Relationship between gut microbiota and circulating metabolites in population-based cohorts

Dina Vojinovic*, Djawad Radjabzadeh*, Alexander Kurilshikov*, Najaf Amin, Cisca Wijmenga, Lude Franke, Andre G. Uitterlinden, Alexandra Zhernakova, Jingyaun Fu**,Robert Kraaij**, Cornelia M. van Dujin**

*These authors contributed equally to this work

**These senior authors contributed equally to this work

This chapter is in preparation.

The supplemental information for this paper is available at https://drive.google.com/file/d/1MJW0tieyfMGBWLnqS7xTAIxq_cEzNuXb/view?usp=sharing

ABSTRACT

Gut microbiota has been implicated in the major diseases affecting the human population and has also been linked to triglycerides and high-density lipoprotein levels in the circulation. As recent development in metabolomics allows to classify the lipoprotein particles into more details, we aim to examine the impact of gut microbiota on circulating metabolites measured by Nuclear Magnetic Resonance (^{1}H-NMR) technology in 2,309 individuals from the Rotterdam Study and LifeLines DEEP cohort in whom gut microbiota was profiled using 16S rRNA gene sequencing. The relationship between gut microbiota and metabolites was assessed by linear regression analysis while adjusting for age, gender, body-mass index, technical covariates and medication use. Our analysis revealed association of 32 microbial families and genera with very-low-density and high-density lipoprotein subfractions, serum lipid measures, glycolysis-related metabolites, amino acids, and acute phase reaction markers. These observations provide novel insights into the role of microbiota in host metabolism and support the potential of gut microbiota as a target for therapeutic and preventive interventions.

INTRODUCTION

There is increasing interest in the role of the gut microbiota in the major diseases affecting the human population. For a large part, these association can be attributed to metabolic and immune signals of the microbiota that enter the circulation.[1] The gut microbiota has been implicated in obesity and diabetes,[2] but recently Zhernakova *et al.* have shown that the microbiota is also a major driver of circulating lipid levels, including triglycerides and high-density lipoproteins (HDL).[3] The association with low-density lipoprotein (LDL) cholesterol levels, the major target for treatment of dyslipidemia, or total cholesterol was weaker.[3,4] Recent development in metabolomics allows subclassifying the lipoprotein classes into more detail based on their particle size, composition, and concentration. Various studies further linked the gut microbiota to various amino acids, which have been implicated in diabetes and cardiovascular disease.[5-9]

To provide novel insights into the relation of gut microbiota and circulating metabolites, we have performed an in-depth study of the metabolome characterized by nuclear magnetic resonance (^{1}H-NMR) technology and the microbiota. To obtain sufficient power, we combined the data of two large population-based prospective studies, which have a rich amount of data on risk factors and treatment of disease.

METHODS

Study population

Our study population included participants from Rotterdam Study and LifeLines-DEEP cohort.

The Rotterdam Study is a prospective population-based cohort study that includes participants from the well-defined district of Rotterdam.[10] The initial cohort was defined among 7,983 persons, aged 55 years or older in 1990 (RS-I).[10] The cohort was further extended in 2000/2001 by additional 3,011 individuals, aged 55 years and older (RS-II), and in 2006/2008 by adding 3,932 individuals, aged 45 years and older (RS-III).[10] All participants provided written informed consent. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, approved the study.

The LifeLines-DEEP cohort is a sub-cohort of LifeLines study, a prospective population-based cohort study in the north of the Netherlands.[11] The LifeLines cohort was established in 2006 among participants aged 20-50 years.[12] After completion of inclusion in 2013, the cohort includes 165,000 participants.[11] A subset of approximately 1,500 Life-

Lines participants participated in Lifelines-DEEP.[12] The LifeLines-DEEP study is approved by the Ethical Committee of the University Medical Center Groningen.[12] All participants provided written informed consent.

Metabolite profiling

Quantification of small compounds in fasting plasma samples was performed using ^{1}H-NMR technology in both participating studies.[13-15] Simultaneous quantification of a wide range of metabolites, including amino acids, glycolysis-related metabolites, ketone bodies, fatty acids, routine lipids and lipoprotein subclasses was done using the Nightingale Health metabolomics platform (Helsinki, Finland). The detailed description of the method can be found elsewhere.[13,16] In total there were 145 non-derived metabolite measures quantified in absolute concentration units across the participating studies (**Supplementary Table 1**).

Gut microbiota profiling

In order to study gut microbiota, fecal samples were collected from participants of Rotterdam Study and LifeLines-DEEP study. 16S rRNA gene sequencing of the V4 variable region was performed using the Illumina MiSeq platform.[12] A closed reference Operational Taxonomic Unit (OTU) mapped to a Silva (128) database as implemented by RDP classifier (2.12) was used to infer taxonomy.[12] Detail information regarding the gut microbiota profiling is described elsewhere.[12] Absolute values of taxonomy abundance were used. Furthermore, the microbial Shannon diversity index was calculated. Gut microbiota composition dataset included 1,427 participants from the RS-III cohort that participated in the second examination round at the study center. Metabolite measurements were available for 1,390 RS-III participants. In the LifeLines-DEEP study, gut microbiota composition dataset included 1,248 participants and metabolite measurements were available for 915 participants.[12]

Statistical analysis

Prior to the analysis, metabolites were natural logarithmic transformed to obtain approximately normal distribution. To deal with metabolite concentration of zero, half of the minimum detectable value of the metabolite was added to metabolites before the transformation. The metabolite measures were scaled to standard deviation units (SD). Similarly, to obtain approximately normal distribution of microbial taxa, we first added 1 to the abundance values and subsequently performed natural logarithmic transformation.

The relationship between metabolites and microbial taxa was assessed by linear regression analysis while adjusting for age, gender, body-mass index (BMI), technical covariates

including time in mail and storage time, and medication use including lipid-lowering medication, protein-pump inhibitors, and metformin. Furthermore, the analyses were adjusted for smoking and alcohol consumption. Participants using antibiotics were excluded from the analysis. The summary statistics of participating studies were combined using inverse variance-weighted fixed-effect meta-analysis in R (https://www.r-project.org/). In total, 145 overlapping metabolite measures and 455 overlapping microbial taxa were tested for association. As measurements in both metabolomics and gut microbiota datasets are highly correlated, we used a method of Li and Ji to calculate a number of independent tests.[17] There were 37 independent tests among the metabolite measures and 152 independent tests among microbial taxa. The significance threshold was set at $0.05/(37 \times 152) = 8.89 \times 10^{-6}$.

The relationship between metabolites and microbial diversity was also assessed by linear regression analysis while adjusting for age, gender, BMI, technical covariates and medication use (lipid-lowering medication, protein-pump inhibitors, and metformin) in each of the participating studies and summary statistics results were combined using inverse variance-weighted fixed-effect meta-analysis.

RESULTS

Participants from Rotterdam Study (n = 1,390, mean age 56.9±5.9, 57.5% women) were older compared to the participants from LifeLines-DEEP study (n = 915, mean age 44±13.9, 58.7% women), while gender distribution in the two cohorts was comparable.

The results of association analysis between circulating metabolites and composition of gut microbiota are shown in **Supplementary Table 2.** Multiple significant associations were detected for very low-density lipoprotein (VLDL) particles of various sizes (extra small, small, medium, large, very large, extremely large) and HDL particles (small, medium, large, very large) when adjusting for age, gender, BMI, medication use, technical covariates, and multiple testing (**Figure 1A**). When adjusting for smoking and alcohol intake in addition, similar association pattern was observed (**Figure 1B**). Family *Christensenellaceae* and genera *Christensenellaceae R7 group*, *Ruminococcaceae UCG-005,* and *Eubacterium xylanophilum group* were found to be generically associated with VLDL particles of various sizes, small HDL particles and triglycerides in medium HDL. Of note is that the association pattern of very large and large HDL particles including concentration of particles and its total lipids, cholesterol, free cholesterol, cholesterol esters was opposite compared to the association pattern of small and medium HDL (**Figure 1**). Monounsaturated fatty acids (MUFA), serum triglycerides (TG), saturated fatty acids

(SFA), and total fatty acids (TotFA) followed the same direction of association of VLDL (**Figure 1**). Focusing further on the lipoprotein particles, we found that genus *Clostridium sensu stricto 1*, family *Clostridiaceae1*, and one unknown family and genus followed a similar pattern as described above, i.e., being inversely associated to VLDL particles or various size, small HDL subfractions, and triglycerides in medium HDL and positively associated to very large and large HDL particles (**Figure 1B)**. Genera *Ruminococcaceae UCG-003*, *Ruminococcaceae UCG-002,* and *Ruminococcaceae UCG-010, Marvinbryantia* and *Lachnospiraceae FCS020 group* were again inversely associated with VLDL particles of various size and small HDL but the positive association to very large and large HDL was not significant when adjusting for multiple testing (**Figure 1B**). In addition to these generic effect, there are more targeted associations, for instance of family *Lachnospiraceae* and genus *Blautia* with small HDL particles and genus *Ruminococcus gnavus group*

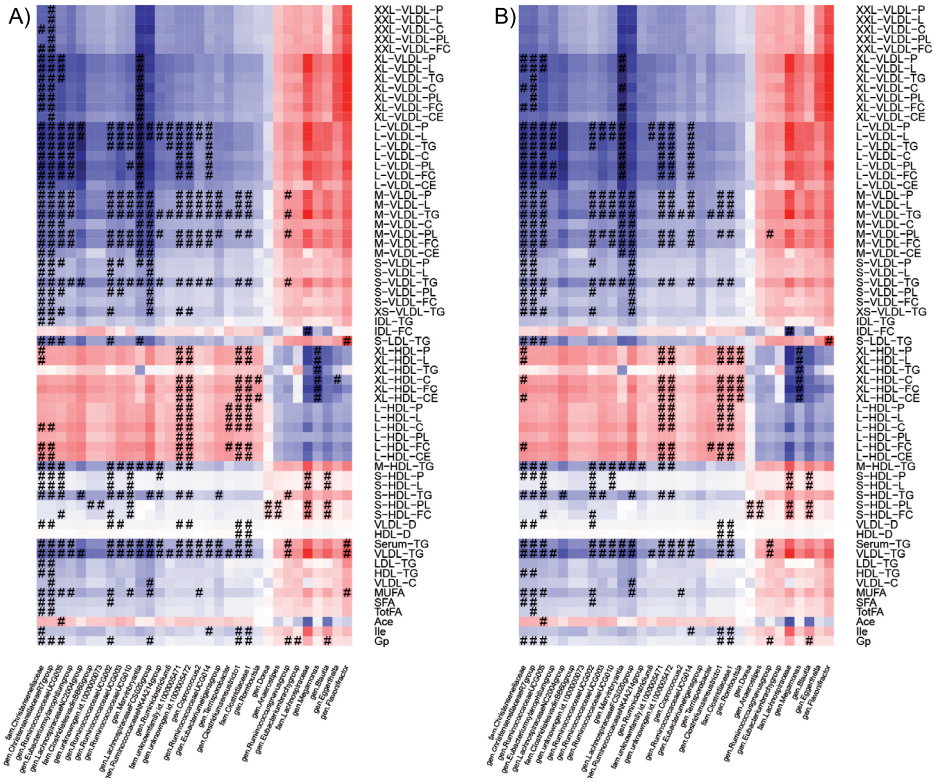

Figure 1. A) Results of association analysis between metabolites and microbial genera and families. The colors represent effect estimates of the metabolites and microbial taxa after adjustment for age, gender, body-mass index, technical covariates and medication use. Blue color stands for inverse association. Red color denotes positive associations. Symbols on the plot represent the level of significance with hash denoting Bonferroni significant associations. B) Association between metabolites and microbial genera and families after additional adjustment for smoking and alcohol consumption.

to phospholipids in medium VLDL, triglycerides in VLDL and serum triglycerides (**Figure 1B**). Family *Clostridiaceae1* and genus *Clostridium sensu stricto 1* were associated with both the HDL diameter and VLDL diameter (**Figure 1B**). The VLDL diameter was further associated with family *Christensenellaceae* and genera *Christensenellaceae R7 group* and *Ruminococcaceae UCG-002*. There is further a specific association of free cholesterol in IDL with family *Lachnospiraceae* and triglycerides in small LDL with genus *Flavonifractor*.

Beyond the lipoprotein fractions, we found three other metabolites, including the ketone body acetate, amino acid isoleucine, and acute phase reaction marker glycoprotein acetyl (mainly alpha 1), to be significantly associated with the microbiota when adjusting for multiple testing and age, sex, BMI, technical covariates, medication, smoking, and alcohol consumption. Genus *Ruminococcaceae UCG-005* was associated to acetate levels, family *Clostridiaceae1* and genera *Clostridium sensu stricto 1* and *Ruminococcaceae UCG-014* with isoleucine and genera *Clostridium sensu stricto1*, *Christensenellaceae R7 group, Ruminococcaceae UCG-005, Ruminococcus gnavus group*, *Blautia* and families *Clostridiaceae1* and *Christensenellaceae*, all associated to glycoprotein levels.

We next determined whether microbial diversity of gut microbiota was associated with lipoprotein particles or other metabolites (**Figure 2**). When adjusting for multiple testing and age, sex, BMI, technical covariates, and medication use, the pattern emerging is that higher microbiome diversity is significantly associated with lower levels of VLDL particles (small, large, medium, very large, extra-large), TotFA, MUFA, and SFA and increased levels of large and extra-large HDL particles and an increased diameter of HDL (**Figure 2**). As to the other metabolites, higher microbiome diversity is significantly associated with lower levels of glycoprotein acetyl, alanine, isoleucine, and lactate (**Figure 2)**.

DISCUSSION

We have examined the impact of gut microbiota on host circulating metabolites in 2,300 individuals from Rotterdam Study and LifeLines-DEEP cohort using ^{1}H-NMR technology. We identified associations between the gut microbiota composition and various metabolites including specific VLDL and HDL lipoprotein subfractions, serum lipid measures including triglycerides and fatty acids, glycolysis-related metabolite lactate, ketone body acetate, amino acids including alanine and isoleucine, and acute phase reaction marker including the glycoprotein acetyls independent on age, gender, BMI, and medication use. No associations were found to LDL subfractions and glucose levels measured by ^{1}H-NMR.

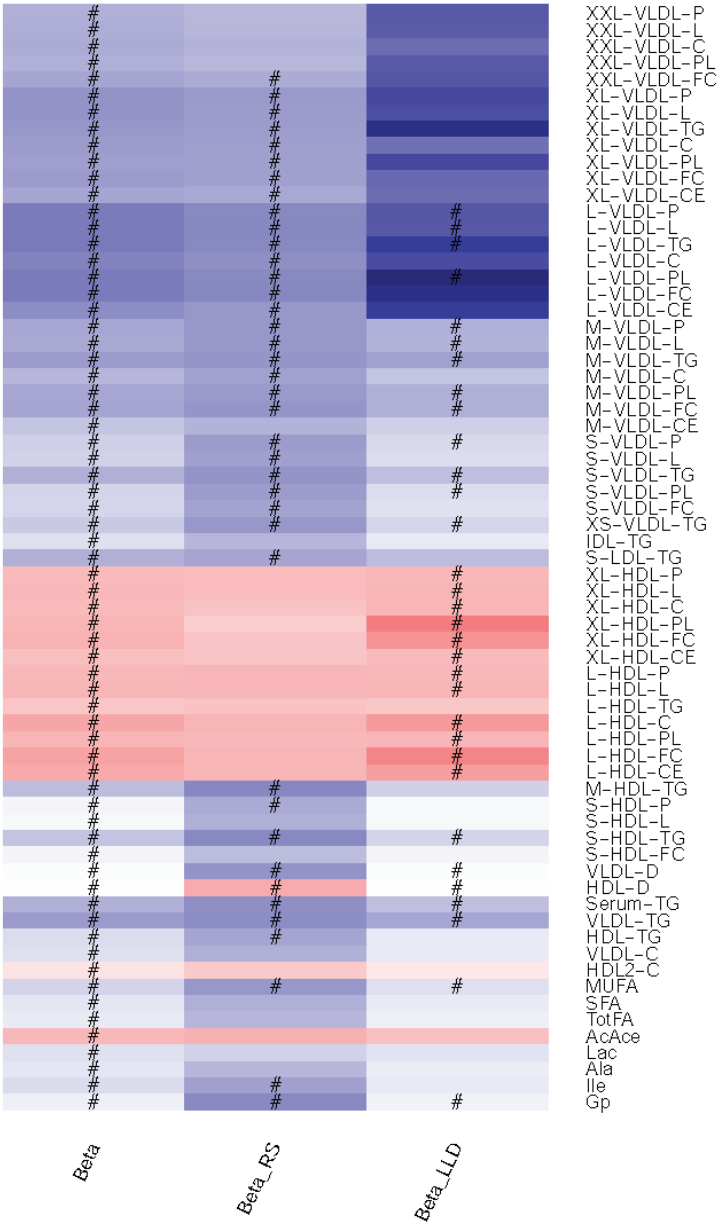

Figure 2. Results of association analysis between metabolites and alpha diversity. The colors represent effect estimates of the metabolites with alpha diversity. Effect estimates from meta-analysis (Beta), and in each of the participating studies are shown (effect estimate in Rotterdam Study - Beta_RS, effect estimate in LifeLines DEEP - Beta_LLD). Blue color stands for inverse association. Red color denotes positive associations. Symbols on the plot represent level of significance with hash denoting Bonferroni significant associations.

Our results based on two large population-based studies identified novel associations between the gut microbiota composition and various lipoprotein particles. We observed inverse association of family *Christensenellaceae* with VLDL particles of various sizes, small HDL particles, and triglycerides in medium HDL (**Figure 1B**). The family *Christensenellaceae* was previously linked to BMI and was associated with the reduced weight gain as reported in the mice study in which germfree mice were inoculated with lean and obese human fecal samples.[18] Furthermore, the family *Christensenellaceae* was reported to be the most heritable microbial taxon in the study by Goodrich *et al.* independently of the effect of BMI.[18]

Interestingly, the gut microbiota composition showed association with VLDL and HDL particles of various sizes, however weak association has been found for LDL and IDL particles suggesting that gut microbiota affects distinct classes of lipoproteins.[19] While VLDL particles of various sizes showed the same pattern of association, differences were noticed between large, medium, and small HDL particles suggesting that they are heterogeneous structures.[20] Small HDL particles are dense, protein-rich, and lipid-poor, whereas large HDL particles are large, lipid-rich particles.[21,22] Despite the fact that HDL is consistently associated with a reduced risk of cardiovascular disease, the past decade has seen major controversies on the clinical relevance of HDL interventions. Most trials aiming to increase HDL levels in the aggregate have been unsuccessful and were even stopped because of adverse effects.[23-25] The heterogeneity of HDL classes has been long recognized but can now be assessed on a large scale. This compositional heterogeneity of HDL results in functional heterogeneity such that small and large HDL particles are negatively correlated and display inverse relationship with various diseases including cardiovascular disease, as reported previously.[20,21] As observed in our study the small HDL particles were driven by genus *Blautia* and family *Lachnospiraceae* and were associated with lower diversity. Indeed the higher levels of small lipoprotein particle concentration have previously been associated with increased risk of stroke as reported in a recently published study of Holmes *et al.*, while the large and extra-large HDL particles that were driven by family *Clostridiacae1,* genus *Clostridium sensu stricto 1* and unknown family and genus and were associated with decreased risk of cardiovascular disease and stroke.[6] Interestingly, family *Clostridiacae1* was previously inversely correlated with BMI, serum triglycerides and is known to be involved in bile acid metabolism.[4,26]

Furthermore, we confirmed association of genus *Ruminococcus gnavus group* and serum triglycerides level,[27] and additionally reported association with triglycerides in VLDL particles and phospholipids in medium VLDL. *Ruminococcus gnavus group* was previously associated with low gut microbial richness[28] and its abundance was higher in patients with atherosclerotic cardiovascular disease.[29]

In addition to circulating lipids and lipoprotein particles, an association was found between gut microbiota and ketone bodies including acetate, amino acids including isoleucine, and acute phase reaction marker including glycoprotein acetyls mainly alpha 1. Circulating levels of acetate were specifically associated with genus *Ruminococcaceae UCG-005*. Acetate is the most common short-chain fatty acid (SCFA) formed by bacterial species in the colon.[30] SCFA can serve as an energy source, predominately via metabolism in liver.[31,32] Previous studies suggested that acetate mediates a microbiota-brain axis and promotes metabolic syndrome.[33] Circulating levels of isoleucine, an essential branched-chain amino acid, were inversely associated with family *Clostridiaceae1* and genera *Clostridium sensu stricto 1* and *Ruminococcaceae UCG-014* in our sample. Recent studies reported association of circulating levels of isoleucine with diabetes and cardiovascular disease.[7,34] Furthermore, isoleucine was reported to be negatively associated with *Christensenellaceae* and positively with *Blautia*.[35] Even though we observed the same pattern of association between isoleucine and these taxa, the associations did not reach the significance threshold. Also recently, a study focusing on relation of fecal metabolites using mass spectroscopy (Metabolon) and the gut microbiota was published.[5] Even though the overlap of measured metabolites is limited, amino acids are measured on both platforms. Other amino acids showed a strong association with the gut microbiota but not isoleucine.[5] However, the concentration of metabolite levels in feces and blood may differ. This an important field of future research. Lastly, glycoprotein acetyls, a composite marker that integrates protein levels and glycosylation states of the most abundant acute phase proteins in circulation,[36,37] was positively associated with genus *Blautia* and *Ruminococcus gnavus group*. Genus *Blautia* is one of the microbial taxa with substantial heritability in twin study,[18] and showed strong association with the host genetic determinants which has been associated with BMI and obesity.[38] Glycoprotein acetyls are associated with other common markers of inflammation.[36,37] Circulating level of glycoprotein acetyls have been implicated in inflammatory diseases and cancer, and have been associated with mortality and cardiovascular disease.[6,7,39,40]

The strengths of our study are large sample size, population-based study design, extensive phenotyping of study participants, and harmonized analysis in participating studies while correcting for factors such as use of medication and BMI. Merging the data of two large population-based studies allowed us to internally validate the findings. However, our study has also limitations. When exploring circulating molecules, we focused on metabolites measured by Nightingale platform which covers a wide range of circulating compounds.[14] However, these compounds represent a limited proportion of circulating metabolites, therefore, future studies should focus on metabolites detected by other more detailed techniques.[41] Further, the gut microbial composition was determined from fecal samples. As gut microbial composition varies throughout the gut with

respect to the anatomic location along the gut and at the given site, more complete picture of the gut microbiota could be obtained by getting samples from different locations along the intestines in the future.[19,42] Furthermore, when exploring gut microbiota, we focused on 16S rRNA sequencing. Even though broad shifts in community diversity could be captured by 16S rRNA, metagenomics approaches provide better resolution and sensitivity.[43] With the decreasing costs of metagenome sequencing, our knowledge can be extended in the future. Finally, although our analyses were adjusted for various known confounders, residual confounding remains possible.

To conclude, we found association between gut microbiota composition and various circulating metabolites including lipoprotein subfractions, serum lipid measures, glycolysis-related metabolites, ketone bodies, amino acids, and acute phase reaction markers. Association between gut microbiota and specific lipoprotein subfractions of VLDL and HDL particles provides novel insights into the role of microbiota in influencing host lipid levels. These observations support the potential of gut microbiota as a target for therapeutic and preventive interventions.

REFERENCES

1. Holmes, E., Li, J.V., Marchesi, J.R. & Nicholson, J.K. Gut microbiota composition and activity in relation to host metabolic phenotype and disease risk. *Cell Metab* **16**, 559-64 (2012).
2. Komaroff, A.L. The Microbiome and Risk for Obesity and Diabetes. *JAMA* **317**, 355-356 (2017).
3. Zhernakova, A. *et al.* Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. *Science* **352**, 565-9 (2016).
4. Fu, J. *et al.* The Gut Microbiome Contributes to a Substantial Proportion of the Variation in Blood Lipids. *Circ Res* **117**, 817-24 (2015).
5. Zierer, J. *et al.* The fecal metabolome as a functional readout of the gut microbiome. *Nature Genetics* **50**, 790-+ (2018).
6. Holmes, M.V. *et al.* Lipids, Lipoproteins, and Metabolites and Risk of Myocardial Infarction and Stroke. *J Am Coll Cardiol* **71**, 620-632 (2018).
7. Wurtz, P. *et al.* Metabolite profiling and cardiovascular event risk: a prospective study of 3 population-based cohorts. *Circulation* **131**, 774-85 (2015).
8. Nakamura, H. *et al.* Plasma amino acid profiles are associated with insulin, C-peptide and adiponectin levels in type 2 diabetic patients. *Nutrition & Diabetes* **4** (2014).
9. Magnusson, M. *et al.* A diabetes-predictive amino acid score and future cardiovascular disease. *European Heart Journal* **34**, 1982-1989 (2013).
10. Ikram, M.A. *et al.* The Rotterdam Study: 2018 update on objectives, design and main results. *Eur J Epidemiol* **32**, 807-850 (2017).
11. Scholtens, S. *et al.* Cohort Profile: LifeLines, a three-generation cohort study and biobank. *Int J Epidemiol* **44**, 1172-80 (2015).
12. Tigchelaar, E.F. *et al.* Cohort profile: LifeLines DEEP, a prospective, general population cohort study in the northern Netherlands: study design and baseline characteristics. *BMJ Open* **5**, e006772 (2015).
13. Soininen, P. *et al.* High-throughput serum NMR metabonomics for cost-effective holistic studies on systemic metabolism. *Analyst* **134**, 1781-5 (2009).
14. van der Lee, S.J. *et al.* Circulating metabolites and general cognitive ability and dementia: Evidence from 11 cohort studies. *Alzheimers Dement* (2018).
15. Vojinovic, D. *et al.* Metabolic profiling of intra- and extracranial carotid artery atherosclerosis. *Atherosclerosis* **272**, 60-65 (2018).
16. Soininen, P., Kangas, A.J., Wurtz, P., Suna, T. & Ala-Korpela, M. Quantitative serum nuclear magnetic resonance metabolomics in cardiovascular epidemiology and genetics. *Circ Cardiovasc Genet* **8**, 192-206 (2015).
17. Li, J. & Ji, L. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. *Heredity (Edinb)* **95**, 221-7 (2005).
18. Goodrich, J.K. *et al.* Human genetics shape the gut microbiome. *Cell* **159**, 789-99 (2014).
19. Ghazalpour, A., Cespedes, I., Bennett, B.J. & Allayee, H. Expanding role of gut microbiota in lipid metabolism. *Curr Opin Lipidol* **27**, 141-7 (2016).
20. Kontush, A. HDL particle number and size as predictors of cardiovascular disease. *Front Pharmacol* **6**, 218 (2015).
21. Camont, L., Chapman, M.J. & Kontush, A. Biological activities of HDL subpopulations and their relevance to cardiovascular disease. *Trends in Molecular Medicine* **17**, 594-603 (2011).
22. Camont, L. *et al.* Small, Dense High-Density Lipoprotein-3 Particles Are Enriched in Negatively Charged Phospholipids Relevance to Cellular Cholesterol Efflux, Antioxidative, Antithrombotic,

Anti-Inflammatory, and Antiapoptotic Functionalities. *Arteriosclerosis Thrombosis and Vascular Biology* **33**, 2715-2723 (2013).
23. Clark, R.W. *et al.* Raising high-density lipoprotein in humans through inhibition of cholesteryl ester transfer protein: An initial multidose study of torcetrapib. *Arteriosclerosis Thrombosis and Vascular Biology* **24**, 490-497 (2004).
24. Keene, D., Price, C., Shun-Shin, M.J. & Francis, D.P. Effect on cardiovascular risk of high density lipoprotein targeted drug treatments niacin, fibrates, and CETP inhibitors: meta-analysis of randomised controlled trials including 117 411 patients. *Bmj-British Medical Journal* **349** (2014).
25. Berenson, A. Pfizer Ends Studies on Drug for Heart Disease. in *The New York Times* (2006).
26. Caspi, R. *et al.* The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Research* **40**, D742-D753 (2012).
27. Lahti, L. *et al.* Associations between the human intestinal microbiota, Lactobacillus rhamnosus GG and serum lipids indicated by integrated analysis of high-throughput profiling data. *Peerj* **1** (2013).
28. Le Chatelier, E. *et al.* Richness of human gut microbiome correlates with metabolic markers. *Nature* **500**, 541-6 (2013).
29. Jie, Z. *et al.* The gut microbiome in atherosclerotic cardiovascular disease. *Nat Commun* **8**, 845 (2017).
30. Louis, P., Scott, K.P., Duncan, S.H. & Flint, H.J. Understanding the effects of diet on bacterial metabolism in the large intestine. *J Appl Microbiol* **102**, 1197-208 (2007).
31. Rios-Covian, D. *et al.* Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. *Frontiers in Microbiology* **7** (2016).
32. Turnbaugh, P.J. *et al.* An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027-1031 (2006).
33. Perry, R.J. *et al.* Acetate mediates a microbiome-brain-beta-cell axis to promote metabolic syndrome. *Nature* **534**, 213-7 (2016).
34. Wang, T.J. *et al.* Metabolite profiles and the risk of developing diabetes. *Nat Med* **17**, 448-53 (2011).
35. Org, E. *et al.* Relationships between gut microbiota, plasma metabolites, and metabolic syndrome traits in the METSIM cohort. *Genome Biology* **18** (2017).
36. Otvos, J.D. *et al.* GlycA: A Composite Nuclear Magnetic Resonance Biomarker of Systemic Inflammation. *Clin Chem* **61**, 714-23 (2015).
37. Connelly, M.A. *et al.* GlycA, a marker of acute phase glycoproteins, and the risk of incident type 2 diabetes mellitus: PREVEND study. *Clin Chim Acta* **452**, 10-7 (2016).
38. Bonder, M.J. *et al.* The effect of host genetics on the gut microbiome. *Nat Genet* **48**, 1407-1412 (2016).
39. Connelly, M.A., Gruppen, E.G., Otvos, J.D. & Dullaart, R.P.F. Inflammatory glycoproteins in cardiometabolic disorders, autoimmune diseases and cancer. *Clinica Chimica Acta* **459**, 177-186 (2016).
40. Lawler, P.R. *et al.* Circulating N-Linked Glycoprotein Acetyls and Longitudinal Mortality Risk. *Circulation Research* **118**, 1106-1115 (2016).
41. Wishart, D.S. *et al.* HMDB 3.0-The Human Metabolome Database in 2013. *Nucleic Acids Research* **41**, D801-D807 (2013).
42. Allayee, H. & Hazen, S.L. Contribution of Gut Bacteria to Lipid Levels Another Metabolic Role for Microbes? *Circulation Research* **117**, 750-754 (2015).
43. Poretsky, R., Rodriguez, R.L., Luo, C., Tsementzi, D. & Konstantinidis, K.T. Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. *PLoS One* **9**, e93827 (2014).

SUPPLEMETARY TABLES

Supplementary Table 1. List of all circulating metabolites tested for association with gut microbiota.

Supplementary Table 2. Results of association analysis between gut microbiota and circulating metabolites.