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Environment-host-microbe interactions shape human metabolism

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American Heart Association

Circulation Research

Chapter 2

Gut microbial associations to plasma metabolites linked to cardiovascular phenotypes and risk

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Circulation Research, 2019, 124(12): 1808-1820. Cover story

Microbial Association of Plasma Metabolome and CVD *p 1808*
Ndufs2 Mediates Hypoxic Pulmonary Vasoconstriction *p 1727*
Propentdyopents and Cerebral Vascular Diameter *p e101*
Microvascular Dysfunction and Mortality in ESRD *p 1796*

Abstract

Altered gut microbial composition has been linked to cardiovascular diseases (CVD), but its functional links to host metabolism and immunity in relation to CVD development remain unclear. To systematically assess functional links between the microbiome and the plasma metabolome, cardiometabolic phenotypes and CVD risk and to identify diet-microbe-metabolism-immune interactions in well-documented cohorts. We assessed metagenomics-based microbial associations between 231 plasma metabolites and microbial species and pathways in the population-based Lifelines-DEEP cohort (n=978) and a clinical obesity cohort (n=297). After correcting for age, gender and BMI, the gut microbiome could explain up to 11.1% and 16.4% of the variation in plasma metabolites in the population-based and obesity cohorts, respectively. Obese-specific microbial associations were found for lipid compositions in the VLDL, IDL and LDL lipoprotein subclasses. Bacterial L-methionine biosynthesis and a *Ruminococcus* species were associated to cardiovascular phenotypes in obese individuals, namely atherosclerosis and liver fat content, respectively. Integration of microbiome-diet-inflammation analysis in relation to metabolic risk score of CVD in the population cohort revealed 48 microbial pathways associated to CVD risk that were largely independent of diet and inflammation. Our data also showed that plasma levels rather than fecal levels of short chain fatty acids were relevant to inflammation and CVD risk. This study presents the largest metagenome-based association study on plasma metabolism and microbiome relevance to diet, inflammation, CVD risk and cardiometabolic phenotypes in both population-based and clinical obesity cohorts. Our findings identified novel bacterial species and pathways that associated to specific lipoprotein subclasses and revealed functional links between the gut microbiome and host health that provide a basis for developing microbiome-targeted therapy for disease prevention and treatment.

Introduction

The human gut is colonized by a highly complex community of microorganisms called the microbiome. The gut microbiome interacts closely with the host and is involved in digestion and degradation of nutrients, maintenance of digestive tract integrity, stimulation of the immune system and modulation of the host metabolism[1,2]. Recent studies indicate a strong link between the gut microbiota and the development of various human diseases, including obesity[3,4], insulin resistance and type 2 diabetes[5,6], as well as gastrointestinal[7,8], autoimmune[9–11], and cardiovascular[12,13] disorders (CVD). Various studies have provided evidence that host-microbe interactions contribute to the etiology of many of these diseases through their impact on metabolism and immunity. Lower bacterial richness (a reduction in the number of different species or bacterial genes) has been associated with an overall increase in adiposity, insulin resistance, dyslipidemia and inflammatory phenotypes3. The distinct gut microbiome profile found in overweight individuals has been shown to have an increased capacity to harvest nutrients from food[14]. Moreover, the gut microbiome is also associated with an individual's cytokine production capacity in response to different pathogens[15]. Despite a large body of evidence from cross-sectional association studies, the underlying mechanisms are largely unknown and several putative mechanisms and functional links have been proposed. For instance, the impact of the gut microbiome on insulin sensitivity and glucose homeostasis may be mediated via microbial biosynthesis of branched-chain amino acids5, short-chain fatty acids (SCFAs) and N-acyl amides[16,17]. The fermentation products of dietary fibers, in particular SCFAs, also have potential roles in the host's innate and adaptive immunity through modulation of cell proliferation and differentiation[18,19], hormone secretion[20], G protein-coupled receptor activation and regulation of colonic Treg cell homeostasis[21–23]. Finally, inhibition of gut-microbiome-induced trimethylamine-N-oxide (TMAO) production can attenuate atherosclerosis development in mice[12,24].

However, our understanding of the functions of gut microbes and of diet-microbe-metabolism-immune interactions in CVD remains limited, leaving a knowledge gap that greatly delays clinical translation. Evaluating the complex interactions between the gut microbiome, host metabolism and immune system-as affected by intrinsic host and external factors (diet, medication)-requires multi-omics, systems-biology-based approaches[25].

Table 1. Summary characteristics of the cohorts used in the study.

Phenotype	LifeLines-DEEP	300-OB
Number of participants	978	297
Cohort design	Population-representative	Age > 55, BMI > 27
Relatedness	Unrelated	279 unrelated; 9 pairs of family members (see description)
Ethnicity	Dutch	Dutch
Males/Females	411/576	166/131
Age	44.5 (13.3)	67.1 (5.4)
BMI	25.1 (4.1)	30.7 (3.5)
Time of sample collection	April-August 2013	2014-2016
Place of sampling	Groningen, the Netherlands	Nijmegen, the Netherlands
Time lag between fecal and blood sampling	<2 weeks	<2 days

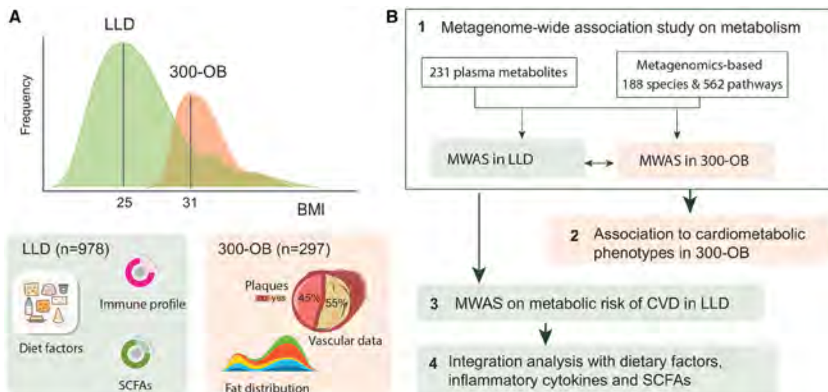


Figure 1. Study overview. A. This study included a population-representative cohort (LLD) with an average BMI of 25 kg/m² and a cohort of overweight and obese individuals (300-OB study) with an average BMI of 31 kg/m². In addition to metagenomics-sequencing data and plasma metabolomics generated for all individuals, unique phenotypic information was collected in each cohort. In the population-representative LLD, detailed lifestyle

and phenotypic information was collected, including 78 dietary factors, 12 inflammatory markers and stool levels of 5 short-chain fatty acids (SCFAs). In the 300-OB study, detailed cardiometabolic phenotyping was conducted, including assessment of carotid artery plaques and the amount of subcutaneous and visceral adipose tissue and liver fat measured using magnetic resonance imaging. B. Analysis scheme. The whole analysis can be divided into four steps: 1) a metagenome-wide association study (MWAS) to explore pair-wise association between 231 metabolic traits and metagenomics-based 188 species and 562 pathways in LLD and 300-OB, respectively, 2) an MWAS to assess the relevance of metabolome-associated microbial features to cardiometabolic phenotypes in the 300-OB cohort, 3) an MWAS to identify microbial factors associated with the metabolic risk score (MRS) of CVD in LLD, which was constructed using 33 metabolic biomarkers, and 4) an integration analysis to assess the relevance of 78 dietary factors, inflammatory cytokines, and SCFAs in microbial association of MRS in LLD.

To do so, we examined both a population-representative and an overweight patient cohort, collectively comprising 1,275 individuals. The obese cohort was deeply phenotyped for cardiometabolic traits, fat distribution and plasma level of TMAO, while the population-based cohort was deeply phenotyped for inflammation, diet and SCFAs (Figure 1A). We first aimed to identify microbial species and metabolic pathways associated with plasma metabolite profiles (Figure 1B). We then identified the relevance of these metabolism-related microbial factors to cardiometabolic phenotypes in the obese patient cohort. Finally, we assessed individual metabolic risk of CVD in the population-based cohort, and identified bacterial pathways associated to the CVD risk score and assessed the diet-microbe-metabolism-immune interactions in CVD risk (Figure 1B).

Results

Gut microbiome associated with plasma metabolomics in healthy and obese individuals

This study included 1,275 individuals from two independent Dutch cohorts: 978 subjects from the population-based cohort LifeLines-DEEP in the Northern part of the Netherlands (the provinces of Groningen, Drenthe and Friesland), with an average age of 44.5 years (18-81 years), an average BMI of 25 (16-45) and 42% male and 297 individuals were from the 300-OB cohort on the territory of Nijmegen province, with an average age of 67 (54-81 years), an average BMI of 30.7 (26.3-45.5) and 55% male (Table 1). Both the LLD and 300OB cohorts followed cohort-specific, disease- and drug-specific exclusion criteria (see Methods). We measured both serum metabolomics and gut metagenomics profiles in both cohorts (Figure 1A).

After quality check and imputation of ~2.2% missing values, a total of 188 microbial species (Online Table II), 562 bacterial metabolic pathways (Online Table III) and 231 metabolic traits (Online Table IV) were subjected to association analysis. After correcting for age, gender and BMI, microbial associations were detected for 210 metabolites in LLD at FDR 0.05 level, with 64 associations to 12 species and 4,135 associations to 308 pathways. To evaluate if imputation of missing values in the metabolite data (see Methods) introduced any systematic bias, we re-conducted association analyses by removing missing values following two approaches and found concordant results (Online Figure II). For instance, after removing missing values, 3,952 significant associations were detected at FDR 0.05 level, and 3,597 of these overlapped with 4,135 associations revealed by missing values imputation. In the 300-OB cohort, microbial associations were detected for 42 metabolites, with one association to species and 105 associations to 19 pathways. All significant associations at FDR<0.05 level can be downloaded from https://github.com/alexa-kur/NMR_microbiome. Most of the microbial factors identified showed very modest effects and could jointly explain, on average, 3.7% of the variation in LLD and 7.7% of the variation in 300-OB (Figure 2). The highest levels of variation explained were 11.1% for glycoprotein N-acetyls (Gp) in LLD and 16.4% for XS_VLDL_C_percent in 300-OB. The smaller number of associations in 300-OB was likely due to its smaller sample size, but our data also show that the microbial factors identified in LLD generally had lower predictive value for metabolic variation in 300-OB (Figure 2). This indicates there are some genuine differences in microbial associations between the population and the obese cohort. We further compared association strength and directions

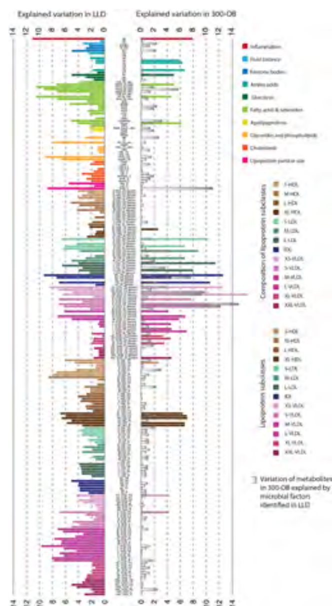
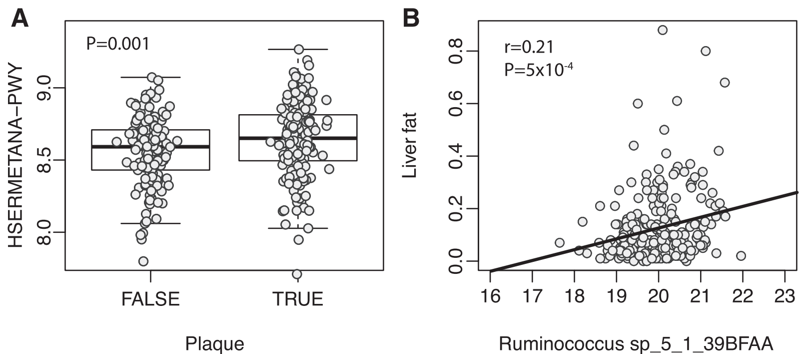


Figure 2. Variation of plasma metabolites explained by the identified gut microbiome. Bar plot shows the variation of 231 metabolites explained by identified microbial factors in LLD (left) and in 300-OB (right). Each bar represents one metabolite. Bar color indicates type of metabolite according to the color key at right. We also assessed to what extent the microbial factors identified in the LLD cohort could explain the metabolic variation in the 300-OB cohort. This data is shown by the gray bars on the right.

Metabolism-related microbial factors linked to clinical phenotypes in obese subjects

To investigate whether the microbial factors found to be associated with plasma metabolites were related to end-point phenotypes, we focused on the top associated species and pathways from both cohorts (Online Table V-VI) and further tested their associations to 26 cardiometabolic phenotypes in the 300-OB cohort (Figure 1B). After correcting for age, sex and BMI, two microbial factors were identified with FDR<0.05 (Online Table VII): higher abundance of bacterial L-methionine biosynthesis (HSERMETANA-PWY) was significantly associated with the presence of plaque (P=0.001) and maximum stenosis (P=0.001), and *Ruminococcus sp_5_1_39BFAA* was positively associated to hepatic fat content (Liver-fat) (r=0.21, P=5.0x10^{-4}) (Figure 3). Previous studies have suggested that gut-microbiome-derived TMAO can increase CVD risk. We observed that plasma level of TMAO was positively associated to visceral fat (r=0.167, P=0.002) but not to atherosclerosis phenotypes and hepatic fat (Online Table VIII), nor was it associated to metabolite-associated species and bacterial pathways (Online Table IX). After correcting for TMAO and its related metabolites (L-carnitine, choline and betaine), associations of L-methionine biosynthesis and *Ruminococcus sp_5_1_39BFAA* to cardiometabolic phenotypes remained similar (Online Table VII).

Figure 3. Associations of gut microbial pathways and species with clinical outcomes in the 300-Obese cohort. A. Abundance of the L-methionine synthesis pathway (HSERMETANA-PWY) is significantly higher in individuals with carotid atherosclerotic plaques. B. *Ruminococcus sp_5_1_39BFAA* is significantly correlated with liver fat content measured by magnetic resonance spectroscopy. Each dot presents one individual and the fitted line is shown in black.



Microbial pathways linked to metabolic risk of CVD in the LLD general population cohort

We further assessed the association of the gut microbiome to individual MRSs of CVD in LLD (Figure 1B). To do so, we constructed CVD MRSs using 33 established CVD metabolic biomarkers that have been associated with future CVD incidents and that were independent of other known risk factors like age, gender, BMI and smoking status[36]. At FDR 0.05 level, 48 associations of MRS with microbial pathways were detected (Figure 4, Online Table X). The top associated pathways were related to GDP-mannose biosynthesis (PWY-7323), which was negatively associated with CVD MRS (beta=-0.122, $P=7.5\times10^{-6}$). Interestingly, a large number of associated pathways were involved in amino acid (AA) metabolism, including metabolism of glutamate-family AAs (L-proline, L-arginine, L-histidine, L-histidine), branched-chain AAs (L-valine), hydrophobic AAs (L-threonine), aromatic AAs (L-phenylalanine and L-tyrosine) and sulfur-containing AAs (L-methionine). Most AA pathways were associated to lower MRS score, except for positive associations detected for L-methionine and L-threonine. Other associated pathways were mostly involved in, among others, fermentation, carbohydrates and sugar derivatives metabolism (Online Table X).

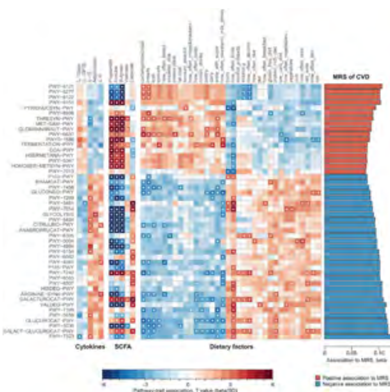


Figure 4. Association of 48 microbial pathways with metabolic risk score of CVD, SCFAs in stool, cytokines and dietary factors. Bar plot on the right shows the associations of 48 microbial metabolic pathways with MRS of CVD. Y-axis refers to the association strength in terms of beta-value. Red bars indicate positive associations and blue bars indicate negative associations. The 3-panel heatmap on the left shows the associations of MRS-associated pathways with plasma levels of cytokines, stool levels of SCFAs and dietary factors, respectively. Blue cells indicate negative associations. Red cells indicate positive associations. White stars indicate significance at FDR<0.05. The color key at the bottom shows association strength and direction in terms of t-value.

We also assessed to what extent specific bacterial taxa can drive MRS-related microbial pathways. For this purpose, we identified the top taxon for each pathway that showed the strongest association between the abundances

of the taxon and the pathway (Online Table X). What we found is that the relative contribution of the top taxa varied greatly: the correlation coefficients between top taxa and pathways ranged from 0.26 to 0.89, with an average value of 0.60. This suggests that some pathways are driven by one dominant bacterial player, while others may be attributed to many different players. For instance, phylum Bacteroidetes-including class Bacteroidia and families Bacteroidaceae and Rikenellaceae-is the major player in 17 of 31 lower-MRS-associated pathways, in particular GDP-mannose biosynthesis and glutamate-family AAs (r>0.8). In contrast, the top players in L-methionine metabolism, the *Ruminococcus* genus and Actinobacteria phylum, only contributed a very modest effect (Online Table X).

The linkage of MRS-related pathways to inflammation and diet

To gain deeper insight into the contribution of host-microbe-diet interactions to metabolism and inflammation, which both underlie susceptibility to CVD, we conducted a systematic integration analysis between the 48 MRS-associated bacterial pathways and the plasma level of 12 cytokines (as a read-out of low-grade inflammation, Online Table XI) and 78 dietary factors (see Methods). After adjustment for age, sex and BMI, 14 associations between 12 pathways and 5 cytokines were significant at FDR 0.05 level (Figure 4, Online Table XI). The associations detected were also largely independent of MRS and remained significant after adjusting for MRS (Online Table XII). Most associations were found to interleukins members, namely 8 associations to IL-10, 3 associations to IL-6, one to IL-12P70 and one to IL-18bp. Elevated levels of these interleukin members have previously been linked to increased risk of CVD[42]. The pathways associated with these interleukins were related to bacterial AA biosynthesis (proline, ornithine, threonine, citrulline, tyrosine, arginine), although IL-10 and IL-18bp were also associated to GDP-mannose metabolism glycolysis and homolactic fermentation. Moreover, the bacterial glycolysis pathway (GLYCOYSIS) was positively associated to plasma level of adiponectin, which is known to be involved in glucose metabolism regulation. This finding shows a possible interaction between the host and the gut microbiome in glycose metabolism.

Diet is known to be an important factor that affects metabolism, CVD risk and the gut microbiome. Among 78 dietary factors, 34 were associated to MRS-associated microbial pathways at FDR 0.05 level, after adjustment for age, sex and BMI and smoking (Online Table XIII). The dietary factors linked with lower-CVD-associated-pathways included higher intake of fruits, vegetables, nuts, fish, tea and red wine and a protein-rich or gluten-free diet. Higher intakes of carbohydrates, fat, total calories, sweetened drinks, bread and dairy products were associated with microbial pathways linked to higher CVD risk (Figure 4).

To further estimate to what extent the microbiome-MRS associations were dependent on effects of diet and inflammation, we included diet and cytokines in the stepwise regression model and selected the best model with the highest AICs (see Methods). This analysis showed that 43 of 48 pathways survived feature selection and were included as predictors, which indicates that they were significantly associated to MRS variation independent of diet and inflammation (Online Table XIV).

Plasma levels rather than stool levels of SCFAs are more relevant to CVD

As fruit and vegetable intake were mostly associated to MRS-associated pathways, we hypothesized that bacterial production of SCFAs by fiber fermentation may underlie the mechanism. We measured stool levels of five different SCFAs (Online Table XI) and found they were associated to most of the MRS-associated microbial pathways (Figure 4). To our surprise, the association directions we observed were in contrast to the previously suggested beneficial effect of SCFAs. Furthermore, no significant associations were found between stool levels of SCFAs and MRS (Online Table XV). Since 95% of the SCFAs produced in the gut are rapidly absorbed by colonocytes, and only 5% are actually secreted into the feces[18], we hypothesized that blood levels of SCFAs might be more relevant to CVD risk. We therefore extracted the plasma level of acetate, the most abundant SCFA, from the NMR-based metabolic profiling. Indeed, the plasma level of acetate was associated to lower MRS (Beta=-0.09, $P=7.2 \times 10^{-4}$) and to 29 MRS-associated pathways at $P<0.05$, all with the expected effect directions (Online Table XVI).

Discussion

This study presents a comprehensive exploration of the relationship of the gut microbiome with plasma metabolites, metabolic risk of CVD and cardiometabolic phenotypes in 1,275 individuals from a population cohort and a cohort of overweight individuals with cardiovascular and metabolic complications. We conducted an integrated analysis of diet, metagenome, plasma metabolites, inflammatory cytokines and stool SCFAs. Previously, relationships of the gut microbiome to plasma metabolites and metabolic traits were investigated using 16S rRNA sequencing[43]. To our knowledge, this study is the largest metagenome-based study to date and provides richer information into the functional link between the gut microbiome and CVD risk. It is also the first study to simultaneously address microbial association with cardiometabolic phenotypes in obesity and with metabolic risk for future development of CVD in a population-based non-patient cohort. Due to

the scope of the NMR-based metabolic platform, this study primarily focused on lipid-related traits, including various lipoprotein subclasses and fatty acids. Microbial associations to a broader spectrum of metabolism therefore still need to be identified.

While our group had previously established the association of the gut microbiome with routine blood lipid level, our current data show that it is not only levels of lipoproteins but also their size and composition that are important and differentially associated with microbiome taxonomic and functional composition. After correcting for age, gender, BMI and smoking, microbial associations were identified for 210 metabolic traits in the LLD population cohort and for 35 metabolic traits in the 300-OB obesity cohort. The microbial factors identified showed modest effects, jointly explaining up to 11.1% of the variation in plasma level of glycoprotein acetyls in LLD and up to 16.4% of variation in XS_VLDL_C_percent in 300-OB. Our data also show obese-specific microbial associations, in particular for lipid compositions in VLDL, IDL and LDL lipoprotein subclasses. Furthermore, our data identified that bacterial L-methionine biosynthesis and the species *Ruminococcus_5_1_39BFAA* were associated to atherosclerosis and liver fat content, respectively, in our obese cohort, and that 48 bacterial pathways were associated to metabolic risk score of CVD in our population cohort. Finally, we integrated the microbiome and metabolomics with diet, SCFAs and low-grade inflammation, with most associations being detected to fruit and vegetable intake, plasma levels of adiponectin and several interleukin family members (IL-10, IL-6, IL-2P70 and IL-18bp). We also observed that the microbiome-MRS associations identified were largely independent of diet and cytokines. However, the current dietary information was obtained from food questionnaires, and the inaccuracy of self-reported data can attenuate the power in dietary analysis. Our data further shows that plasma level of SCFAs is more relevant to CVD risk than stool SCFA levels. We did not confirm the previously observed association of TMAO to CVD in 300OB cohort. Similar negative associations were reported in a young adults study (CARDIA cohort)[44]. In contrast, Andrianariosa et al reported the significant association between cIMT and TMAO in a Germany cohort[45]. The substantial loss of TMAO-cIMT association was observed if adjusted for age. Moreover, our study revealed association of plasma TMAO level with visceral fat, which supported previous fidning[46]. Notable, when comparing microbial associations in different human cohorts, it is important to keep in mind those differences in diet, genetic background and environmental exposures can affect the gut microbiome, TMAO production and CVD risk, thereby resulting in controversial findings. For instance, 300OB cohort contained only elderly, obese Dutch individuals. We found that plasma level of TMAO, at average 5.18 umol/l, was higher than those reported in other cohorts[44].

Among the bacterial associations we identified, the bacterial pathways of

L-methionine biosynthesis showed consistent links with plasma metabolites, MRS of CVD and atherosclerotic plaques, and these pathways were driven by lower fruit intake. These observations are consistent with some previous findings. For instance, supplementation of the glutamine-family amino acids is predicted to have a beneficial effect towards decreasing CVD risk[47,48], while L-methionine and its metabolic products S-adenosyl-L-methionine (SAM-e) and L-homocysteine have been associated with CVD incidence and complication[49]. Methionine has an essential role in a number of cellular processes, including the initiation of protein synthesis, the methylation of DNA and metabolism of xenobiotics. It is also a crucial factor in the biosynthesis of cysteine, phospholipids and polyamine[50]. It is hypothesized that L-methionine induces atherosclerosis by increasing plasma homocysteine levels, as L-methionine can be converted to homocysteine directly or through SAM-e. Hyperhomocysteinemia has also been related to CVD development[51,52]. A recent meta-analysis addressing the effects of low homocysteine by folic acid supplementation found a 10% reduction in risk for stroke and a 4% reduction in risk for CVD[53]. Individuals with homocysteinuria, a genetic disorder characterized by severe hyperhomocysteinemia, suffer from severe atherosclerotic disease at a young age[54]. A number of mechanisms have been proposed to explain the induction of atherosclerosis by elevated homocysteine levels, e.g., through endothelial dysfunction, an increase in proliferation of vascular smooth muscle cells, oxidative damage with deterioration of arterial wall elastic material[55], and a reduction of HDL-cholesterol levels[56]. Furthermore, homocysteinemia has been shown to promote the attraction of monocytes and production of pro-inflammatory cells[57]. Homocysteine also induces macrophage maturation in vessel walls with enhanced vascular inflammation[58]. Recently, Wang et al. revealed a pro-inflammatory status via NLRP3 inflammasome activation in hyperhomocysteinemia induced by a high methionine diet in apoE-deficient mice[59.] Our data thus highlight that bacterial metabolism of L-methionine is also associated to the development of CVD in humans, an observation that was confirmed in both our population-representative cohort and the CVD-enriched cohort of overweight individuals. The association between bacterial metabolism of L-methionine and atherosclerotic plaques was observed to be independent of TMAO metabolism (L-carnitine, choline, betaine and TMAO) and BMI.

Our study also identified several functional links between the gut microbiome and metabolic profile that may predict future CVD events, in particular the association of bacterial pathways related to metabolism of amino acids, carbohydrates and polysaccharides (specifically, GDP-mannose) with MRS. This highlights the potential of microbiome-targeting therapy for CVD prevention and treatment. Some of these pathways are dominantly driven by a specific taxon such as class Bacteroidia. However,

there are several pathways, including bacterial pathways of L-methionine metabolism, which are driven by many different taxa, each with modest or small effect. This suggests potential applications of different microbiome-targeting approaches in controlling a certain taxon or bacterial pathway, e.g., through personalized dietary control combined with prebiotic and 'post-biotic' treatment[60,61]. Interestingly, the top players in MRS-related bacterial metabolism we identified were in line with previous findings. For instance, genus *Faecalibacterium, Subdoligranulum,* species *Methanobrevibacter smithii, Eubacterium eligens* and others were top players in lower-MRS associated pathways. Previous studies have suggested their health-promoting properties: *Faecalibacterium* members have been associated to lower intestinal and adipose-tissue inflammation[62,63], lower levels of members of *Rikenellaceae* family have been associated to liver disease and obesity[64,65], and levels of different members of *Bacteroidaceae* family have been associated with numerous host properties, acting as both mutualistic and pathogenic cohabitants[66].

We acknowledge several limitations in our study. Firstly, both the LLD and 300-OB cohorts comprised of participants of Dutch ethnicity. The reported results are thus likely biased towards a region-specific genetic background and diet, and both are known to affect both metabolism and the gut microbiome. Secondly, this was an association analysis based on a cross-sectional design, which means that the underlying causality and mechanism remain unclear. The Mendelian randomization (MR) approach is considered to be a powerful method to assess causality. However, several recent studies have shown that genetics and microbiome likely exert independent additive effects on CVD-related phenotypes that include, among others, blood lipid levels, CVD-related proteins and BMI[67–69]. This may limit the power of our MR analysis to illustrate underlying causality. Longitudinal studies and further functional studies are thus essential to reveal the underlying mechanism and causality.

Our study presents an integrated analysis of the relationship between the gut microbiome and host metabolomics, cardiometabolic phenotypes and metabolic risk of CVD in humans. Importantly, we investigated microbial association in both a population-based and an obese cohort. We identified numerous associations of functional properties and microbial species in the gut microbiome with plasma metabolic traits, including lipoprotein particle composition, fatty acid saturation and glycoprotein N-acetyls. Some of the microbial factors identified were also linked with clinical outcomes in obese subjects, including hepatic fat content and atherosclerosis. In our population-representative cohort, the combined metabolic risk that represents the probability of having a CVD event in future is associated with numerous microbiome functional parameters such as biosynthesis and degradation of amino acids, fermentation, and carbohydrate and sugar

derivative metabolism.

Altogether, our study highlights microbial associations to current and future clinical outcomes related to CVD. The microbial factors identified and their interactions with diet and inflammation, such as association of bacterial L-methionine biosynthesis with CVD risk and current atherosclerosis, provide a rationale for the future studies, including intervention and prospective experiments. These may contribute to the development of preventive or therapeutic strategies aimed at modulating the microbiome to reduce the burden of cardiovascular events.

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Declaration of interests

None.

Materials and methods

Cohorts

LifeLines-DEEP cohort (LLD): LLD is a sub-cohort of the large, prospective, population-based LifeLines cohort (167,729 subjects) from the north of the Netherlands[26]. A subset of 1,539 participants with deep omics profiling makes up LLD[13,27]. Participants volunteered to participate in LLD from April to August 2013, with blood and fecal samples collected in the same period (within 2 weeks). High quality metagenomic data and detailed dietary (78 dietary factors) information are available for 1,135 LLD participants[13], and 1,046 LLD individuals were profiled for plasma metabolites and inflammation data (see sections below). We further excluded 57 participants who were taking antibiotics or lipid-lowering medication and 11 non-fasting subjects. This left 978 subjects (411 males and 567 females) for further analyses. The morbidity in LLD cohort is similar to general population of the Northern Netherlands. The most prevalent CVD-related diseases in LLD are heart rhythm problems (232 cases), ever reported high blood pressure (169 cases), and diabetes (10 cases).

300-Obesity cohort (300-OB): Between 2014 and 2016, 302 individuals aged 55 to 80 years were enrolled in the 300-OB study at the Radboud University Medical Center (RUMC), Nijmegen, the Netherlands. All had a body mass index (BMI) >27 kg/m2, and the majority (n=227) had participated in the Nijmegen Biomedical Study–Non-Invasive Measurements of Atherosclerosis 1 (NBS-NIMA1) study, a population-based survey of Nijmegen residents[28]. We recruited another 75 participants, acquaintances of previously-included subjects, who fulfilled the inclusion criteria of age >55 years and BMI >27 kg/m2. Most of these new participants were unrelated subjects, with only nine being family members of previously-included subjects; we therefore did not separately evaluate or incorporate the potential clustering of subjects. The most prevalent morbidities include atherosclerotic plaques (55%), hypertension (44%) and type 2 diabetes (12%). Other morbidities include cancer (11%) intermittent claudication (10%), gout (9%), and thyroid condition (4%). Subjects with a recent cardiovascular event (myocardial infarction, transient ischemic attack, stroke <6 months), a history of bariatric surgery or bowel resection, inflammatory bowel disease, renal dysfunction, increased bleeding tendency, use of oral or subcutaneous anti-coagulant therapy, use of thrombocyte aggregation inhibitors other than acetylsalicylic acid and carbasalate calcium, or a contra-indication for magnetic resonance imaging (MRI) were excluded from the study. Participants who used lipid-lowering therapy temporarily discontinued this medication 4 weeks prior to the measurements. All women were postmenopausal and did not use hormonal replacement therapy. All subjects completed questionnaires about lifestyle, medication use and previous diagnosis of hypertension and

diabetes. For all participants, blood samples for metabolomics analysis were collected in the morning following an overnight fast. All underwent comprehensive assessment of cardiovascular profile and fat distribution, as detailed below. Five samples of metagenomic data failed to pass the quality control, leaving 297 individuals for further analyses.

Cardiovascular phenotyping in the 300-OB cohort

Cardiovascular assessment was performed at Radboud UMC (see Online Table I). Vascular studies included the measurement of carotid intima-medial thickness (cIMT), carotid plaque presence and maximum plaque thickness. Measurements were performed after an overnight fast or in the afternoon 6 hours after a standardized breakfast. Participants were asked to abstain from caffeinated products for at least 12 hours and to not smoke for 12 hours before the visit. Testing was performed in a quiet temperature-controlled room with patients in supine position. After a resting period of at least 30 minutes, baseline resting diameter, distensibility and wall thickness of the carotid artery were assessed by a well-trained sonographer using a 7.5-MHz transducer of a Mylab Class C ultrasound device (Esaote Biomedica, Genoa, Italy) connected to a computer with a data acquisition board (Art. lab, Esaote Europe BV, Maastricht, Netherlands). Ultrasound parameters were set to optimize longitudinal B-mode images of the lumen/arterial wall interface. The cIMT and diameter measurements were performed in the proximal 1cm straight portion of the carotid artery in three different angles (90°, 120° and 180°) for 6 heartbeats. Measurements were recorded during the diastolic phase. Measurement of cIMT was performed using an automatic boundary detection system based on RF processing-based measurement (Art. lab)[29]. The primary outcome variable was defined as the mean cIMT of the different angles[30].

Subsequently the presence of plaque and the maximum thickness of plaques in the common carotid, internal carotid, external carotid artery and at the carotid bulbus were measured. Presence of plaque was defined as focal thickening of the wall of at least 1.5x the mean IMT or an IMT >1.5mm, according to the Mannheim intima-media thickness consensus[31]. Furthermore, fat distribution was assessed using MRI, including volumes of visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT), divided into deep and superficial subcutaneous adipose tissue (dSAT and sSAT), respectively. Hepatic fat content was quantified using localized proton magnetic resonance spectroscopy (1H-MRS) (detailed methods see Online Information).

Microbiome data profiling

Metagenomic shotgun sequencing: High quality metagenomics data were already available for the 1,135 LLD participants[13]. For this study, we performed metagenomic sequencing of the 300-OB cohort using a similar protocol and analysis pipeline. In brief, fecal and blood samples were collected within 2 weeks for LLD participants and within 1-2 days for 300-OB participants in order to reduce potential bias introduced by sampling. Further processing of all sample sets was identical to LLD. In brief, DNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany; cat. #80204) with the addition of mechanical lysis. Metagenomic shotgun sequencing was performed using the Illumina HiSeq platform (Illumina, San Diego, California), with an average of 3.0 Gb data (around 32.3 million reads) obtained per sample. Reads were quality-filtered using our in-house pipeline. Sequencing adapters were removed using Trimmomatic (v.0.32)[32]. Human reads were removed by mapping the data to the human reference genome (version NCBI37) with Bowtie2 (v.2.1.0).

Identifying microbial taxa abundances: The profile of microbial composition was determined using MetaPhlan 2.2[33], and it reported 1,772 microbial taxonomies in our data. We normalized the taxonomy data using log- and inverse rank sum transformation and further corrected for age and sex with linear regression in R language (v.3.4.3). We confined the analysis to the 188 common microbial species (Online Table II) present in >10% of samples.

Identifying abundances of bacterial metabolic pathways: The abundance of metabolic pathways was determined using HUMAnN2 (http://huttenhower.sph.harvard.edu/humann2), which maps reads to a customized database of functionally annotated pan-genomes. This analysis mapped reads to UniProt Reference Clusters (UniRef50, http:/www.uniprot.org), then further grouped them to 773 pathways from the MetaCyc metabolic pathway database (www.metacyc.org). Only pathways present in >25% of samples (562 pathways, Online Table III) were used in our downstream analysis. For the non-zero gene counts per MetaCyc pathway, we performed log- and inverse rank sum transformations, followed by correction for the effects of age and sex using linear regression.

Plasma metabolome profiling

For both cohorts, we profiled a wide range of plasma metabolites using nuclear magnetic resonance (NMR) and Nightingale's Biomarker Analysis Platform[34]. This platform provides measures of 231 plasma metabolome traits (Online Table IV), including total lipid concentrations and relative compositions of 14 lipoprotein subclasses, lipoprotein particle sizes,

apolipoproteins, cholesterol, glycerides and phospholipid concentrations, various glycolysis components, fatty acid composition, inflammation, fluid balance, ketone bodies and amino acids. The NMR metabolomics platform has recently been used in several epidemiological, genome-wide association and functional genetic studies[35–37]. To further validate platform precision, we compared NMR measures of several traits with corresponding routine lipid measurements, including concentrations of HDL, LDL, triglycerides and total cholesterol (Online Figure I). For these traits we observed very high correlation rates (R>0.89), in agreement with earlier platform validation results[37]. For LLD, all measures were performed in one batch. For 300OB, metabolomics profiling was performed in two batches, thus we corrected 300OB data for batch effects using linear regression before downstream analysis. There were ~2.2% missing values across all data. Given the high correlation structure of metabolites, missing values could be imputed using the Principal Components Imputation method implemented in the "missMDA" package (v.1.12) for R, using the first 10 principal components. Prior to microbiome-metabolome association analysis, due to non-linear dependency of metabolomic traits to covariates, we used locally weighted scatterplot smoothing (LOESS) to correct for sex-dependent age and BMI effects.

Adipokine and cytokine profiling in LLD

The panel of cytokines for the LLD cohorts (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α) was measured by ProcartaPlexTM multiplex immunoassay (eBioscience, San Diego, California, USA) according to protocols described before[38,39]. Other inflammation markers were measured by using commercially available sandwich ELISA kits (R&D systems, Minneapolis, Minnesota, USA), including leptin, adiponectin, IL-18, IL-18BP, resistin and alpha-1 antitrypsin (AAT).

SCFA profiling in LLD

For LLD, we measured fecal levels of acetate, propionate, butyrate, valerate and caproate by gas chromatography-mass spectrometry following the method of Garciá-Villalba *et al* (2012)[40]. The abundance of acetate in plasma was obtained through plasma metabolome profiling using NMR. All SCFA measurements were corrected for age and gender using LOESS.

TMAO, choline, betaine and citrulline profiling in 300-OB

TMAO, choline, L-carnitine, betaine and γ-butyrobetaine in plasma were

analyzed by ultra-high performance liquid chromatography in combination with isotope dilution tandem mass spectrometry (UPLC-MS/MS). In short, 25µL plasma was pipetted into 96-well plates, 25µL internal standard solution was added (containing TMAO-D9, choline-D9, L-carnitine-D3 and betaine-D11), followed by 300µL 80% acetonitrile (ACN) and 1% formic acid (FA) in Millipore water. Mass spectrometric detection was performed on a XEVO TQ-s system (Waters). Analytes were detected in positive mode and selected reaction monitoring mode. The respective quantifier ion transitions were as follows: m/z 76.15 > 58.3 for TMAO, m/z 104.2>60.3 for choline, m/z 162.2 > 103.25 for L-carnitine, m/z 118.2 > 59.3 for betaine and m/z 146.25>60.3 for γ-butyrobetaine. All analytes were baseline separated from each other.

Statistical analysis

All statistical analyses were performed using R statistical language (v.3.4.3). Microbiome-metabolome association and explained variance: (a) To identify the associations between metabolic and microbial factors, association analyses were performed in both LLD and 300-OB. Microbiome features included both microbial pathways and species. After adjusting for age, sex, BMI and smoking, Spearman correlation of metabolic traits and microbiome features was used to identify associations. The analysis was confined to pair-wise non-zero values. False discovery rate (FDR) was estimated using 100x permutations. We also calculated the permutation-based family-wise error rate (FWER) and report the top association at FWER <0.05 level. (b) To estimate the proportion of variation in plasma metabolism explained by microbial factors, we used a LASSO shrinkage model[41] from R package 'glmnet' (v.2.0.16) that included all identified microbial pathways and species as predictors. The independent and most dominant microbial features were selected automatically, and the variation of each metabolite explained by the selected microbial factors was then estimated by LASSO. Moreover, we used the same LASSO algorithm to estimate the proportion of metabolic variation in 300-OB explained by microbial factors identified in LLD.

Association to cardiometabolic phenotypes: The bacterial pathways and species identified were further tested for association with cardiometabolic phenotypes in 300-OB, including fat distribution and atherosclerosis phenotypes. The analysis was performed using linear regression, with cardiometabolic phenotype as outcome and microbiome feature as predictor, and treating age, sex and BMI as covariates. Quantitative outcomes were adjusted using inverse rank-sum transformation; for binary outcomes, logistic regression was used instead of linear regression. Furthermore, to assess to what extent bacterial-derived TMAO and its relevant metabolites could underlie microbial associations to cardiometabolic phenotypes, we

performed extra analysis with adjustment for the plasma levels of TMAO, choline, betaine and L-carnitine. Significant associations for each phenotypic trait are reported at empirical FDR <0.05 level based on 100x permutations.

Microbiome association to metabolic risk of CVD: To estimate individual metabolic risk for CVD development in the population cohort, we used 33 established metabolic biomarkers for CVD measured using the same NMR platform and associated with future CVD incidents in three perspective cohorts[36]. We first constructed each individual's CVD metabolic risk score (MRS) using a weighted risk model:

$$MRS = \sum_{i=1}^{33} b_i M_i$$

where Mi is the scaled level of the i-th metabolic marker in serum, not adjusted for phenotypes, and bi is a hazard ratio for the corresponding effect of each metabolic marker on the CVD risk as reported by Würtz *et al*[36]. The MRS score showed a normal distribution. We then tested MRS association to microbial pathways and species, adjusting for age, sex, BMI and smoking. The significance was controlled at FDR<0.05 based on 100x permutations. For microbiome pathways, we also examined to what extent these pathways are driven by specific taxa by calculating Spearman correlations between pathways abundance and taxa abundance.

Integration association with dietary factors, inflammatory markers and stool levels of SCFAs: To better understand the functional properties of the MRS-associated bacterial pathways in relation to metabolism, inflammation, diet and SCFAs, we conducted an integration analysis with 12 inflammatory markers, 78 dietary factors and stool levels of 5 SCFAs.

First, we computed pair-wise associations between MRS-associated pathways and each of these factors, using linear regression adjusted for age, sex, BMI and smoking and controlling for FDR 0.05 using 100x permutations per dataset (cytokines, diet, SCFA) separately. All traits were transformed using inverse rank sum transformation prior to analysis.

Second, to elaborate if pathway-cytokine associations were dependent on MRS, we recalculated these associations using linear regression, additionally adjusting for MRS as a covariate.

Finally, to explore if the microbial pathways were associated to variance of MRS independently of inflammatory markers and diet, we performed stepwise model selection for each pathway, with MRS as outcome and pathway, age, sex, BMI, smoking, all pathway-associated cytokines and all pathway-associated diet categories as predictors. This was done using the stepAIC function from R package 'MASS' (v.7.3.50). At each selection step,

predictors were selected by both forward and backward direction using AIC value (Akaike information criterion) as an indicator of goodness-of-fit. The model with the highest AIC was selected as the best model. Pathways that survived in the best-fit model were considered independent predictors.

Data and code availability

All metagenomics and metabolism data have been made publicly available at the European Genomics-phenome Archive (EGA) at accession number EGAS00001001704 for the LifeLines-DEEP cohort and EGAS00001003508 for the 300-Obese cohort. Because of the sensitive nature of clinic data collected for this study, requests to access clinical phenotypic data of the Lifelines-DEEP cohort and 300-Obese cohort may be sent to the LifeLines cohort study at research@lifelines.nl and to the Human Functional Genomics project at martin.jaeger@radboudumc.nl, respectively.

Supplementary information

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