Gut Flora Metabolism of Phosphatidylcholine Promotes Cardiovascular Disease

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Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease

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Metabolomics studies hold promise for the discovery of pathways linked to disease processes. Cardiovascular disease (CVD) represents the leading cause of death and morbidity worldwide. Here we used a metabolomics approach to generate unbiased small-molecule metabolic profiles in plasma that predict risk for CVD. Three metabolites of the dietary lipid phosphatidylcholine—choline, trimethylamine N-oxide (TMAO) and betaine—were identified and then shown to predict risk for CVD in an independent large clinical cohort. Dietary supplementation of mice with choline, TMAO or betaine promoted upregulation of multiple macrophage scavenger receptors linked to atherosclerosis, and supplementation with choline or TMAO promoted atherosclerosis. Studies using germ-free mice confirmed a critical role for dietary choline and gut flora in TMAO production, augmented macrophage cholesterol accumulation and foam cell formation. Suppression of intestinal microflora in atherosclerosis-prone mice inhibited dietary-choline-enhanced atherosclerosis. Genetic variations controlling expression of flavin monooxygenases, an enzymatic source of TMAO, segregated with atherosclerosis in hyperlipidaemic mice. Discovery of a relationship between gut-flora-dependent metabolism of dietary phosphatidylcholine and CVD pathogenesis provides opportunities for the development of new diagnostic tests and therapeutic approaches for atherosclerotic heart disease.

The pathogenesis of CVD includes genetic and environmental factors. A known environmental risk factor for the development of CVD is a diet rich in lipids. A relationship between blood cholesterol and triglyceride levels and cardiovascular risk is well established. However, less is known about the role of the third major category of lipids, phospholipids, in atherosclerotic heart disease pathogenesis.

Another potential yet controversial environmental factor in the development or progression of atherosclerotic heart disease is inflammation due to infectious agents. Some studies have reported associations between coronary disease and pathogens such as cytomegalovirus (CMV), Helicobacter pylori, and Chlamydia pneumoniae. However, prospective randomized trials with antibiotics in humans have thus far failed to demonstrate cardiovascular benefit and studies with germ-free hyperlipidaemic mice confirm that infectious agents are not necessary for murine atherosclerotic plaque development. Although a definite cause and effect relationship between a bacterial or viral pathogen and atherosclerosis in humans has not yet been established, the prospect of a role for microbes in atherosclerosis susceptibility remains enticing.

The intestinal microbiota (‘gut flora’), comprised of trillions of typically non pathogenic commensal organisms, serve as a filter for our greatest environmental exposure—what we eat. Gut flora have an essential role, aiding in the digestion and absorption of many nutrients. Animal studies have recently shown that intestinal microbial communities can influence the efficiency of harvesting energy from diet, and consequently influence susceptibility to obesity. Metabolomics studies of inbred mouse strains have also recently shown that gut microbiota may have an active role in the development of complex metabolic abnormalities, such as susceptibility to insulin resistance and non alcoholic fatty liver disease. A link between gut flora dependent phospholipid metabolism and atherosclerosis risk through generation of pro atherosclerotic metabolites has not yet been reported.

Metabolomics studies identify markers of CVD

In initial studies we sought to discover unbiased small molecule metabolic profiles in plasma that predict increased risk for CVD. An initial ‘Learning Cohort’ was used comprising plasma from stable patients undergoing elective cardiac evaluation who subsequently experienced a heart attack (myocardial infarction), stroke or death over the ensuing three year period versus age and gender matched subjects who did not. Liquid chromatography with on line mass spectrometry (LC/MS) analysis of plasma was performed to define analytes associated with cardiac risk as described in Methods. Of an initial 2,000+ analytes monitored, 40 met all acceptability criteria within the Learning Cohort. Subsequent studies within an independent ‘Validation Cohort’ led to identification of 18 analytes that met acceptability criteria in both Learning and Validation Cohorts (Fig. 1a, b, Supplementary Fig. 1a and Supplementary Table 1).

The structural identity of the 18 small molecules in plasma, the levels of which track with cardiac risks, was not known, as the compounds were screened on the basis of retention time and mass to charge ratio (m/z) when analysed by LC/MS. Among the 18 analytes, those with m/z 76, 104 and 118 demonstrated significant (P < 0.001) correlations among one another, suggesting a potential relationship via a common biochemical pathway (Supplementary Fig. 1b). We therefore initially sought to structurally define these three analytes.

Phosphatidylcholine metabolites are linked to CVD

The candidate compound in plasma with an m/z of 76 associated with CVD risks was isolated and unambiguously identified as TMAO using
Gut flora is needed to form TMAO from dietary PC
Intestinal microflora have a role in TMAO formation from dietary free choline\(^1\), so we therefore proposed that commensal organisms (gut flora) might also have an obligate role in TMAO formation from dietary PC. To test this, deuterated PC was synthesized whereby the choline methyl groups were deuterium labelled (that is, d9 PC) and

![Image]

**Figure 1** Strategy for metabolomics studies to identify plasma analytes associated with cardiovascular risk. a. Overall schematic to identify plasma analytes associated with cardiovascular risk over the ensuing 3 year period. CVA, cerebrovascular accident; HPLC, high performance liquid chromatography; MI, myocardial infarction. b. Odds ratio (OR) and 95% confidence intervals (CI) of incident (3 year) risk for MI, CVA or death of the 18 plasma analytes that met all selection criteria in both Learning and Validation Cohorts; odds ratio and 95% confidence intervals shown are for the highest versus lowest quartile for each analyte. Filled circles represent the analytes (m/z = 76, 104, 118) focused on in this study. m/z, mass to charge ratio.

multinuclear nuclear magnetic resonance (NMR), multi-stage mass spectrometry (MS\(^n\)), liquid chromatography with tandem mass spectrometry (LC/MS/MS) and gas chromatography with tandem mass spectrometry (GC/MS/MS) after multiple derivatization strategies (see Methods, Supplementary Figs 2a, d, and Supplementary Table 2). TMAO, an oxidation product of trimethylamine (TMA), is a relatively common metabolite of choline in animals\(^2,3\). Foods rich in the lipid phosphatidylcholine (PC, also called lecithin), which predominantly include eggs, milk, liver, red meat, poultry, shellfish and fish, are believed to be the major dietary sources for choline, and hence TMAO production\(^4\). Bifidobacteria contain a high proportion of choline and other trimethylamine containing species (for example, betaine) by intestinal microbes forms the gas TMA\(^3\), which is efficiently absorbed and rapidly metabolized by at least one member of the hepatic flavin monoxygenase (FMO) family of enzymes, FMO3, to form TMAO\(^5,6\). Identification of the plasma analyte associated with CVD risk with an m/z of 76 as TMAO therefore indicated that the plasma analyte with an m/z of 104 might be choline. Further, these results also indicated that the plasma analyte with an m/z of 118 associated with CVD might be related to PC (choline) metabolism.

To test the hypothesis that the plasma analytes with m/z 76 (TMAO), 104 and 118 might all be derived from the major dietary lipid PC, mice were fed egg yolk PC (through oral gavage) and plasma levels of analytes over time were monitored. In both male and female mice, analytes with the same m/z (76, 104 and 118) and the same retention times as the corresponding analytes of interest were observed in human plasma all increased after oral PC feeding (Supplementary Fig. 3a, b), strongly indicating that the m/z 104 analyte was choline, and the analyte at m/z 118 was derived from PC. Confirmation that the plasma analyte (m/z 104) associated with CVD risk was choline was achieved by MS\(^n\), LC/MS/MS and GC/MS/MS after multiple derivatization strategies (Supplementary Fig. 4a d and Supplementary Table 3).

We next studied the plasma analyte with m/z 118. We proposed that the analyte was either betaine or one of several potential methylated metabolites of choline (see Supplementary Fig. 5a for structures and strategy for discrimination among these isomers). To distinguish between these species, and explore a role for intestinal generation of the various metabolites, different isotope labelled choline precursors were administered to mice either through an oral (gavage) or a parenteral (intraperitoneal, i.p.) route. The observed m/z of new isoformically labelled analytes at the appropriate retention times identified in plasma after these isotope tracer studies are summarized in Fig. 2a. Oral administration of non labelled choline resulted in time dependent increases in plasma levels of analytes with m/z 76, 104 and 118, consistent with TMAO, choline and either betaine or a methylated choline species (Supplementary Fig. 6a). Use of selectively deuterated choline species at either the trimethylamine moiety (d9 isopropyl) or the methyl moiety (d4 isopropyl) unambiguously confirmed the m/z 118 analyte as betaine (Fig. 2a and Supplementary Fig. 6b). Further confirmation was acquired by observing the same retention time in LC/MS and an identical collision induced dissociation (CID) mass spectrum (Supplementary Fig. 5b). Moreover, supplementation of PC or choline isopropyls via gavage and i.p. injection showed an absolute requirement for the oral route in TMAO production, whereas betaine production from PC or choline was formed via both oral and i.p. routes (Fig. 2 and Supplementary Fig. 7a).

![Image]

**Figure 2** Identification of metabolites of dietary PC and an obligatory role for gut flora in generation of plasma analytes associated with CVD risks. a. Summary schematic indicating structure of metabolites and routes (oral or i.p.) of formation observed in choline challenge studies in mice using the indicated isotope labelled choline. The m/z in plasma observed for the isopropyls of the choline metabolites are shown. b. Plasma levels of d9 metabolites after i.p. challenge with d9(trimethyl) dipalmitoylphosphatidylcholine (d9 DPPC). c, d. TMAO production after oral d9 DPPC administration in mice, following suppression of gut flora with antibiotics (3 weeks), and then following placement (4 weeks) into conventional cages with non sterile mice ("conventionalized"). Data are presented as mean ± standard error (s.e.) from four independent replicates.
used as isotope tracer for feeding studies. When mice were fed through oral gavage with d9 PC, the time dependent appearance of the anticipated d9 isotopomer of TMAO was observed in plasma (Fig. 2c). Interestingly, pretreatment of mice with a three week course of broad spectrum antibiotics to suppress intestinal flora completely suppressed the appearance of d9 TMAO in plasma after oral d9 PC administration (Fig. 2c). A similar pattern was observed after oral administration of d9 choline to mice, with d9 TMAO produced in untreated mice, but not in the same mice after a 3 week course of broad spectrum antibiotics (Supplementary Fig. 7b), or in germ free mice born sterilely by Caesarean section (Supplementary Fig. 7c). In a final series of studies, mice with suppressed intestinal microflora after antibiotics were placed in conventional cages with normal (non germ free) mice to permit intestinal colonization with microbes. After four weeks, repeat oral d9 PC challenge of the new ‘conventio nalized’ mice resulted in readily detectable plasma levels of d9 TMAO (Fig. 2c). Similar results were observed after conventionalization of germ free mice and oral d9 choline (Supplementary Fig. 7c). Collectively, these results show an obligate role for intestinal microbiota in the generation of TMAO from the dietary lipid PC. They also reveal the following metabolic pathway for dietary PC producing TMAO: PC → choline → TMA → TMAO.

Dietary PC metabolites predict CVD risk

We next sought to independently confirm the prognostic value of monitoring fasting plasma levels of TMAO, choline and betaine in a large independent clinical cohort distinct from subjects examined in both the Learning and Validation Cohorts. Stable subjects (N = 1,876) undergoing elective cardiac evaluations were enrolled. Clinical, demographic and laboratory characteristics of the cohort are provided in Supplementary Table 4a. Fasting plasma levels of TMAO, choline and betaine were quantified by LC/MS/MS using methods specific for each analyte (Supplementary Fig. 8). Increasing levels of choline, TMAO and betaine were all observed to show dose dependent associations with the presence of CVD (Fig. 3a c) and multiple individual CVD phenotypes including peripheral artery disease (PAD), coronary artery disease (CAD), and history of myocardial infarction (see Supplementary Table 5a d for multilogistic regression models, and Supplementary Table 5e for Somers’ Dxy correlation). The associations between increased risk of all CVD phenotypes monitored and elevated systemic levels of the three PC metabolites held true after adjustments for tradi tional cardiac risk factors and medication usage (Fig. 3a c and Supplementary Table 5a e).

Dietary choline or TMAO promotes atherosclerosis

We next investigated whether the strong associations noted between plasma levels of the dietary PC metabolites and CVD risk reflected some hidden underlying pro atherosclerotic mechanism. Atherosclerosis prone mice (C57BL/6J Apoe−/−) at time of weaning were placed on either normal chow diet (contains 0.8% 0.09% total choline, wt/wt) or normal chow diet supplemented with intermediate (0.5%) or high amounts of additional choline (1.0%) or TMAO (0.12%). At 20 weeks of age increased total aortic root atherosclerotic plaque area was noted in both male and female mice on diets supplemented with either choline or TMAO (Fig. 3d and Supplementary Fig. 9a). Analysis of plasma levels of choline and TMAO in each of the dietary arms showed nominal changes in plasma levels of choline, but significant increases of TMAO in mice receiving either choline or TMAO supplementation (Supplementary Fig. 10). Parallel examination of plasma cholesterol, triglycerides, lipoproteins, glucose levels and hepatic triglyceride content in the mice failed to show significant increases that could account for the enhanced atherosclerosis (Supplementary Table 6 and Supplementary Fig. 11). Interestingly, all dietary groups of mice revealed a sig nificant positive correlation between plasma levels of TMAO and atherosclerotic plaque size (Fig. 3e and Supplementary Fig. 9b). Of note, plasma TMAO levels observed within the female mice (which get enhanced atherosclerosis relative to their male counterparts), even on normal chow diet, were significantly higher than those observed among male mice (Supplementary Fig. 10). No significant gender differences in plasma levels of TMAO were observed in humans (P = 0.47); however, a clear dose response relationship was observed between TMAO levels and clinical atherosclerotic plaque burden in subjects undergoing coronary angiography (Fig. 3f).

Hepatic FMOs, TMAO and atherosclerosis

Hepatic FMO3 is a known enzymatic source for TMAO in humans, based on the recent recognition of the aetiology of an uncommon genetic disorder called trimethylaminuria (also known as fish malodour syndrome15,17). Subjects with this metabolic condition have impaired capacity to convert TMA, which smells like rotting fish, into TMAO, an odourless stable oxidation product17. We therefore sought to identify possible sources of genetic regulation and the role of Fmo3 in atherosclerosis using integrative genetics in mice18. Expression levels of Fmo3 were determined by microarray analysis in the livers of mice from an F2 intercross between atherosclerosis prone C57BL/6J Apoe−/− mice fed with chow diet supplemented with the indicated amounts (wt/wt) of choline or TMAO from time of weaning (4 weeks). e, Relationship between plasma TMAO levels and aortic lesion area. f, Relationship between fasting plasma levels of TMAO versus CAD burden among subjects (N = 1,020). Boxes represent 25th, 50th and 75th percentile, and whiskers 5th and 95th percentile plasma levels. Single, double and triple coronary vessel disease refers to number of major coronary vessels demonstrating ≥50% stenosis on diagnostic coronary angiography.

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Figure 3 | Plasma levels of choline, TMAO and betaine are associated with atherosclerosis risks in humans and promote atherosclerosis in mice.

a, c, Spline models of the logistic regression analyses reflecting risk of CVD (with 95% CI) according to plasma levels of choline, TMAO and betaine in the entire cohort (n = 1,876 subjects). d, Comparison in aortic lesion area among 20 week old female C57BL/6J Apoe−/− mice fed with chow diet supplemented with the indicated amounts (wt/wt) of choline or TMAO from time of weaning (4 weeks). e, Relationship between plasma TMAO levels and aortic lesion area. f, Relationship between fasting plasma levels of TMAO versus CAD burden among subjects (N = 1,020). Boxes represent 25th, 50th and 75th percentile, and whiskers 5th and 95th percentile plasma levels. Single, double and triple coronary vessel disease refers to number of major coronary vessels demonstrating ≥50% stenosis on diagnostic coronary angiography.

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Atherosclerosis in male mice from the C57BL6/J Apoe\textsuperscript{HeJ}/ F2 intercross. The F2 intercross revealed that multiple FMO genes monitored, only a trend towards positive association was noted between hepatic expression of FMO3 and plasma TMAO levels (Fig. 4d and Supplementary Fig. 17).

Next, we focused on the genetic regulation of hepatic FMO3 expression (and other FMO genes) using expression quantitative trait locus (eQTL) analyses in the F2 mouse intercross. The eQTL plot for FMO3 messenger RNA levels is shown in Supplementary Fig. 18, and demon-

strates a strongly suggestive cis locus (lod score 5.9) on mouse chro-

mosome 1 at 151 Mb. Fmo3 (and several other FMO genes) is located at 164.8 Mb in a region identified as non identical by descent between C3H/HeJ and C57BL/6 (http://mouse.cs.ucla.edu/perlegen/). This region is just distal to the 95% confidence interval of a previously reported murine atherosclerosis susceptibility locus\textsuperscript{20}. Examining the effect of the closest single nucleotide polymorphism (SNP) to Fmo3 (rs3689151) as a function of alleles inherited from either parental strain indicated a strong effect on atherosclerosis in both genders of the F2 mice (Kruskal Wallis test, $P < 10^{-6}$). Bonferroni corrected pair wise comparisons indicated a dose dependent significant increase in atherosclerosis in F2 mice heterozygous or homozygous for the C57BL/6J allele (Fig. 4e). Although the resolution on average for an F2 intercross of this size is in excess of 20 Mb and thus does not provide ‘gene level’ resolution, these data show that the locus encompassing the Fmo gene cluster on chromosome 1 is associated with atherosclerotic lesion size. Collectively, these results indicate that: (1) hepatic expression levels of multiple FMO genes are linked to plasma TMAO levels in mice; (2) hepatic expression levels of multiple FMO genes are associated with both the extent of aortic atherosclerosis and HDL cholesterol levels in mice; (3) hepatic expression levels of FMO3 indi-

cate an association with plasma TMAO levels in humans; and (4) a genetic locus containing the Fmo gene cluster on chromosome 1 in mice has a strong effect on atherosclerosis.

**Figure 4** Hepatic FMO genes are linked to atherosclerosis and dietary PC metabolites enhance macrophage scavenger receptor expression.

**a**, Correlation between hepatic Fmo3 expression and aortic lesion, plasma HDL cholesterol and TMAO in female mice from the F2 intercross between atherosclerosis prone C57BL/6J Apoe\textsuperscript{−/−} and atherosclerosis resistant C3H/HeJ Apoe\textsuperscript{−/−} mice. **b**, Correlation between human hepatic FMO3 expression and plasma TMAO. **c**, Effect of Fmo3 genotype (SNP rs3689151) on aortic sinus atherosclerosis in male mice from the C57BL/6J Apoe\textsuperscript{−/−} and C3H/HeJ Apoe\textsuperscript{−/−} F2 intercross. **d**, Quantification of scavenger receptor CD36 and SR A1 surface protein levels in macrophages harvested from C57BL/6J mice (13 weeks) after three weeks of standard chow versus chow supplemented with the indicated amounts (wt/wt) of choline, TMAO or betaine. Data are presented as mean ± s.e. from the indicated numbers of mice in each group.

FMO3 is one member of a family of FMO enzymes, the majority of which are physically located as a cluster of genes on chromosome 1 in both humans and mice. The various FMOs share sequence homology and overlapping substrate specificities. Further, although rare mutations in or near the FMO3 gene have been identified in individuals with trimethylaminuria\textsuperscript{21}, the impact of these mutations on other FMO genes remains unknown. Examination of the hepatic expression levels of the various FMO genes revealed that many are highly correlated with each other in both mice and humans (Supplementary Table 7). Examination of hepatic expression levels of additional FMO genes in mice from the atherosclerosis F2 intercross revealed that multiple FMO genes are significantly correlated with aortic lesion formation, HDL cholesterol concentrations and plasma TMAO levels (Supplementary Figs 14, 16), suggesting that several members of the FMO family of enzymes may participate in atherosclerosis and the PC → TMAO metabolic pathway. To explore the relationship between hepatic FMOs and plasma TMAO levels in humans, paired samples of liver and plasma from subjects undergoing elective liver biopsy were examined. Among all of the human FMO genes monitored, only a trend towards positive association was noted between hepatic expression of FMO3 and plasma TMAO levels (Fig. 4d and Supplementary Fig. 17).

**Diet and gut flora alter macrophage phenotype**

To explore potential mechanisms through which dietary choline and its metabolites might exert their pro atherosclerotic effects, C57BL/6J Apoe\textsuperscript{−/−} mice at time of weaning were placed on a normal chow diet supplemented with either choline, TMAO or betaine (for >3 weeks). Both mRNA levels (Supplementary Fig. 19) and surface protein levels (Fig. 4f, g and Supplementary Fig. 20) of two macrophage scavenger receptors implicated in atherosclerosis, CD36 and SR A1, were then determined in peritoneal macrophages. Relative to normal chow diet, mice supplemented with either choline, TMAO or betaine all showed enhanced macrophage levels of CD36 and SR A1. We next examined the impact of dietary choline and gut flora on endogenous formation of cholesterol laden macrophage foam cells, one of the earliest cellular hallmarks of the atherosclerotic process. Hyperlipidaemic C57BL/6J Apoe\textsuperscript{−/−} mice were fed diets with defined levels of choline as follows: (1) ‘control’ (0.07 0.08%, wt/wt), which is similar to the choline content of normal chow (0.08 0.09%); versus (2) high ‘choline’, corresponding to a >10 fold higher level of choline (1.0%, wt/wt) than normal chow. Concomitantly, half of the mice were administered broad spectrum antibiotics for 3 weeks to suppress intestinal microflora, which was confirmed by the reduction of plasma TMAO levels by >100 fold (plasma TMAO concentrations in groups receiving antibiotics were <100 nM). Whereas mice on the control diet showed modest evidence of endogenous macrophage foam cell formation, as indicated by Oil red O staining of peritoneal macrophages, mice on the 1% choline supplemented diet showed markedly enhanced lipid laden macrophage development (Fig. 5a). In contrast, suppression of intestinal flora...
significantly inhibited dietary choline induced macrophage foam cell formation (Fig. 5a, b). These results were confirmed by microscopic quantification of endogenous foam cell levels (Fig. 5b) and analytical quantification of the cholesterol content of recovered macrophages (Fig. 5c). Histopathological and biochemical studies of livers recovered from these mice showed no evidence of steatosis (Supplementary Fig. 21). Parallel analyses of plasma PC metabolites also demonstrated no significant changes in choline or betaine levels between the different dietary groups, and significant increases of plasma TMAO levels only in mice on the high choline diet in the absence of antibiotics (males, control versus choline diet, 2.5 ± 0.1 µM versus 28.3 ± 2.4 µM, P < 0.001; for females, control versus choline diet, 4.0 ± 0.5 µM versus 158.6 ± 32.9 µM, P < 0.001).

Gut flora promote diet-induced atherosclerosis

In additional studies, we sought to test whether gut flora are involved in dietary choline induced atherosclerosis. At the time of weaning (4 weeks old), atherosclerosis prone C57BL/6j Apoe−/− mice were placed on either a control diet (0.08 ± 0.01%, wt/wt choline) or a diet supplemented with 1% choline (wt/wt, choline diet). Half of the mice were also treated with broad-spectrum antibiotics to suppress intestinal microflora. Serial plasma measurements confirmed suppression of TMAO levels to virtually non-detectable levels (<100 nM) throughout the duration of the study. At 20 weeks of age, mice were killed and aortic root lesion development was quantified. In the absence of antibiotics (i.e., with preserved intestinal microflora), choline supplementation augmented atherosclerosis in both male and female mice nearly threefold (Figs 5d and Supplementary Figs 22, 23). Both histopathological and biochemical examination of liver sections from mice showed no evidence of steatosis or altered neutral lipid (triglyceride or cholesterol/cholesterol ester) levels on either diet in the absence or presence of antibiotics (Supplementary Fig. 21 and Supplementary Table 8). Finally, the structural specificity of PC metabolites in promoting a proatherogenic macrophage phenotype was examined. Mice fed diets supplemented with trimethylamine species (choline or TMAO) showed increased peritoneal macrophage cholesterol content and raised plasma levels of TMAO. In contrast, dietary supplementation with the choline analogue 3,3-dimethylbutanol (DMB), where the quaternary amine nitrogen of choline is replaced with a carbon, resulted in no TMAO increase and no increase in cholesterol in macrophages (Supplementary Fig. 24).

Discussion

Using a targeted metabolomics approach aimed at identifying plasma metabolites of the levels of which predict risk of CVD in subjects, we have identified a novel pathway linking dietary lipid intake, intestinal microflora and atherosclerosis (Fig. 6). The pathway identified (dietary PC, choline → gut flora formed TMA → hepatic FMO3 formed TMAO) represents a unique additional nutritional contribution to the pathogenesis of CVD that involves PC and choline metabolism, an obligate role for the intestinal microbial community, and regulation of surface expression levels of macrophage scavenger receptors known to participate in the atherosclerotic process. The pro-atherogenic gut flora generated metabolite TMAO is formed in a two-step process initiated by gut flora dependent cleavage of a trimethylamine species (for example, PC, choline, betaine) generating the precursor TMA, and subsequent oxidation by FMO3 and possibly other FMOs (Fig. 6). PC is by far the most abundant dietary source of choline in most humans. The present results indicate that both environmental exposure (dietary lipid from predominantly animal products) and microbial flora participate in TMAO formation and producing a pro-atherogenic macrophage phenotype. Although the present genetic studies also indicate a role for hepatic expression levels of one or more FMO genes in both enhanced atherosclerotic plaque and decreased HDL levels in mice, the participation of FMO genes in human atherosclerosis and HDL cholesterol levels remains to be established. Strong associations between systemic TMAO levels and both angiographic measures of coronary artery atherosclerotic burden and cardiac risk were observed among subjects; however, no correlation was observed between plasma TMAO levels and HDL cholesterol levels in subjects. It remains to be determined whether genetic impairment in FMO3 alone or in
combination with other FMO genes is protective for CVD. No phenotype other than the objectionable odour accompanying this disorder is known. In fact, individuals with trimethylaminuria often become vegans, as reducing the ingestion of dietary animal products rich in lipids decreases TMA production and the associated noxious odour. Little is also known about the biological functions of TMAO in humans. TMAO apparently serves as an osmolite in the freeze avoidance response of some species. In vitro it can function as a small molecule chaperone, affecting the folding and functioning of some proteins. In addition, TMAO and TMA accumulate in plasma of subjects on maintenance haemodialysis, raising the possibility that TMAO may contribute to the well-established enhanced CVD risk noted among subjects with end stage renal disease.

Choline is an essential nutrient that is usually grouped within the vitamin B complex. Choline and its metabolite betaine are methyl donors, along with folate, and are metabolically linked to transmethylation pathways, including the synthesis of the CV risk factor homocysteine. Deficiency in both choline and betaine have been suggested to produce epigenetic changes in genes linked to atherosclerosis, and acute choline and methionine deficiency in rodent models causes lipid accumulation in liver (steatohepatitis), heart and arterial tissue. Alteratively, some studies have reported an association between increased whole blood levels of total choline and cardiovascular disease. Few clinical studies have examined the relationship between choline intake and CVD, probably because accurate measures of the choline content of most foods has only recently become available. The association between dietary choline and (alternative trimethyl) amine containing species) and atherosclerosis will be complex because, as the present studies show, it will be influenced by interindividual differences in the composition of the intestinal microflora.

The human intestinal microbial community is an enormous and diverse ecosystem with known functions in nutrition, gut epithelial cell health, and innate immunity. Intestinal flora recently also has been implicated in the development of some metabolic phenotypes such as obesity and insulin resistance, as well as alterations in immune responses. To our knowledge, the present studies are the first to identify a direct link between intestinal microflora, dietary PC and CVD risk. These results indicate that an appropriately designed probiotic intervention may serve as a therapeutic strategy for CVD. Interestingly, production of TMAO can be altered by probiotic administration. Thus, in addition to the current clinical recommendation for a general reduction in dietary lipids, manipulation of commensal microbial composition may be a novel therapeutic approach for the prevention and treatment of atherosclerotic heart disease and its complications. The present studies also suggest a further novel treatment for atherosclerosis blocking the presumed pathogenic biochemical pathway at the level of the gut flora through use of a non-systemically absorbed inhibitor.

METHODS SUMMARY

Plasma samples and associated clinical study data were identified in patients referred for cardiac evaluation at a tertiary care centre. All subjects gave written informed consent and the Institutional Review Board of the Cleveland Clinic approved all study protocols. Urinary metabolic profiling was performed using LC/MS. Target analyte structural identification was achieved using a combination of LC/MS/MS, LC/MS², tandem mass NMR, GC/MS and choline isotope tracer feeding studies in mice as outlined in Methods. Statistical analyses were performed using R (version 2.10.1). Intestinal microflora were suppressed by supplementing drinking water with a cocktail of broad spectrum antibiotics. Germ free mice were purchased from Taconic SWIG, QTL analyses to identify atherosclerosis related genes were performed on F2 mice generated by crossing atherosclerosis prone C57BL/6J Apo e−/− mice and atherosclerosis resistant C3H/HeJ Apo e−/− mice. mRNA expression was assayed by microarray analysis and real time PCR. Aortic root lesion area in mice was quantified by microscopy after staining. Mouse peritoneal macrophages were collected by lavage for foam cell quantification and cholesterol accumulation assay. Surface protein levels of scavenger receptors CD36 and SR-A1 were determined by flow cytometry.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

General procedures. Lipids were extracted by chloroform:methanol (2:1, v/v)45.
Cholesterol was quantified by GC/MS44. Triglyceride was quantified by GPO reagent
set (Pointe Scientific)45. Cell DNA content was quantified by PicoGreen46. RNA was
isolated by TRIZOL reagent (Invitrogen) and RNeasy Mini Kit (Qiagen). All
reagents were purchased from Sigma unless otherwise specified.

Research subjects. Plasma samples and associated clinical data were collected as
part of two studies involving stable non symptomatic subjects undergoing elec-
tive cardiac evaluations at a tertiary care centre. The first study, GeneBank, is a
large well characterized tissue repository with longitudinal data from subjects
undergoing elective diagnostic left heart catheterization or elective coronary
computed tomography angiography44. The second study, BioBank, includes sub-
jects undergoing cardiac risk factor evaluation/modification in a preventive car-
diology clinic47. CAD included adjudicated diagnoses of stable or unstable angina,
myocardial infarction or angiographic evidence of ≥50% stenosis of one or more
ericardial vessels. PAD was defined as any evidence of extra coronary athero-
sclerosis. Atherosclerotic CVD was defined as the presence of either CAD or
PAD. All subjects gave written informed consent and the Institutional Review
Board of the Cleveland Clinic approved all study protocols.

Discovery metabolomic analyses began with an unbiased search for plasma
(fasting, EDTA purple top tube) analytes linked to CVD risk using a case control
design (GeneBank cohort, N = 50 cases and 50 controls). Cases were randomly
selected from GeneBank subjects who experienced a myocardial infarction, stroke
or death over the ensuing 3 year period. An age and gender matched control
group was randomly selected from GeneBank subjects that did not experience a
CVD event. An independent non overlapping Validation Cohort (N = 25 cases
and 25 controls) was also from GeneBank. A third large (N = 1,876) independent
study comprised of non overlapping subjects then evaluated clinical associations
of identified analytes. Approximately half (N = 1,020) of the subjects enrolled
were from GeneBank and the remaining (N = 856) were from BioBank. Similar
patient characteristics within each cohort and the combined cohort are observed,
as shown in Supplementary Table 4a, b. The association of each PC metabolite
and various cardiovascular phenotypes within each cohort (GeneBank and
BioBank) are also similar (Supplementary Tables 4c e). All subjects in the large
independent clinical study had similar inclusion and exclusion criteria, negative
cardiac enzymes (troponin I < 0.03 ng ml⁻¹) and no recent history of myocardial
infarction or coronary artery bypass graft. Estimate of glomerular filtration rate
was calculated using the MDRD formula46. Fasting blood glucose, C reactive protein,
troponin I and lipid profiles were measured on the Abbott ARCHITECT plate-
form (Abbott MetaboLomics).

Metabolomic analyses. Plasma proteins were precipitated with four volumes of
ice cold methanol and small molecule analytes within supernatants were ana-
lysed after injection onto a phenyl column (4.6 x 250 mm, 5 μm Rextrom
Phenyl, Regis) at a flow rate of 0.8 ml min⁻¹ using a Coghes HPLC interfaced
with a PE Sciex API 365 triple quadrupole mass spectrometer (Applied
Biosystems) with Ionics HSD+, EPI10+, X+ designed source and collision
cell as upages in positive MS1 mode. LC gradient (LC1) starting from 10 mM
ammonium formate over 0.5 min, then to 5 mM ammonium formate, 25% meth
anol and 0.1% formic acid over 3 min, held for 8 min, followed by 100% methanol
and water washing for 5 min at a flow rate of 0.8 ml min⁻¹ was used to resolve
analytes. Spectra were continuously acquired after the initial 4 min. Peaks within
reconstructed ion chromatograms at 1 AMU increments were integrated and both
retention times and m/z of analytes were used for statistical analyses.

Selection criteria for determining analytes of interest were based on the com-
posite of MACE as the clinical phenotype, defined as incident myocardial infarc-
tion, stroke or death at 3 years, and included: (1) demonstration of a statistically
significant difference between cases versus controls using a Bonferroni adjusted
m/z test (P < 0.05); (2) evidence of a significant (P < 0.05) dose response
relationship between analyte level and clinical phenotype using Cochran
Armitage trend test; and (3) a minimal signal to noise ratio of 3:1 for a given
analyte.

Chemical characterization of unknown metabolites. To chemically define the
structures of the plasma analytes selected for further investigation (that is, ana-
ytes with m/z 76, 104 and 118 in positive MS1 mode), multiple approaches were
used. Analytes of interest were isolated by HPLC, vacuum dried, re dissolved in
water and injected onto the same phenyl column with a distinct HPLC gradient
(LC2, flow rate: 0.8 ml min⁻¹) starting from 0.2% formic acid over 2 min, then
linearly to 18% acetonitrile containing 0.2% formic acid over 16 min and further
to 100% acetonitrile containing 0.2% formic acid over 3 min. The targeted ana-
ytes were identified by their m/z and the appropriate fractions recovered. After
removal of solvent, dry analytes were used for structural identification.

Samples analysed by GC/MS were derivatized using Sylon HTTP kit (HMDS +
TMCS + Pyridine (3: 1: 9), Supelco). Derivatization of TMAO and the plasma
analyte at m/z 76 also included initial reduction by titanium (III) chloride48 and
further reaction with 2,2,2-trichloroethylchloroformate49. Analyses were per
formed on the Agilent Technologies 6890/5973 GC/MS in positive ion chemical
ionization mode. The GC/MS analyses used a J&W Scientific DB 1 column (30 m,
0.25 mm inner diameter, 0.25 μm film thickness) for separations.

Quantification of TMAO, choline and betaine. Stable isotope dilution LC/MS/
MS was used for quantification of TMAO, choline and betaine. TMAO, choline
and betaine were monitored in positive MRM MS mode using characteristic
precursor product ion transitions: m/z 76→58, m/z 104→60 and m/z 118→59,
respectively. The internal standards TMAO trimethyl d₉ (d₉ TMAO) and choline
trimethyl d₉ (d₉ choline), were added to plasma samples before protein precipita-
tion, and were similarly monitored in MRM mode at m/z 75→68 and m/z 113→69,
respectively. Various concentrations of TMAO, choline and betaine standards
and a fixed amount of internal standards were spiked into control plasma sample
and used to prepare the calibration curves for quantification of plasma analytes.
TMA was similarly quantified from acidified plasma by LC/MS/MS using
MRM mode.

Aortic root lesion quantification. Apolipoprotein E knockout mice (C57BL/6J
Apo−/−) were weaned at 4 weeks of age and fed with either standard chow
control diet (Teklad 2018) or a custom diet comprised of normal chow supple-
mented with 0.5% choline (Teklad TD.07863), 1.0% choline (Teklad TD.07864)
and 0.15% choline (Teklad TD.07867) for 16 weeks. Mice were treated with ketamine/xylazine before cardiac puncture to collect blood. Hearts were fixed
and stored in 4% paraformaldehyde before frozen OCT sectioning and staining
with Oil red O and haematoxylin. Aortic root lesion area was quantified as the mean
value of six sections48. Aortic sections were immunostained with rat anti mouse
F4/80 antibody (ab6640, Abcam) followed by goat anti rat IgG FITC antibody
(sc 2011, Santa Cruz) and FITC conjugated CD36 monoclonal antibody
(Camayan Chemical) for F4/80 and CD36, respectively. Sections were mounted in
Vegetashield DAPI (H 1200, Vectashield) to take pictures under a Leica DMR
microscope (W. Nuhsbaum) equipped with a Q Imaging Retiga EX camera. We
used Image Pro Plus Version 7.0 (MediaCybernetics) to integrate the positive
staining area of F4/80 and CD36 in a blinded fashion.

Flow cytometry assays on scavenger receptors. Cell surface expression of
scavenger receptors SR A1 and CD36 were quantified on peritoneal macrophages
from female mice by flow cytometry after immunostaining with fluorochrome
coujugated antibodies. Fluorescence intensity was quantified on a FACSCalibur
flow cytometry instrument with FlowJo software (BD Biosciences). More than
10,000 total events were acquired to obtain adequate macrophages numbers.
The following antibodies were used to stain macrophages: CD36 monoclonal
antibody FITC (Camayan Chemical), anti mouse SR A1/MSA1 (R&D Systems),
anti goat anti IgG FITC (Santa Cruz Biotechnolog), Alexa Fluor 647 anti mouse
F4/80 (eBioscience), Alexa Fluor 647 anti mouse CD11b (eBioscience) and the
isotype controls, Alexa Fluor 647 rat IgG2b (eBioscience). Alexa Fluor 647 rat
IgG2b (eBioscience), normal mouse IgG2b (eBioscience), normal mouse IgG2b
(eBioscience), normal mouse IgG2b (eBioscience) and were incubated with antibodies for 30 min at 4°C washed with 0.1% BSA in PBS. Cells with
double positive staining for F4/80 and CD11b were gated as macrophage50 51 for
the quantification of fluorescence intensity for CD36 and SR A1 (Supplementary
Fig. 20), with results normalized to F4/80.

eQTL studies. C57BL/6J and/or C3H/HeJ (60 mice each) were purchased from
the Jackson Laboratory and C3H/HeJ (60 mice each) were bred by backcrossing
B6 Apo−/− to C3H/HeJ for 10 generations. F2 mice were generated by crossing B6 Apo−/− with C3H/HeJ Apo−/− and subsequently intercrossing the F1
mice. Mice were fed Purina Chow containing 4% fat until 8 weeks of age, and
then transferred to a Western diet (Teklad 88137) containing 42% fat and 0.15%
cholesterol for 16 weeks. Mice were anesthetized with ketamine/xylazine before
removal from the germ free microisolator shipper. After the choline or PC challenge,
the germ free microisolator shipper. After the choline or PC challenge, the
germ free mice were placed in conventional cages with non sterile C57BL/6J
female mice to facilitate transfer of commensal organisms. Four weeks later,
the conventionalized mice underwent a second choline or PC challenge.
In vivo macrophage studies. C57BL/6J mice or B6 Apoe⁻/⁻ mice were fed with either standard chow diet control (Teklad 2018) or a custom diet supplemented with 1.0% betaine (Teklad TD.08122), 1.0% choline (Teklad TD.07864), 0.12% TMAO (Teklad TD.07865) or 1.0% dimethylbutanol (DMB) supplemented in drinking water for at least 3 weeks. Elicited mouse peritoneal macrophages (MPMs) were harvested by peritoneal lavage with ice cold PBS 3 days after i.p. injection of 1 ml 4% thioglycollate. Some studies with mice were performed using a custom diet with low but sufficient choline content (0.07% total; Teklad TD.09040) versus high choline diet (1.0% total; Teklad TD.09041) in the presence or absence of antibiotics. Choline content of all diets was confirmed by LC/MS/MS.

Foam cell staining. Foam cells were identified by microscopy cultured peritoneal macrophages on glass coverslips after 6 h in RPMI 1640 medium supplemented with 3% lipopolysaccharide deficient serum. Cells were fixed with paraformaldehyde and stained with Oil red O/haematoxylin53. Cells containing >10 lipid droplets were scored as foam cells54. At least 10 fields and 500 cells per condition were counted.

Real time PCR. Real time PCR of Cld36, Sr a1 and flavin monoxygenases (mice Fmos) was performed using Brilliant II SYBR Green qRT PCR kit (Stratene). The forward and reverse primers Cld36, Gapdh, Sr a1, mouse Fmos and F4/80 were synthesized by IDT based on sequences reported54,55. RT-PCR of human FMOs was similarly performed using primers specific for the sequence of each of the indicated human FMOs.

d9 DPPC synthesis and vesicle preparation. d9 DPPC was synthesized by reaction of 1,2 dipalmitoyl sn glycero 3 phosphoethanolamine (Genzyme Pharmaceuticals) with per deuteromethylchloride (CD1, Cambridge isotope Laboratories)56. The product was purified by preparative silica gel TLC and confirmed by both MS and NMR. Egg yolk lecithin (Avanti Polar Lipids) and d9 DPPC liposomes used for gavage feeding and i.p. injection of mice were prepared by the method of extrusion through polycarbonate filters58.

Metabolic challenges in mice. C57BL/6J mice were administered (gavage) unlabelled or the indicated stable isotope labelled choline or PC (egg yolk lecithin or d9 DPPC) using a 1.5 inch 20 gauge intubation needle. Choline challenge: gavage consisted of 150 μl of 150 mM d9 choline. PC challenge: gavage or i.p. injection of 300 μl 5 mg/ml of unlabelled PC or labelled d9 DPPC. Mice were fasted 12 h before PC challenge. Plasma (50 μl) was collected via the saphenous vein from mice at baseline and after gavage or i.p. injection time points.

Statistical analysis. Student’s t test and Wilcoxon rank sum test were employed to compare group means.26Pearson correlation, Spearman rank correlation and Somers’ D correlation were used to investigate the correlation between two variables26.4. Comparison of categorical measures between independent groups was done using χ² tests26. Odds ratios and 95% confidence intervals for cardiac vascular phenotypes (history of myocardial infarction, CAD, PAD, CVD and CAD+PAD) were calculated with R, version 2.10.1 (http://www.cran.r-project.org), using logistic regression26 with case status as the dependent variable and plasma analyte as independent variable. Trend tests in frequencies across quartiles were done using Cochran Armitage trend tests68. Levels of analytes were adjusted for analyte as independent variable. Trend tests in frequencies across quartiles were done using logistic regression67 with case status as the dependent variable and plasma analyte as independent variable. Mann-Whitney test, Wilcoxon rank sum test were employed to compare group means.

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