Gut microbial prediction and therapeutic avenues for cardiovascular disease

PROJECT PURPOSE AND BACKGROUND

Cardiovascular diseases (CVDs) are the number one cause of death globally: more people die annually from CVDs than from any other cause. Today, almost 1.8 million individuals in Sweden are living with CVDs, and a large amount of these individuals suffer from coronary heart disease (CHD). Diabetes is a prominent risk factor for CVD; if you have diabetes, you are two to four times more likely to develop cardiovascular disease than people without diabetes. Although modern preventive strategies (e.g., statin treatment) have reduced the burden of CVDs over the past decades, our modern lifestyle with a high intake of refined foods, saturated fats, and fast carbohydrates, maintains a high incidence of CVD. Experimental and epidemiological studies suggest that stressing psychosocial work conditions further aggravate this unhealthy behavior. Our team has, over the past decade, identified the gut microbiota, the collection of microorganisms residing in the gut, as an essential environmental factor strategically located at the interface between diet and metabolism. We have also demonstrated that the gut microbiota is altered in patients with stroke and type 2 diabetes. Importantly, several other factors such as stressing psychosocial work conditions may affect the gut microbiota. This hypothesis is supported by animal work demonstrating that stress exposure alters the microbiota, which in turn increases intestinal permeability, a feature associated with metabolic disease. Here we will use two separate human cohorts, one population-based (SCAPIS) and a risk-enriched cohort (local at Sahlgrenska university hospital/academy) *to evaluate if stressing psychosocial work conditions modulate the gut microbiota and whether such alterations are associated with epicardial fat accumulation, atherosclerosis, stroke, and myocardial infarction*. Pathological enlargement of epicardial fat can induce myocardial inflammation and dysfunction as well as left ventricular hypertrophy and coronary artery disease through paracrine actions. Expertise in determining epicardial fat volume through advanced imaging allows us to address novel questions employing the teams' strengths in microbiota, lipid metabolism, and CVD. Furthermore, using animal models we will determine if the disease-associated microbiota causatively contributes to disease and elucidate the mechanisms.

To address these objectives, we will investigate three aims:

1. Test the hypothesis that stressing psychosocial work conditions contributes to CVD by altering the gut microbiota.
2. Test the hypothesis that individuals with elevated epicardial fat have an altered microbiota
3. Test whether the microbiota can be causally linked to CVD.

Project implementation

Overall remarks: Overall we have managed to address the main aims that we set forth in the application despite delays in recruitment of the study population and slower analyses of imaging data, as well as limited physical interactions in the team due to COVID-related restrictions. Furthermore, the complex nature of the data has required significantly more analyses and interpretation that combined has slowed down our publications. We still have the ambition to submit our main findings to high-ranked journals and have several papers resubmitted/under consideration in top-ranked journals in the field.

Aim 1

Background: Experienced strain induced by psychosocial exposure in the work environment and stressing psychosocial work conditions may alter environmental factors such as dietary habits, exercise, and sedentary behavior that in turn may have a great impact on gut microbiota. However, there is limited knowledge on how work-related stress affects the gut microbiota and if it can be related to CVD in humans. Furthermore, we previously demonstrated that the microbiota is altered in patients that have had stroke [1], and accordingly we here wanted to investigate if a stress-microbiota-CVD axis exists.

Approach/results: To address this aim we used two separate cohorts SCAPIS and a similar cohort collected in Gothenburg [2]. During the first part of the grant, we worked with Mia Söderblom to analyze work-related stress and stratified participants with a complete dataset in the cohort (n=1129) according to their stressing psychosocial work conditions JDC (defined by job control and job demand and divided into 4 classes accordingly, from worst to best: JDC1: control < 20, demand > 12.5. JDC2: control >= 20, demand > 12.5. JDC3: control < 20, demand < 12.5. JDC4: control >= 20, demand < 12.5). Differences between the groups in terms of anthropometric variables (gender, body mass index (BMI), waist-to-hip ratio (whr), age, physical activity (vigorous and moderate (vpa, mpa) and diet (intakes of fibers and alcohol, and anti-inflammatory dietary index (AIDI [3])) were evaluated and a significant difference between the JDC-groups were found for: gender, age, and variables related to physical activity (**Table 1**).

In parallel we extracted, sequenced and processed the metagenome sequences from the IGT_microbiota study (N=1864), but also realized that we needed to update the bioinformatic pipeline to have the most current methodology, which was accomplished last year. Thus, we were in the position to finalize the analyses. In addition to the work-related stress, we also interrogated how factors such as age, gender, BMI, whr, physical activity, and diet significantly may explain gut microbiota variation between individuals in our risk-enriched cohort.

Our results are in line with previous observations in similarly sized European populations [4-6] and show that individual variables have relatively low explanatory effects. We also observe that stressing psychosocial work conditions do not have significant explanatory value for the variation of the gut microbiota in our cohort. A principal coordinate analysis of the Bray-Curtis dissimilarity distances was performed (**Figure 1A**) alongside a permutational-ANOVA test for significance (adonis2), the test did not find the stress variables (JobControl, JobDemand, and JDC) to explain a significant fraction of the variation in the gut microbiota. A multivariate distance-based redundancy analysis (db-RDA) model using forward selection was created to explain the inter-individual variation in the gut microbiota using the variables related to stress, diet, physical activity, diabetes status (NGT, normal glucose tolerance; hrNGT, high risk normal glucose tolerance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; and T2D, type-2 diabetes), and anthropomorphism (**Figure 1B-C**). Variables not selected by the db-RDA model were individually evaluated using the same method and tested for significance (**Figure 1D**). The model showed that intakes of fiber and alcohol as well as AIDI and physical activity added explanatory value beyond the anthropometric variables and also beyond the diagnose of prediabetes and diabetes (indicated as Diabetes status in **Figure 1C**. *Unfortunately, we have to conclude that work-related stress does not appear to be associated with over all altered gut microbiota.*

Table 1: Characteristics of the participants according to their stressing psychosocial work conditions

	JDC1 (276)	JDC2 (273)	JDC3 (297)	JDC4 (283)	Significance
Gender (M/F)	90/186	129/144	132/165	156/127	***
Age	56.55 ± 0.26	57.12 ± 0.25	57.44 ± 0.25	57.71 ± 0.26	*
Findrisc score	12.74 ± 0.26	12.07 ± 0.29	12.25 ± 0.28	11.92 ± 0.27	ns
BMI	28.25 ± 0.27	27.59 ± 0.24	27.47 ± 0.24	27.37 ± 0.24	ns
whr	0.91 ± 0.006	0.92 ± 0.005	0.93 ± 0.005	0.92 ± 0.005	ns
Recgroup (1/2/3/4)	21/95/79/81	14/60/107/92	15/85/106/91	14/71/116/82	*
sed	484.10 ± 6.51	506.37 ± 5.48	478.50 ± 5.18	503.22 ± 5.14	***
lpa	342.75 ± 5.00	311.96 ± 4.40	342.50 ± 4.78	317.42 ± 4.57	***
mpa	49.04 ± 1.45	51.72 ± 1.31	51.87 ± 1.44	51.22 ± 1.35	ns
vpa	3.72 ± 0.42	5.74 ± 0.52	4.59 ± 0.45	5.45 ± 0.53	***
Physical activity	52.76 ± 1.58	57.46 ± 1.51	56.46 ± 1.61	56.67 ± 1.51	*
AIDI	5.92 ± 0.10	6.10 ± 0.10	5.96 ± 0.10	6.07 ± 0.10	ns
Fibrer.(g)	18.54 ± 0.69	20.14 ± 0.66	18.43 ± 0.55	18.90 ± 0.64	ns
Alkohol.(g)	7.05 ± 0.38	7.74 ± 0.39	7.68 ± 0.36	7.84 ± 0.37	ns

Values describe the means for the JDC-groups ± standard errors (number of individuals in each group are also displayed). The Wilcox rank-sum test was used for testing significance for continuous variables and Chi-squared test for categorical variables (gender, Recgroup). Acronyms: sed, time spent sedentary; lpa, light physical activity; mpa, medium physical activity; vpa, vigorous physical activity; physical activity, the sum of mpa and vpa; recgoup, classification based on amount of physical activity.

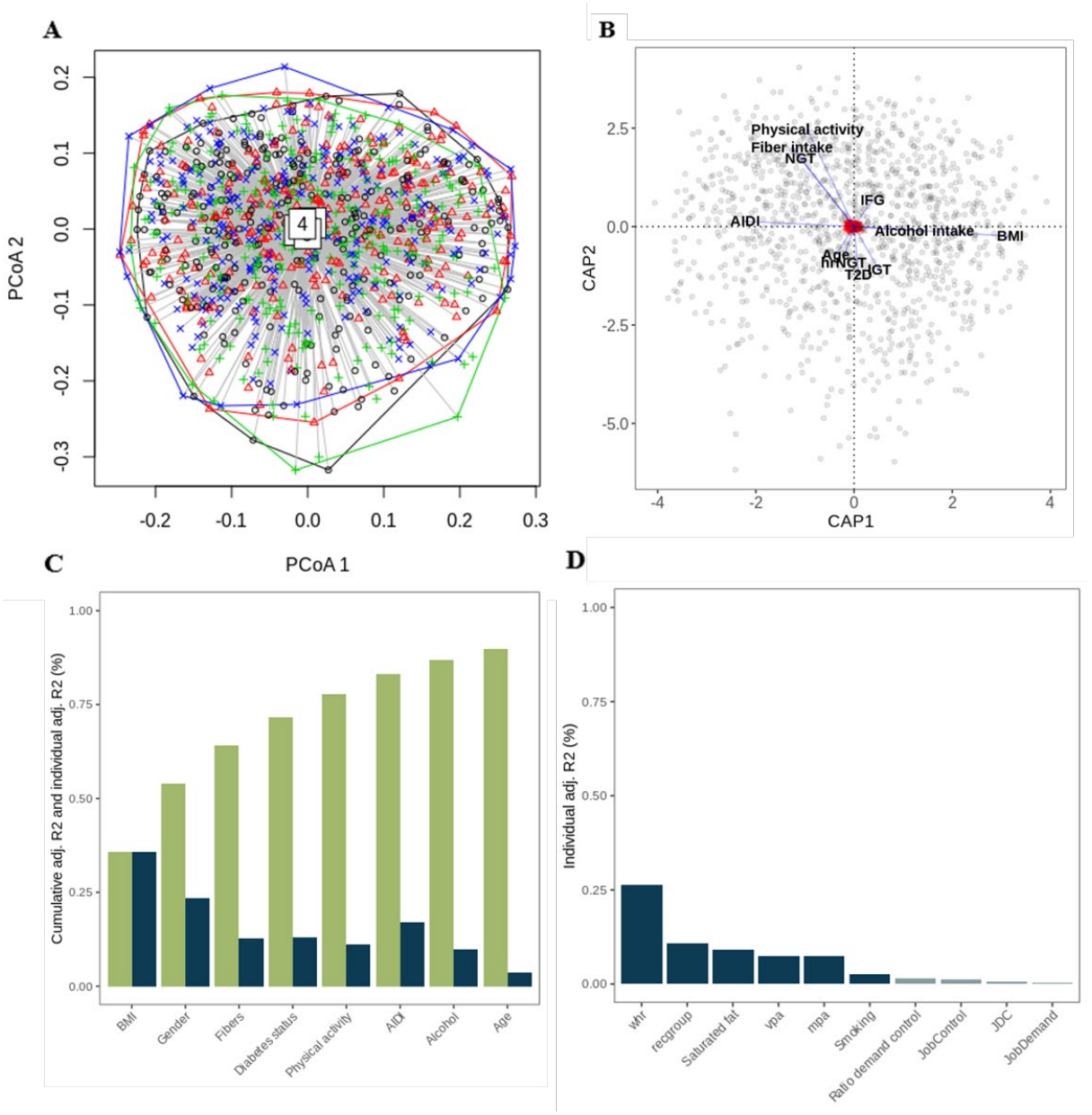

Figure 1: Effect of anthropometric and lifestyle variables on gut microbiota composition in the risk enriched Swedish population. (A) Principle coordinate analysis using Bray-Curtis dissimilarity index with the samples colored and shaped by the JDC-categories. (B) db-RDA showing explanatory variables related to the variation in the gut microbiome. (C) The individual (blue) and cumulative (green) effect sizes of variables selected by the forward-selection db-RDA model displayed as adjusted R2. (D) The individual effect size of variables not selected by the forward-selection db-RDA model; blue being significant variables and light-blur being non-significant.

Together with AFA we then decided to explore how different environmental and physiological feature could affect the microbiota. Considering that the main feature contributing to the microbiota composition was BMI (**Figure 1C**) and the wide impact of BMI has on all host features including its nonredundant impact on the microbiome, we next explored how these spaces connect with each other alone and in combination. For this aim, we leveraged a subgroup of 1408 subjects free of CVD, who had complete clinical, metabolome, and microbiome data

as well as data related to body composition to delve into obesity phenotypes. From 1392 of those participants, levels of 1462 proteins related to inflammation, cardiometabolic disease, neurological and oncological disorders, were measured using the Olink Proteomics platform with unique PEA oligonucleotide probes labeled antibodies. To understand the connections between the microbiome, metabolome, and proteome signatures on the one hand and several host metadata categories on the other, we interrogated the amount of variance explained for each of the available metadata variables. To this aim, ridge regression models with nested 10-fold cross-validation were used to calculate the variance explained for each variable from each feature space (metadata category) using microbial species abundances (CLR transformed), scaled Gut Microbial Modules (GMMs), KEGG modules, metabolome, and proteome data as well as diet information. Feature spaces included anthropometric data, body composition, blood components, diet estimates of food intake, food items and dietary indices, job-related stress, kidney function, medication intake, metabolic parameters, microbiome richness, physical activity, measured proteomics, and cardiovascular risk indices. If variables were to be estimated by a particular model, this model was to exclude the entire feature space containing the variable (e.g., no metabolome data were used to predict single circulating metabolites). Ten R2 values were retained for each covariate and predictor-specific R2 distributions (bacterial species/MAGs, GMMs, KEGG modules, proteome, metabolome, and diet) were plotted for each feature space (**Figure 2A**). We observed that the variance explained was similarly distributed between metabolome and gut bacterial species for variables related to body composition, cardiovascular risk, and medication intake as well as circulating proteins. The microbiome explained higher variance in variables related to diet and work-related stress, while also largely contributing to the variance explained of measured metabolites including xenobiotic metabolites (**Figure 2A,B**).

Looking at all available metadata, we further found that bacterial species explain the highest amounts of variation for 2878 single variables. While GMMs contributed to the variance of more variables (2890), the overall variance explained was much lower, with similar results for KEGGs (median R2 33.6%, 4.2%, 6.5% for bacterial species, GMMs and KEGGs respectively, **Figure 2C**). Interestingly and perhaps counterintuitively, while the highest amounts of single variable variance were estimated for metabolites (including xenobiotic metabolites) and medication intake, GMMs explained the largest amount of variance in gut observed gene richness at 81% compared to bacterial species at 74%, linking bacterial metabolism to overall gene richness, a measure of microbiota health, which is tightly connected to host metabolic health.

Moreover, available species contributed similarly to the variance of diet estimates, circulating metabolites and body composition at around 35%, suggesting that these features are particularly intimately regulated in our cohort (**Figure 2C**). Variables solely explained by single gut features (**Figure 2D**) were mainly quantifications of drug intake, whereas GMMs seem to contribute singly to several measured xenobiotic metabolites from central acting drugs such as 2-propyl-2-pentenoate (2-ene-valproate), paroxetine, fexofenadine, and THC carboxylic acid glucuronide as well as the cardiac drug metabolite warfarin. GMMs were also the only gut feature, which co-varied with intake of drugs for constipation. Considering the close connection between gene richness and stool consistency, our results establish a connection between host factors impacting stool consistency, GMMs distribution and gut microbiome gene richness.

Although work-related stress was not estimated to significantly contribute to overall microbiome composition, bacterial taxa contributed to 45% of variance in the variables from this metadata category hinting at a clear covariance between gut microbiome species and perceived work-related stress. *This was an exciting turn of events which is attributed to that we know could perform more detailed analyses.*

We further investigated the impact of all host variables, including lifestyle, diet, metabolic markers, and markers of body composition and medication intake on overall microbiome composition using dbRDA for the selection of features with a nonredundant contribution to the microbial community. We found that a central adipose tissue distribution had the highest impact on overall microbiome composition (**Figure 2E**). Diet and lifestyle variables such as smoking, fiber, and fruit intake, anti-inflammatory diet, alcohol intake as well as medication intake (PPI treatment and beta-blocker intake) also significantly shape the microbiome, although these factors explain together 4.9% of the variance of overall microbiome composition. Investigating gene richness as a broad summary feature of microbiome structure and health and after correcting for Age, sex, and BMI and multiple testing, we found microbial gene richness to be associated significantly and inversely with proteins related to macrophage differentiation (CPM), to circulating furin endoprotease, which governs phospholipid transfer and levels of plasma lipids and to other phenotypic and metabolic risk factors such as triglyceride-glucose index (TyG), circulating LDL-receptor, and abdominal adipose tissue area. A wide range of variables correlated positively with gut microbiome gene richness, most notably many metabolites. Gene richness also correlated positively with circulating phospholipase A2 (PLA2G10), PON3 (HDL binding and inhibition of LDL), with dietary metabolites such as carotene diol, urolithin B, and the coffee metabolite quinate, as well as with characteristics of adipose tissue such as abdominal vat attenuation and liver attenuation (**Figure 2F**).

Conclusion: Our results corroborate the existence of a wide interconnectedness across omics features with microbial species tracking intimately with the metabolome and show for the first time that both these highly dynamic feature spaces can similarly contribute to the more stable and highly prognostic proteome space.

While work-related stress does not seem to impact microbiome composition, microbiome features contributed to 45% of the variance explained in JobDemand, JobControl, and JDC, suggesting that this connection is a potential niche-to-system effect, whereby the microbiome can impact work-related stress directly or indirectly. We further evidence that the microbiome, metabolome, and proteome are closely linked to markers of central adiposity such as adipose tissue distribution but more importantly with qualitative characteristics of adipose tissue and liver which are reflected in circulating proteins and associated with insulin resistance and cardiovascular risk in our cohort.

Results reported under Aim 1 has been summarized in a manuscript that will be submitted to Nature Metabolism in the next month. wide impact of BMI on all host features including its nonredundant impact on the microbiome, and the divergent associations of omics and lifestyle features with metabolically heterogeneous obesity phenotypes, we next explored how these spaces connect with each other alone and in combination. For this aim, we leveraged a subgroup of 1408 subjects free of CVD, who had complete clinical, metabolome, and microbiome data as well as data related to body composition to delve into obesity phenotypes. From 1392 of those participants, levels of 1462 proteins related to inflammation, cardiometabolic disease, neurological and oncological disorders, were measured using the Olink Proteomics platform with unique PEA oligonucleotide probes labeled antibodies. To understand the connections between the microbiome, metabolome, and proteome signatures on the one hand and several host metadata categories on the other, we interrogated the amount of variance explained for each of the available metadata variables. To this aim, ridge regression models with nested 10-fold cross-validation were used to calculate the variance explained for each variable from each feature space (metadata category) using microbial species abundances (CLR transformed), scaled Gut microbial modules (GMMs), KEGG modules, metabolome, and proteome data as well as diet information. Feature spaces included anthropometric data, body composition, blood components, diet estimates of food intake, food items and dietary indices, job-related stress,

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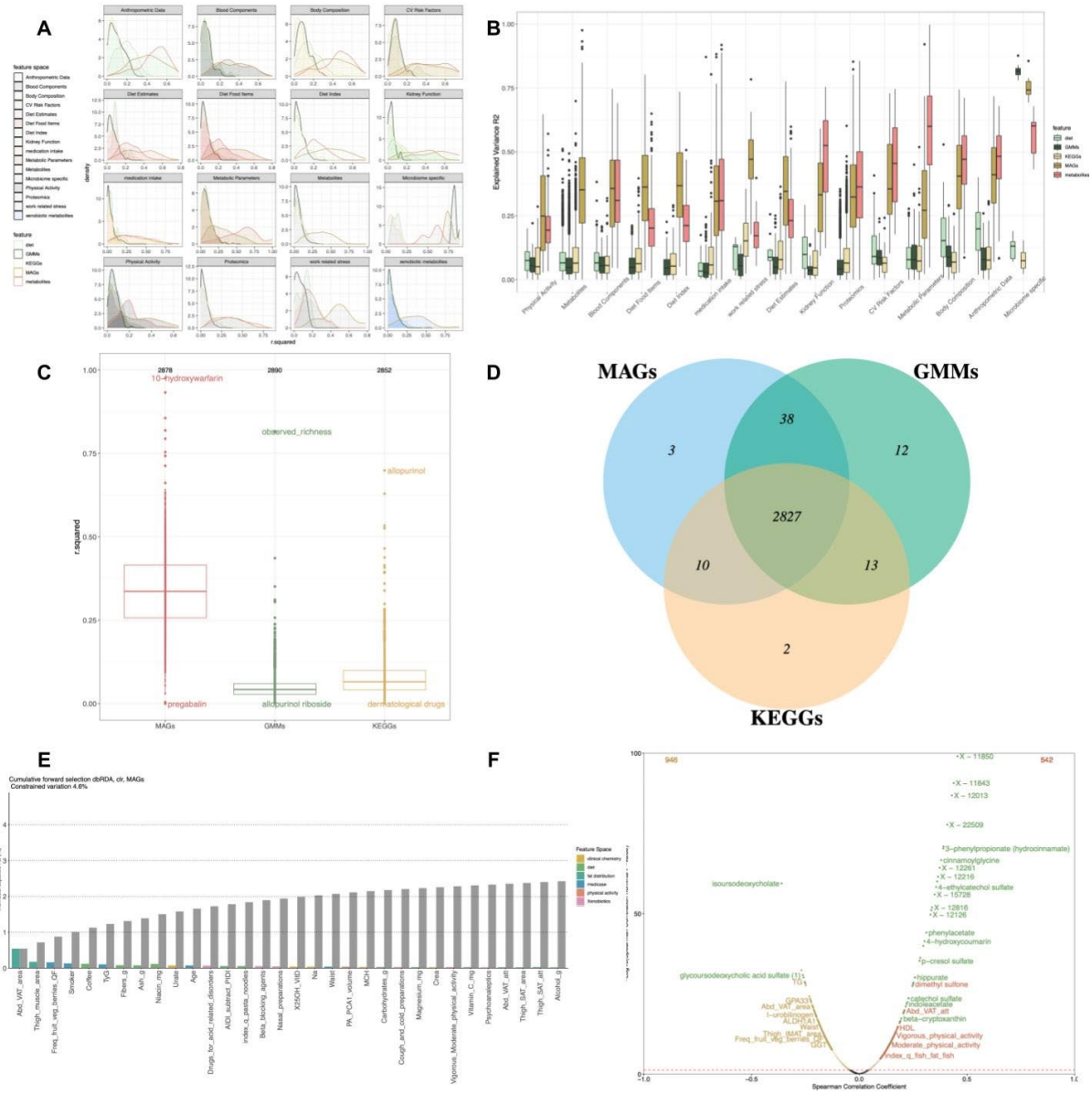

Figure 2: the interconnectedness of omics features in a non-disease at risk population-based cohort (A) density plots depict the distribution of R2 by microbial species, GMMs, KEGG modules, metabolome, and diet on variables from all feature spaces, outlines are colored by contributing omics feature and feature spaces are depicted in separate boxes. (B) boxplots showing the distribution of variance explained for metabolome, diet and bacterial features (C) Distrution of variance explained for bacterial species (MAGs), GMMs and KEGG modules. Variables with highest and lowest variance explained as well as the number of features are labeled (D) quantification of overlapping and specific variance explained for microbiome related features (E) db-RDA showing explanatory variables with individual nonredundant and cumulative contribution to the variation in the gut microbiome ordered by feature importance. (F) age, sex, and BMI corrected correlations of metadata, metabolome, and proteome data with gene richness.

Aim 2

Background: Epicardial adipose tissue is part of the visceral adipose tissue distributed around the viscus or hollow muscular organs of the body and is associated with coronary artery disease. Moreover, excess epicardial fat is associated with left atrium enlargement, with lower ejection fraction, increased left ventricular mass, and abnormal diastolic function. Since we previously demonstrated that the gut microbiota contributes to adiposity [7] we will here test the hypothesis that the gut microbiota is altered in patients with increased amounts of epicardial fat compared with individuals with normal volumes.

Approach/results: We have analyzed cardiac CT images and quantified epicardial fat as total volume (EAT) and density (attenuation, EAT_HU) in 1787 of 1868 included individuals and used the microbiome data from the study in Aim 1. EAT attenuation is an independent predictor for CAD and is considered more sensitive than EAT volume. We first investigated which anthropometric and clinical features that contributed to variation in EAT attenuation by using Lasso regression feature selection model (**Figure 3A**). The model was based on 1678 samples with no missing values for the clinical variables and identified 39 clinical features that significantly contributed to EAT attenuation and together explained to 58.6% of the variance in the EAT attenuation. EAT volume, body weight, visceral and sub-cutaneous fats as well as smoking status were the top features explaining EAT attenuation.

Next, we observed that the fecal microbiota contributed to 9.8% variance in EAT attenuation using lasso regression. Gut microbiota can be influenced by various lifestyle and clinical factors and to further investigate how the variance in the gut microbiota that can be explained by the 39 clinical features that significantly contributed to EAT attenuation we performed a distance-based redundancy analysis (dbRDA) on the gut microbiota profile (**Figure 3B**). We observed that 16 features contributed significantly to the explained variance in the microbiota (**Figure 3B**). The results revealed a specific contribution to EAT attenuation compared to EAT volume, VAT and BMI, suggesting a specific contribution independent of VAT and BMI. Variation partitioning analysis of the four variables (EAT, VAT, SAT and BMI) on the dbRDA shows significant EAT attenuation contribution with adjusted R2=0.0015.

Next, we stratified the cohort into quartiles after EAT attenuation to investigate the microbiome difference between individuals with low epicardial fat (quartile 1) and individuals with high EAT attenuation (quartile 4) (**Figure 3C**). We observed that individuals in quartile 4 with low EAT attenuation had higher EAT volume compared with individuals with high EAT attenuation (quartile 1). Beta diversity ordination plot based on Bray-curtis dissimilarity matrix revealed significant differences in the gut microbiota of the four quartile groups (**Figure 3D**). The medium two quartiles 2 and 3 did not demonstrate any significant differences in the microbiota. The gene richness profile, a marker of gut microbiome health, decreased from quartile 1 to quartile 4 with the medium two quartiles not significantly differing in gene richness (**Figure 3E**).

To identify microbial taxa which significantly differ between individuals with high or low EAT attenuation using ANCOM-BC between EAT quartile 1 and quartile 4. We performed a binomial logistic regression model between quartile 4 and quartile 1 adjusting for identified covariates that contributed to microbiome composition: VAT, SAT, BMI, smoking status, triglycerides, fibre intake, gender, age and HOMA-IR (**Figure 3B**). We also adjusted for medications e.g. statins, PPI, laxatives, SSRI, antihypertensives, anti-thrombotics were adjusted for using the MetadeconfoundR pipeline. 114 taxa were differentially abundant between quartile 4 and quartile 1, even after adjustment for all covariates. Most of these taxa/metagenome assembled genomes (MAGs) are represented by single reference genome with same taxonomic lineage. Therefore, representative taxa are selected based on common

lineage and completeness and contamination score of the MAG. 69 of the representative taxa are shown in the barplot (**Figure 4**). The top taxa enriched in quartile 4 belong to the family Lachnospiraceae and are genus *Blautia*, *Clostridium*, *Ruminococcus*, *Tyzzerella* and additional other enriched taxa belonged to the families Anaerovoraceae, Bacteroidaceae and Coriobacteriaceae. These taxa also have a steady variation across four quartiles in the population and most of the taxa enriched in quartile 4 are positively correlated (based on spearman correlation) to epicardial fat and triglycerides, visceral obesity (VAT, SAT, BMI , waist-hip ratio), insulin resistance and inflammation while being negatively correlated to fiber intake, AIDI and gut gene richness across the population irrespective of quartile division, as seen in the heatmap (**Figure 4**). The species *Blautia producta*, Lachnospiraceae_UC5−1−2E3 sp001304875, *Dorea faecis* and Sellimonas sp002161525 were the only enriched taxa positively associated with EAT attenuation and not with other covariates such as VAT, SAT, BMI and EAT volume, suggesting potential direct interaction with EAT attenuation. The depleted taxa in high epicardial attenuation belonged to the order Christensenellales, family Oscillospiraceae, Acutalibacteraceae, Ruminococcaceae and Rikenellaceae and class Clostridia of which the majority are butyrate producers, which are associated with anti-inflammation and reduced adiposity.

Some microbes can perform similar functions in the gut, which is also known as functional redundancy. To further explore function potential of the gut microbiome in individuals with varying epicardial fat, we mapped the gut microbial genes to a customized set of 117 human gut metabolic modules (GMM) which were manually curated. These modules represent the functional potential of the gut bacterial and archaeal metabolism. Differentially abundant analysis on the abundance count of these functional modules resulted in a set of 52 GMMs which were significantly different between quartile1 and 4. Among these GMMs, the 17 modules with the highest correlation o EAT attenuation across the population, here defined as EAT attenuation associated. The top enriched modules were related to tyrosine degradation II, nitrate reduction(dissimilatory), glyoxylate bypass, Entner-Doudoroff pathway, pentose phosphate pathway(oxidative), aspartate degradation II and lactose degradation. These modules are also associated to markers of dyslipidemia suggesting an altered lipid profile as seen in the metabolites and oxidative stress. The top depleted GMMs negatively associated to EAT attenuation were feruloyl esterase, glutamate degradation III, triacylglycerol degradation, pyruvate dehydrogenase complex, methionine degradation II, lysine degradation II, cinnamate conversion, hippurate hydrolase, 4-aminobutyrate degradation and succinate consumption. These depleted modules are also negatively correlated to markers of dyslipidemia, visceral obesity and systemic inflammation while positively correlated to fiber intake, AIDI and gut gene richness profile. Among these, modules such as lysine degradation and 4-aminobutyrate degradation are pathways known to produce butyrate from amino acids suggesting a role of decreased butyrate production in high epicardial fat.

As evident from the microbes above, most the depleted taxa are butyrate producers, which is also reflected in the depleted gut metabolic modules. To study the butyrate production potential of the gut microbiota with relation to epicardial fat, we quantified the relative abundance of five terminal genes involved in butyrate biosynthesis from both carbohydrates (i.e., but and buk) and proteins (i.e., atoA/D and 4hbt). We observed that the combined abundance of all five genes was decreased across the EAT attenuation quartiles. A similar pattern was observed for buk, 4hbt/but, AtoA/D genes suggesting the butyrate production potential is reduced in high epicardial fat.

In conclusion, our results clearly show that an altered microbiota is associated with EAT attenuation.

Compared with the application we expanded the metabolomics to cover all individuals using untargeted metabolomics plasma samples to investigate how the systemic metabolite profile with respect to changes in quality of EAT. Differential abundance analysis, using wilcoxon rank-sum test with FDR correction, was used to identify metabolites that differed between quartile 4 and quartile 1. Metabolites were adjusted for covariates and medications using the logistic regression model and MetadeconfoundR pipeline and identified 40 metabolites to be differentially abundant between groups. The enriched plasma metabolites are sphinolipids, primarily sphingomyelins and ceramides known to be associated with atherogenesis and CVD risk. We also observed that glutamate, gamma-glutamyltyrosine, 1−stearoyl−2−arachidonoyl−GPC (a phophotidylcholine) and cis−3,4−methyleneheptanoylcarnitine were enriched metabolties. The depleted metabolites were amino acids N-acetylyglycine, N-acetylaspartate, antioxidant carotene diol(1) and fatty acid components hydroxy-CMPF and 3-methyladipate.

We furthermore conclude that also circulating metabolites are associated with EAT. Results reported under Aim 2 has been summarized in a manuscript that will be submitted to Nature Metabolism in the next month.

Table 2: Characteristics of the participants in each EAT quartile

	Low_EAT	LoMed_EAT	HiMed_EAT	High_EAT
EAT_volume Quartile range	23.67 - 84.89	84.89 - 110.62	110.62 - 143.9	143.9 - 313.6
EAT_volume	65.99 (13.09)	98.22 (7.66)	126.8 (9.17)	175.83 (27.73)
EAT_HUmean	-70.29 (2.95)	-73.11 (2.8)	-75.19 (2.96)	-77.41 (3.12)
Gender	M=118 ,F=336	M=168 ,F=285	M=218 ,F=235	M=319 ,F=135
Age	56.31 (4.61)	57.54 (4.51)	57.9 (4.36)	58.7 (4.38)
BMI	24.39 (3.06)	27.11 (3.46)	28.72 (3.96)	30.92 (4.16)
Waist	86.66 (8.34)	95.34 (8.73)	101.61 (10.45)	109 (10.41)
sbp	122.19 (16.81)	128.82 (16.5)	130.77 (16.63)	133.59 (17.98)
dbp	78.27 (9.75)	82.76 (9.7)	83.94 (9.55)	85.85 (10.8)
ogtt_0gl	5.44 (0.69)	5.55 (0.7)	5.75 (0.9)	5.85 (0.9)
ogtt_120gl	7.22 (1.85)	7.38 (1.9)	7.53 (1.97)	7.98 (2.16)
HOMA-IR	1.18 (0.8)	1.54 (1.1)	1.83 (1.31)	2.38 (1.58)

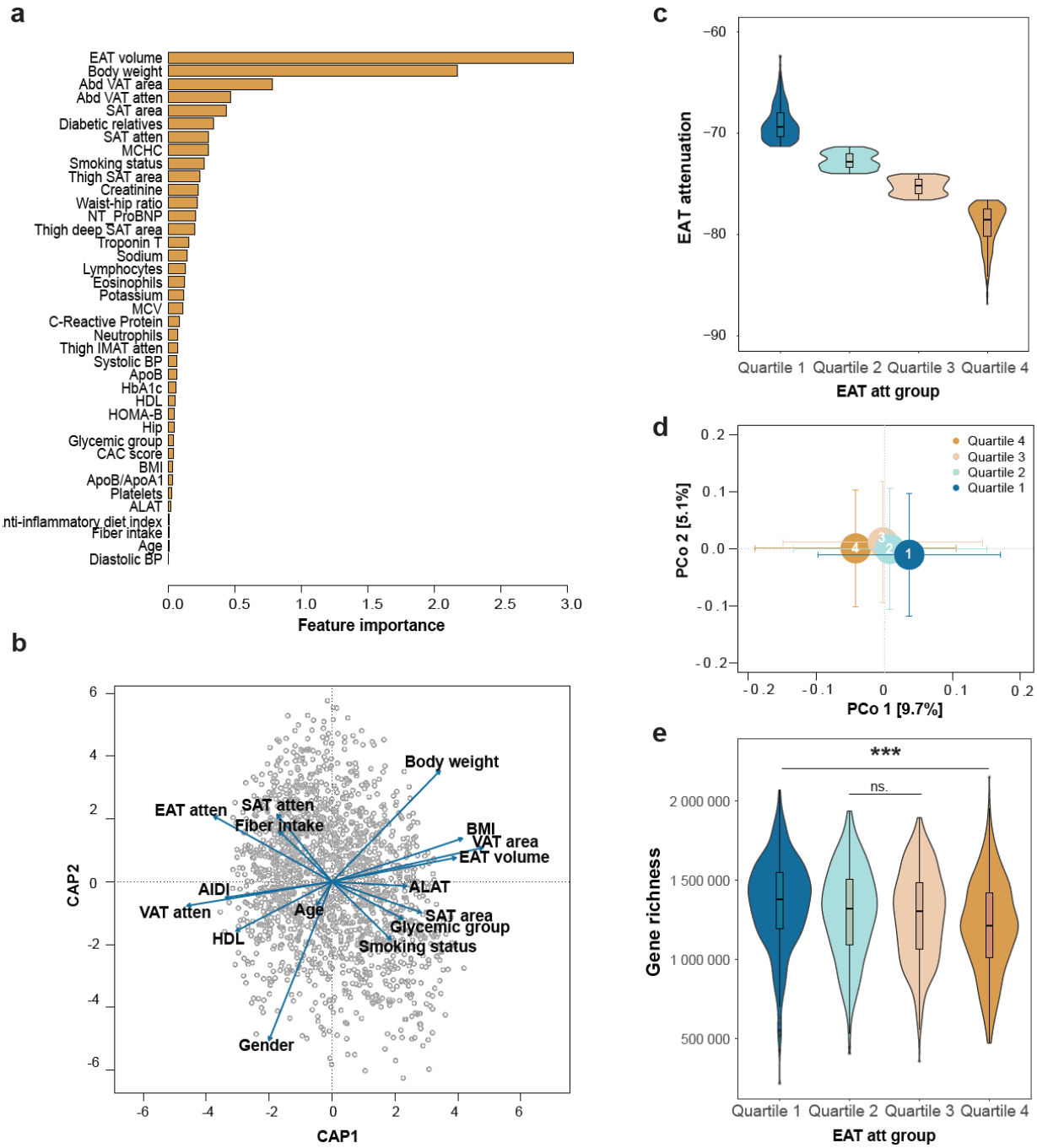

Figure 3: Epicardial adipose tissue (EAT) attenuation affects microbiome composition. a, Bar plot of Lasso regression model against EAT attenuation idntifies important clinical features explaining microbiota variation. b, distance-based Redundancy analysis based on Bray-Curtis dissimilarity matrix showing the 16 significant clinical features contributing to EAT attenuation. c, Violin plot showing the EAT attenuation quartile distribution with quartile 1 having low and quartile 4 having high EAT attenuation. d, Ordination plot for beta diversity of the gut microbiome profile separating quartiles of EAT attenuation. e, Violin plot of gut gene richness profile for each EAT attenuation quartile; P-value ns > 0.05, ***p < 0.001. Significance across the groups was tested using Kruskal-Wallis test (p-value < 0.0001) and between group using Wilcoxon rank sum test (quartile1~quartile2 p-value=0.0005; quartile1~quartile3 p-value < 0.0001; quartile2~quartile3 p-value=0.258; quartile2~quartile4 p-value < 0.0001; quartile3~quartile4 p-value=0.0012).

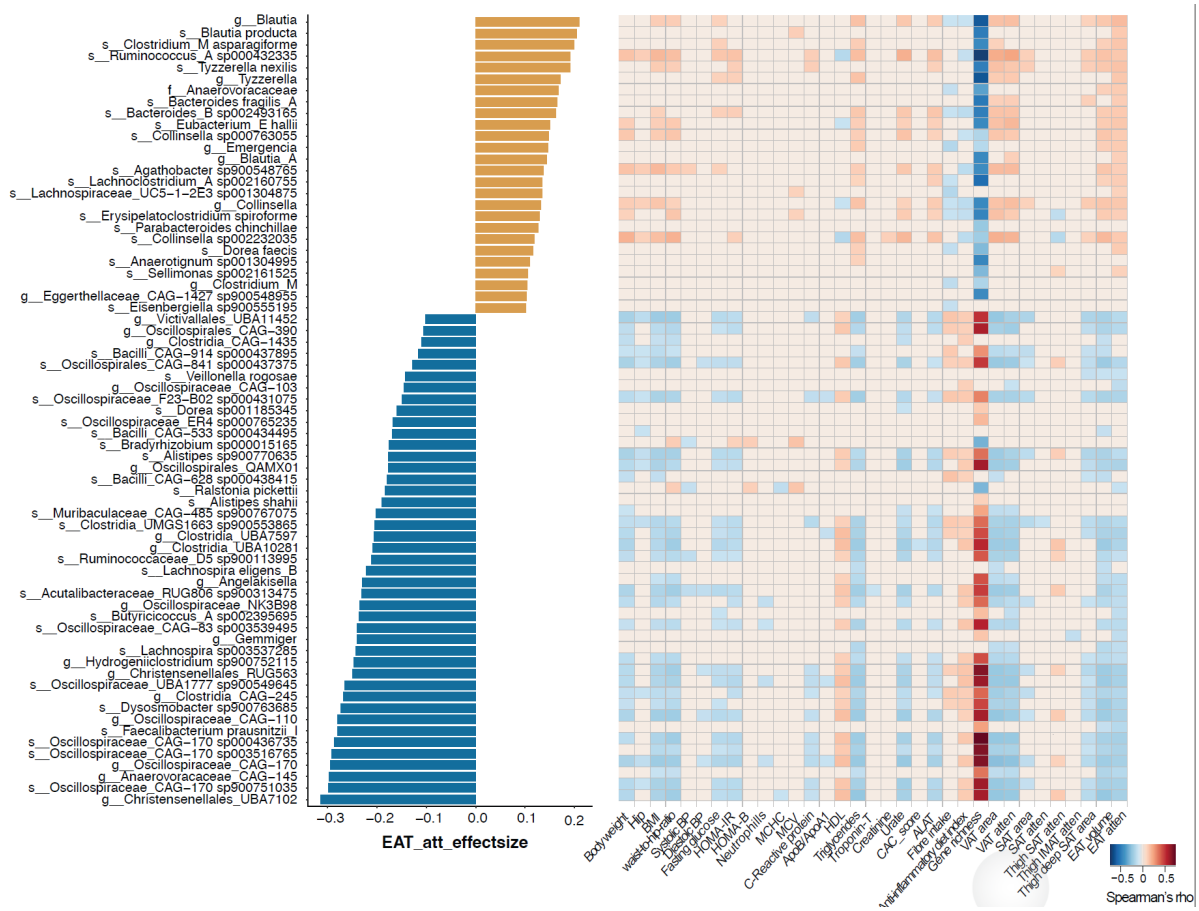

Figure 4: Differentially abundant microbial taxa associated with high and low EAT attenuation. Left panel, Bar plot showing the cliff's delta effect size of significant differentially abundant taxa after adjustment of covariates and medication. Orange bar indicates taxa positively associated and blue bar indicates taxa negatively associated with attenuation.

Right panel, Heatmap showing the spearman correlations of the 69 taxa with EAT attenuation identified using Lasso regression of the whole population. Only significant rho coefficients >=0.1 (FDR adjusted p-value > 0.05) are plotted in the heatmap.

Aim 3

Background: It will be essential to investigate if correlative findings from the patient can be translated to a functional impact of the microbiota in the above phenotypes. To address this hypothesis we will perform animal experiments using germ-free mice.

Approach/results: We assessed a number of different models for CVD and made several important observations, but since we did not observe any connection between work related stress and microbiota this was not further addressed. First, we analysed if microbiota was associated with atherosclerosis and observed that microbial butyrate production was protective against atherosclerosis development [8]. Second, we investigated if we could perform experiments assessing if the gut microbiota was associated with myocardial infarction but observed that the surgery was associated with weight loss prohibited further studies since myocardial infarction was associated with weight loss rather than colonization.

During the tenure of the grant we made observations that the gut microbiota in the presence of fiber not only produces protective butyrate [8] but also secondary bile acids that improve cardiometabolic feature [9]. In contrast we observed that a microbially produced metabolite, imidazole propionate (ImP), was associated with type 2 diabetes [10]. Further analyses identified that ImP is associated with CVD and even more so in patients with heart failure in Europeans and North Americans (**Figure 6A**). Furthermore, ImP levels could predict major cardiovascular events and death (**Figure 6B,C**). *These initial data have been resubmitted to JACC heart failure*. Since one of the main aims was to mechanistically explore if and how the gut microbiota can contribute to the CVD we further explored these findings and found that treatment of mice with the metabolite produced 'white areas' on the heart, which likely are necrotic/fibrotic tissue (**Figure 6D**). ImP treatment was associated with increased fibrosis in the heart muscle and may thus provide a mechanistic explanation for how the gut microbiota may contribute to heart failure. Followed by negative data in the beginning of the project we found the connection between microbially produced ImP and CVD and demonstrated causality. Taken together our data suggests that inhibiting microbial production of ImP may provide a novel strategy for treating/preventing cardiometabolic diseases.

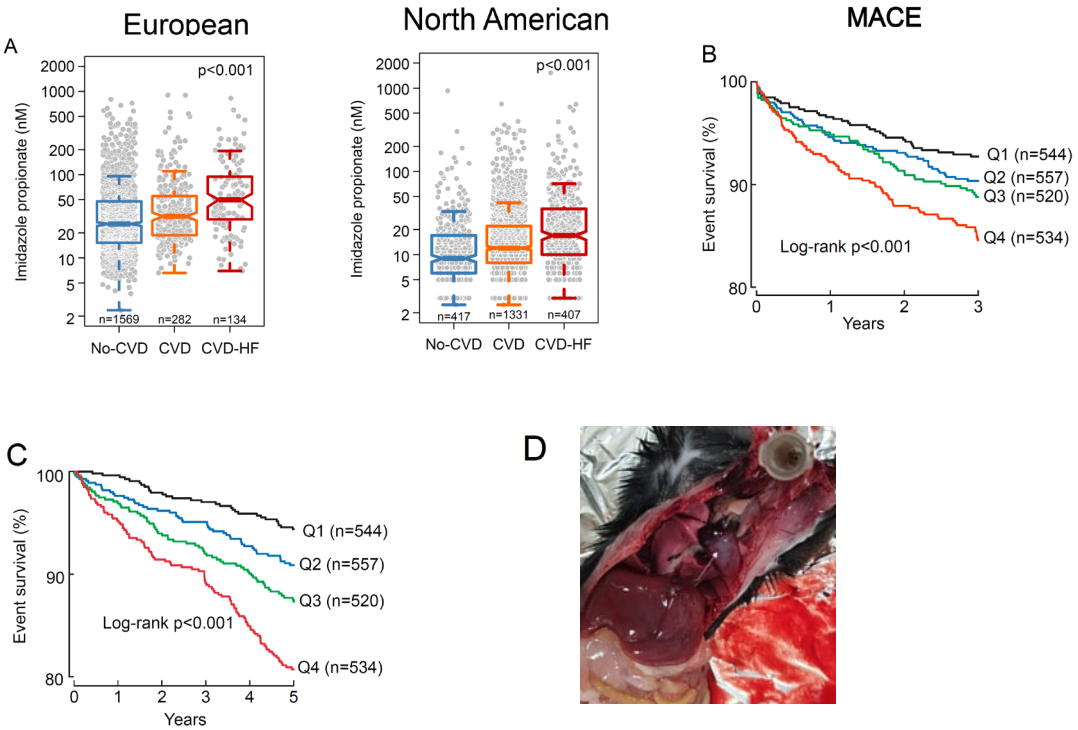

Figure 5: Imidazole propionate is increased in individuals with cardiovascular diseases and may contribute to major cardiovascular events and death. (**A**) Serum levels of imidazole propionate in individuals without cardiovascular diseases (CVD) (n=1596), CVD (n=282) and heart failure (CVD-HF; n=134) (p values were calculated with linear regression) in Europeans (n=2012) and in North Americans (n=2155). Kaplan-Meier estimates **(B)** for the risk of MACE at 3-years follow-up and **(C)** death at 5-years follow-up according to quartiles (Q) of ImP. (**D**) picture of a mouse heart with infarcted/necrotic tissue after 5 weeks of chronical ImP administration

Utilization of data

We have performed the analyses outlined in Aim 1 and 2 that resulted in two different manuscripts, which will be submitted for publication in the next month. In the attempt to provide causality by demonstrating that butyrate production from the gut microbiota can protect against atherosclerotic disease (published from this study) and then validated by our collaborator Federic Rey [11]. Furthermore, we identified how the microbiota in the presence of dietary fibers, not only produces butyrate but also produces secondary bile acids that may mediate some of the beneficial effects following fiber supplementation [9]. We also observed that the microbiota associated with CVD produces increased levels of imidazole propionate (ImP) and that ImP contribute to fibrosis development that can contribute to heart failure. These findings were confirmed using independent cohorts showing that imidazole propionate is an independent risk factor for heart failure and furthermore could demonstrate that administration of this metabolite causes scarring on the heart. We also found that imidazole propionate predicts MACE and death and in collaborative studies found that ImP is also associated with blood pressure.

These data have been presented at scientific conferences and have or will soon be submitted to scientific journals and we expect them to be well appreciated by the scientific community. In additional experiments we have started to develop inhibitors to block the production of ImP.

Several studies, including ours, have associated butyrate producing bacteria with cardiometabolic health. However, butyrate administration did not improve insulin sensitivity [12]. Thus we isolated butyrate producing bacteria and started to develop these into therapy and have resubmitted the paper to Nature (Khan et al., submitted).

In addition to the research, we have continued to develop a platform for dissemination of our results and communication to the general public. This is becoming increasingly important as there is much noise in the field, and recommendations in media are not always scientifically built on facts. Here we provide updates on the five pillars we established last year:

1. We have published a new web site to communicate our research to scientists internationally (English; www.backhedlab.com), but also a site in Swedish (www.backhedlab.se) with a target audience of the interested general public as well as physicians, nurses, dieticians etc. These two web pages also highlight the scientists in the group.

2. We have developed a web site with basic information on the gut microbiota targeting the interested general public as a fact-based source of knowledge (www.livetitarmen.se).

3. We continued to be active on Twitter (~1500 followers) where we communicate our research to the research community and have as of September 2021 started an Instagram

account, which matches www.livetitarmen.se. The Instagram has just reached 336 followers.

4. We have produced seven short movies on the field for YouTube and social media. This will target a younger crowd and feature key components of the microbiome field. These are now available at www.livetitarmen.se and have also engaged two schools to explore the possibility to develop a course curriculum. This work has been extended together with a collaboration together with Universeum to continue disseminate our findings.

5. We have been giving several popular science talks at the city library and other venues to the public.

6. We have resumed work with the popular science book were a scientific writer, Nathalie von der Lehr, has been recruited.

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