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

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Gut microbiota has been implicated in major diseases affecting the human population and has also been linked to triglycerides and high-density lipoprotein levels in the circulation. Recent development in metabolomics allows classifying the lipoprotein particles into more details. Here, we examine the impact of gut microbiota on circulating metabolites measured by Nuclear Magnetic Resonance technology in 2309 individuals from the Rotterdam Study and the LifeLines-DEEP cohort. We assess the relationship between gut microbiota and metabolites by linear regression analysis while adjusting for age, sex, body-mass index, technical covariates, medication use, and multiple testing. We report an association of 32 microbial families and genera with very-low-density and high-density subfractions, serum lipid measures, glycolysis-related metabolites, ketone bodies, amino acids, and acute-phase reaction markers. These observations provide insights into the role of microbiota in host metabolism and support the potential of gut microbiota as a target for therapeutic and preventive interventions.
The gut microbiota has been implicated in obesity and diabetes, while recently it was also shown that the microbiota is also a substantial driver of circulating lipid levels, including triglycerides and high-density lipoproteins (HDL)\(^\text{1,2}\). The association with low-density lipoprotein (LDL) cholesterol levels, the major target for treatment of dyslipidemia, or total cholesterol was weaker than the association with triglycerides and HDL\(^\text{3,4}\). 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 diseases\(^\text{5–10}\).

To provide novel insights into the relation of gut microbiota and circulating metabolites, we perform an in-depth study of the metabolome characterized by nuclear magnetic resonance (\(^1\)H-NMR) technology in two large population-based prospective studies which have a rich amount of data on risk factors and disease. We identify 32 microbial families and genera associated with very-low-density and high-density subfractions, serum lipid measures, glycolysis-related metabolites, amino acids, and acute-phase reaction markers.

**Results**

**Characteristics of study population.** Our study is embedded within the Rotterdam Study and LifeLines-DEEP cohort. The Rotterdam Study is a prospective population-based cohort study that started in 1990 among individuals from the well-defined district of Rotterdam\(^\text{11}\), while LifeLines-DEEP cohort is a sub-cohort of LifeLines study, a prospective population-based cohort study in the north of the Netherlands established in 2006\(^\text{12}\). Participants from the Rotterdam Study (\(n=1390\), 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 sex distribution in the two cohorts was comparable.

**Association of gut microbiota with circulating metabolites.** The results of association analysis between circulating metabolites (Supplementary Data 1) and composition of gut microbiota (Supplementary Data 2) are illustrated in Fig. 1. There were 32 microbial families and genera associated with various circulating metabolites after adjusting for age, sex, body mass index (BMI), medication use, including lipid-lowering medication, protein-pump inhibitors, and metformin, technical covariates, and multiple testing (Fig. 1, Source Data). The variables corrected for in the analysis were selected according to previous literature findings\(^\text{3,13}\). The multiple testing correction included Bonferroni correction which was applied for the number of independent tests in both metabolomics and gut microbiota datasets calculated by a method of Li and Ji\(^\text{14}\) (0.05/(37 independent metabolite measures × 274 independent microbial taxa = 4.93 × 10\(^{-6}\)). After additional adjustment for smoking and alcohol intake, similar association pattern was observed (Fig. 1b, Source Data). The direction of effect size across the cohorts was generally concordant (Supplementary Figs. 1−6). After adjustment, the direction of effect size across the cohorts was generally concordant (Supplementary Figs. 1−6). The coherence (Supplementary Figs. 1−6). The coherence (Supplementary Figs. 1−6).

We detected significant associations between 18 microbial families and genera and very-low-density (VLDL) particles of various sizes (extra small, small, medium, large, very large, and extremely large) and 22 microbial families and genera and HDL particles (small, medium, large, and very large) (Source Data). Abnormalities in VLDL particle distribution were reported to be associated with metabolic disease etiology, cardiovascular diseases, and type 2 diabetes\(^\text{15–17}\), while inverse association of very large and large HDL particles and small and medium HDL particles was reported in relation to disease risk\(^\text{7}\). There were 13 microbial families and genera associated with both VLDL and HDL particles subclasses. For example, family Christensenellaceae and genera Christensenella and Ewingella presented with HDL particles of medium and small size and Clostridium sensu stricto was associated with medium and large HDL particles (Source Data), whereas family Clostridiaceae and genus Clostridium sensu stricto was associated with very large and large HDL particles (Fig. 1b).

We also identified an association between family Lachnospiraceae and its genus Blautia with small HDL particles (Fig. 1b). Genera from family Lachnospiraceae, one of the major taxonomic groups of the human gut microbiota, have been associated with the maintenance of gut health, and genus Blautia was associated with obesity and reported to be involved in conversion of primary bile acids into secondary bile acid\(^\text{18–20}\). Correlation coefficient between family Lachnospiraceae and genus Blautia that belongs to this family was 0.82 (Supplementary Data 3). Family Clostridiaceae and genus Clostridium sensu stricto was associated with diameter of both HDL and VLDL particles (Fig. 1b). The VLDL diameter was further associated with family Christensenellaceae and genera Christensenella and Ruminococcaceae UCG-002. Interestingly, diameter of HDL particles was linked to cardiovascular disease\(^\text{7,8}\).

Beyond the lipoprotein fractions, six microbial families and genera were associated with fatty acids, including monounsaturated (MUFA), saturated (SFA), and total fatty acids (TotFA), while eight microbial families and genera were associated with three other metabolites, including the ketone body acetate, amino acid isoleucine, and acute-phase reaction marker glycoprotein acetyl (mainly alpha 1) (Fig. 1b, Source Data). Genus Ruminococcaceae UCG-005 was associated with acetate, while family Clostridiaceae and genus Clostridium sensu stricto was associated with VLDL and HDL particles (Source Data). Positive correlation of small and medium HDL particles with glycoprotein acetyl levels which are known to be associated

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**T**here is increasing interest in the role of the gut microbiota in the major diseases affecting the human population. For a large part, these associations can be attributed to metabolic and immune signals of the microbiota that enter the circulation\(^\text{1}\). The gut microbiota has been implicated in obesity and diabetes\(^\text{9}\), while recently it was also shown that the microbiota is also a substantial driver of circulating lipid levels, including triglycerides and high-density lipoproteins (HDL)\(^\text{1,2}\). The association with low-density lipoprotein (LDL) cholesterol levels, the major target for treatment of dyslipidemia, or total cholesterol was weaker than the association with triglycerides and HDL\(^\text{3,4}\). 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 diseases\(^\text{5–10}\).

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with other common markers of inflammation and have been implicated in inflammatory diseases and cancer\(^7,8,27,28\). Genera from family *Ruminococcaceae* and *Lachnospiraceae* including genus *Blautia* are reported to be involved in conversion of primary bile acids into secondary bile acids and/or production of short-chain fatty acids (SCFAs)\(^{25,29,30}\).

**Association of microbial diversity with circulating metabolites.**

We next determined whether microbial diversity of gut microbiota was associated with lipoprotein particles or other metabolites (Fig. 2). When adjusting for multiple testing and age, sex, BMI, and medication use, the pattern emerging was that higher microbiome diversity was significantly associated with lower levels of VLDL particles (small, large, medium, very large, and extremely large), serum triglycerides, TotFA, MUFA, and SFA, and increased levels of large and extra-large HDL particles and an increased diameter of HDL (Fig. 2). As to the other metabolites, higher microbiome diversity was significantly associated with lower levels of glycoprotein acetyl, alanine, isoleucine, and lactate (Fig. 2). The strongest association was observed with triglycerides in VLDL particles ($p$ value $= 8.48 \times 10^{-10}$).

**Discussion**

We have examined the impact of gut microbiota on host circulating metabolites in 2309 individuals from the 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 metabolites, including lactate; ketone bodies, including acetate; amino acids, including alanine and isoleucine; and acute-phase reaction marker, including the glycoprotein acetyl independent on age, sex, BMI, and medication use. No associations were found to LDL subfractions except for triglycerides in small LDL and glucose levels measured by \(^1\)H-NMR.

Our results based on two large population-based studies identified associations between the gut microbiota composition and various lipoprotein particles. We observed an inverse association of family *Christensenellaceae* with VLDL particles of various sizes, small HDL particles, and triglycerides in medium HDL (Fig. 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
Interestingly, family Christensenellaceae was previously associated with low gut microbial richness and its abundance was higher in patients with atherosclerotic cardiovascular disease. This genus showed positive correlation with family Lachnospiraceae and inverse correlation with genera from family Ruminococcaceae. This is in line with earlier misclassification as a Ruminococcaceae (now a Lachnospiraceae) microbial taxa that showed association with serum triglycerides showed association with other lipoprotein particles as well (Supplementary Fig. 13). However, we also observed microbial taxa that were exclusively associated with VLDL (two microbial genera), HDL (nine microbial genera and families) and LDL particles (one genus) suggesting that lipoprotein particles are important and not just a spillover effect of circulating triglycerides.

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 SCFA formed by bacterial species in the colon and can serve as an energy source, predominately via metabolism in liver. Previous studies suggested that acetate mediates a microbiota-brain axis and promotes metabolic syndrome. Circulating levels of isoleucine, an essential branched-chain amino acid, were inversely associated with three microbial taxa in our sample. Recent studies reported association of circulating levels of isoleucine with diabetes and cardiovascular disease. Furthermore, isoleucine was reported to be negatively associated with Christensenellaceae and microbial diversity and positively with Blautia. Even though we observe 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. 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. However, the concentration of metabolite levels in feces and blood may

Fig. 2 Results of association analysis between metabolites and alpha diversity (n = 2309). Metabolites are displayed on x-axis whereas microbial diversity in the RS, LLD, and combined meta-analysis is shown on y-axis. Lipoprotein classes, include very-low-density lipoprotein particles (VLDL), low-density lipoprotein particles (LDL), and high-density lipoprotein particles (HDL) of very low (XS), low (S), medium (M), large (L), very large (XL), and extremely large (XXL) size. The colors represent effect estimates. 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.
and cancer, and have been associated with mortality and cardi-

Potential mechanisms through which gut microbiota may affect circulating lipid levels may involve bile acids and SCFAs. Some of the microbial taxa identified in our study are involved in conversion of primary to secondary bile acids and production of SCFAs. Previous studies demonstrated that hepatic and/or systemic lipid and glucose metabolism can be modulated by bacte-

The strengths of our study are large sample size, population-based study design, and harmonized analysis in participating studies while correcting for factors such as use of medication and BMI. Combining the data of two large population-based studies allowed us to improve the statistical power of the study and internally cross-check consistency of the findings. However, our study has also limitations. When exploring circulating molecules, we focused on metabolites measured by NMR and provided insights in association with HDL particles. Previous studies reported positive association between microbial diversity and HDL, however, advanced analysis on lipoprotein subfractions revealed that large and extra-large HDL particles were positively associated with microbial diversity while negative association was found for medium and small HDL. Lower microbial diversity has been found in autoimmune diseases, obesity, and cardiometabolic conditions.

The methods of this study are described in Supplementary Data 1. Moreover, additional biological materials and information on health status were collected for these participants. The LifeLines-DEEP study is a sub-cohort of LifeLines study, a prospective population-based cohort study in the north of the Netherlands established. The LifeLines cohort was established in 2006 among participants aged from 6 months to 93 years in order to gain insights into the etiology of healthy aging. At the baseline, the participants filled in extensive questionnaires and visited of one of the LifeLines Research Sites twice for physical examinations. After completion of inclusion in 2013, the cohort includes 165,000 participants. A follow-up questionnaire was sent to each participant every 18 months and follow-up measurements of the health parameters were performed every 5 years. A subset of approximately 1500 LifeLines participants aged 18–81 years was included in the LifeLines-DEEP. These participants were examined more thoroughly, specifically with respect to molecular data. Additional biological materials and information on health status were collected for these participants. All participants provided written informed consent.

Metabolite profiling. Quantification of small compounds in fasting plasma samples was performed using H-NMR technology in both participating studies. Plasma samples of Rotterdam Study participants were collected in EDTA coated tubes during the visit to the research facility in the center of the district, while the plasma samples of LifeLines-DEEP participants were collected during participant’s second visit to the site. Similar 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). Detailed description of the method can be found elsewhere. In total there were 145 nonderided metabolite measures quantified in absolute concentration units across the participating studies.

Gut microbiota profiling. In order to study gut microbiota, fecal samples were collected from participants of Rotterdam Study and LifeLines-DEEP study. Fecal samples of Rotterdam Study participants were collected at home and sent through regular email to the Erasmus MC. Upon arrival at Erasmus MC, samples were stored at −20 °C. After transport on dry ice, all samples were stored at −80 °C. Aliquots were made, and DNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen; cat. #80204). Isolated DNA was sequenced at the Broad Institute, Boston, using Illumina MiSeq sequencing (MiSeq Reagent Kit v3, 2 × 300 bp). Fecal samples of LifeLines-DEEP participants were collected at home and stored immediately at −20 °C. After transport on dry ice, all samples were stored at −80 °C. Aliquots were made, and DNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen; cat. #80204). Isolated DNA was sequenced at the Broad Institute, Boston, using Illumina MiSeq paired-ends sequencing. The reads were processed using QIIME pipeline, and the taxonomy was assigned using the SILVA database (release 128) with RDP classifier (2.12).
genera, using vegan package in R (https://www.r-project.org/). Gut microbiota composition dataset included 1427 participants from the RS-III cohort that participated in the second examination round at the study center. Metabolite measurements were available for 1390 Rotterdam Study (RSIII-2) participants. In the LifeLines-DEEP study, gut microbiota composition dataset, included 1186 participants; from them the metabolite measurements were available for 915 participants.

**Statistical analysis.** Prior to the analysis, all metabolites were natural logarithmic transformed to reduce skewness of traits distributions. To deal with values under detectable limit (reported as zeros) we added half of the minimum detectable value of the corresponding metabolite prior to transformation. The metabolite measures were then centered and scaled to mean of 0 and standard deviation of 1. Similarly, to reduce skewness of the distribution of microbial taxa counts, we first added 1 to all taxonomy counts and then performed natural log transformation. Correlation between microbial taxa was assessed by Spearman correlation.

The relationship between metabolites and microbial taxa was assessed by linear regression analysis while adjusting for age, sex, BMI, technical covariates including time in mail and DNA batch effect (only in Rotterdam Study) and regular medication use (yes/no) including lipid-lowering medication (395 users in Rotterdam Study and 34 in Lifelines-DEEP), proton-pump inhibitors (258 users in Rotterdam Study and 72 in Lifelines-DEEP), and metformin (67 users in Rotterdam Study and 8 in Lifelines-DEEP). The analyses were further adjusted for smoking status defined as never, former or current and daily alcohol consumption (in grams per day). Participants using antibiotics were excluded from the analysis.

The summary statistics of participating studies were combined using inverse variance-weighted fixed-effect meta-analysis using meta package in R (https://cran.r-project.org/web/packages/meta/index.html, https://www.r-project.org/). In total, 145 overlapping metabolite measures and 345 overlapping microbial taxa at taxonomic level of family and genera were tested for association. These microbial taxa were present in at least three samples. As measurements in both metabolomics and gut microbiota datasets are highly correlated, we used a method of Li and Ji14 to calculate a number of independent tests. There were 37 independent tests among the metabolite measures and 274 independent tests among microbial taxa. The significance threshold was thus set at 0.05/ (37 × 274) = 4.93 × 10^{-6}.

The relationship between metabolites and microbial diversity was also assessed by linear regression analysis while adjusting for age, sex, 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 using meta package in R.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All relevant data supporting the key findings of this study are available within the article and its supplementary information files. Data underlying Fig. 1 and Supplementary Figs. 1–13 are provided as Source Data file. Other data are available from the corresponding author upon reasonable requests. Due to ethical and legal restrictions, individual-level data of the Rotterdam Study (RS) cannot be made publicly available. Data are available upon request to the data manager of the Rotterdam Study Frank van Rooyen (fr van rooyen@erasusmc.nl) and subject to local rules and regulations. This includes submitting a proposal to the management team of RS, where upon approval, analysis needs to be done on a local server with protected access, complying with GDPR regulations. The LifeLines-DEEP metagenomics sequencing data are available at the European Genome-phenome Archive under accession EGAS00001001704.

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Author contributions

Conception and design of the study: D.V., D.R., A.K., J.F., R.K., C.M.v.D. Collection of the data: D.R., R.K., A.K., A.Z., M.A.I., A.G.U., N.A., R.K., J.F., C.M.v.D. Drafting of the paper: D.V., C.M.v.D. All authors read, revised, and approved the final draft.

Competing interests

The authors declare no competing interests.

Additional information

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