Microbiome and metabolome features of the cardiometabolic disease spectrum

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Previous microbiome and metabolome analyses exploring non-communicable diseases have paid scant attention to major confounders of study outcomes, such as common, pre-morbid and co-morbid conditions, or polypharmacy. Here, in the context of ischemic heart disease (IHD), we used a study design that recapitulates disease initiation, escalation and response to treatment over time, mirroring a longitudinal study that would otherwise be difficult to perform given the protracted nature of IHD pathogenesis. We recruited 1,241 middle-aged Europeans, including healthy individuals, individuals with dysmetabolic morbidities (obesity and type 2 diabetes) but lacking overt IHD diagnosis and individuals with IHD at three distinct clinical stages—acute coronary syndrome, chronic IHD and IHD with heart failure—and characterized their phenome, gut metagenome and serum and urine metabolome. We found that about 75% of microbiome and metabolome features that distinguish individuals with IHD from healthy individuals after adjustment for effects of medication and lifestyle are present in individuals exhibiting dysmetabolism, suggesting that major alterations of the gut microbiome and metabolome might begin long before clinical onset of IHD. We further categorized microbiome and metabolome signatures related to prodromal dysmetabolism, specific to IHD in general or to each of its three subtypes or related to escalation or de-escalation of IHD. Discriminant analysis based on specific IHD microbiome and metabolome features could better differentiate individuals with IHD from healthy individuals or metabolically matched individuals as compared to the conventional risk markers, pointing to a pathophysiological relevance of these features.

Epidemiological and genetic studies in humans and experimental studies in animals have shown that the pathogenesis of most common chronic non-communicable diseases involves a complex interplay among polygenic susceptibility, aging, sex and a multitude of environmental exposures. Intriguingly, environmental components, such as diet, physical activity and smoking, might exert some of their pathogenic effect via modification of the intestinal microbiome. Therefore, a first logical step in exploration of the intestinal microbiome as a putative chronic disease co-trigger appears to be the conduct of studies integrating epidemiology and various -omics analyses. However, for the reliability of such study outcomes and for the planning of subsequent clinical interventions and mechanistic experiments, disease-specific microbiome and linked metabolome features need to be separated from confounders introduced by pre-morbidities and co-morbidities and by multifactorial treatment. Commonly prescribed drugs, for example, widely influence the gut microbiome and host metabolome and can confuse for, or even mask, genuine disease signatures. Accordingly, a recent report argues for extensive adjustments for confounders that influence the human gut microbiome to avoid spurious associations and to identify genuine disease-specific variance.

The present microbiome and metabolome study is focused on IHD, a leading cause of mortality worldwide. Previous reports comparing microbiome and metabolome markers of IHD cases and controls usually failed to adjust for the massive confounding by polypharmacy and the effect of metabolic abnormalities occurring...
during a long prodromal phase before diagnosis of IHD\textsuperscript{11–13}. Such common metabolic dysfunctions include overweight and obesity\textsuperscript{14,15}, type 2 diabetes (T2D)\textsuperscript{16}, hypertension\textsuperscript{17} and dyslipidaemia\textsuperscript{18} (collectively termed ‘dysmetabolism’ in the present study), all of which have been shown to exhibit both shared and disease-specific aberrations in microbiome and metabolome profiles. Individuals with the metabolic syndrome or overt T2D have vastly increased risk of IHD\textsuperscript{19}, and asymptomatic T2D is often coincidentally found at IHD diagnosis\textsuperscript{20}, highlighting these pre-morbidities to be a clinically relevant baseline for studying overt IHD. Most studies to date have overlooked this aspect by either comparing individuals with IHD with healthy, lean individuals\textsuperscript{21} or not focusing on IHD per se but on various forms of atherosclerotic organ damage\textsuperscript{22,23,24}. Thus, segregating IHD-specific changes in gut microbial and metabolic features from such potential confounders remains an utmost priority.

In the MetaCardis consortium, we designed the present cross-sectional study including healthy individuals, individuals with dysmetabolic morbidities and individuals with IHD at three distinct clinical stages, capturing a wide spectrum of gut microbiome and plasma and urine metabolome signatures for cardiometabolic diseases (CMDs). With our approach for integrative analysis of the -omics data, we adjust for confounding by polypharmacy and the effect of metabolic abnormalities occurring during the prodromal phase before diagnosis of IHD. Furthermore, we categorize microbiome and metabolome pathophysiological signatures related to dysmetabolism or to escalation, de-escalation or stabilization of IHD and its subtypes.

**Results**

**Study design, in-depth phenotyping and multi-omics profiling.**

This study encompassed 372 individuals with IHD, including 112 with acute coronary syndrome (ACS), 158 with chronic ischemic heart disease (CIHD) and 102 with IHD and heart failure (HF). In addition, we included 275 healthy controls (HCS) matched on demographics, age and sex and 222 untreated metabolically matched controls (UMMCs)—that is, individuals with features of the metabolic syndrome and, thus, at increased risk of IHD but receiving no lipid-lowering or anti-diabetic or anti-hypertensive drugs. Finally, we included 372 controls matched with individuals with IHD in terms of T2D status and body mass index (BMI), thereafter termed metabolically matched controls (MMCs) (Fig. 1).

We profiled their serum and urine metabolome (1,558 metabolites and lipids) and examined their intestinal microbiome, considering inter-individual variations in absolute fecal bacterial cell density, a factor potentially reflecting the disease state and obscuring genuine microbiome involvement\textsuperscript{9}. Inclusion of MMC and UMMC groups allowed for the differentiation of the gut microbial and metabolomic signatures of IHD from the often-accompanied metabolic dysfunctions and related drug intake.

As expected from inclusion criteria, we found increasing CMD phenotype severity and related drug intake along the implied progression from HCs through treated and untreated metabolically matched controls (MMCs and UMMCs, respectively) to overt IHD cases (Extended Data Fig. 1 and Supplementary Tables 1–3). Despite matching for country, age, sex, BMI and T2D status, individuals with IHD remained phenotypically distinct from MMCs. They displayed increased visceral fat ($P=0.048$), worse glycemia (HbA1c; $P=0.005$ and fasting plasma glucose; $P=0.006$), higher plasma concentration of liver enzymes (aspartate aminotransferase, alanine aminotransferase and γ-glutamyl transferase; $P<0.001$) and increased prevalence and severity of hypertension ($P<0.001$) (Supplementary Tables 1 and 2). Similarly, individuals with IHD had decreased heart contractility mirrored in reduced left ventricular ejection fraction (LVEF) and increased plasma pro-atrial natriuretic peptide (pro-ANP) levels relative to both HCs and MMCs ($P<0.001$), which was further altered in the HF subgroup relative to ACS and CHD ($P<0.001$) (Extended Data Fig. 1 and Supplementary Table 2).

**Diet and physical activity variation across study groups.** Diet affects microbiome composition and IHD risk\textsuperscript{25}. We found that HCs reported healthier diets than individuals in the IHD and MMC groups, with higher values of composite metrics, such as alternative healthy eating index (aHEFI) (HC versus IHD, $P<0.001$), diet diversity score (DDS\textsuperscript{26}) (HC versus IHD, $P=0.001$), dietary approaches to stop hypertension (DASH\textsuperscript{27}) score (HC versus IHD, $P=0.013$) and lower overall daily energy intake (HC versus IHD, $P=0.013$). HCs consumed significantly less fatty animal-based food and meat and more plant-based food rich in non-digestible polysaccharides (Supplementary Table 4). They further reported higher physical activity levels (Extended Data Fig. 1), more often being in manual work and undertaking more frequent moderate to vigorous leisure time activities than individuals with IHD or MMCs (Supplementary Table 4). Some of the microbiome differences between MMCs and individuals with IHD as opposed to HCs might also reflect a less healthy lifestyle.

**Microbiome and metabolome changes related to dysmetabolism.** Both the taxonomy and functional potential of the gut microbiome as well as the metabolome differed significantly between individuals with IHD and HCs in accordance with previous reports\textsuperscript{11–14}. Remarkably, comparing HCs to MMCs revealed even more differential features than comparing HCs to individuals with IHD (Fig. 2a and Supplementary Tables 5–8). Moreover, the discriminatory potential of microbiome and metabolome features was significantly higher between individuals with IHD and HCs than between individuals with IHD and MMCs (Fig. 2b). We recovered most previously published IHD-related gut microbiome findings (Extended Data Fig. 2 and Supplementary Tables 15 and 16), primarily by contrasting HCs and individuals with IHD. However, most were already significant in MMC versus HC comparisons, suggesting that previous studies might have erroneously reported dysmetabolism features as bona fide IHD features. These might contribute to increased risk of IHD, but our analyses indicate that they are not specific for IHD.

At higher microbiome architecture levels, there was a significant shift from the *Bacteroides* 1 and *Ruminococcus* enterotypes toward the low bacterial cell-count-associated *Bacteroides* 2 as disease worsened\textsuperscript{28} (Fig. 2c). These findings mirror significant loss of microbial gene richness (Fig. 2d) and absolute gut bacterial cell load (that is, microbial load) in both MMCs and individuals with IHD to HCs. In contrast, no differences were found when individuals with IHD were compared to MMCs (Supplementary Table 5). Bacterial gene depletion and *Bacteroides* 2 prevalence were even more exacerbated in UMMCs, possibly due to drugs not yet being prescribed and the presence of a more obese phenotype in this group\textsuperscript{14}. Consistently, the total number of gut microbiome and metabolome features significantly differential in abundance was higher when HCs were compared to UMMCs relative to MMCs (Extended Data Fig. 3).

**Microbiome and metabolome signatures of IHD.** We consider the identification of genuine microbiome and metabolome signatures of IHD—that is, disease features not better explained as indirect associations via drugs and demographics—to be a major contribution of our study. Additionally, we further differentiate IHD features from their metabolic morbidities by categorizing them according to their signatures among the various group comparisons across the CMD spectrum, focusing qualitatively on condition specificity and quantitatively on effect size (Fig. 3 and Extended Data Fig. 4). We identify features as being specific to dysmetabolism (Fig. 3a,b) or IHD (Fig. 3a,c) by exhibiting a significant change only under the respective condition—that is, HCs versus MMCs/MMCs for...
Feature classification
Based on the statistical significance and directional congruence among various group comparisons
(applied to features with non-confounded status)

Effect of metabolic dysregulation segregated from IHD using metabolic matching

Study design
Comparisons
Methodological strategies
Analytical framework

Fig. 1 | Overview of the study design. Top: the 1,241 individuals studied here are a subset of individuals from the European MetaCardis cohort, in which participants underwent deep bioclinical phenotyping combined with gut microbiome and serum and urine metabolome profiling. Participants were classified as being HCs \( n = 275 \), healthy by self-report and no intake of lipid-lowering, anti-diabetic or anti-hypertensive drugs) and a combined group of patients diagnosed with IHD \( n = 372 \), on various drugs). The IHD group included cases with ACS \( n = 112 \), CIHD \( n = 158 \) and HF \( n = 102 \) due to CIHD.

Two additional control groups were included: MMCs without diagnosed IHD \( n = 372 \), matched on age, BMI and T2D status of the individuals with IHD, some of whom were prescribed lipid-lowering, anti-diabetic and anti-hypertensive medication but no IHD-related drugs) and untreated (non-medicated) metabolically matched non-IHD controls \( n = 222 \), no intake of lipid-lowering, anti-diabetic, anti-hypertensive or IHD drugs). Bottom: microbiome and metabolome features were segregated into four categories, as indicated. The human icons were adapted from https://smart.servier.com/.
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Articles uncharacterized Ruminococcus depleted in IHD, was an IHD escalation marker (Fig. 4b); ruminococci include butyrate producers, known to improve cardiovascular health, exhibited the above depletion pattern, whereas glutathione metabolism and markers of oxidant properties were among the top metabolites whose depletion constitutes markers specific for IHD (Fig. 4a). Notably, similar patterns remained in IHD subtype-specific analyses (Extended Data Figs. 6–8 and Supplementary Table 17).

Most IHD escalation features represented by the metabolome exhibited an initial depletion upon dysmetabolism, which continued after IHD diagnosis (Fig. 4b and Supplementary Table 17). Besides several complex phospholipids, including sphingomyelins and glylycerophospholipids, several carotenoids (for example, carotene diols and β-cryptoxanthin) and ergothioneine, which are known to improve cardiovascular health, exhibited the above depletion pattern, whereas glutathione metabolism and markers of oxidative stress (for example, cystathionine and cyst-gly oxidized) instead escalated. Ergothioneine, in particular, has been associated with reduced cardiovascular and overall mortality and was also identified as a key metabolite exhibiting a positive correlation with SGB 4712 (that is, both SGB 4712 and ergothioneine exhibiting depletion) in ACS cases relative to controls in the companion paper.

Consistently, in the present study, a reduction in circulating levels of ergothioneine was also observed in individuals with ACS and HF relative to HCs (Supplementary Table 17). In contrast, 4-cresol exhibited an enrichment pattern from dysmetabolism to IHD (Fig. 4b). 4-cresol is a bacterial product of colonic fermentation of phenylalanine and tyrosine and a precursor of 4-oxo-2-heptenal, a pro-inflammatory agent associated with atherosclerosis.

In parallel, the metabolome reporting most of IHD-specific markers showed a marked enrichment, with only 50 of 203 IHD-specific markers (25%) being depleted in IHD relative to HC (Fig. 4 and Supplementary Table 17). We identified enrichment of a range of IHD-specific metabolites, including intermediaries of the choline and carnitine pathways quantified by ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS)—that is, choline, betaine-aldehyde, 4-hydroxybutanate, linoleoylcarnitine and trimethylamine (TMA), the precursor of trimethylamine N-oxide (TMAO), which is known to modulate IHD risk. Other such carnitine metabolites included medium-chain and long-chain fatty acyl carnitines, suggesting an increase in transport into the mitochondria through the carnitine shuttle, typically for β-oxidation. In particular, microbial aromatic acids, such as phenyllactate, reported to be inversely associated with species-level genome bin (SGB) 4712 in the companion paper, or benzoate, follow a similar process, producing phenylacetylcarnitine or benzoylecarnitine. They undergo conjugation with amino acids to form, for instance, phenylacetylglutamate or hippurate, of which both phenylacetylcarnitine and phenylacetylglutamate are IHD-specific markers in our study (Fig. 4a).

Along the same lines, we observed an increase in pro-inflammatory lipids derived from arachidonic acid (C20:4) starting with arachidonoylcarnitine, 5-hydroxyeicosatetraenoic acid (5-HETE) as well as leukotriene B4 and 9-/13-hydroxyoctadecadienoic acid (9-HODE/13-HODE), which are known mediators of inflammation and atherogenesis. In contrast, fatty acid methyl esters, including methyl hexadecanoate, methyl linolenate and methyl oleate, along with alpha-tocopherol, known for vasoprotective and antioxidant properties, respectively, were among the top metabolites whose depletion constitutes markers specific for IHD (Fig. 4a).

Notably, similar patterns remained in IHD subtype-specific analyses (Extended Data Figs. 6–8 and Supplementary Table 17). Most IHD escalation features represented by the metabolome exhibited an initial depletion upon dysmetabolism, which continued after IHD diagnosis (Fig. 4b and Supplementary Table 17). Besides several complex phospholipids, including sphingomyelins and glycerophospholipids, several carotenoids (for example, carotene diols and β-cryptoxanthin) and ergothioneine, which are known to improve cardiovascular health, exhibited the above depletion pattern, whereas glutathione metabolism and markers of oxidative stress (for example, cystathionine and cyst-gly oxidized) instead escalated. Ergothioneine, in particular, has been associated with reduced cardiovascular and overall mortality and was also identified as a key metabolite exhibiting a positive correlation with SGB 4712 (that is, both SGB 4712 and ergothioneine exhibiting depletion) in ACS cases relative to controls in the companion paper.

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Fig. 2 | Alterations of gut microbiome and metabolome features along the natural history of IHD. a. Violin plots representing the distribution of significant gut microbiome and metabolome features among various group comparisons before and after data being subjected to the drug deconfounding pipeline (lower line, lower quartile; medium line, median; upper line, upper quartile). Numbers below each subplot represent total features in the respective group comparison (shown as x axis) that retained significance (FDR ≤ 0.1) plotted against the Cliff’s delta (y axis) for each set of features before (uncorrected) or after drug deconfounding (corrected). b. Box plots showing classifier performance comparison using HCs or MMCs as controls relative to individuals with IHD, based either on all microbial features (left) or on quantified metabolome features (right) as input (center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range; points, outliers). Two-sided MWU P values are included for each comparison. c. Pie chart (right) comparing the percent (shown as numbers) distribution of four enterotypes among various study groups. Table (left) shows the chi-squared value for each study group relative to the three control groups—that is, HC, MMC and UMMC. d. Box plots (upper) comparing gut bacterial gene richness among the indicated study groups (violin, distribution; center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range; points, outliers). Table (below) shows the two-sided MWU P values for each study group relative to the three control groups—that is, HC, MMC and UMMC.

Two-sided MWU and chi-squared tests were used for assessing the significance of group-wise comparisons in a, b, d and e, respectively, using HC (n = 275), MMC (n = 372), UMMC (n = 222), IHD (n = 372), ACS (n = 112), CHD (n = 158) and HF (n = 102) groups. Multiple testing corrections were done using the Benjamini–Hochberg method, and FDR ≤ 0.1 was considered significant. NS, not significant.
for uremic toxin 4-cresylsulfate. Similarly, phenylacetylglutamine, another uremic toxin derived from microbial phenylacetate and that acts through adrenergic receptors, showed an enrichment pattern from dysmetabolism to IHD. It was also shown (by ref. 25) to be inversely associated with SGB 4712. The findings implicate these metabolites as key targets for early intervention. 4-Cresol, in particular, has been found in lower concentrations in the blood of vegetarians than of omnivores; it has also been shown to inhibit...
colonocyte oxygen consumption and to be reduced once fat intake is curbed. In our study, this compound appeared as an ACS and CIHD escalation feature, and it was also one of the top markers specifically enriched in the blood of individuals with HF, likely related to its role in uremia, with dysregulation of fluid homeostasis being a key feature of HF (Extended Data Fig. 7). Interestingly, we also observe in another MetaCardis study that 4-cresol plays a causal role in the gut microbiome–kidney–heart axis in HCs, culminating in increased pro-ANP levels (Chechi et al., in revision).

Most DSCFs (89% and 100% for metabolites and predicted microbiome functions, respectively) exhibited the pattern of initial depletion at the stage of dysmetabolism but an apparent reversal at the stage of treated IHD (Fig. 4c). For instance, O-acetylsalicylate, the active component in aspirin, appeared as an archetypal DSCF putatively due to patient treatment compliance in IHD. Similarly, several catecholamine intermediates and end-products, bilirubin products, bile acids and odd-chain lipids with bacterial origin were identified as DSCFs. Moreover, TMA production (MC0022) and butyrate production II (MF0089) as gut microbial functional features exhibited a depletion at the dysmetabolism stage but an apparent restoration at the IHD stage (Extended Data Fig. 5). Overall, these observations might point toward a responsiveness of both microbiome and metabolome features to long-term multifactorial treatment, plausibly contributing to stabilization of IHD. In addition, achieving a stabilized IHD state appeared to involve restoring lost gut microbial cell density (Fig. 4c) alongside a capacity to degrade BCAAs and galactose while restoring lost capacity for butyrate and acetate production (Extended Data Fig. 5).

Microbiome and metabolome markers of IHD sub-phenotypes. Detailed analysis of ACS-, CIHD- and IHD-caused HF groups provided more granularity for relative shifts in microbiome and metabolome features (Fig. 5, Extended Data Figs. 6–9 and Supplementary Table 17). The total number of features typical for each IHD subgroup compared to controls was highest for CIHD, followed by HF and ACS. CIHD exhibited the most differential changes in the gut microbiome functional potentials (Extended Data Fig. 9), whereas ACS exhibited predominantly differential changes in metabolome features (Fig. 5, Extended Data Figs. 6–9 and Supplementary Table 17).
**Fig. 4 | Microbiome and metabolome features linked with IHD and its dysmetabolic pre-morbidities.** Using the categorization scheme described in Fig. 3 and Extended Data Fig. 4, gut microbiome and metabolome markers were categorized as DMFs, IHDs, ESCFs or DSCFs, of which IHDFs (a), ESCFs (b) and DSCFs (c) are displayed here. In each panel, arrow length shows effect sizes (Cliff’s delta) for respective group comparisons. Cliff’s delta for HC versus IHD comparisons are displayed for IHDs (a), whereas Cliff’s delta for both HC versus MMC and MMC versus IHD are displayed for ESCFs (b) and DSCFs (c), with arrowhead pointing to the direction of change. Only features exhibiting an absolute effect size greater than 0.1 are displayed, inclusive of serum metabolites, metagenomic species and microbial density indices (see Supplementary Table 17 for a description of effect sizes and confounding status). Two-sided MWU was used for assessing the significance of group-wise comparisons using HC (n = 275), MMC (n = 222) and IHD (n = 372) groups. Multiple testing corrections were done using the Benjamini–Hochberg method, and FDR ≤ 0.1 was considered significant.

*The metabolite was not validated by an internal standard but confirmed with great confidence according to information from Metabolon (Methods) who performed the analysis.* **An internal standard for the metabolite was not available but was confirmed with reasonable confidence according to information from Metabolon (Methods) who performed the analysis.*
Most (69%) of the dysmetabolism-linked species found by IHD versus HC comparisons were also present in comparisons of IHD subgroups versus HC, suggesting that the major disruption of the microbiome, which appears to be related to metabolic dysfunction, might persist throughout the various stages of IHD.

Strikingly, for the ACS subgroup, besides the 91 dysmetabolism-related species, no other species markers (ACS-specific, ESCF-related or DSCF-related) were found (Supplementary Table 17).

In the same ACS group, the pattern was very different for serum metabolites where only 55% of markers were related to dysmetabolism, whereas 25% were ACS-specific (Supplementary Table 17). We, thus, observed the acute disease phase being characterized by microbiome alterations almost exclusively related to dysmetabolism, presumably accumulating during the long prodromal stage, as well as host metabolome perturbations unrelated to dysmetabolism, presumably beginning only shortly before the ACS event. It is
tempting to suggest that the conjunction of the two might be conducive to some of the decompensation observed in ACS.

When considering the metabolome markers specific to ACS, eight of the top ten metabolites were drug analytes or drug metabolites, related to aspirin, metoprolol and atorvastatin. There was also an increase in pro-inflammatory metabolites such as 5- and 12-hydroxyeicosatetraenoic acid (HETE), leukotriene B4 and B5, as well as products of microbial–host phenylalanine co-metabolism (phenylacetyl carnitine, phenylacetylglutamate and 2-hydroxyphenylacetate), followed by indoxylsulfate and TMA, which is consistent with the identified overall IHD-specific signature. Likewise, some of the ACS-specific depleted metabolites were also less abundant in IHD, including health beneficial metabolites such as alpha-tocopherol, ergothioneine, methyl oleate and methyl hexadecanolate (Extended Data Fig. 6 and Supplementary Table 17).

In contrast to the findings in ACS, 19 and 31 specific species markers were found for CIHD and HF, respectively, indicating additional microbiome changes in the chronic phases of IHD. Noticeably, these changes affected genera represented by only a few species: eight of 14 depleted and 11 of 17 enriched species in HF cases, respectively, belonged to genera represented by no more than six species ($P = 2.9 \times 10^{-7}$) as estimated by the number of species belonging to different genera found in our study (Extended Data Figs. 7 and 8 and Supplementary Table 17).

Most CIHD-specific features were found in cases over controls (Extended Data Figs. 7 and 9 and Supplementary Table 17). This was particularly the case for microbiome functional potentials for amino acid biosynthesis, including BCAA (KEGG modules M00619, M00570 and M00432), methionine (KEGG module M00017) and lysine (KEGG module M00030) (Extended Data Fig. 9). Similarly, enhanced degradation of aromatic amino acids phenylalanine and tyrosine (GMM modules MF0027 and MF0026) was reflected by increased abundance of phenylacetate metabolites (phenylacetyl carnitine and phenylacetylglutamate). We also observed increased abundance of methionine and two of its metabolites ($\gamma$-amino-$\beta$-phenylpropionate and $\gamma$-glutamylmethionine), which are known to be associated with cardiovascular phenotypes.

Of interest, the gut microbiome-derived $\gamma$-methionine biosynthesis pathway was recently directly associated with atherosclerotic plaque burden and enhanced metabolic risk score for cardiovascular disease, whereas $\gamma$-methionine sulfoxide as a product of protein methionine oxidation might influence thrombosis and vascular function (Extended Data Figs. 7 and 9 and Supplementary Table 17). In addition, the abundance of multiple UPLC–MS/MS-quantified carnitines, including decanoylcarnitine and oleoylcarnitine, was elevated in CIHD.

Some metabolite features also exhibited HF specificity with an enrichment of 4-cresol, 4-cresyl sulfate (also called $p$-cresol sulfate), 4-cresylglucuronide (also called $p$-cresol glucuronide), choline and TMA as well as several carnitines (3-methylglutaryl carnitine, suberylcarnitine (C8), octadecanediocarnitine (C18) and levulinyl carnitine, including microbiome-derived carnitines (benzoylcarnitine and phenylacetylcarnitine)). In contrast, metabolites, such as alpha-tocopherol, ergothioneine and 3-indoleglyoxylic

**Fig. 6 | Validation of markers for ACS.** a-c. For the gut microbial and plasma metabolome features common to both MetaCardis and Israeli cohorts, a Spearman correlation analysis (a) was conducted between the effect sizes (Cliff’s delta) for HC versus ACS in each study after recalculating Cliff’s deltas in the Israeli population. Next, ROC curves depicting the classifier performance (AUROC) of five-fold cross-validated O-PLS-DA models based on the overlapped set of ACS biomarkers in three settings are shown for MetaCardis as the training population (b) and Israeli cohort as the test population (c). Model 1 included nine clinical ACS risk variables—that is, age, sex, BMI, systolic blood pressure, diastolic blood pressure, glycated hemoglobin (factorized as $5.5, 5.7–6.4$ and $6.6–7.6$ mmol l$^{-1}$), smoking status, fasting total cholesterol and HDL cholesterol (mmol l$^{-1}$). Model 2 included ACS-specific biomarkers identified in our study that were also found in ref. $^{25}$ (118 variables), whereas model 3 involved all variables considered for model 1 and model 2 (that is, 127 variables). Two-sided MWU was used for assessing the significance of group-wise comparisons using HC ($n = 275$) and ACS ($n = 112$) in MetaCardis population and HC ($n = 473$) versus ACS ($n = 156$) in the Israeli population. Multiple testing corrections were done using the Benjamini–Hochberg method, and FDR $\leq 0.1$ was considered significant.
acid, exhibited HF-specific depletion (Extended Data Fig. 8 and Supplementary Table 17). These findings point toward altered fatty acid metabolism, which is known to play a crucial role in HF pathogenesis\(^\text{31}\).

Classification of participants into clinical subgroups. Robustness of our microbiome and metabolome signatures was evaluated by comparing the performance of orthogonal partial least squares discriminant analysis (O-PLS-DA) models for classifying ACS \((n = 112)\), CIHD \((n = 158)\) and HF \((n = 102)\) relative to HC \((n = 275)\) and MMC \((n = 372)\) (Extended Data Fig. 10). Classification was based on (1) clinical markers routinely assessed during IHD diagnosis; (2) deconfounded microbiome and metabolome markers specific for each IHD subtype identified in the current study; and (3) a combination of the two. Models were built by randomly splitting our MetaCardis study population into groups of 70% and 30%, respectively, and using the former for training and the latter for testing; the process was iterated 1,000 times to minimize overfitting. The performance of the specific -omics markers on the testing sets yielded area under the curve (AUC) values greater than 0.7 in all cases and was systematically higher than that of clinical markers only, in particular for classification relative to the MMC group. Combination of the two marker types did not improve classification relative to MMC and only marginally improved classification relative to HC (Extended Data Fig. 10).

To validate our classification models further, we took advantage of the independent dataset from the companion paper\(^\text{25}\), focusing on our ACS subgroup to match the pathology of the Israeli study sample. ACS-specific metabolomics markers from the two studies were highly correlated (Cliff’s delta values computed relative to HC are shown in Fig. 6a and Supplementary Table 18), confirming that similar changes were observed in the two studies and validating a large fraction of our ACS-specific metabolome features. Notably, our markers exhibited strong discriminatory potential when employed in O-PLS-DA models trained in our population and tested in the independent Israeli population\(^\text{32}\). Models based on our ACS-specific metabolome markers with clinical variables (model 3, area under the receiver operating characteristic curve \((\text{AUROC} = 0.87)\) or without clinical variables (model 2, \text{AUROC} = 0.85) performed substantially better than a model based on clinical variables alone (\text{AUROC} = 0.764) (Fig. 6c).

Altogether, our work confirmed the robustness of the discriminatory potential of our deconfounded microbiome and metabolome markers in a clinical setting (Fig. 6 (metabolome markers) and Extended Data Fig. 10 (microbiome and metabolome markers)).

Discussion

We show that a vast majority of the intestinal microbiome and circulating and urine metabolome signatures that were previously reported as characteristic of IHD and that do not reduce to drug treatment effects are, in fact, already present in individuals with common dysmetabolic phenotypes, such as obesity and T2D. Our observations further align with the presence of a reduced gut bacterial cell density and changes in the abundance of multiple species and microbial functional potentials. Accounting for bacterial cell density, we identify the low-cell-count Bacteroides 2 enterotype\(^\text{33}\) as a biomarker both in individuals with dysmetabolism and in individuals diagnosed with IHD. We particularly highlight low gut bacterial cell count as one of the microbiome features linked with IHD, which appears to reverse in treated IHD cases. Interestingly, both the present paper and another recent MetaCardis publication\(^\text{12}\) suggest that statin drugs widely prescribed to individuals with CMD might help restore gut bacterial cell load. These results are particularly relevant because several statins and their drug metabolites (mostly related to atorvastatin) and β-blockers (metoprolol and its metabolites) are reflected in the here-identified specific signatures of IHD and its subtypes.

In individuals with diagnosed IHD and treatment-induced improvement of vascular, inflammatory and lipid health markers, we found less aberrant microbiome and metabolome profiles when compared to healthy individuals. Still, we found bacterial species specifically altered in IHD cases, and most of them were depleted in agreement with findings of the companion paper\(^\text{11}\). Similarly, we observed a depletion of IHD-specific metabolites, including the fatty acid esters, ergothioneine and alpha-tocopherol, known for vasoprotective\(^\text{1}\) and antioxidant properties\(^\text{2}\), whereas metabolites enriched in individuals with IHD included intermediates related to TMA and compounds derived from tryptophan and phenylalanine metabolism. Finally, 4-cresol and phenylacetylglutamine stood out as representatives of ESCF, potentially mirroring disease severity.

In IHD subtype analyses, we identified multiple dysmetabolism-related gut microbiome changes in individuals with ACS, further strengthening our hypothesis that gut microbiome alterations take place in the prodromal stages before the onset of IHD. In contrast, a substantial fraction of altered host metabolites (45%) in individuals with ACS was unrelated to dysmetabolism. In addition, we found alterations of the microbiome and metabolome that were specific for CIHD and HF, putatively conditioned by a conjunction of intervention and disease worsening.

Of relevance for actionable targets in future preclinical and clinical trials, we confirm reduced microbiome potentials for biosynthesis of SCFAs and increased production of BCAAs\(^\text{52}\) in individuals at increased risk of asymptomatic coronary atherosclerosis before IHD diagnosis. In the later phases of IHD pathogenesis, we show an overwhelming role for microbial–host metabolism of aromatic amino acids derived from phenylalanine and tyrosine—that is, emerging from phenylacetate and cresol co-metabolism. Thus, our findings suggest that, beyond diminishing microbial–host production of TMAO, future interventions aiming to delay or prevent IHD might be directed at increasing microbial SCFA biosynthesis but lowering microbial production of aromatic amino acids and BCCAs. Finally, the identified microbiome and metabolome features allowed us to stratify individuals with IHD from healthy individuals or metabolically matching individuals at levels above those achieved with conventional risk markers, pointing to their pathophysiological relevance.

In conclusion, at prodromal dysmetabolic stages and at both early and late clinical manifestations of IHD, multiple deconfounded microbiome and metabolome alterations are present, reflecting distinct metabolic pathways. Several of these are modifiable and might be targets for future mechanistic experiments and clinical trials aiming at IHD prevention.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41591-022-01688-4.

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Methods

Study design and participants. The MetaCardis project included HCs and individuals at increasing stages of dysmetabolism and IHD severity, aged 18–75 years old and recruited from Denmark, France and Germany between 2013 and 2015. IHD cases were classified into patients with first case of ACS (<15 days); patients CIHD with normal heart function; and patients with documented HF and IHD as demonstrated by echocardiography—evaluated LVEF less than 45%. Our study encompassed 372 individuals with IHD (112 with ACS, 158 with CIHD and 102 with HF caused by CIHD). In addition, 275 HCs were matched on demographic characteristics, as well as 222 UMMS participants with features of the metabolic syndrome but receiving no lipid-lowering, anti-diabetic or anti-hypertensive drugs. Finally, we included 372 controls matched with individuals with IHD in terms of T2D status and BMI (referred to as MMGs).

Exclusion criteria were known confounders of the gut microbiome—that is, antibiotic use in the 3 months before inclusion, past history of abdominal cancer with and without radiation therapy, intestinal resection except for appendectomy and inflammatory or infectious diseases, including hepatitis B, hepatitis C or HIV.

Additionally, patients with a history of organ transplantation, patients receiving immunosuppressants, patients with estimated glomerular filtration rate (eGFR) <50 ml/min/1.73 m² and patients with drug or alcohol addiction were excluded. Ethical approval was obtained from the Ethics Committee CPP Ille-de-France, the Ethics Committee of the Capital Region of Denmark (H-3-2013-145) and the Ethics Committee at the Medical Faculty at the University of Leipzig (047-13-28012013). All study participants provided written informed consent, and all clinical investigations were undertaken according to Helsinki Declaration II.

Bioscientific variables. Clinical measurements were made using standardized operating procedures concluded before patient recruitment. Bioscientific variables included age, sex, BMI, smoking status, dietary intake, physical activity and drug intake. We obtained habitual dietary information using food frequency questionnaires adapted to cultural habits of each of country of recruitment. Smoking status was obtained from a standardized questionnaire, and information on physical activity was assessed using the Recent Physical Activity Questionnaire. Drug intake was assessed either by recall or from medication list, and a medical specialist interviewed study participants about adherence to prescribed medication. T2D was defined as fasting plasma glucose ≥7.0 mmol/l and/or HbA1c ≥6.5% and/or individuals taking any glucose-lowering agents. Hypertension was defined as systolic blood pressure >140 mmHg and/or diastolic blood pressure <90 mmHg and/or individuals taking anti-hypertensive drugs. Echocardiography enabled the measurement of LVEF for diagnosis of HF (LVEF <45%). Renal function was assessed via eGFR calculated using the Modification of Diet in Renal Disease equation. Blood was collected in the morning after an overnight fast. Plasma and serum samples were stored at the clinical centers at −80°C until shipment to a central laboratory facility. Fasting plasma glucose, total and HDL cholesterol, triglycerides, creatinine and HbA1c levels were measured using standard enzymatic methods. LDL cholesterol concentrations were measured enzymatically for German participants or by the Friedwald equation for French individuals or by the Fermi Laboratory equation for Danish participants. Alanine aminotransferase, aspartate aminotransferase and γ-glutamyltransferase were measured by an enzyme-coupled kinetic method. Ultra-sensitive C-reactive protein was measured using an Image Automatic Immunoassay System (Beckman Coulter). High-sensitivity IL-6 was measured using the Human IL-6 Quantikine ELISA Kit (R&D Systems). IFN-γ-induced protein 10 (IP-10) and C-X-C motif chemokine ligand 5 (CXCL5), CCL2, Eotaxin, IL7, MIF, MIP1b, SDF1 and VEGFa were measured using a Luminex assay (ProcartaPlex Mix&Match Human 13-plex, eBioscience). Plasma pro-ANP was measured using a processing-independent assay.

Stool sample collection. Stool samples were processed according to International Human Microbiome Standards (HMS) guidelines (SOP 03 V1). Samples were collected by study participants at home and immediately stored at −20°C until they were transported on dry ice and frozen 4–24 h later at −80°C in plastic tubes at the biobanks of corresponding recruitment centers.

Microbial load measurement by flow cytometry. Microbial loads of fecal samples were processed and analyzed as described. Briefly, 0.2 g-frozen (−80°C) aliquots were suspended in physiological solution to a total volume of 100 ml (8.5 g l⁻¹ NaCl, VWR); the slurry was diluted 1:1000 times; and samples were filtered using a sterile syringe filter (pore size 5 μm, Sartorius). Next, 1 ml of the microbial cell suspension was stained with 1 µl of SYBR Green I (1:100 dilution in DMSO, shacked 15 min incubation at 37°C, 10,000 concentration, Thermo Fischer Scientific). The flow cytometry analysis was performed with a C6 Accuri flow cytometer (BD Biosciences) based on Prest et al. Events were monitored using the FL1 533/30-nm and FL3 670-nm detectors. Instrument and gating settings were kept identical for all samples (fixed staining/gating strategy), and cell counts were converted to microbial loads per gram of fecal material (microbial load index).

Stool sample processing and metagenomic analyses. Total fecal DNA was extracted following the IHMS guidelines (SOP 07 V2 H). Samples were sequenced in a non-randomized order using ion proton technology (Thermo Fisher Scientific) resulting in 23.3 ± 4.0 million (mean ± s.d.) single-end short reads with an on-average length of 150 bases. Sequencing was carried out with standardized protocols at a single site (Metagenerome) over a period of 18 months. There was no significant bias in sequencing data for different Metacardis groups (Kruskal–Wallis P value of 0.4 for HC, MMC, UMMS and IHD groups). Reads were cleaned using Alien Trimmer (version 0.4.0) to (1) remove resilient sequencing adapters and (2) trim low-quality nucleotides at the 3’ side using a quality and length cutoff of 20 bp and 45 bp, respectively. Cleaned reads were subsampled and filtered from human and other possible fungal contamination using HMM using human genome RCR37-p10, Bos taurus and Arabidopsis thaliana with an identity score threshold of 97%. Gene abundance profiling was performed using the 9.9 million gene integrated reference catalog of the human microbiome. Filtered high-quality reads were mapped with an identity threshold of 95% to the 9.9 million gene catalog using Bowtie2 (version 2.3.4) included in METEOR version 3.2 included in METEOR. Filtered high-quality reads were mapped with an identity threshold of 95% to the 9.9 million gene catalog using Bowtie2 (version 2.3.4) included in METEOR version 3.2. First, reads mapped to a unique gene in the catalog were attributed according to the ratio of their unique mapping counts. The gene abundance table was processed for rarefaction and normalization and further analysis using the MetaOMineR (momr, version 1.31) R package. To decrease technical bias due to different sequencing depth and to avoid any artifacts of sample size on low-abundance genes, read counts were rarefied. The gene abundance table was rarefied to 10 million reads per sample by random sampling of 10 million mapped reads with replacement. The resulting rarefied gene abundance table was rarefied according to fragments per kilobase of transcript per million mapped reads (FPKM) approach to give the gene abundance profile table. Metagenomic species (MGS) are co-abundant gene groups with more than 50 genes corresponding to microbial species. In total, 1,436 MGS were clustered from 1,267 human gut microbiome samples used to construct the 9.9 million gene catalog. MGS abundances were estimated as the mean abundance of the 50 genes defining a robust centroid of the cluster (if more than 10% of these genes gave positive signals). Abundances were corrected for bacterial cell count by multiplying by an index factor calculated as the bacterial cell count of the sample divided by the mean value of this bacterial cell count over the dataset as a whole. MGS taxonomical annotation was performed using all genes by sequence similarity using NCBI blast Nt, a species-level assignment was given if more than 50% of the genes matched the same reference genome of the NCBI database (November 2016 version) at a threshold of 95% of identity and 90% of gene length coverage. Remaining MGS were assigned to a given taxonomical level from genus to super-kingdom level, if more than 50% of their genes had the same level of assignment. MGS richness (MGS count) was calculated directly from the rarefied MGS abundance matrix. Bacterial gene richness (gene count) was calculated by counting the number of genes detected at least once in a given sample, using the average number of genes counted in ten independent rarefaction experiments. MGS richness (MGS count) was calculated directly from the rarefied MGS abundance matrix.

Customized microbial module analysis. Customized module sets included previously described GMMs covering bacterial and archaeal metabolism specific to the human gut environment with a focus on anaerobic fermentation processes, expanded with a specific set of six modules focusing on in bacterial TMA metabolism. Additionally, after a previously published strategy to build a manually curated gut-specific metabolic modules, we constructed a novel set of 20 modules describing microbial phenylpropanoid metabolism (phenylpropanoid metabolism modules) from shotgun metagenomic data. Abundances of customized modules were derived from the ortholog abundance tables using Omixer-RPM version 1.0 (https://github.com/caeslab/omixer-rpm). The coverage of each metabolic variant encoded in a module was calculated as the number of steps for which at least one of the orthologous groups was found in a metagenome, divided by the total number of steps constituting the variant. The coverage of the GMM was defined as equal to the one of the variants with maximum coverage. Module presence/absence was identified with a detection threshold of more than 95% coverage to provide ortholog annotations and protein identification in metagenomes. Module abundance was calculated as the median of KEGG orthology abundance in the pathway with maximum coverage. Abundances were corrected for bacterial cell count similarly to MGSs.

Metabolic profiling. We deployed a comprehensive metabolic phenotyping strategy combining in-house analysis by proton nuclear magnetic resonance (1H-NMR) spectroscopy, gas chromatography coupled to mass spectrometry (GC–MS) and targeted UPLC–MS/MS data generated by Metabolon, as described in detail below:

1H-NMR spectroscopy. 1H-NMR experiments were carried out using a Bruker Avance spectrometer operating at 600 MHz, as reported. Structural assignment was performed using data from literature, the Human Metabolome Database (http://www.hmdb.ca/), S-Base (Bruker) and in-house databases. 1H-NMR
spectra were pre-processed and exported to MATLAB for multivariate statistical analyses using O-PLS-DA, as previously reported. Absolute metabolite quantifications were also derived using Bruker’s In Vitro Diagnostics for research (IVDr) quantification algorithms.

**GC-MS semi-targeted profiling.** Sample sera were prepared, analyzed and processed using standard protocols. In brief, serum samples (100 μl) were cleaned up with methanol protein precipitation, evaporated to dryness, derivatized and injected to an Agilent 7890B-5977B Inert Plus GC–MS system. The chromatographic column was an Agilent ZORBAX DB5-MS (30 m x 0.25 mm x 0.25 μm + 10 m Duragard). The temperature gradient was 37.5 min long, and the mass analyzer was operated in full-scan mode between 50 m/z and 600 m/z. Peaks were annotated with the use of the Fiehn library (Agilent G1676AA Fiehn GC/MS Metabolomics RTL Library, User Guide, Agilent Technologies, https://www.agilent.com/cms/content/us/en/products/public/G1676-90019_fiehn.pdf). Metabolites were reported with high reproducibility (CV < 30%) and linearity (that is, dilution signal rho > 0.9 and false discovery rate (FDR)–corrected P < 0.05 (one-tailed Spearman)) were kept in the final dataset, resulting in 102 annotated metabolic features.

**UPLC–MS/MS isotopic quantification of methylamines and carnitines.** UPLC–MS/MS was employed for the determination of absolute concentrations for TMA, TMAO, choline, betaine, γ-butyrobetaine, betaine-aldehyde, butyryl-carnitine, isovaleryl-carnitine, OH-isovaleryl-carnitine, stearoyl-carnitine, oleoyl-carnitine, linoleoyl-carnitine, myristoyl-carnitine, lauroyl-carnitine and decanoyl-carnitine. Serum samples (50 μl) were prepared as follows: (1) samples were spiked with 10μl of Internal Standard solution (14C/15N-TMA, d₃-TMAO, d₂-choline, d₄-isovaleryl carnitine and d₂-betaine in water,1 μg l⁻¹; Sigma–Aldrich); (2) 30 μl of ethyl-2-bromoacetate solution (22.5 g l⁻¹ of ethyl-2-bromoacetate and 1.4% NH₄OH in acetonitrile) was added, and derivatization of trimethylamines (TMA and 14C/15N-TMA) to their ethoxy-analges was completed after 30 min at room temperature; and (3) 910 μl of protein/liquid precipitation solution (94% acetonitrile:5% water (v/v) + 1% formic acid) was added; samples were centrifuged for 20 min (4°C at 20,000 g), and 400 μl of the supernatants was transferred to UPLC autosampler 500-μl-well plates. Sample injections (5 μl, full loop) were performed to a Waters Acuity UPLC-Xevo TQ-S UPLC–MS/MS system equipped with an Acquity BEH HILIC (2.1 x 100 mm, 17.5 μm) chromatographic column. An isocratic elution was applied with 10mM ammonium formate in 95:5 (v/v) acetonitrile:water for 11.5 min at 500 μl/min, then (2) 910 μl of methanol protein precipitation, evaporated to dryness, derivatized and injected to an Agilent 7890B-5977B Inert Plus GC–MS system. The temperature gradient was 37.5 min long, and the mass analyzer was operated in full-scan mode between 50 m/z and 600 m/z. Peaks were annotated with the use of the Fiehn library (Agilent G1676AA Fiehn GC/MS Metabolomics RTL Library, User Guide, Agilent Technologies, https://www.agilent.com/cms/content/us/en/products/public/G1676-90019_fiehn.pdf). Metabolites were reported with high reproducibility (CV < 30%) and linearity (that is, dilution signal rho > 0.9 and false discovery rate (FDR)–corrected P < 0.05 (one-tailed Spearman)) were kept in the final dataset, resulting in 102 annotated metabolic features.

**Statistical analyses.** Downsampled microbiome functional profile and taxonomic composition data, metabolite and quantitative clinical phenotype measurements were assessed between and within groups using non-parametric tests (MWU and Spearman test) corrected for multiple testing using the Benjamini–Hochberg approach. For each feature significantly (FDR < 0.1) associated with defined phenotype status, it is checked whether it has significant associations with any potential confounder. If not, it is considered trivially unconfounded (not confounded (NC)). If at least one covariate also has significant association with the phenotype, then, for each such covariate, a post hoc test for confounding is applied. This test takes the form of nested linear model comparisons (likelihood ratio test for P values), where the dependent variable is the feature (X), and the independent variables are the disease status (A) and the tested covariate (B) versus a model containing only the covariate (B), thus testing whether disease status (A) adds explanatory statistical power beyond the covariate (B). If this holds (likelihood ratio test (LRT) P < 0.05) for all covariates (B), then disease status is confidently deconfounded (CD) concerning its effect on feature X; it cannot be reduced to any confounding factor. For each covariate (B) where significance is lost, a complementary modeling test is performed of the null model (X = A) versus the full model (X = A + B), thus testing whether the covariate (B) in turn is equally reducible to (A). If for at least one such covariate (B), (B) has independent effect (LRT P > 0.05) on top of (A), then the feature (X) is considered confounded by (B). However, if none of the pairwise tests the original significance holds, then (A) and (B) are considered so correlated that their relative influence cannot be disentangled. We consider these cases laxly deconfounded (LD), in the sense that, for these cases, clear confounding influence can neither be concluded nor ruled out. The R package was applied to the present dataset considering medication status either as binary variables or as normalized dosages.

Our deconfounding pipeline takes into account linear effects related to drug categories. Still, we were not able to control for every possibly lifestyle confounding factor, making a lack of full confounding adjustment a limitation of our study.

**Supplementary information on data availability is linked to the online version of the paper at www.nature.com/nature. Raw shotgun sequencing data that support the findings of this study have been deposited in the European Nucleotide Archive with accession codes PRJEB37249, PRJEB38742, PRJEB43131 and PRJEB43132.**
Code availability
The novel drug-aware univariate biomarker testing pipeline, described in full elsewhere, is available as an R package (metadefconfoundR; Birker et al., manuscript in preparation) on GitHub (https://github.com/TillBirkner/metadefconfoundR) and also at https://doi.org/10.5281/zenodo.7241078. The latest version (0.1.8) of this package was used to generate the data shown in this publication. In addition, the scripts used to perform the analysis presented here are available at https://doi.org/10.5281/zenodo.5516219.

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present study. K.C. has held a collaborative research contract with Danone Research in the context of the MetaCardis project. The other authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | Overview of selected bio-clinical variables of the various groups. Box plots (above) representing the distribution of key bio-clinical variables in various study groups (lower line, lower quartile; medium line, median; upper line, upper quartile). Table (below) shows the two-sided MWu P for respective group comparisons using HC (n = 275), MMC (n = 372), UMMC (n = 222), IHD (n = 372), ACS (n = 112), CIHD (n = 158), HF (n = 102). IHD: ischemic heart disease patients, HC: healthy controls, MMC: metabolically matched controls, UMMC unmedicated metabolically matched controls, ACS: acute coronary syndrome, CIHD: chronic IHD, HF: heart failure due to CIHD, BMI: body mass index; HbA1c: glycated haemoglobin, pro-ANP: pro-atrial natriuretic peptide, MWu: Mann-Whitney U.
Extended Data Fig. 2 | Microbiome findings from the literature. Cuneiform plot shows literature review of gut microbial taxonomic and predicted functional features reported to be associated with IHD, while highlighting their individual replication in the present MetaCardis study group either as a general dysmetabolism biomarker (seen only in case of HC versus MMC), or as an IHD biomarker (seen also in case of MMC versus IHD) (Supplementary Table 15). The literature review was performed as a keyword search in PubMed (Medline) using combinations of the words ‘microbiota’ and ‘microbiome’ with the word ‘atherosclerosis’, ‘cardiovascular disease’, ‘coronary artery disease’, ‘ischemic heart disease’, ‘myocardial infarction’, ‘acute coronary syndrome’, ‘angina pectoris’ and ‘heart failure’. Studies61–72 were identified that met the following criteria: 1) published during the recent 15 years, 2) reporting data from human studies with at least 15 participants, 3) using culture-independent methods for microbiota profiling and 4) evaluating the link between human microbiota and manifestations of impaired heart disease (Supplementary Table 16). Results on functional features were derived from four studies using whole-genome shotgun sequencing68,71,72. Results imputed from 16S rRNA gene analyses were not included. Point marker color and size reflect MetaCardis findings (Cliff’s delta), with arrows displaying direction of effects. Literature findings are shown at a uniform effect size. Markers are shown only for features significantly different in abundance (FDr < 0.1) and have a bold border if they cannot be reduced to the confounding influence of any drug or drug combination prescribed to treat dysmetabolism. While the majority of literature findings are recaptured in our study when comparing HC and IHD, relatively fewer were found in MMC and IHD comparisons, implying them to be general markers of dysmetabolism rather than being IHD-only microbiome markers. Two-sided MWu tests were used for assessing the significance of group-wise comparisons using HC (n = 275), MMC (n = 372), uMMC (n = 222) and IHD (n = 372) groups. Multiple testing corrections were done using Benjamini-Hochberg method and FDr < 0.1 was considered significant. IHD: ischemic heart disease patients, HC: healthy controls, MMC: metabolically matched controls, MWu: Mann-Whitney-U tests, FDr: false-discovery rate.
Extended Data Fig. 3 | Distribution of differential features among various group comparisons pre- and post- deconfounding. (a) Venn diagrams showing the comparative shift in the number of gut microbiome and metabolome features that remain differentially abundant (FDr < 0.1) in various group comparisons when healthy individuals (HC) and drug-treated IHD cases are compared to untreated metabolically matched controls (UMMC) or (b) drug-treated metabolically matched controls (MMC) without any adjustments for potential confounders followed by (c) drug-deconfounding. Two-sided MWu tests were used for assessing the significance of group-wise comparisons using HC (n = 275), MMC (n = 372), UMMC (n = 222) and IHD (n = 372) groups. Multiple testing corrections were done using Benjamini-Hochberg method and FDr <= 0.1 was considered significant. IHD: ischemic heart disease patients, MWU: Mann-Whitney-U tests, FDR: false-discovery rate.
Extended Data Fig. 4 | Operational classification of microbiome and metabolome features from the perspective of IHD pathology. A classification tree was constructed based on significance and alignment of effect size and directionality of microbiome and metabolome features in the various group comparisons leading to the identification of: Features that reflect metabolic dysregulation in the individual but are not associated with diagnosed IHD: dysmetabolism features (DMF). Features that are significantly associated with IHD but are also significantly altered in metabolically dysregulated individuals in the same direction; we suggest that these features are early markers of IHD pathogenesis in individuals with metabolic dysregulation: IHD escalation features (ESCF). Features that are significantly associated with IHD but are also significantly altered in metabolically dysregulated individuals in the reverse direction; we suggest that these features are early markers of IHD seen in metabolically dysregulated individuals. However, they exhibit reversibility. This may plausibly be due to 1) long-term drug-treatment and improvement in overall lifestyle of the IHD individuals, 2) a compensatory response to the initiation of disease or 3) a trajectory-associated differential response to disease development. We propose that some of these features contribute to the stabilization of IHD and dysmetabolism and we coin those IHD de-escalation features (DSCF). IHD-specific features (IHDF) that achieve a significant shift only under IHD diagnoses. Two-sided MWU tests were used for assessing the significance of group-wise comparisons using HC (n = 275), MMC (n = 372), uMMC (n = 222), IHD (n = 372), ACS (n = 112), CIHD (n = 158), HF (n = 102) groups. Multiple testing corrections were done using Benjamini-Hochberg method and FDR < 0.1 was considered significant. HC: healthy controls, MMC: metabolically matched controls, uMMC: unmedicated metabolically matched controls, IHD: ischemic heart disease, ACS: acute coronary syndrome, CIHD: chronic IHD, HF: heart failure due to IHD, MWU: Mann-Whitney U, FDR: false-discovery rate.
Extended Data Fig. 5 | Gut microbial functional features categorization. Gut microbial functional features (GMM and KEGG modules) categorized as escalation-, de-escalation-, and IHD-specific biomarkers when features classification scheme (as shown in Fig. 3, Extended Data Fig. 4 and described in supplementary methods) was applied to various group comparisons involving HC, MMC and IHD subjects. HC: healthy controls, MMC: metabolically matched controls, IHD: ischemic heart disease.
Extended Data Fig. 6 | Features categorization for ACS subgroup. Microbiome and metabolome features categorized as escalation-, de-escalation-, and ACS-specific biomarkers when features classification scheme (as shown in Fig. 3, Extended Data Fig. 4 and described in supplementary methods) was applied to various group comparisons involving HC, MMC and ACS groups. HC: healthy controls, MMC: metabolically matched controls, ACS: acute coronary syndrome, eSCF: escalation features, DSCF: De-escalation features. Gut microbiome features included taxonomic (prefix: Taxon) and microbiome density indices, whereas metabolome features included serum and urinary metabolites. Only features exhibiting absolute effect size > 0.1 are displayed whereas the full list is given in Supplementary Table 17).
**Extended Data Fig. 7 | Features categorization for CIHD subgroup.** Microbiome and metabolome features categorized as escalation-, de-escalation- and CIHD-specific biomarkers when features classification scheme (as shown in Fig. 3, Extended Data Fig. 4 and described in supplementary methods) was applied to various group comparisons involving HC, MMC and CIHD groups. HC: healthy controls, MMC: metabolically matched controls, CIHD: chronic IHD. eSCF: escalation features, DSCF: De-escalation features. Gut microbiome features included both taxonomic (prefix: T axon) and microbiome density indices, whereas metabolome features included serum and urinary metabolites. Only features exhibiting absolute effect size > 0.1 are displayed whereas the full list is given in Supplementary Table 17.)
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Features categorization for HF subgroup. Microbiome and metabolome features categorized as escalation-, de-escalation- and HF-specific biomarkers when features classification scheme (as shown in Fig. 3, Extended Data Fig. 4 and described in supplementary methods) was applied to various group comparisons involving HC, MMC and HF groups. HC: healthy controls, MMC: metabolically matched controls, HF: heart failure due to CHD. ESCF: escalation features, DSCF: De-escalation features. Gut microbiome features included both taxonomic (prefix: Taxon) and microbiome density indices, whereas metabolome features included serum and urinary metabolites. Only features exhibiting absolute effect size > 0.1 are displayed whereas the full list is given in Supplementary Table 17).
Extended Data Fig. 9 | Gut microbial functional features categorization for IHD subgroups. Microbial functional features (GMM and KEG modules) categorized as escalation-, de-escalation- and subtype-specific biomarkers when features classification scheme (as shown in Fig. 3, Extended Data Fig. 4 and described in supplementary methods) was applied to various group comparisons involving HC, MMC and IHD subgroups (that is, ACS, CIHD and HF). HC: healthy controls, MMC: metabolically matched controls, ACS: acute coronary syndrome, CIHD: chronic IHD, HF: heart failure due to CIHD. Only features exhibiting absolute effect size > 0.1 are displayed whereas the full list is given in Supplementary Table 17).
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Discriminatory potential IHD subtype-specific features. We compared clinical variables assessed for risk prediction in the companion paper25 (Model 1) with our IHD subgroup-specific gut microbiome and metabolomic features (Model 2) and a combination of the two (Model 3) for their discriminatory potentials using orthogonal partial least squares- discriminant analysis (O-PLS-DA; ropls r package). Model 1 included ten variables (that is age, sex, body mass index, waist circumference, hip circumference, waist to hip ratio, systolic blood pressure, diastolic blood pressure, glycated haemoglobin (factored as > 5.7, 5.7-6.4 and < 6.4 mmol/l) and smoking status). Model 2 included each IHD subgroup-specific metagenomic species and fasting serum metabolites. Model 3 involved a combination of model 1 and 2 variables. OPLS-DA models were trained on 70% of the subgroup specific sample and then tested in 30% of the remaining subgroup sample using 1000 iterations of random sampling (bootstrapping). Boxplots represent the distribution (center line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range; points, outliers) of area under the receiver operating characteristic (ROC) curves derived from 1000 bootstraps based on these models in the training set (A) and test set (B) using both healthy controls (HC, n=275) and metabolically matched controls (MMC, n=372) relative to the IHD subtype cases (ACS, n=112, CIHD n=158 and HF n=102). Models were compared using Kruskal-Wallis test and Dunn’s pairwise multiple comparisons post hoc testing with Bonferroni correction. Dunn’s test P are shown for each comparison. As expected, the model performance improves significantly for model 2 and 3 relative to model 1, respectively, when either HC or MMCs are used as controls for IHD cases in test samples. HC: healthy controls, MMC: metabolically matched controls, IHD: ischaemic heart disease. ACS: acute coronary syndrome, CIHD: chronic IHD, HF: heart failure due to CIHD.
Reporting Summary

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
See MS and Online Methods for details; METEOR v3.2 (https://forgemia.inra.fr/metagenopolis/meteor), Allentrimmer v0.4.0, Bowtie2 v2.3.4, MetaOMiner (momr, v1.31), Gmixer-RPM (v1.0) were each used to process microbiome data. MasslynxTM (Waters corporation; Version 4.2) software was used for UPLC-MS/MS data acquisition and analysis.

Data analysis
Most analysis was conducted using the R statistical language as described in Methods and Online Methods. In particular the package metaconfoundR (v0.1.8 - see https://github.com/tilibirkner/metaconfoundR or https://doi.org/10.5281/zenodo.4721078) was employed. In addition we applied custom R and Perl scripts (see Code Availability or https://doi.org/10.5281/zenodo.3556219).

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Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
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- A description of any restrictions on data availability

Supplementary information on data availability is linked to the online version of the paper at www.nature.com/nature. Raw shotgun sequencing data that support the findings of this study have been deposited in European Nucleotide Archive with accession codes PRJEB37249, PRJEB38742, PRJEB41311, and PRJEB46098 with public access. Metabolome data have been uploaded to Metabolights and MassIVE with respective accession numbers i.e., serum UPLCMS, serum NMR and urine NMR with accession number MTBLS3429, serum GCMS with accession number MassIVE MSV000088042, and additional isotopically quantified serum metabolites.
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
No prior power calculation was carried out but sample size was selected so as to exceed that of the MetaHIT study, which was adequately powered.

**Data exclusions**
No subjects for which data was available was excluded during analysis.

**Replication**
The study was hypothesis generating and observational, not experimental. Reproducibility of findings was assessed by comparing present findings with 1) prior literature and 2) another ACS cohort from the back to back Talmor-Bar et al. paper 3) assessment of whether each association is reproduced [significant or trending] in the different disease subcohorts. Where testable, most findings replicate in these assessments. In more detail, as we report several hundred findings, the subsequent reproduction/consistency status of each is listed in the manuscript itself as well as in Supplementary Table 15, 16 and 18, respectively.

**Randomization**
As no intervention or experiment was made, only observation, there is no intervention to randomize and as such randomization is neither well-defined, applicable, meaningful or relevant.

**Blinding**
Investigators (clinicians and study nurses) were aware of clinical diagnosis by necessity and default, but blinded to any laboratory, clinical or -omics data as that was generated by others from biosamples. Analysts (data managers, statisticians, bioinformaticians) were blinded by having access only to pseudonymized data, and performed no manual analyses - all statistics and visualization were undertaken using computer software not taking any group allocation into account except where testing for associations to it. As such, no analysts awareness of any group allocation (diagnosis) affected outcomes of any statistical analysis, and only results of such analysis in aggregate were used to draw conclusions and for interpretations of results. In this sense analysis is as blinded as is at all possible in an -omics biomarker study, and in line with standards of the field.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a  | Involved in the study |
|------|------------------------|
| ☒    | Antibodies            |
| ☒    | Eukaryotic cell lines  |
| ☒    | Palaeontology and archaeology |
| ☒    | Animals and other organisms |
| ☒    | Human research participants |
| ☒    | Clinical data         |
| ☒    | Dual use research of concern |

### Methods

| n/a  | Involved in the study |
|------|------------------------|
| ☒    | ChiP-seq               |
| ☒    | Flow cytometry         |
| ☒    | MRI-based neuroimaging |

Human research participants

Policy information about [studies involving human research participants](https://www.nature.com/articles/d41586-020-00024-z)

**Population characteristics**

The European MetaCardis project included healthy control individuals and individuals at increasing stages of dysmetabolism and ischaemic heart disease (IHD) severity, aged 18–75 years old, and recruited from Denmark, France and Germany between 2013 and 2015. IHD cases were recruited solely in Denmark and France and were classified into: patients with first case of acute coronary syndrome (ACS), patients with chronic IHD but normal heart function and another similar group with documented heart failure (HF) and IHD as demonstrated by left ventricular ejection fraction (LVEF) < 45% evaluated with echocardiography. In total, the study encompassed 372 IHD cases including 112 with acute coronary syndrome (ACS), 158 with chronic ischaemic heart disease (CHD) and 102 with combined ischaemic heart disease and heart failure (HF). In addition, we included 275 healthy controls (HC) matched on demographics, age and sex, and 222 untreated metabolically matched controls (UMMC); i.e. individuals with features of the metabolic syndrome and thus at increased risk
of IHD but receiving no lipid-lowering or anti-diabetic or anti-hypertensive drugs. Finally, we included 372 controls matched with IHD cases on T2D status and body mass index (BMI), thereafter termed metabolically matched controls (MMC). A large number of covariate-relevant population characteristics was tracked. These are described in Supplementary Tables 1-4.

Recruitment
The applied recruitment scheme resulted in a proband/patient population that match individuals who do/do not require care for the diseases in question, which does not credibly introduce any biases with bearing on the specific questions the study asks. While there is an uneven distribution of individuals from different clinical groups between the study sites, study site (France, Germany, Denmark) was included as a covariate, with findings reducible to the influence of this variable filtered out. As such, the recruitment strategy is not likely to bias the results.

Ethics oversight
The study protocol was approved by the Ethics Committee at the Medical Faculty at the University of Leipzig, the Ethical Committee of the Capital Region of Denmark and the Ethics Committee CPP île-de France. All participants provided written informed consent and all investigations were conducted according to Helsinki declaration.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data
Policy information about clinical studies
All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration
The study protocol was registered at clinicaltrial.gov (NCT02059538).

Study protocol
Available from the study promoter: Assistance Publique-Hôpitaux de Paris (AP-HP).

Data collection
This is described in greater detail in the manuscript and companion manuscripts, but involve hospital regions of Paris, Copenhagen and Leipzig during period 2012-2016.

Outcomes
No outcomes were tracked; cross-sectional study.