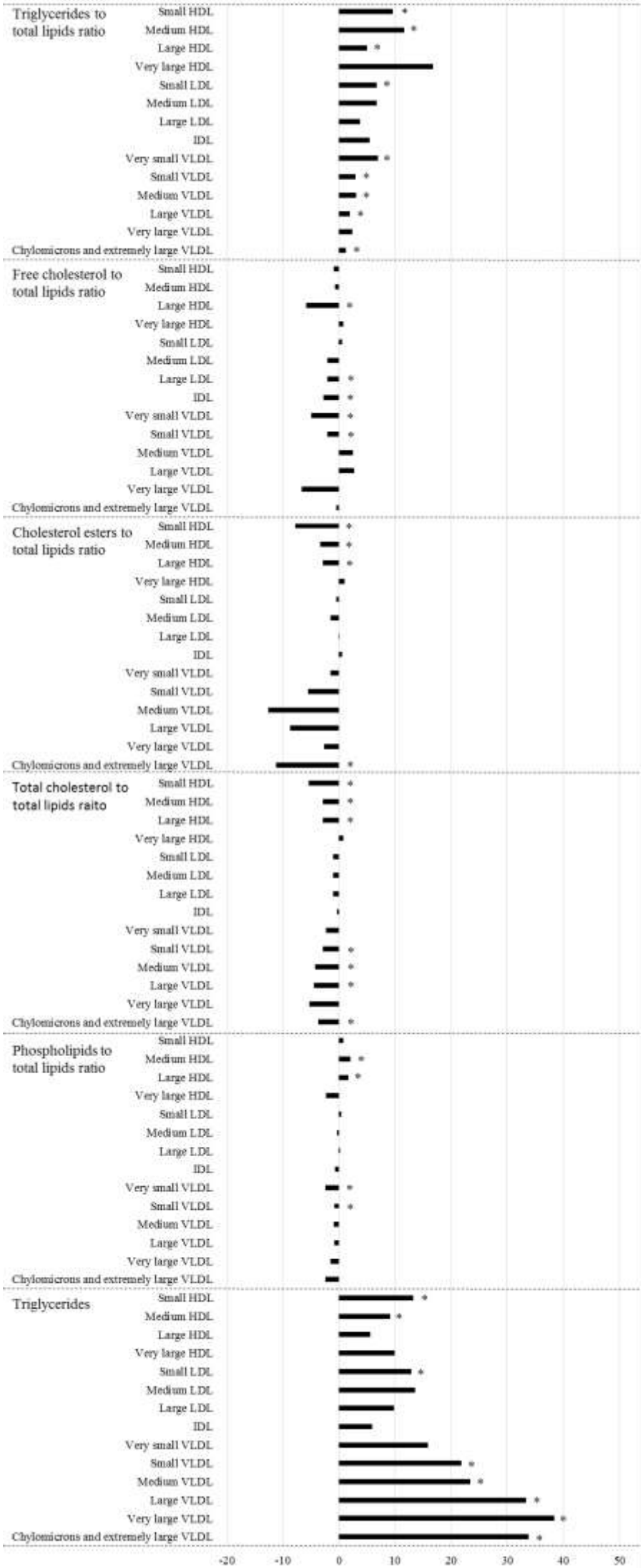
Table 3. Association of prepregnancy BMI and metabolites with HOMA2-IR and GlycA in stepwise linear regression in overweight and obese pregnant women.

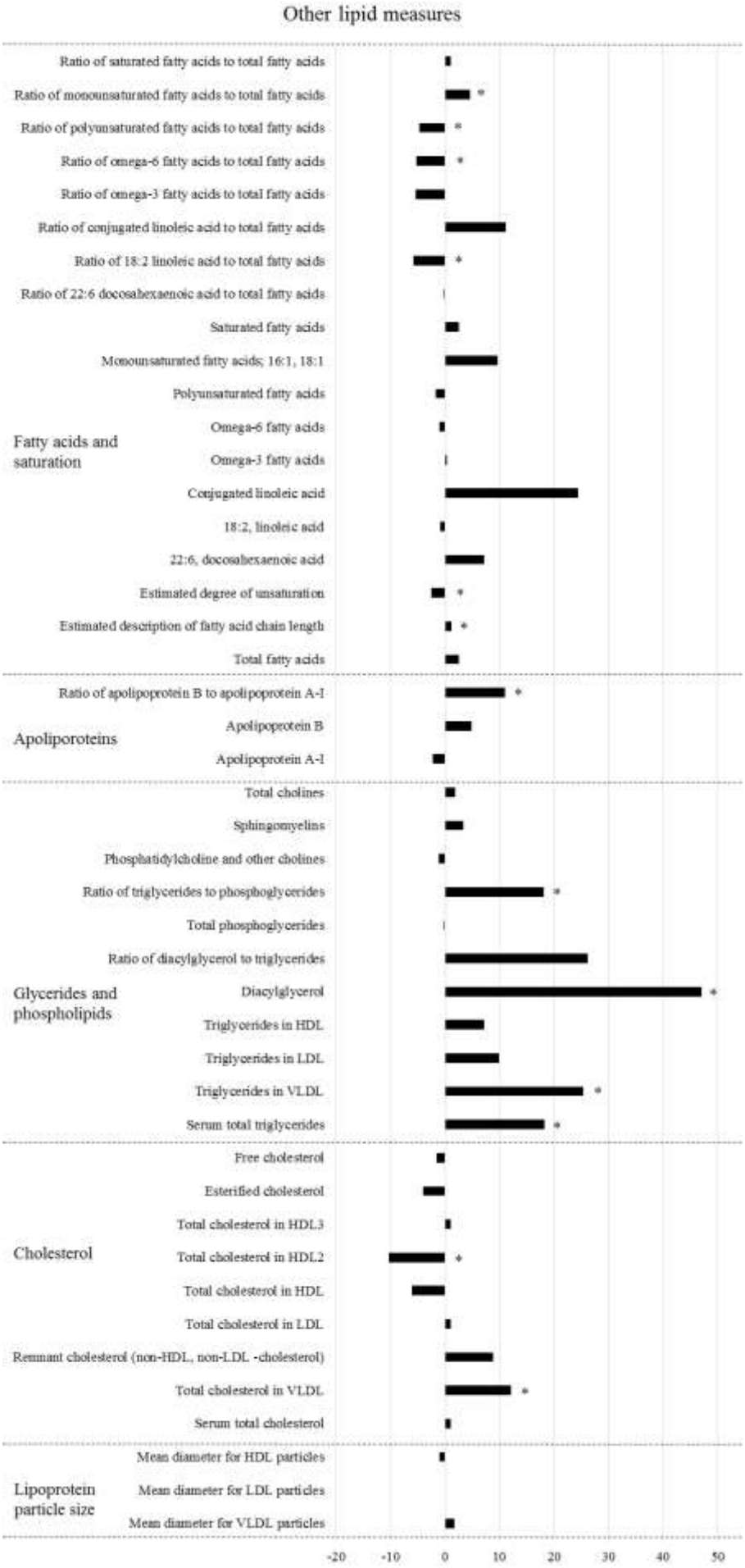
	β (95% CI) BMI	β (95% CI) Very large VLDL particle	β (95% CI) Valine	β (95% CI) Phenylanaline	β (95% CI) Leucine	β (95% CI) Isoleucine
HOMA2-IR	0.072 (0.046. 0.099)	9.58 (5.65, 13.51)[1]	6.22 (1.63, 10.80)	-	-	-
P-value	< 0.001	< 0.001	0.008	-	-	-
GlycA	-	3.15, (2.15, 4.14)[1]	-	5.96 (4.10, 7.82)	-8.42 (-12.75, -4,09)	9.65 (3.74, 15.57)
P-value	-	< 0.001	-	< 0.001	< 0.001	0.002

One unit change in HOMA2-IR and GlycA is shown as regression coefficient (β) with the change in prepregnancy BMI and metabolites. 95% CI: 95 % confidence interval for β. [1]1e-8.

FIGURES

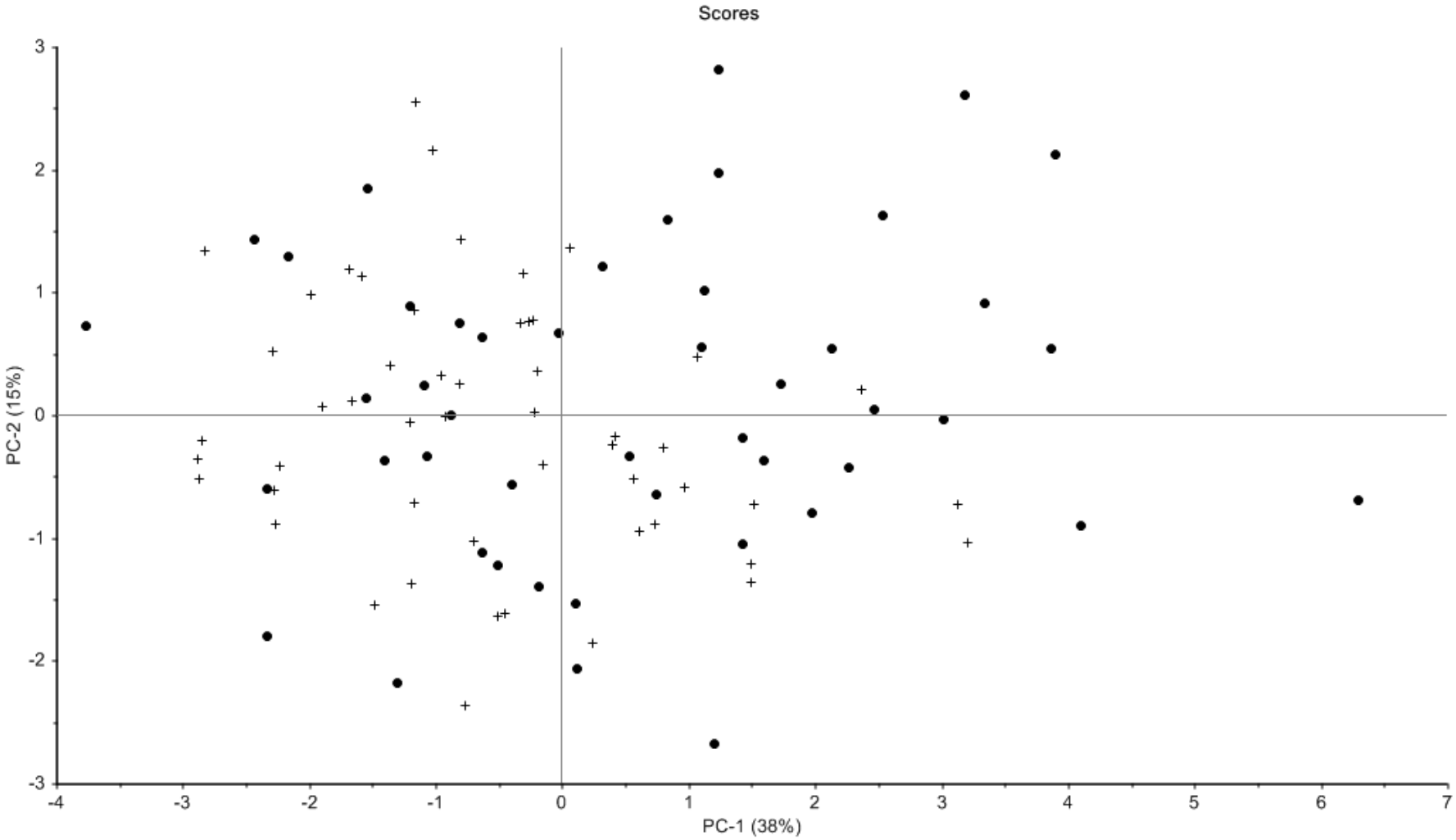

Lipid measures for each lipoprotein subclass
Free cholesterol
Cholesterol esters
Total cholesterol
Phospholipids
Total lipids
Concentration
Small HDL
Medium HDL
Large HDL
Very large HDL
Small LDL
Medium LDL
Large LDL
IDL
Very small VLDL
Small VLDL
Medium VLDL
Large VLDL
Very large VLDL
Chylomicrons and extremely large VLDL
-20
-10
0
10
20
30
40
50


Triglycerides to total lipids ratio
Small HDL
Medium HDL
Large HDL
Very large HDL
Small LDL
Medium LDL
Large LDL
IDL
Very small VLDL
Small VLDL
Medium VLDL
Large VLDL
Very large VLDL
Chylomicrons and extremely large VLDL
Free cholesterol to total lipids ratio
Small HDL
Medium HDL
Large HDL
Very large HDL
Small LDL
Medium LDL
Large LDL
IDL
Very small VLDL
Small VLDL
Medium VLDL
Large VLDL
Very large VLDL
Chylomicrons and extremely large VLDL
Cholesterol esters to total lipids ratio
Small HDL
Medium HDL
Large HDL
Very large HDL
Small LDL
Medium LDL
Large LDL
IDL
Very small VLDL
Small VLDL
Medium VLDL
Large VLDL
Very large VLDL
Chylomicrons and extremely large VLDL
Total cholesterol to total lipids raito
Small HDL
Medium HDL
Large HDL
Very large HDL
Small LDL
Medium LDL
Large LDL
IDL
Very small VLDL
Small VLDL
Medium VLDL
Large VLDL
Very large VLDL
Chylomicrons and extremely large VLDL
Phospholipids to total lipids ratio
Small HDL
Medium HDL
Large HDL
Very large HDL
Small LDL
Medium LDL
Large LDL
IDL
Very small VLDL
Small VLDL
Medium VLDL
Large VLDL
Very large VLDL
Chylomicrons and extremely large VLDL
Triglycerides
Small HDL
Medium HDL
Large HDL
Very large HDL
Small LDL
Medium LDL
Large LDL
IDL
Very small VLDL
Small VLDL
Medium VLDL
Large VLDL
Very large VLDL
Chylomicrons and extremely large VLDL
-20
-10
0
10
20
30
40
50


Other lipid measures
Ratio of saturated fatty acids to total fatty acids
Ratio of monounsaturated fatty acids to total fatty acids
Ratio of polyunsaturated fatty acids to total fatty acids
Ratio of omega-6 fatty acids to total fatty acids
Ratio of omega-3 fatty acids to total fatty acids
Ratio of conjugated linoleic acid to total fatty acids
Ratio of 18:2 linoleic acid to total fatty acids
Ratio of 22:6 docosahexaenoic acid to total fatty acids
Saturated fatty acids
Monounsaturated fatty acids; 16:1, 18:1
Polyunsaturated fatty acids
Omega-6 fatty acids
Omega-3 fatty acids
Fatty acids and saturation
Conjugated linoleic acid
18:2, linoleic acid
22:6, docosahexaenoic acid
Estimated degree of unsaturation
Estimated description of fatty acid chain length
Total fatty acids
Ratio of apolipoprotein B to apolipoprotein A-I
Apolipoprotein B
Apolipoprotein A-I
Apoliporoteins
Total cholines
Sphingomyelins
Phosphatidylcholine and other cholines
Ratio of triglycerides to phosphoglycerides
Total phosphoglycerides
Ratio of diacylglycerol to triglycerides
Diacylglycerol
Triglycerides in HDL
Triglycerides in LDL
Triglycerides in VLDL
Serum total triglycerides
Glycerides and phospholipids
Free cholesterol
Esterified cholesterol
Total cholesterol in HDL3
Total cholesterol in HDL2
Total cholesterol in HDL
Total cholesterol in LDL
Remnant cholesterol (non-HDL, non-LDL -cholesterol)
Total cholesterol in VLDL
Serum total cholesterol
Cholesterol
Mean diameter for HDL particles
Mean diameter for LDL particles
Mean diameter for VLDL particles
Lipoprotein particle size
-20
-10
0
10
20
30
40
50

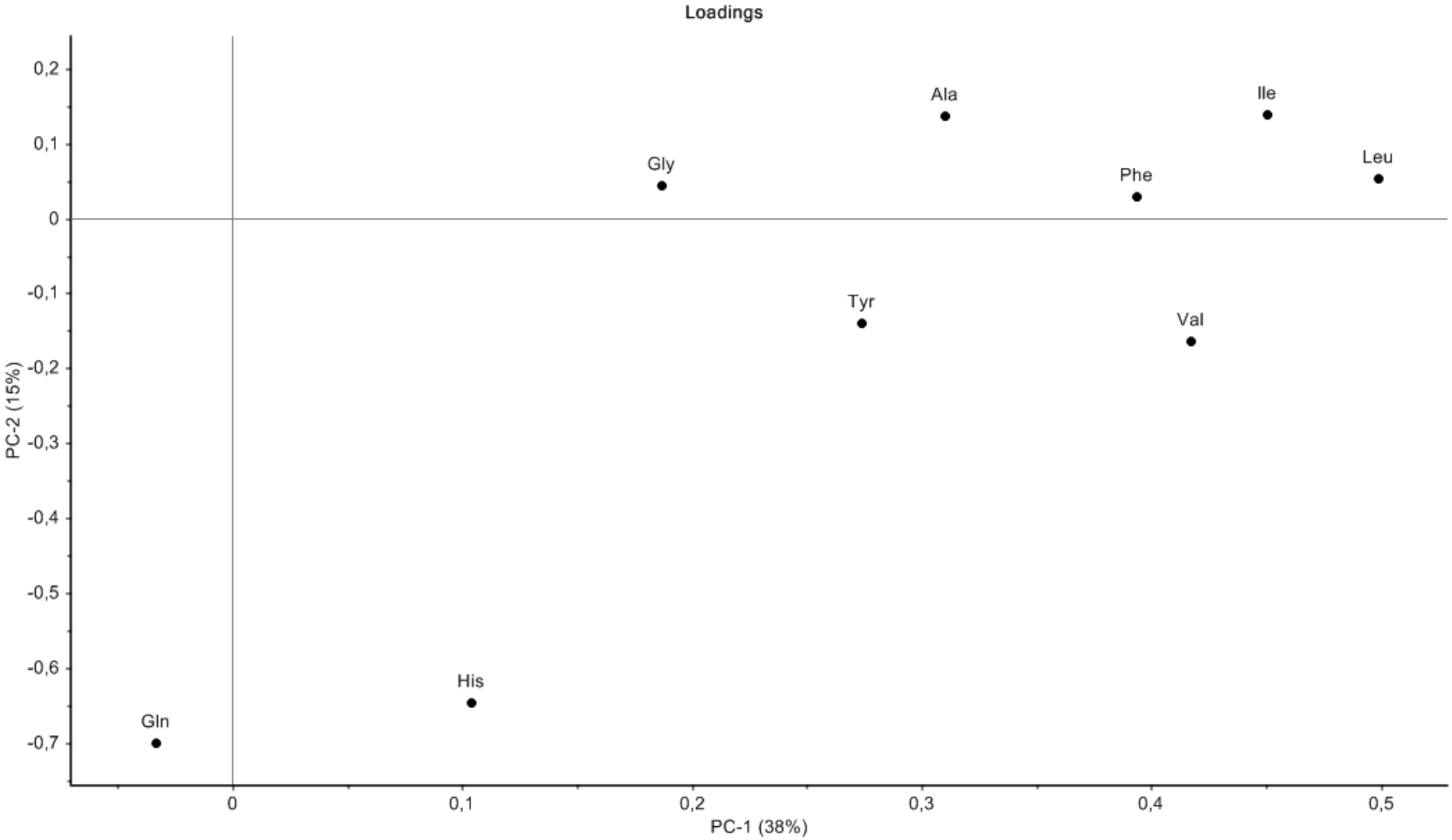
629 Figure 1. Median percentage differences in the measures reflecting lipids between overweight
630 and obese pregnant women. Mann-Whitney U test. The p-values denote statistical significance
631 after correcting for multiple testing (Benjamini-Hochberg procedure), *$p < 0.12$.

632 a)



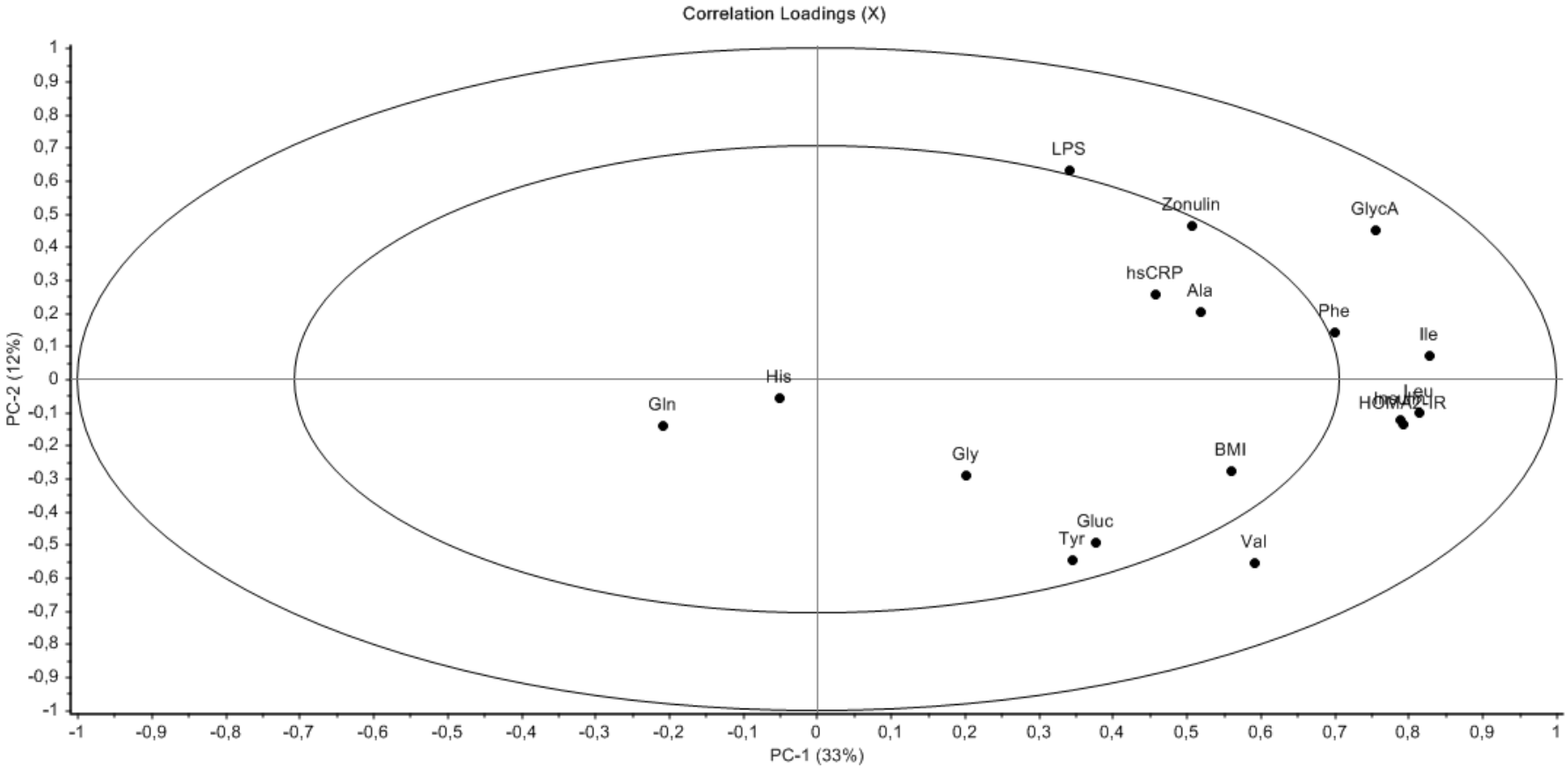
633

634 b)


Loadings
0,2
0,1
0
-0,1
-0,2
-0,3
-0,4
-0,5
-0,6
-0,7
PC-2 (15%)
Ala
Ile
Gly
Leu
Phe
Tyr
Val
His
Gln
0
0,1
0,2
0,3
0,4
0,5
PC-1 (38%)

635

636 c)


Correlation Loadings (X)
PC-2 (12%)
PC-1 (33%)
LPS
Zonulin
GlycA
hsCRP
Ala
Phe
Ile
His
Gln
Leu
HOMA2IR
Gly
BMI
Gluc
Tyr
Val

637

Figure 2. Principal component analysis plot of amino acids of overweight and obese pregnant women, a) Scores, b) Loadings and c) Correlation loadings of amino acids and metabolic risk markers, Insulin and HOMA2-IR are located similarly (on top of each other), Gln: glutamate, His: histidine, Gly: glycine, Tyr: tyrosine, Ala: alanine, Phe: phenylalanine, Val: valine, Ile: isoleucine, Leu: leucine, Cross: Overweight pregnant women, dot: obese pregnant women, hsCRP: high-sensitivity C-reactive protein, GlycA: a1-acid glycoprotein, Insu: insulin, Gluc: Glucose preBMI: prepregnancy BMI, PC-1: principal component 1, PC-2: principal component 2.