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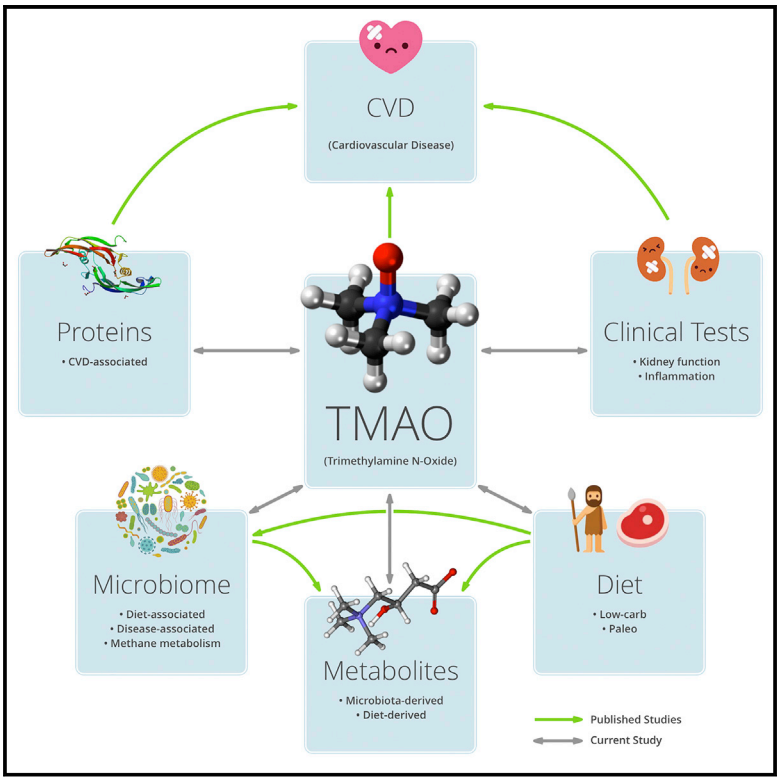
A Multi-omic Association Study of Trimethylamine N-Oxide

Graphical Abstract



Highlights

- TMAO is independently associated with demographic factors and kidney function
- Gut microbiota-derived metabolites are significantly associated with TMAO
- Diet- and disease-associated metabolites are significantly associated with TMAO
- Proteins linked to cardiovascular and kidney disease are correlated with TMAO

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In Brief

Trimethylamine N-oxide (TMAO) is a circulating metabolite that has been implicated in the development of atherosclerosis and cardiovascular disease. Manor et al. identify blood markers, metabolites, proteins, gut microbiota patterns, and diets that are significantly associated with levels of plasma TMAO.

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A Multi-omic Association Study of Trimethylamine N-Oxide

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SUMMARY

Trimethylamine N-oxide (TMAO) is a circulating metabolite that has been implicated in the development of atherosclerosis and cardiovascular disease (CVD). In this paper, we identify blood markers, metabolites, proteins, gut microbiota patterns, and diets that are significantly associated with levels of plasma TMAO. We find that kidney markers are strongly associated with TMAO and identify CVD-related proteins that are positively correlated with TMAO. We show that metabolites derived by the gut microbiota are strongly correlated with TMAO and that the magnitude of this correlation varies with kidney function. Moreover, we identify diet-associated patterns in the microbiome that are correlated with TMAO. These findings suggest that both the process of TMAO accumulation and the mechanism by which TMAO promotes atherosclerosis are a complex interplay between diet and the microbiome on one hand and other system-level factors such as circulating proteins, metabolites, and kidney function.

INTRODUCTION

The metabolite trimethylamine N-oxide (TMAO) has been recently implicated in humans as being proatherogenic and as an independent prognostic marker for developing cardiovascular disease (CVD), a leading cause of disability and mortality (Koeth et al., 2013; Tang et al., 2015; Zhu et al., 2016). In animal models, TMAO has been shown to directly cause atherosclerosis and thrombosis (Chen et al., 2016; Koeth et al., 2013, 2014; Tang et al., 2015). Although the mechanisms behind these outcomes are not well understood, evidence from human and mouse studies suggests one possible mechanism being that TMAO promotes platelet aggregation (Zhu et al., 2016, 2017).

There are two major sources contributing to the levels of circulating TMAO. First, it can be acquired directly from diet, for example, from certain seafood that contains TMAO (Cho et al., 2017). Second, the gut microbiota can metabolize certain substrates such as carnitine and choline into trimethylamine (TMA), which is then oxidized by liver enzymes to form TMAO. These substrates are found mainly in animal-based products such as red meat, eggs, and dairy, which may explain why TMAO levels in omnivores have been shown to be elevated compared to vegans and vegetarians (De Filippis et al., 2016; Koeth et al., 2013). In addition to these input sources, the kidneys filter out TMAO from the blood and excrete it through urine, and TMAO has been shown to promote kidney damage, explaining the observed strong association between lower kidney function and elevated TMAO levels (Stubbs et al., 2016; Tang et al., 2015).

Previous studies have been performed to elucidate the pathogenic impact of elevated TMAO levels on the risk for developing cardiovascular disease, and a number of studies measured the levels of TMAO in humans in combination with other omic types. For example, Koeth et al. (2013) measured TMAO and profiled the gut microbiome of 53 individuals, and Cho et al. (2017) measured TMAO and profiled the gut microbiome of 40 individuals. Randrianarisoa et al. (2016) and Stubbs et al. (2016) measured TMAO and clinical laboratory tests (including kidney function) in 220 and 104 individuals, respectively. However, these studies lack the sample size and breadth of multiple omic types to both discover novel associations and to identify patterns that span multiple omics. Here, we present a large-scale, multi-omic, cross-sectional study of TMAO in 648 individuals. We identify significant associations between TMAO and other data types, including blood markers, plasma metabolites, plasma proteins, the gut microbiome, and dietary patterns. In addition, to understand the predictive power of these associations, we use a machine-learning algorithm to predict levels of TMAO in individuals. Finally, we use a clustering-based approach to assess whether individuals with elevated TMAO display different metabolic profiles.

RESULTS

Characteristics of the Multi-omic Cohort

The data presented in this study were collected from 648 individuals participating in a commercially available wellness program (see Experimental Procedures). Participants were 52% female and 75% white, with an average age of 50 ± 13 years, average body mass index (BMI) of 26 ± 5.4, average glomerular filtration rate (GFR) of 89 ± 16 mL/min/1.73 m^2, and average blood urea

Table 1. Summary of Demographics and Relevant Biomarkers in Individuals

		TMAO Quartiles				
Feature	Overall	Q1	Q2	Q3	Q4	p Value Q1 versus Q4
Number of Individuals	648	162	162	162	162	N/S
Sex (% female)	52%	57%	54%	53%	45%	0.035
Age	50 ± 13	45 ± 11	50 ± 12	53 ± 13	54 ± 12	4.86E-10
Ethnicity (% white)	75%	63%	72%	83%	81%	0.0002
BMI	26 ± 5.4	26 ± 5	26 ± 4.8	27 ± 5.9	27 ± 5.6	N/S
GFR	89 ± 16	95 ± 14	89 ± 16	87 ± 15	84 ± 17	1.28E-09
BUN/CREAT	17 ± 4.1	16 ± 3.9	17 ± 3.8	18 ± 4.4	18 ± 4	0.0001

CREAT, creatinine.

nitrogen (BUN)-to-creatinine ratio of 17 ± 4.1 (Table 1). For each participant, 918 biomarkers were measured from blood samples taken at baseline, including 71 clinical laboratory tests, 619 metabolites, and 228 proteins (Table S1). In addition, individuals collected a baseline stool sample that was assayed using 16S-based sequencing to profile the composition of the gut microbiome.

TMAO Is Independently Associated with Demographic Factors and Kidney Function

We first compared the levels of circulating TMAO with the levels of the 71 clinical labs across all individuals (Table S1) and found that 27 of them were significantly associated with TMAO (Table S1; false discovery rate [FDR]-corrected $p < 0.05$, Spearman correlation test). The most significant negative and positive associations were with the kidney markers GFR and BUN ($\rho = -0.21$, $p < 10^{-5}$; $\rho = 0.26$, $p < 10^{-8}$, respectively; see Figure 1). The association between kidney function and TMAO is not surprising since the kidneys filter out TMAO from the blood and previous studies have reported this association (Randrianarisoa et al., 2016). Interestingly, adjusting TMAO and the clinical lab values for the age, sex, and race of individuals attenuated the significance of these associations but most remained significant (Table S1). In addition, we found that TMAO level at baseline was a prognostic marker for kidney function (as measured by GFR) in a follow-up measurement (6 months later), adjusting for baseline GFR, sex, and age ($p < 0.001$; see Figure S1). This result is consistent with previous studies that showed that elevated circulating TMAO can directly cause kidney damage in animal models suggesting that elevated TMAO can also damage the kidneys (Tang et al., 2015). Notably, 97% of the individuals in our study had normal kidney function (i.e., GFR > 60), highlighting the predictive power of TMAO even within the normal range of kidney function.

Comparing TMAO levels to the age, sex, and BMI of individuals, we found that older age and being male were significantly correlated with higher levels of TMAO ($\rho = 0.28$, $p < 10^{-12}$; $\rho = 0.08$, $p < 0.05$; respectively), while higher BMI was positively correlated but not significant ($\rho = 0.07$, $p = 0.054$). Importantly, after adjusting for differences in GFR, age and sex remained independently and significantly correlated with TMAO levels ($\rho = 0.18$, $p < 10^{-5}$; $\rho = 0.09$, $p < 0.05$, for age and sex, respectively).

TMAO Is Associated with Nutrition, Inflammation, and Blood Protein Markers

In addition to the association we identified between TMAO levels and kidney markers, we further identified 16 significant sex- and age-adjusted associations between TMAO and clinical labs (FDR-corrected $p < 0.05$; Table S1). Interestingly, the levels of omega-3 fatty acids were significantly positively correlated with TMAO ($\rho = 0.135$, $p < 0.05$). One possible explanation for this finding is that a major source of omega-3 fatty acids is seafood, some types of which also contain high levels of TMAO (Zhang et al., 1999). Strengthening this hypothesis, we found that both mercury and vitamin D levels, which are known to increase with consumption of certain fish (Bradley et al., 2017; Lehmann et al., 2015), were also significantly positively correlated with TMAO levels ($\rho = 0.11$, $\rho = 0.13$, respectively; $p < 0.05$). In addition, we found that interleukin-8 was significantly positively correlated with TMAO ($\rho = 0.16$, $p < 0.005$). Finally, we found that both the total protein and globulin levels were significantly negatively correlated with TMAO levels ($\rho = -0.13$, $\rho = -0.12$; $p < 0.02$, $p < 0.02$, respectively). Lower levels of total protein and globulin have been previously linked to decreased kidney function (Levey et al., 2003), which might explain this association.

Gut Microbiota-Derived Metabolites Are Associated with TMAO

To explore the relationship between the metabolome and TMAO, we calculated the Spearman correlation between TMAO and 618 circulating metabolites. Using a significance threshold of $p < 0.05$ after FDR correction, the levels of 252 metabolites were found to be significantly correlated with TMAO levels (Table S1). Remarkably, among the top six metabolites positively correlated with TMAO were five metabolites that are a direct product or derivative of the gut microbiota (Barrios et al., 2015; Meyer and Hostetter, 2012; Tanaka et al., 2015): hydroxyindole sulfate ($\rho = 0.37$, $p < 10^{-19}$), phenylacetylglutamine (PCG) ($\rho = 0.4$, $p < 10^{-23}$), indoxyl-sulfate (IS) ($\rho = 0.32$, $p < 10^{-14}$), p-cresol-sulfate (PCS) ($\rho = 0.31$, $p < 10^{-13}$), and 4-methylcatechol sulfate ($\rho = 0.32$, $p < 10^{-14}$). Intriguingly, in previous studies of patients with chronic kidney disease, three of these metabolites were found to be associated with cardiovascular disease (Lin et al., 2015; Poesen et al., 2016), suggesting a link between gut microbiome-derived metabolites, TMAO, and atherosclerosis.

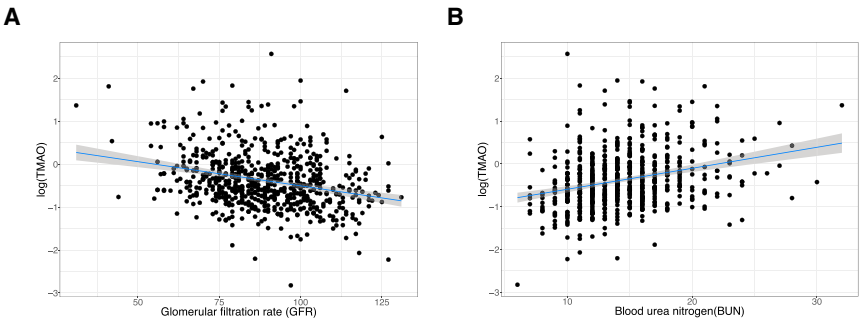

Figure 1. TMAO Levels Are Correlated with Kidney Markers

Shown are the scatterplots of TMAO levels (y axis) versus levels of the kidney markers (x axis) glomerular filtration rate (GFR) (A) and blood urea nitrogen (BUN) (B). The blue line shows a linear fit to the scatterplot (with gray band showing confidence interval).

In addition, PCS and IS production was shown to be markedly reduced in vegetarians compared to omnivores (Patel et al., 2012), again linking animal-based diets to elevated levels of TMAO and these metabolites. To test whether these associations were only a by-product of lower kidney function, we conducted two additional analyses. In the first analysis, we re-calculated the correlations restricting our samples only to individuals with normal kidney function (GFR $\geq$ 90) and the associations between TMAO and microbiome-derived metabolites remained highly significant (Table S1). In the second analysis, we adjusted the levels of each metabolite (including TMAO) across individuals by their value of GFR using a linear model (Experimental Procedures) and we re-calculated the correlations on the residuals of these models. We again found that even after adjusting for GFR value, the associations between TMAO and microbiome-derived metabolites remained highly significant (Table S1).

To further explore the relationship between metabolites, TMAO, and kidney function, we defined a group of individuals with high kidney function (HKF) (upper quartile of GFR; n = 162; median GFR of 107), and a group with low kidney function (LKF) (bottom quartile of GFR; n = 162; median GFR of 71). Next, within each group, we compared the levels of all 619 metabolites between individuals with reduced levels of TMAO and individuals with elevated TMAO (i.e., individuals in the bottom or upper quartile of TMAO values) and corrected for multiple hypotheses using FDR. In the LKF group, no metabolite showed a significant difference between the elevated versus low TMAO groups except for TMAO itself. However, in the HKF group, there were five metabolites that showed significant differences with respect to TMAO levels. Out of these five metabolites, four were the same microbiota-derived metabolites discussed above (Figure 2), suggesting that in individuals with elevated TMAO but high kidney function, the microbiome plays a key role in TMAO levels. The additional metabolite found (methionine sulfone) is an oxidized form of methionine. Methionine is a precursor for carnitine biosynthesis and was shown to be correlated with carnitine in plasma samples (Krajcovicová-Kudlácková et al., 2000), suggesting a possible link to TMAO.

Diet- and Disease-Associated Metabolites Are Associated with TMAO

Additional metabolites were identified in the analysis above to be associated with TMAO. Specifically, we found that the metabolites choline and carnitine, which are present mainly in animal products are strongly correlated with levels of circulating TMAO. Importantly, these metabolites are the most common substrates of microbiome-derived TMAO. Moreover, carnitine-containing metabolites had a significantly stronger positive correlation with TMAO compared to all other metabolites ($p < 0.01$, Wilcoxon rank-sum test). The metabolite 1-methylhistidine, a marker of meat consumption (Cross et al., 2014), was the third most correlated with TMAO, suggesting again that diet is a strong driver of metabolic associations with TMAO.

In addition, several metabolites containing glycerylphosphorylcholine (GPC) were found to be significantly positively correlated with TMAO. Interestingly, acetyl GPC is a known platelet aggregation factor (Albert and Snyder, 1983), which is one of the proposed mechanisms by which TMAO promotes atherosclerosis. Another metabolite found to be significantly positively correlated with TMAO was dimethylguanosine ($\rho = 0.25$, $p < 10^{-8}$), which was shown to be significantly elevated in the urine of colorectal cancer patients (Zheng et al., 2005), an intriguing finding given that TMAO has been recently linked to colorectal cancer (Xu et al., 2015).

Proteins Associated with Cardiovascular and Kidney Disease Are Correlated with TMAO

We calculated the Spearman correlation between levels of 228 circulating proteins and TMAO, and after adjusting for multiple tests using an FDR cutoff of $p < 0.05$, 40 proteins remained significantly associated with TMAO (Table S1). The most positively correlated protein was growth/differentiation factor 15 (GDF-15) ($\rho = 0.23$, $p < 10^{-5}$), which remained significant even when accounting for kidney function. Interestingly, GDF-15 has been shown to predict all-cause mortality in a large cohort of stable coronary heart disease patients, even after adjusting for known prognostic biomarkers (Hagström et al., 2017). As TMAO was also shown to be an independent prognostic marker of mortality and cardiac events (Li et al., 2017; Tang et al., 2013; Wang et al., 2011, 2014), this strong association with GDF-15 is intriguing and necessitates further research.

Several proteins from the galectin family were found to be significantly correlated with TMAO, including galectin-9 and galectin-3 ($\rho = 0.18$, $p < 10^{-3}$; $\rho = 0.12$, $p < 0.05$, respectively), which were both shown to be higher in the serum of atherosclerotic stroke patients compared to controls (He et al., 2017). Additional significantly associated proteins were adrenomedullin ($\rho = 0.18$, $p < 10^{-3}$), a protein reported to be higher in patients suffering from cardiovascular or kidney disease (Bunton et al., 2004; Ishimitsu et al., 1994), and kidney injury molecule 1 ($\rho = 0.17$, $p < 10^{-2}$), a protein that has been shown to be induced

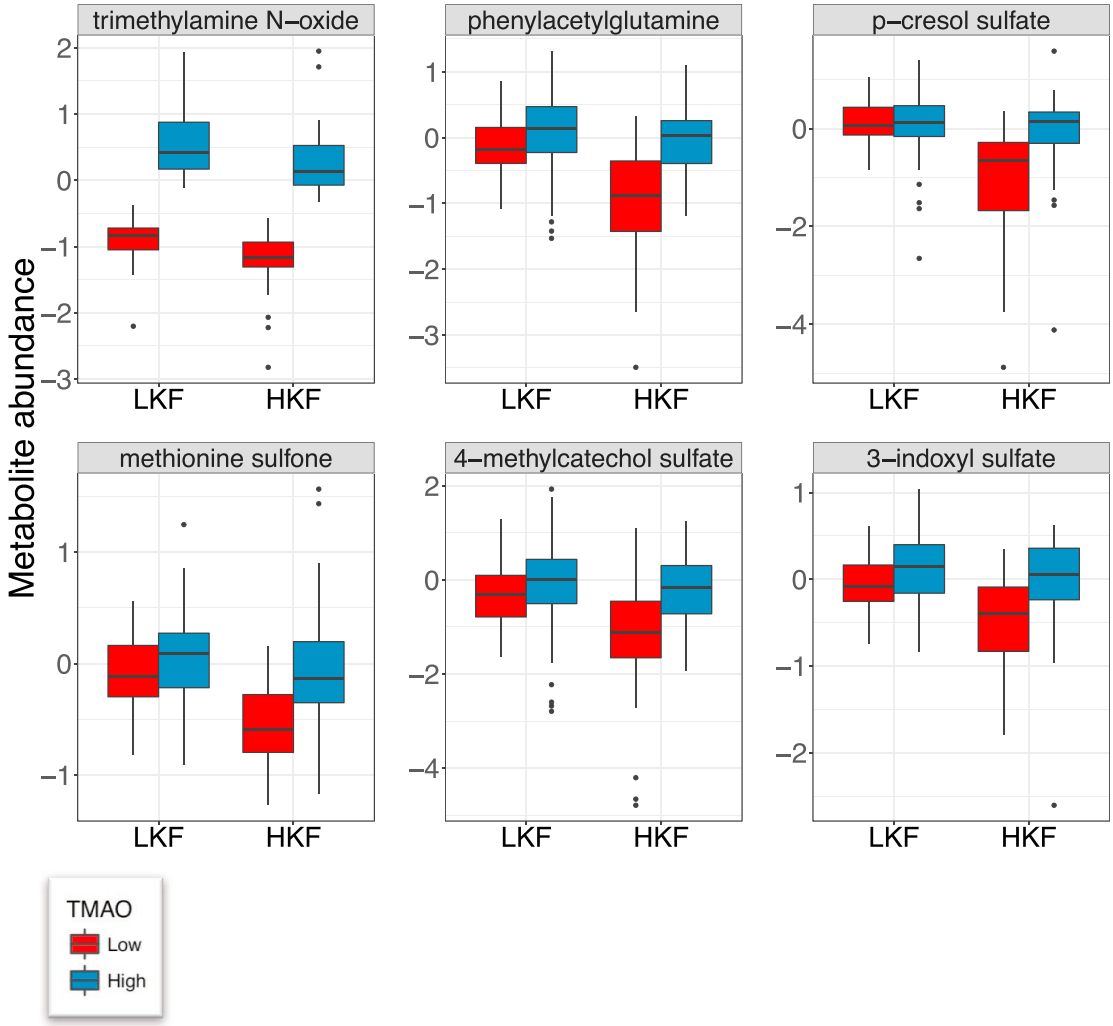

Figure 2. Metabolites that Are Significantly Higher Only in High Kidney Function Individuals with Elevated TMAO Levels

Shown are the boxplots representing the distributions of metabolite values (log-transformed) across four different groups of individuals: low kidney function (LKF) and low TMAO (left pair; red box), low kidney function and high TMAO (left pair; blue box), high kidney function (HKF) and low TMAO (right pair; red box), and high kidney function and high TMAO (right pair; blue box). TMAO (top left metabolite) is shown as an example of a metabolite that is significantly different in both the low kidney function groups and the high kidney function groups, while the other five metabolites are only significantly different in the high kidney function pair. Whiskers extend to the lowest and highest values within 1.5 times of the interquartile range (IQR).

in experimental kidney injury and in biopsies of renal disease (van Timmeren et al., 2007; Waanders et al., 2010).

Microbial Clades that Are Correlated with TMAO Are Diet and Disease Associated

Since the composition of the gut microbiome has been shown to be associated with the levels of circulating TMAO (Koeth et al., 2013), we examined the relationship between TMAO and the microbiome in our cohort. Specifically, we calculated the correlation between TMAO and the relative abundance of microbial clades and identified clades that are significantly positively or negatively correlated with TMAO levels (Table S1). Of note, species found to be negatively correlated with TMAO were mostly of the family *Bifidobacteriaceae* or its genus *Bifidobacterium* (e.g., *B. longum*). This family has been previously reported to be negatively correlated with TMAO in mouse (Chen et al., 2016) and human (Zhang et al., 2015) studies, and the genus *Bifidobacterium* was reported to significantly decrease in individuals consuming an animal-based diet (David et al., 2014). Additional microbial clades were found to be positively correlated with the levels of TMAO, many of which have been previously reported to correlate with TMAO levels across individuals, to contain enzymes that can produce TMA from choline, to increase in abundance in symptomatic CVD patients, or to associate with an animal-based diet (Table 2).

Methane Metabolism in the Microbiome Is Positively Correlated with TMAO

Having identified microbial clades that are correlated with TMAO, we next examined whether certain community-wide

Table 2. Selected Microbial Clades that Are Significantly Correlated with TMAO

Clade	Direction	Previous Findings
Bifidobacterium	negative	reported to be negatively correlated with TMAO in mice and human, and to decrease in individuals consuming an animal-based diet (Chen et al., 2016; David et al., 2014; Zhang et al., 2015)
Treponema spp.	negative	treponemal infection was shown to produce antibodies that are cardioprotective and reduce the rates of atherosclerosis (Agmon-Levin et al., 2009)
Desulfovibrio	positive	multiple member species have been shown to produce TMA from choline (Craciun and Balskus, 2012)
Dehalobacterium	positive	reported to be positively correlated with TMAO (Wang et al., 2015)
Shuttleworthia	positive	belongs to the family *Lachnospiraceae*, which was shown to be positively correlated with TMAO (Chen et al., 2016; Cho et al., 2017), to decrease in plant-based diets, and to increase in animal-based diets (David et al., 2014)
Synergistes	positive	belongs to the phylum Synergistetes, which was shown to contain species that encode TMA-related enzymes (Jameson et al., 2016)
Neisseriaceae	positive	reported to be more abundant in the atherosclerotic plaques of symptomatic patients than in those of asymptomatic ones (Mitra et al., 2015)
Coriobacteriaceae	positive	reported to be positively correlated with TMAO (Wang et al., 2015; Zhu et al., 2016), to encode enzymes that can convert choline to TMA (Martínez-del Campo et al., 2015), and to increase in an omnivore diet (De Filippis et al., 2016)
Clostridiaceae	positive	reported to increase in omnivores and to be positively correlated with TMAO (Koeth et al., 2013)
Peptococcaceae	positive	reported to be positively correlated with TMAO (Chen et al., 2016; Org et al., 2017) and to contain genera that are higher in omnivores (Wu et al., 2016)
Christensenellaceae	positive	reported to be positively correlated with TMAO (Bell, 2016)

functional capacities are associated with TMAO levels. We used PICRUSt (Langille et al., 2013) to predict the functional composition (represented as Kyoto Encyclopedia of Genes and Genomes [KEGG] orthology groups [KOs]) of each sample from the 16S-based taxonomic composition. We then calculated the Spearman correlation between the abundance of each KO and TMAO across all individuals and corrected the p values for multiple hypotheses using FDR. We identified a total of 895 KOs that were significantly positively (762 KOs) or negatively (133 KOs) correlated with levels of TMAO (FDR-corrected $p < 0.05$; Table S1). To examine whether there are broader functional categories (e.g., pathways) that are associated with TMAO levels, we took two different approaches. In the first approach, we calculated pathway abundances for each sample as the sum of corresponding KO abundances and calculated the correlation between these pathway sums and TMAO levels across all samples. After correcting for multiple hypotheses, no pathway showed significant association with TMAO.

In the second approach, we initially calculated the correlation between the abundance of each KO and TMAO across all samples and defined a subset of KOs that are significantly associated with TMAO. Next, we examined whether there are pathways that contain a significantly higher number of TMAO-associated KOs than expected by chance. Using this approach, we identified the methane metabolism pathway as being significantly enriched for TMAO-associated KOs ($p < 10^{-4}$, hypergeometric test, FDR-corrected $p < 0.05$). Notably, TMA is one of the substrates in methane metabolism. Therefore, one possible explanation for this finding is that elevated levels of circulating TMA promote the growth of methane-producing archaea that can utilize it as a carbon source (Brugère et al., 2014; Hippe et al., 1979).

Paleo and Low-Carbohydrate Diets Are Associated with Elevated Levels of TMAO

As part of this multi-omic study, self-reported adherence to specific diets was collected. Out of 648 individuals, 36, 18, and 50 self-reported as eating a vegetarian, paleo, or low carbohydrate diet, respectively. Although these numbers were relatively small, we found that, among these 104 individuals, diet was significantly associated with the levels of circulating TMAO, even when controlling for age and sex. Specifically, both paleo and low-carbohydrate diets (typically rich in animal-based products) were significantly positively associated with TMAO compared to the vegetarian diet ($p < 10^{-3}$, for both, Student's t test; percentiles of mean TMAO levels were the 72nd percentile, 68th percentile, and 26th percentile for paleo, low-carbohydrate, and vegetarian diets, respectively).

Predicting TMAO Levels Using a Random Forest Algorithm

Having identified multi-omic associations with the levels of TMAO, we sought to understand their combined predictive power. Put differently, we were interested in understanding whether these factors were independent in their association versus co-correlated (which would limit their combined predictive power). In addition, we wanted to assess the amount of variation that is accounted for by these factors versus other unmeasured factors.

We divided our data using a 10-fold cross-validation scheme. In each training set, we re-calculated the correlations between TMAO and the multi-omic data and used these correlations to select a subset of biomarkers as input data for the algorithm (Experimental Procedures). Next, we learned a random forest regression model (Breiman, 2001) with TMAO levels as the outcome (based only on the training data) and tested this model

Table 3. Selected Proteins that Were Important Features in the Random Forest Model

Proteins	Previous Findings
Chitotriosidase-1	implicated in the development of atherosclerosis (Boot et al., 1999; Kitamoto et al., 2013)
P-selectin, IL-8	reported to be significantly higher in unstable coronary heart disease patients in comparison to stable coronary heart disease patients or controls (Romuk et al., 2002)
JAM-A, P-selectin, and MMP-1	reported in different studies to induce platelet activation and to enhance aggregation and thrombus formation (Babinska et al., 2002; Galt et al., 2002; Théorêt et al., 2011)
CDCP1	genetic copy number variants in the CDCP1 gene locus were found to be significantly associated with myocardial infarction (Shia et al., 2011)
CD40 ligand	reported to be mainly derived from activated platelets and to contribute to the pathophysiology of atherosclerosis (Lievens et al., 2010; Pamukcu et al., 2011)
Caspase-3	expressed in atherosclerotic plaques and its levels are correlated with measures of atherosclerosis (Matulevicius et al., 2008)
TNF receptors	reported to be negatively correlated with kidney function and to predict cardiovascular disease in patients with chronic kidney disease (Bae et al., 2017)

TNF, tumor necrosis factor.

on the held-out test data to evaluate its performance. To better understand the predictive power obtained by additional omic types, we applied this learning process first to clinical lab and microbiome data alone, and then evaluated the addition of proteomics and metabolomics data to the predictive power of the model. When only using clinical lab and microbiome data, the model explained on average 14% of TMAO variability in test data (R^2 = 0.14 ± 0.04 across ten cross-validation test sets). Many of the microbiome features found to be important in this random forest model were the same clades we identified to be significantly correlated with TMAO levels (see full list in Table S2). Interestingly, however, a few clades that did not pass our significance threshold for correlation with TMAO, were among the top important features in the random forest model, for example, the order *RF-32* (phylum Proteobacteria) that was previously reported to be positively correlated with TMAO and lesions in mice (Gregory et al., 2015) and to be higher in omnivores (Wu et al., 2016).

When we added the proteomics data to the feature set, the predictive power of the model increased slightly but consistently across different cross-validation sets to 15% variance explained. Notably, the TMAO-associated proteins we identified in the correlation analysis above were not necessarily the top-most important protein features in the random forest model, suggesting that their impact might be captured by other features (e.g., microbiome features). Among the top proteins that were found to be important in the random forest model (Table 3; see full list in Table S2) were proteins reported to be implicated in CVD and atherosclerosis, and specifically to induce platelet activation and aggregation. When we also added the metabolomics data as features (excluding TMAO; with or without the proteomics data), the predictive power increased markedly to 28% variance explained (Figure 3), indicating that the global metabolic profile can explain a substantial fraction of TMAO variability in individuals.

Metabolome-Based Clustering Offers Insight into Different Profiles of Individuals with Elevated TMAO

Having identified various metabolites that are associated with TMAO levels, we were interested to explore whether individuals with elevated levels of TMAO all share these associations, or whether they could be classified into different profiles. To this end, we selected the 162 individuals in the upper quartile of TMAO levels (adjusted for age and sex) and applied hierarchical clustering to their metabolic profiles consisting of 619 metabolites (Figure S2; see Experimental Procedures). We defined the number of clusters to be 5 using a silhouette analysis (Rousseeuw, 1987). Next, to identify the metabolites that distinguished these clusters, we compared the values of each metabolite across clusters and identified 139 metabolites that significantly differed in one of the clusters compared to all other clusters (Table S3; FDR-corrected $p < 0.05$, Wilcoxon rank-sum test). Interestingly, carnitine-containing metabolites were markedly enriched in this list of significantly different metabolites ($p < 10^{-12}$, hypergeometric test). For these 32 carnitine-containing metabolites, we found that cluster 3 had the lowest levels, clusters 4 and 5 had relatively high levels, and clusters 1 and 2 did not show a distinct pattern (Figure S3). As we noted above, another significant precursor for microbiome-derived TMA is dietary choline. We found that choline- or GPC-containing metabolites were also significantly enriched in the list of differentiating metabolites ($p < 0.001$, hypergeometric test). In most of these choline-containing metabolites, cluster 1 had the highest or second-to-highest level (Figure S4), suggesting that for individuals in cluster 1, the high TMAO levels might be driven primarily by choline rather than carnitine. Interestingly, cluster 2, which did not show a strong signal for either carnitine- or choline-containing metabolites, had significantly higher levels for all eight of eight diacylglycerol metabolites that are significantly different in at least one cluster (Figure S5). Diacylglycerol can be produced from an intermediate in the glycolysis pathway (glycerol-3-phosphate) and can be converted to phosphatidylcholine, one of the precursors for TMAO (Hu et al., 2012; Seibel and Walsh, 2002).

Next, we tested whether there were clinical labs or proteins that differed between these metabolome-based clusters. We identified 14 different clinical labs (Table S3; see Figure S6) and 9 proteins (Table S3; see Figure S7) that were significantly different in at least one cluster compared to all others (cluster 5

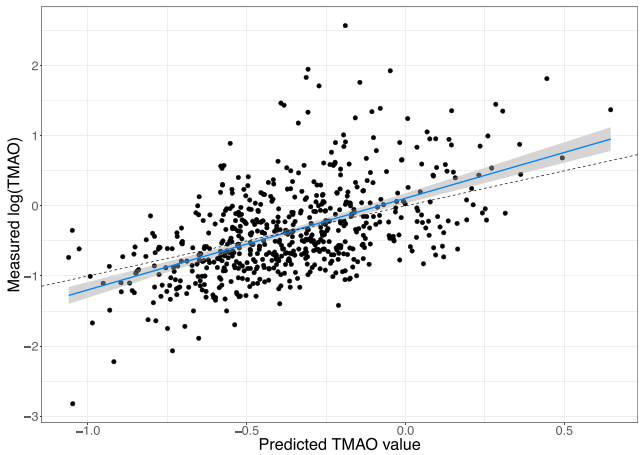

Figure 3. Random Forest Model Predicting TMAO Value

Shown is the scatterplot of predicted TMAO value (x axis) versus the log-transformed measured TMAO value (y axis) across all individuals (Pearson's correlation R = 0.54). Importantly, individuals are plotted only once, for the fold they were part of in the *held-out test set*. The blue line shows a linear fit to the scatterplot (with gray band showing confidence interval), and the dashed line shows the x = y line.

did not show statistical significance due to its small size). Cluster 2 had significantly higher diabetes and cholesterol blood markers (e.g., low-density lipoprotein [LDL] cholesterol and insulin), significantly higher levels of proteins related to these markers (e.g., LDL receptor, PAI-1), and significantly lower levels of the anti-diabetic protein IGFBP-2 (Hedbacker et al., 2010). Notably, when we conducted the same analysis comparing the taxonomic and functional compositions of the microbiome between clusters, we did not identify any feature that was statistically significant after correcting for multiple hypotheses. A summary of the significant differences between clusters is presented in Table 4.

DISCUSSION

TMAO is an emerging risk factor in the development of CVD. Here, we present a large-scale, cross-sectional, multi-omic analysis of the associations between TMAO and different blood markers, metabolites, proteins, diet, and gut microbiome patterns. We validated the previously reported strong association between TMAO and the kidney marker GFR. Notably, we further showed that TMAO is a prognostic biomarker for kidney function independent of age, sex, and baseline GFR (Figure S1) in line with studies showing that elevated levels of TMAO can damage the kidneys. We found that TMAO is correlated with age even after accounting for GFR, suggesting that other age-related mechanisms (beside decrease in kidney function) might be involved in elevated levels of TMAO. Previous studies of humans and animal models have reported mixed results on the relationship between sex and TMAO, with some studies finding higher levels in males (Chen et al., 2015; Mueller et al., 2015; Stubbs et al., 2016), and some in females (Bennett et al., 2013; Obeid et al., 2017). The studies in mice with restricted diets showed that the mechanism by which TMAO levels vary by sex is through hormonal impact on the expression of the *FMO3* gene (which converts TMA to TMAO). In our cohort, males were found to have significantly higher levels of TMAO, even after adjusting for age and kidney function.

Notably, we did not observe any significant correlations between TMAO and established blood markers for CVD such as total cholesterol, LDL, high-density lipoprotein (HDL), or triglycerides (Table S1), even when excluding individuals taking cholesterol-lowering medications (data not shown). This might in part explain why TMAO has been found to be a prognostic marker for CVD beyond traditional risk factors (Li et al., 2017). We also identified an age- and sex-adjusted significant association between TMAO and the inflammatory marker interleukin-8 (IL-8) (in both the clinical labs panel and the independent proteomics panel) that is notable given that a recent study found that TMAO can promote vascular inflammation through the activation of nuclear factor κB (NF-κB) signaling (Seldin et al., 2016).

We additionally found that TMAO is correlated with omega-3 fatty acids, mercury, and vitamin D (Table S1), suggesting a possible link between fish consumption and TMAO levels. We found that consuming animal-rich diets was significantly associated with increased TMAO levels compared to vegetable-rich diets in a small subset of 104 individuals that reported following such diets. These results are consistent with multiple studies that demonstrate that vegetarians have lower TMAO levels compared to non-vegetarians (De Filippis et al., 2016; Koeth et al., 2013; Zhu et al., 2017). Examining levels of circulating proteins, we found many CVD-related proteins that are associated with the levels of TMAO (Table S1). Specifically, several of the TMAO-associated proteins we identified have been implicated in the process of platelet aggregation. As TMAO was also shown to induce platelet aggregation in human cells *in vitro* (Zhu et al., 2016), our findings suggest a mechanistic link between TMAO, proteins, and CVD.

Looking across both metabolomics and gut microbiome composition, we identified three intriguing patterns (Table 2; Table S1). First, we identified several metabolites and microbial clades associated with TMAO that were previously reported to be differentially abundant in individuals following meat- versus plant-based diets, highlighting the impact of diet on both the gut microbiome composition and TMAO. Second, many of the metabolites we identified as associated with TMAO are derivatives of metabolic processes of the gut microbiota. This result is in line with studies showing that TMAO levels are strongly impacted by the composition of the gut microbiome. Third, we found that the association of these microbiota-derived metabolites with TMAO is markedly more significant in individuals with higher kidney function compared to those with lower kidney function. These results suggest that, in individuals with higher kidney function, the elevated levels of TMAO are more strongly affected by microbiota activity, while in individuals with low kidney function, elevated levels of TMAO are more strongly affected by the lack of TMAO filtering by the kidneys.

In addition to examining the taxonomic composition of the gut microbiome, we examined the functional composition using predicted KEGG profiles. We found that the microbial methane metabolism pathway was significantly enriched in genes that are positively correlated with TMAO. One plausible explanation

Table 4. Summary of Significant Differences between Elevated-TMAO Clusters

Cluster	Size	Females	Age	Significantly Higher in	Significantly Lower in
1	47	70%	56 ± 12	choline-containing metabolites	N/S
2	22	36%	52 ± 10	glycerol-containing metabolites; diabetes and cholesterol blood markers; diabetes and cholesterol regulating proteins; BMI	anti-diabetic protein
3	45	69%	44 ± 12	N/S	carnitine-containing metabolites; cholesterol markers; liver and kidney damage markers
4	42	17%	46 ± 12	males; carnitine-containing metabolites; creatinine, ferritin, and hematocrit; the protein MMP3	leptin
5	6	33%	61 ± 1	carnitine-containing metabolites	N/S

for this association is the fact that TMA can be a precursor to methane. Therefore, higher levels of diet- and microbiota-derived TMA would drive both higher levels of TMAO and would promote the growth and utilization of TMA as a substrate in methane production by other microbial clades. Specifically, studies showed that methanogenic archaea can utilize circulating TMA as a carbon source in the production of methane, a finding that has led to a recent suggestion to utilize such archaea as probiotics to decrease levels of TMA and TMAO in the blood (Brugère et al., 2014; Hippe et al., 1979). Our finding that microbial methane metabolism is significantly positively correlated with TMAO levels suggests that simply harboring more methane-producing microbes might not be sufficient to significantly reduce TMA and TMAO levels if that is not accompanied by subsequent dietary changes.

Although we identified numerous significant associations between multiple omics data and TMAO levels, we wanted to understand what fraction of the variability in TMAO levels they can explain in a strict cross-validation setting. In other words, are these associations merely significant in the statistical sense, or are they also predictive? To this end, we trained a random forest model using strict 10-fold cross-validation (Experimental Procedures) and found that we can explain 28% of the variability in TMAO levels on held-out test data. Importantly, the main aim of the random forest analysis was not to claim that such a model could perfectly predict TMAO levels or as means to test for disease susceptibility, but rather to assess how much of the variation is accounted by the factors we measured. Indeed, our results suggest that many of the correlations and associations we identified are indeed valid and predictive, but also point to the fact that other factors have a strong impact on TMAO levels. One obvious factor is dietary intake, which we found to be significantly associated with TMAO in our cohort, with high-meat diets having a higher level of TMAO on average.

As discussed above, we identified many metabolites that are positively associated with increased TMAO levels, some of which have been previously studied (such as high levels of choline and carnitine). However, we were interested to understand whether all individuals with elevated TMAO show an increase in these (or other) metabolites, or whether different subsets of elevated-TMAO individuals have different global metabolic profiles. Such profiles could be possibly linked to different routes of TMAO accumulation in different individuals. To this end, we selected the 162 individuals that displayed the highest level of TMAO in our data and applied hierarchical clustering to their metabolome (Experimental Procedures). Next, we identified metabolites or biomarkers that are strongly associated with one or more of the clusters (i.e., they have significantly different levels across clusters). We found that carnitine- and choline-containing compounds were significantly enriched in the set of metabolites that varied between these clusters. In addition, we found that specific clusters showed an elevated abundance of carnitine-containing metabolites, while other clusters showed higher levels of choline-containing compounds or diacylglycerol-containing compounds, suggesting that different dietary intakes are driving TMAO levels across the clusters. One cluster (cluster 3) was strikingly low in all carnitine-, choline-, and diacylglycerol-containing metabolites. This cluster also had the highest median GFR and lowest median TMAO values across clusters (Figure S7; despite being in the upper quartile of TMAO levels across all samples). In other words, among the elevated-TMAO clusters, cluster 3 seems to be the "healthiest." In contrast, cluster 2, which showed the highest levels of all glycerol-containing metabolites, also showed the highest levels for cholesterol and diabetes markers (e.g., triglycerides, insulin, LDL cholesterol), as well as significantly higher levels of proteins that are known cardiometabolic risk factors (e.g., LDLR, PAI-1). Although this cluster was not the highest for TMAO or lowest for GFR, it was the only cluster with an obese (>30) average BMI among all clusters (Figure S7). Therefore, we can postulate that these individuals might be at the highest risk for atherosclerosis and CVD given their overall profiles, and the question of what differs this cluster in terms of diet or lifestyle is very intriguing. In summary, our clustering analysis of elevated-TMAO individuals suggests that there are different dietary and metabolic routes to the accumulation of TMAO and that certain metabolic profiles might represent individuals at higher risk for TMAO-related diseases.

The present study has the advantage of simultaneously measuring multiple omics in a large cohort of 648 individuals, including clinical labs, metabolites, proteins, and the gut microbiome. There are, however, a few limitations in the present study. One is the lack of follow-up on CVD outcomes. Such a clinical follow-up would allow us to stratify individuals in our analysis by future disease outcomes, as well as better establish the predictive power of TMAO or other multi-omic measurements on disease risk. A second limitation is the cross-sectional nature of our data. Measuring the levels of TMAO longitudinally across

multiple time points would allow both a better assessment of the variability of the measurement, as well as establishing a stronger association between levels of TMAO and other data types. Another limitation is the lack of a well-designed diet questionnaire, and the limited number of individuals who reported their diet. Ideally, such a questionnaire would survey the consumption of all of the foods that were found to be associated with TMAO to allow a deeper understanding of the relationship between diet, the gut microbiome, and TMAO.

In summary, this multi-omic study of TMAO provides an improved understanding of TMAO in a systems context and will hopefully facilitate future discoveries of new therapeutic avenues for preventing CVD.

EXPERIMENTAL PROCEDURES

Institutional Review Board Approval for the Study

Procedures for the current study were run under the Western Institutional Review Board (Institutional Review Board [IRB] Study Number 1178906) at Arivale (Seattle, WA).

Data Used in the Study

De-identified data from individuals in a commercially available lifestyle intervention program were collected starting in July 2015. The Arivale program involves health coaching on exercise, nutrition, stress management, and other wellness goals. Multi-omic data from a total of 648 Arivale program individuals were included in the current study. This research project was performed entirely using de-identified and aggregated data of individuals who had explicitly authorized the use of their data in research.

Clinical Laboratory Tests

Participants' clinical blood laboratory tests were measured up to 3 times (with 2.6 times on average) over a 12-month period (average time interval between measurements, 4.6 months). They were required to avoid alcohol, vigorous exercise, aspartame, and monosodium glutamate 24 hr prior to the blood draw, and to begin fasting 12 hr in advance. To maintain data integrity and quality, blood samples were collected at the LabCorp (North Carolina) or Quest Diagnostics (New Jersey) facility located nearest to participants' geographic location. At the time of each blood draw, weight, height, waist circumference, and blood pressure were measured, and BMI was calculated.

Gut Microbiome Sequencing

Stool specimens were taken at participants' homes using a standardized kit supplied by either Second Genome (California) or DNAgenotek (Ottawa, ON, Canada). Microbial DNA was isolated from 250 μL of homogenized stool, using an automated protocol and MoBio's PowerMag (+ClearMag) microbiome DNA isolation kit on the KingFisher Flex instrument. The extraction protocol involved a precursory bead-beating step with glass beads and plate shaker for recovery of more DNA from a more diverse microbial community. Concentrations of extracted DNA from each sample were determined by Qubit measurement, and an estimate of sample purity was determined with spectrophotometry by measuring the A260/A280 absorbance ratio, Gut microbiome sequencing data in the form of FASTQ files were provided based on either 250-bp paired-end MiSeq profiling of the 16S V4 region (Second Genome) or 300-bp paired-end MiSeq profiling of the 16S V3+V4 regions (Second Genome). Operational taxonomic unit (OTU) read counts were calculated using the QIIME pipeline (Caporaso et al., 2010) (version 1.9.1; default parameters) with closed-reference OTU picking against the Greengenes database (version 13_08). KO abundances were estimated based on OTU abundances using PICRUSt (Langille et al., 2013).

Metabolomics

Metabolon (North Carolina) conducted the metabolomics assays on participant plasma samples. Sample handling, quality control, and data extraction, along with biochemical identification, data curation, quantification, and data normalizations have been previously described (Wittmann et al., 2014); see full details in Supplemental Experimental Procedures.

Proteomics

Plasma concentrations of proteins were measured in baseline EDTA plasma samples using the ProSeek Cardiovascular II, Cardiovascular III, and Inflammation arrays (Olink Biosciences, Uppsala, Sweden), according to the standard protocol (Assarsson et al., 2014). ComBat (Leek et al., 2012) normalization was applied to adjust for potential batch effects (see full details in Supplemental Experimental Procedures).

Statistical Analysis

All correlations reported in the paper are Spearman correlations. Multiple-hypotheses corrections for p values were performed independently for each data type (clinical labs, metabolites, proteins, and microbiome taxa) using the FDR method (Benjamini and Hochberg, 1995) to limit false discoveries per data type. All adjustments for confounding variables such as vendor, age, sex, race, and kidney function (as measured by GFR) were conducted using the "lm" function in R and using the residuals of the linear regression model as values for association testing.

Building a Random Forest Model to Predict TMAO Levels

Taxonomic features were defined as the relative abundances of all taxonomic levels (species, genus, family, order, class, phylum) with a total of 873 such features detected in our samples. Blood marker features were defined as the 71 markers with less than 10% missing data across our blood samples. Protein features were defined as the 238 proteins in our proteomics dataset. Metabolite features were defined as the 618 metabolites in our metabolomics dataset (excluding TMAO). Given the large number of variables measured, a strict cross-validation procedure was followed to minimize the impact of the number of variables on the predictive power of the model. The features (and response defined as the log-transformed TMAO values) were divided in a 10-fold cross-validation scheme. In each fold, only the training data were used to perform feature selection, by calculating the Spearman correlation between each feature value and the response in the training set and selecting features that had an FDR-corrected p value $\leq$ 0.25 (up to a maximum of 500 features). The permissive threshold was selected since the random forest algorithm can further select the important features when building the model. Following feature selection, a random forest model was trained using 1,000 trees, and its performance was evaluated on the held-out test set.

Metabolome-Based Clustering of Individuals with Elevated TMAO Levels

Metabolite values were first log-transformed and then centered and scaled. Hierarchical clustering was applied to the entire set of 619 scaled metabolites measured across individuals, using the Euclidean distance with Ward's clustering algorithm. The number of clusters (five) was selected using the silhouette analysis, where for each sample i, the silhouette width $s(i)$ was defined as $s(i) = (b(i) - a(i))/max(a(i), b(i))$, where $a(i)$ = average distance between sample i and all other samples in the cluster, and $b(i) = min_C\ d(i,C)$, where $d(i,C)$ = average distance between sample i and all other samples in cluster C. Next, for each analyte including metabolites, clinical labs, proteins, and microbiome features, values were compared between each cluster and all other clusters using the Wilcoxon rank-sum test, and biomarkers that are significantly different in at least one cluster (FDR-corrected $p < 0.05$) were reported.

DATA AND SOFTWARE AVAILABILITY

The accession number for the data reported in this paper is SRA: SRP148278.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.06.096.

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AUTHOR CONTRIBUTIONS

O.M., N.Z., M.P.C., X.X., J.E.R., C.E.K., J.C.L., and A.T.M. participated in the study design. O.M. performed the analyses and made interpretation of the results. O.M. and N.Z. wrote the manuscript. O.M., N.Z., M.P.C., X.X., J.E.R., C.E.K., J.C.L., and A.T.M. read and approved the final manuscript.

DECLARATION OF INTERESTS

O.M., N.Z., M.P.C., X.X., J.E.R., C.E.K., J.C.L., and A.T.M. are employees of Arivale, Inc., and have stock options in the company.

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