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Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis

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Intestinal microbiota metabolism of choline and phosphatidylcholine produces trimethylamine (TMA), which is further metabolized to a proatherogenic species, trimethylamine-*N*-oxide (TMAO). We demonstrate here that metabolism by intestinal microbiota of dietary L-carnitine, a trimethylamine abundant in red meat, also produces TMAO and accelerates atherosclerosis in mice. Omnivorous human subjects produced more TMAO than did vegans or vegetarians following ingestion of L-carnitine through a microbiota-dependent mechanism. The presence of specific bacterial taxa in human feces was associated with both plasma TMAO concentration and dietary status. Plasma L-carnitine levels in subjects undergoing cardiac evaluation (*n* = 2,595) predicted increased risks for both prevalent cardiovascular disease (CVD) and incident major adverse cardiac events (myocardial infarction, stroke or death), but only among subjects with concurrently high TMAO levels. Chronic dietary L-carnitine supplementation in mice altered cecal microbial composition, markedly enhanced synthesis of TMA and TMAO, and increased atherosclerosis, but this did not occur if intestinal microbiota was concurrently suppressed. In mice with an intact intestinal microbiota, dietary supplementation with TMAO or either carnitine or choline reduced *in vivo* reverse cholesterol transport. Intestinal microbiota may thus contribute to the well-established link between high levels of red meat consumption and CVD risk.

The high level of meat consumption in the developed world is linked to CVD risk, presumably owing to the large content of saturated fats and cholesterol in meat[1,2]. However, a recent meta-analysis of prospective cohort studies showed no association between dietary saturated fat intake and CVD, prompting the suggestion that other environmental exposures linked to increased meat consumption are responsible[3]. In fact, the suspicion that the cholesterol and saturated fat content of red meat may not be sufficiently high enough to account for the observed association between CVD and meat consumption has stimulated investigation of alternative disease-promoting exposures that accompany dietary meat ingestion, such as high salt content or heterocyclic compounds generated during cooking[4,5]. To our knowledge, no studies have yet explored the participation of commensal intestinal microbiota in modifying the diet-host interaction with reference to red meat consumption.

The microbiota of humans has been linked to intestinal health, immune function, bioactivation of nutrients and vitamins, and, more recently, complex disease phenotypes such as obesity and insulin resistance[6–8]. We recently reported a pathway in both humans and mice linking microbiota metabolism of dietary choline and phosphatidylcholine to CVD pathogenesis[9]. Choline, a trimethylamine-containing compound and part of the head group of phosphatidylcholine, is metabolized by gut microbiota to produce an intermediate compound known as TMA (**Fig. 1a**). TMA is rapidly further oxidized by hepatic flavin monooxygenases to form TMAO, which is proatherogenic and associated with cardiovascular risks. These findings raise the possibility that other dietary nutrients possessing a trimethylamine structure may also generate TMAO from gut microbiota and promote accelerated atherosclerosis. TMAO has been proposed to induce upregulation of macrophage scavenger receptors and thereby potentially contribute to enhanced "forward cholesterol transport."[10]. Whether TMAO is linked to the development of accelerated atherosclerosis through additional mechanisms, and which specific microbial species contribute to TMAO formation, have not been fully clarified.

L-carnitine is an abundant nutrient in red meat and contains a trimethylamine structure similar to that of choline (**Fig. 1a**). Although dietary ingestion is a major source of L-carnitine in omnivores, it is also endogenously produced in mammals from lysine and serves an essential function in transporting fatty acids into the

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mitochondrial compartment[10,11]. L-Carnitine ingestion and supplementation in industrialized societies have markedly increased[12]. Whether there are potential health risks associated with the rapidly growing practice of consuming L-carnitine supplements has not been evaluated.

Herein we examine the gut microbiota–dependent metabolism of L-carnitine to produce TMAO in both rodents and humans (omnivores and vegans or vegetarians). Using isotope tracer studies in humans, clinical studies to examine the effects on cardiovascular disease risk, and animal models including germ-free mice, we demonstrate a role for gut microbiota metabolism of L-carnitine in atherosclerosis pathogenesis. We show that TMAO, and its dietary precursors choline and carnitine, suppress reverse cholesterol transport (RCT) through gut microbiota–dependent mechanisms *in vivo*. Finally, we define microbial taxa in feces of humans whose proportions are associated with both dietary carnitine ingestion and plasma TMAO concentrations. We also show microbial compositional changes in mice associated with chronic carnitine ingestion and a consequent marked enhancement in TMAO synthetic capacity *in vivo*.

RESULTS

Metabolomic studies link L-carnitine with CVD

Given the similarity in structure between L-carnitine and choline (**Fig. 1a**), we hypothesized that dietary L-carnitine in humans, like choline and phosphatidylcholine, might be metabolized to produce TMA and TMAO in a gut microbiota–dependent fashion and be associated with atherosclerosis risk. To test this hypothesis, we initially examined data from our recently published unbiased small-molecule metabolomics analyses of plasma analytes and CVD risks[9].

An analyte with identical molecular weight and retention time to L-carnitine was not in the top tier of analytes that met the stringent *P* value cutoff for association with CVD. However, a hypothesis-driven examination of the data using less stringent criteria (no adjustment for multiple testing) revealed an analyte with the appropriate molecular weight and retention time for L-carnitine that was associated with cardiovascular event risk ($P = 0.04$) (**Supplementary Table 1**). In further studies we were able to confirm the identity of the plasma analyte as L-carnitine and develop a quantitative stable-isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) method for measuring endogenous L-carnitine concentrations in all subsequent investigations (**Supplementary Figs. 1–3**).

Human gut microbiota is required to form TMAO from L-carnitine

The participation of gut microbiota in TMAO production from dietary L-carnitine in humans has not previously been shown. In initial subjects (omnivores), we developed an L-carnitine challenge test in which the subjects were fed a large amount of L-carnitine (an 8-ounce sirloin steak, corresponding to an estimated 180 mg of L-carnitine)[13–15], together with a capsule containing 250 mg of a heavy isotope–labeled L-carnitine (synthetic d3-(methyl)-L-carnitine). At visit 1 post-prandial increases in plasma d3-TMAO and d3- L-carnitine concentrations were readily detected, and 24-h urine collections also revealed the presence of d3-TMAO (**Fig. 1b–e** and **Supplementary Figs. 4** and **5**). **Figure 1** and **Supplementary Figure 4** show tracings from a representative omnivorous subject, of five studied with sequential serial blood draws after carnitine challenge. In most subjects examined, despite clear increases in plasma d3-carnitine and d3-TMAO concentrations over time (**Fig. 1e**), post-prandial changes in endogenous (unlabeled) carnitine and TMAO concentrations were modest (**Supplementary Fig. 5**), consistent with total body pools of carnitine and TMAO that are relatively very large in relation to the amounts of carnitine ingested and TMAO produced from the carnitine challenge.

To examine the potential contribution of gut microbiota to TMAO formation from dietary L-carnitine, we placed the five volunteers studied above on oral broad-spectrum antibiotics to suppress intestinal microbiota for a week and then performed a second L-carnitine challenge (visit 2). We noted near complete suppression of detectable endogenous TMAO in both plasma and urine after a week-long treatment with the antibiotics (visit 2)

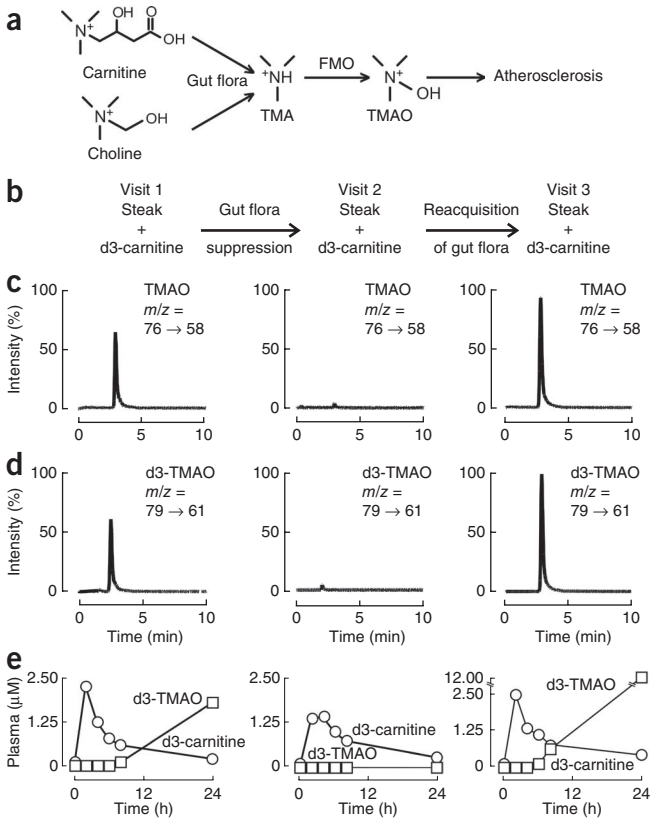

Figure 1 TMAO production from L-carnitine is a microbiota-dependent process in humans. (**a**) Structure of carnitine and scheme of carnitine and choline metabolism to TMAO. L-Carnitine and choline are both dietary trimethylamines that can be metabolized by microbiota to TMA. TMA is then further oxidized to TMAO by flavin monooxygenases (FMOs). (**b**) Scheme of the human L-carnitine challenge test. After a 12-h overnight fast, subjects received a capsule of d3-(methyl)-carnitine (250 mg) alone, or in some cases (as in data for the subject shown) also an 8-ounce steak (estimated 180 mg L-carnitine), whereupon serial plasma and 24-h urine samples were obtained for TMA and TMAO analyses (visit 1). After a weeklong regimen of oral broad-spectrum antibiotics to suppress the intestinal microbiota, the challenge was repeated (visit 2), and then again a final third time after a ≥3-week period to permit repopulation of intestinal microbiota (visit 3). (**c**,**d**) LC-MS/MS chromatograms of plasma TMAO (**c**) and d3-TMAO (**d**) in an omnivorous subject using specific precursor → product ion transitions indicated at $t = 8$ h for each visit. (**e**) Stable-isotope-dilution LC-MS/MS time course measurements of d3-labeled TMAO and carnitine in plasma collected from sequential venous blood draws at the indicated time points. Data shown in **c**–**e** are from a representative female omnivorous subject who underwent carnitine challenge. Data are organized vertically to correspond with the visit schedule indicated in **b**.

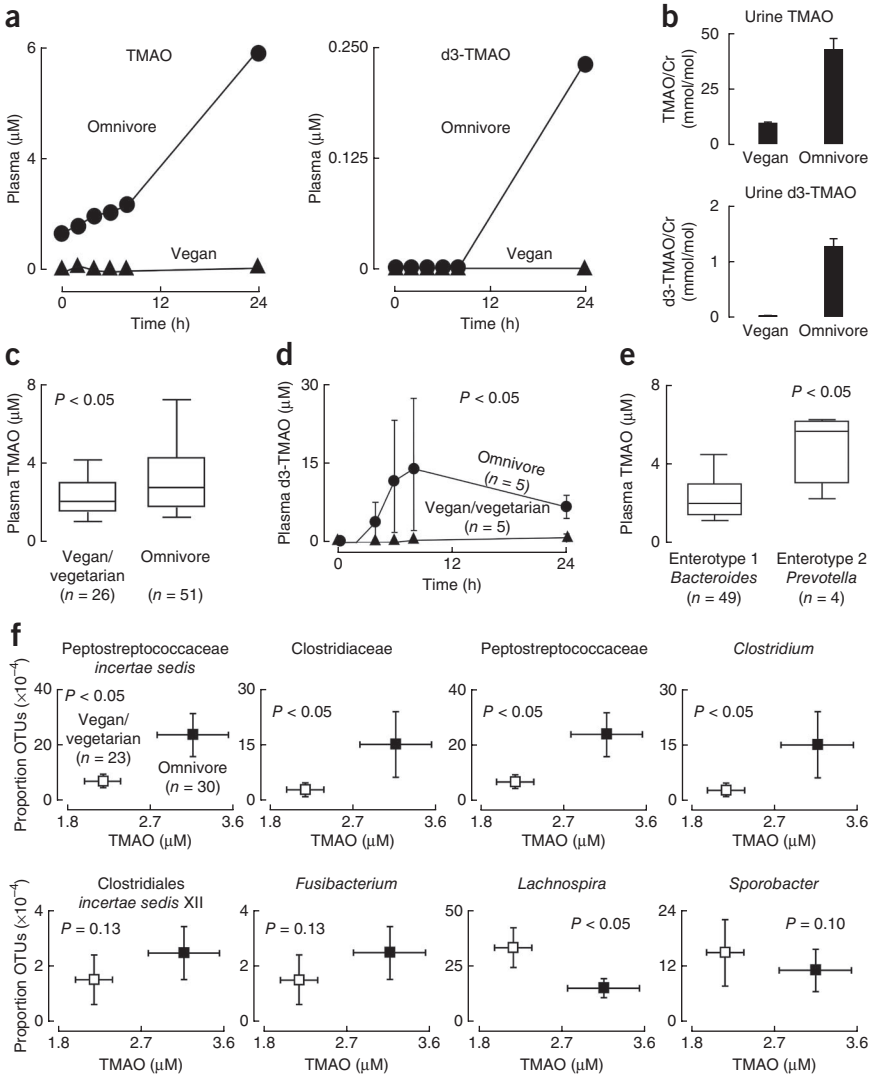

Figure 2 The formation of TMAO from ingested L-carnitine is negligible in vegans, and fecal microbiota composition associates with plasma TMAO concentrations. (**a**,**b**) Data from a male vegan subject in the carnitine challenge consisting of co-administration of 250 mg d3-(methyl)-carnitine and an 8-ounce sirloin steak and, for comparison, a representative female omnivore who frequently consumes red meat. Plasma TMAO and d3-TMAO were quantified after L-carnitine challenge (**a**) and in a 24-h urine collection (**b**). Urine TMAO and d3-TMAO reported as ratio with urinary creatinine (Cr) to adjust for urinary dilution. Data are expressed as means ± s.e.m. (**c**) Baseline fasting plasma concentrations of TMAO and d3-TMAO from male and female vegans and vegetarians ($n = 26$) and omnivores ($n = 51$). Boxes represent the 25th, 50th, and 75th percentiles and whiskers represent the 10th and 90th percentiles. (**d**) Plasma d3-TMAO concentrations in male and female vegans and vegetarians ($n = 5$) and omnivores ($n = 5$) participating in a d3-(methyl)-carnitine (250 mg) challenge without concomitant steak consumption. The *P* value shown is for the comparison of the area under the curve (AUC) of groups using the Wilcoxon nonparametric test. Data points represent mean ± s.e.m. of $n = 5$ per group. (**e**) Baseline TMAO plasma concentrations associate with enterotype 2 (*Prevotella*) in male and female subjects with a characterized gut microbiome enterotype. Boxes represent the 25th, 50th (middle lines) and 75th percentiles, and whiskers represent the 10th and 90th percentiles. (**f**) Plasma TMAO concentrations (plotted on *x* axes) and the proportion of taxonomic operational units (OTUs, plotted on *y* axes), determined as described in **Supplementary Methods**. Subjects were grouped by dietary status as either vegan or vegetarian ($n = 23$) or omnivore ($n = 30$). *P* value shown is for comparisons between dietary groups using a robust Hotelling T^2 test. Data are expressed as means ± s.e.m. for both TMAO concentration (*x* axis) and the proportion of OTUs (*y* axis).

(**Fig. 1b–e** and **Supplementary Fig. 5**). Moreover, we observed virtually no detectable formation of either native or d3-labeled TMAO in all post-prandial plasma samples or 24-h urine samples examined after carnitine challenge, consistent with an obligatory role for gut microbiota in TMAO formation from L-carnitine (**Fig. 1b–e** and **Supplementary Fig. 4**). In contrast, we detected both d3- L-carnitine and unlabeled L-carnitine after the L-carnitine challenge, and there was little change in the overall time course before (visit 1) versus after (visit 2) antibiotic treatment (**Fig. 1e** and **Supplementary Fig. 5**). We rechallenged the same subjects several weeks after discontinuation of antibiotics (visit 3). Baseline and post-L-carnitine challenge plasma and urine samples again showed TMAO and d3-TMAO formation, consistent with intestinal recolonization (**Fig. 1b–e** and **Supplementary Figs. 4** and **5**). Collectively, these data show that TMAO production from dietary L-carnitine in humans is dependent on intestinal microbiota.

Vegans and vegetarians produce less TMAO from L-carnitine

The capacity to produce TMAO (native and d3-labeled) after L-carnitine ingestion was variable among individuals. A *post hoc* nutritional survey that the volunteers completed suggested that antecedent dietary habits (red meat consumption) may influence the capacity to generate TMAO from L-carnitine (data not shown). To test this prospectively, we examined TMAO and d3-TMAO production after the same L-carnitine challenge, first in a long-term (>5 years) vegan who consented to the carnitine challenge (including both steak and d3-(methyl)-carnitine consumption) (**Fig. 2a**). Also shown for comparison are data from a single representative omnivore with self-reported frequent (near daily) dietary consumption of red meat (beef, venison, lamb, mutton, duck or pork). Post-prandially, the omnivore showed increases in TMAO and d3-TMAO concentrations in both sequential plasma measurements (**Fig. 2a**) and in a 24-h urine collection sample (**Fig. 2b**). In contrast, the vegan showed nominal plasma and urine TMAO levels at baseline, and virtually no capacity to generate TMAO or d3-TMAO in plasma after the carnitine challenge (**Fig. 2a**,**b**). The vegan subject also had lower fasting plasma levels of L-carnitine compared to the omnivorous subject (**Supplementary Fig. 6**).

To confirm and extend these findings, we examined additional vegans and vegetarians ($n = 23$) and omnivorous subjects ($n = 51$).

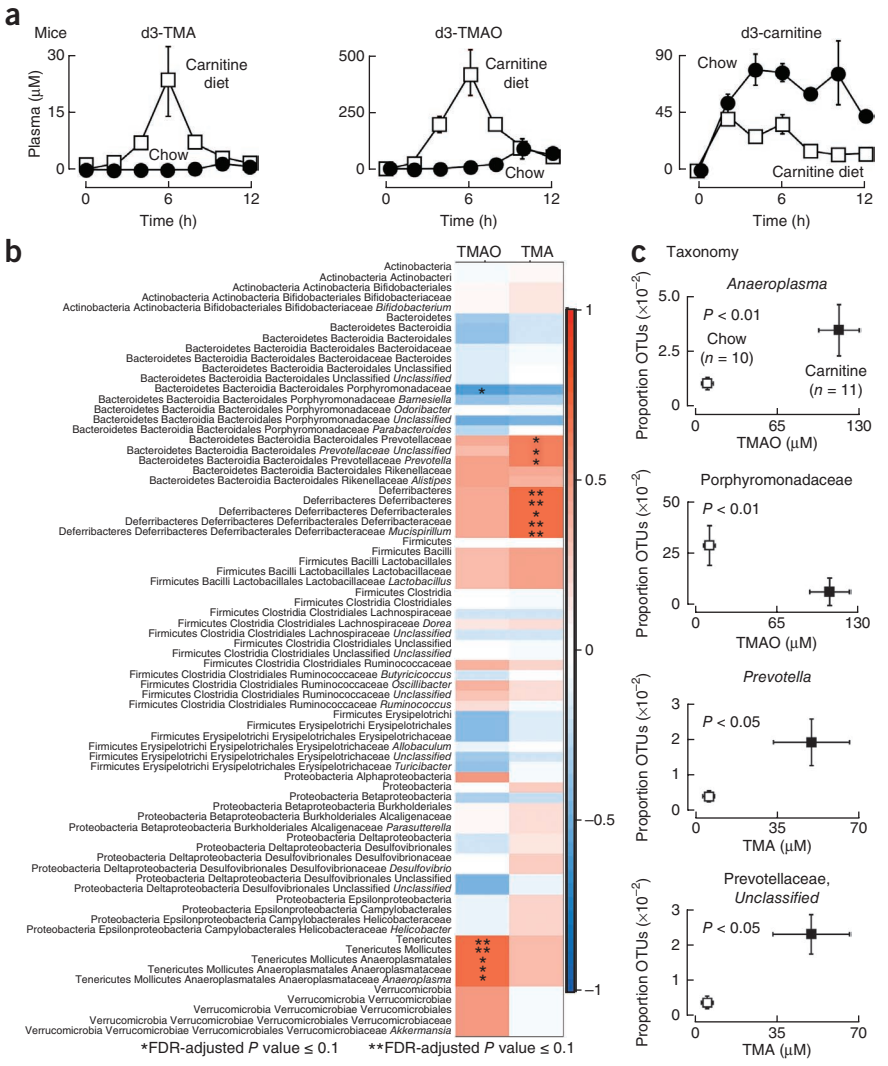

Figure 3 The metabolism of carnitine to TMAO is an inducible trait and associates with microbiota composition. (**a**) d3-carnitine challenge of mice on either an L-carnitine–supplemented diet (1.3%) for 10 weeks and compared to age-matched normal chow–fed controls. Plasma d3-TMA and d3-TMAO were measured at the indicated times after d3-(methyl)-carnitine administration by oral gavage using stable-isotope-dilution LC-MS/MS. Data points represent mean ± s.e.m. of $n = 4$ per group. (**b**) Correlation heat map demonstrating the association between the indicated microbiota taxonomic genera and TMA and TMAO concentrations (all reported as mean ± s.e.m. in μM) of mice grouped by dietary status (chow, $n = 10$ (TMA, 1.3 ± 0.4; TMAO, 17 ± 1.9); and L-carnitine, $n = 11$ (TMA, 50 ± 16; TMAO, 114 ± 16). Red denotes a positive association, blue a negative association, and white no association. A single asterisk indicates a significant FDR-adjusted association of $P \leq 0.1$, and a double asterisk indicates a significant FDR-adjusted association of $P \leq 0.01$. (**c**) Plasma TMAO and TMA concentrations determined by stable-isotope-dilution LC-MS/MS (plotted on *x* axes) and the proportion OTUs (plotted on *y* axes). Statistical and laboratory analyses were performed as described in **Supplementary Methods**. Data are expressed as means ± s.e.m. for both TMAO or TMA concentrations (*x* axis) and the proportion of OTUs (*y* axis).

Fasting baseline TMAO levels were significantly lower among vegan and vegetarian subjects compared to omnivores (**Fig. 2c**). In a subset of these individuals, we performed an oral d3-(methyl)-carnitine challenge (but with no steak) and confirmed that long-term (all >1 year) vegans and vegetarians have a markedly reduced capacity to synthesize TMAO from oral carnitine (**Fig. 2c,d**). Vegans and vegetarians challenged with d3-(methyl)-carnitine also had significantly higher post-challenge plasma concentrations of d3-(methyl)-carnitine compared to omnivorous subjects (**Supplementary Fig. 7**), perhaps due to decreased intestinal microbial metabolism of carnitine before absorption.

TMAO levels are associated with human gut microbial taxa

Dietary habits (for example, vegan or vegetarian versus omnivore or carnivore) are associated with significant alterations in intestinal microbiota composition[16–18]. To determine microbiota composition, we sequenced the gene encoding bacterial 16S rRNA in fecal samples from a subset of the vegans and vegetarians ($n = 23$) and omnivores ($n = 30$) studied above. In parallel, we quantified plasma TMAO, carnitine and choline concentrations by stable-isotope-dilution LC-MS/MS. Global analysis of taxa proportions (**Supplementary Methods**) revealed significant associations with plasma TMAO concentrations ($P = 0.03$), but not with plasma carnitine ($P = 0.77$) or choline ($P = 0.74$) concentrations.

After false discovery rate (FDR) adjustment for multiple comparisons, several bacterial taxa remained significantly (FDR-adjusted $P < 0.10$) associated with plasma TMAO concentration (**Supplementary Fig. 8**). When we classified subjects into previously reported enterotypes[19] on the basis of fecal microbial composition, individuals with an enterotype characterized by enriched proportions of the genus *Prevotella* ($n = 4$) had higher ($P < 0.05$) plasma TMAO concentrations than did subjects with an enterotype notable for enrichment in the *Bacteroides* ($n = 49$) genus (**Fig. 2e**). Examination of the proportion of specific bacterial genera and subject plasma TMAO concentrations revealed several taxa (at the genus level) that simultaneously were significantly associated with both vegan or vegetarian versus omnivore status and plasma TMAO concentration (**Fig. 2f**). These results indicate that preceding dietary habits may modulate both intestinal microbiota composition and ability to synthesize TMA and TMAO from dietary L-carnitine.

TMAO production from dietary L-carnitine is inducible

We next investigated the ability of chronic dietary L-carnitine intake to induce gut microbiota–dependent production of TMA and TMAO in mice. Initial LC-MS/MS studies in germ-free mice showed no detectable plasma d3-(methyl)-TMA or d3-(methyl)-TMAO after oral (gastric gavage) d3-(methyl)-carnitine challenge. However, after a several-week period in conventional cages to allow for microbial colonization ('conventionalization'), previously germ-free mice acquired the capacity to produce both d3-(methyl)-TMA and d3-(methyl)-TMAO following oral d3-(methyl)-carnitine challenge

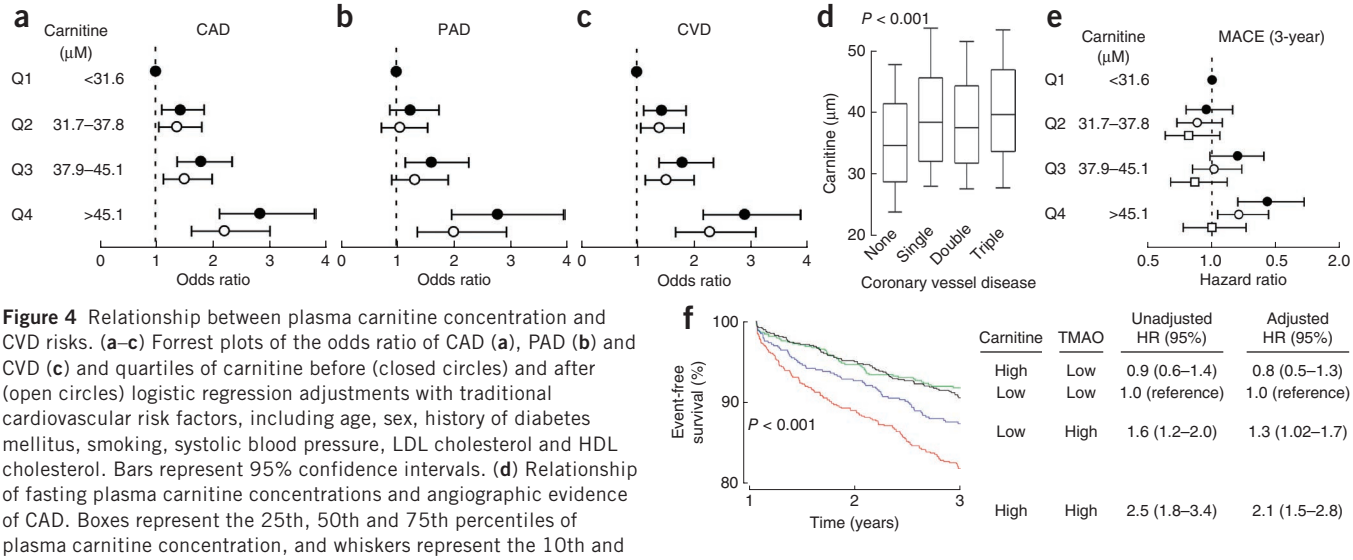

Figure 4 Relationship between plasma carnitine concentration and CVD risks. (**a**–**c**) Forrest plots of the odds ratio of CAD (**a**), PAD (**b**) and CVD (**c**) and quartiles of carnitine before (closed circles) and after (open circles) logistic regression adjustments with traditional cardiovascular risk factors, including age, sex, history of diabetes mellitus, smoking, systolic blood pressure, LDL cholesterol and HDL cholesterol. Bars represent 95% confidence intervals. (**d**) Relationship of fasting plasma carnitine concentrations and angiographic evidence of CAD. Boxes represent the 25th, 50th and 75th percentiles of plasma carnitine concentration, and whiskers represent the 10th and 90th percentiles. The Kruskal-Wallis test was used to assess the degree of CAD (none, single-, double- or triple-vessel disease) association with plasma carnitine concentrations. (**e**) Forrest plot of the hazard ratio of MACE and quartiles of carnitine unadjusted (closed circles) and after adjusting for traditional cardiovascular risk factors (open circles), or traditional cardiac risk factors plus creatinine clearance, history of myocardial infarction, history of CAD, burden of CAD (one-, two- or three-vessel disease), left ventricular ejection fraction, baseline medications (angiotensin-converting enzyme (ACE) inhibitors, statins, beta blockers and aspirin) and TMAO levels (open squares). Bars represent 95% confidence intervals. (**f**) Kaplan-Meier plot and hazard ratios with 95% confidence intervals for unadjusted model, or following adjustments for traditional risk factors as in **e**. Median plasma concentration of carnitine (46.8 μM) and TMAO (4.6 μM) within the cohort were used to stratify subjects as having 'high' (≥median) or 'low' (<median) values.

(**Supplementary Fig. 9**). Parallel studies with non-germ-free ('conventional') *Apoe*$^{-/-}$ mice (lacking apolipoprotein E; on a C57BL/6J background) that had been placed on a cocktail of oral, relatively nonabsorbable broad-spectrum antibiotics previously shown to suppress intestinal microbiota[9,20] showed similar results (complete suppression of both TMA and TMAO formation; **Supplementary Fig. 10**). Collectively, these studies confirm in mice an obligatory role for gut microbiota in TMA and TMAO production from dietary L-carnitine.

To examine whether dietary L-carnitine can induce TMA and TMAO production from intestinal microbiota, we compared the pre- and post-prandial plasma profiles of *Apoe*$^{-/-}$ mice on normal chow diet versus a normal chow diet supplemented with L-carnitine for 15 weeks. The production of both d3-(methyl)TMA and d3-(methyl)TMAO after gastric gavage of d3-(methyl)-carnitine was induced by approximately tenfold in mice on the L-carnitine–supplemented diet (**Fig. 3a**). Furthermore, plasma post-prandial d3-(methyl)-carnitine levels in mice in the L-carnitine–supplemented diet arm were substantially lower than those observed in mice on the L-carnitine–free diet (normal chow), consistent with enhanced microbiota-dependent catabolism before absorption in the L-carnitine–supplemented mice.

Plasma TMA and TMAO associate with mouse gut microbial taxa

The marked effects of an acute L-carnitine challenge (d3-(methyl)-carnitine by gavage) on TMA and TMAO production suggested that chronic L-carnitine supplementation may significantly alter intestinal microbial composition, with an enrichment for taxa better suited for TMA production from L-carnitine. To test this hypothesis, we first identified the cecum as the segment of the entire intestinal tract of mice showing the highest synthetic capacity to form TMA from carnitine (data not shown). We then sequenced 16S rRNA gene amplicons from ceca of mice on either normal chow (n = 10) or L-carnitine-supplemented (n = 11) diets and in parallel quantified plasma concentrations of TMA and TMAO (**Fig. 3b**). Global analyses of the presence of the microbiota taxa revealed that, in general, taxa that were at a relatively high proportion coincident with high TMA plasma concentrations also tended to be a relatively high proportion coincident with high TMAO plasma concentrations. Several bacterial taxa remained significantly associated with plasma TMA and/or TMAO levels after FDR adjustment for multiple comparisons (**Fig. 3b**).

Further analyses revealed several bacterial taxa whose proportion was significantly associated (some positively, others inversely) with dietary L-carnitine and with plasma TMA or TMAO concentrations ($P < 0.05$) (**Fig. 3c** and **Supplementary Fig. 11**). Notably, a direct comparison of taxa associated with plasma TMAO concentrations in humans versus in mice failed to identify common taxa. These results are consistent with prior reports that microbes identified from the distal gut of the mouse represent genera that are typically not detected in humans[16,21].

High plasma L-carnitine concentration is associated with CVD

We next investigated the relationship of fasting plasma concentrations of L-carnitine with CVD risk in an large, independent cohort of stable subjects (n = 2,595) undergoing elective cardiac evaluation. Patient demographics, laboratory values and clinical characteristics are provided in **Supplementary Table 2**. We observed significant dose-dependent associations between carnitine concentration and risks of prevalent coronary artery disease (CAD) ($P < 0.05$), peripheral artery disease (PAD) ($P < 0.05$) and overall CVD ($P < 0.05$) (**Fig. 4a**–**c**). Moreover, these associations remained significant following adjustments for traditional CVD risk factors ($P < 0.05$) (**Fig. 4a**–**c**). Plasma concentrations of carnitine were high in subjects with angiographic evidence of CAD (≥50% stenosis), regardless of the extent (for example, single- versus multivessel) of CAD, as

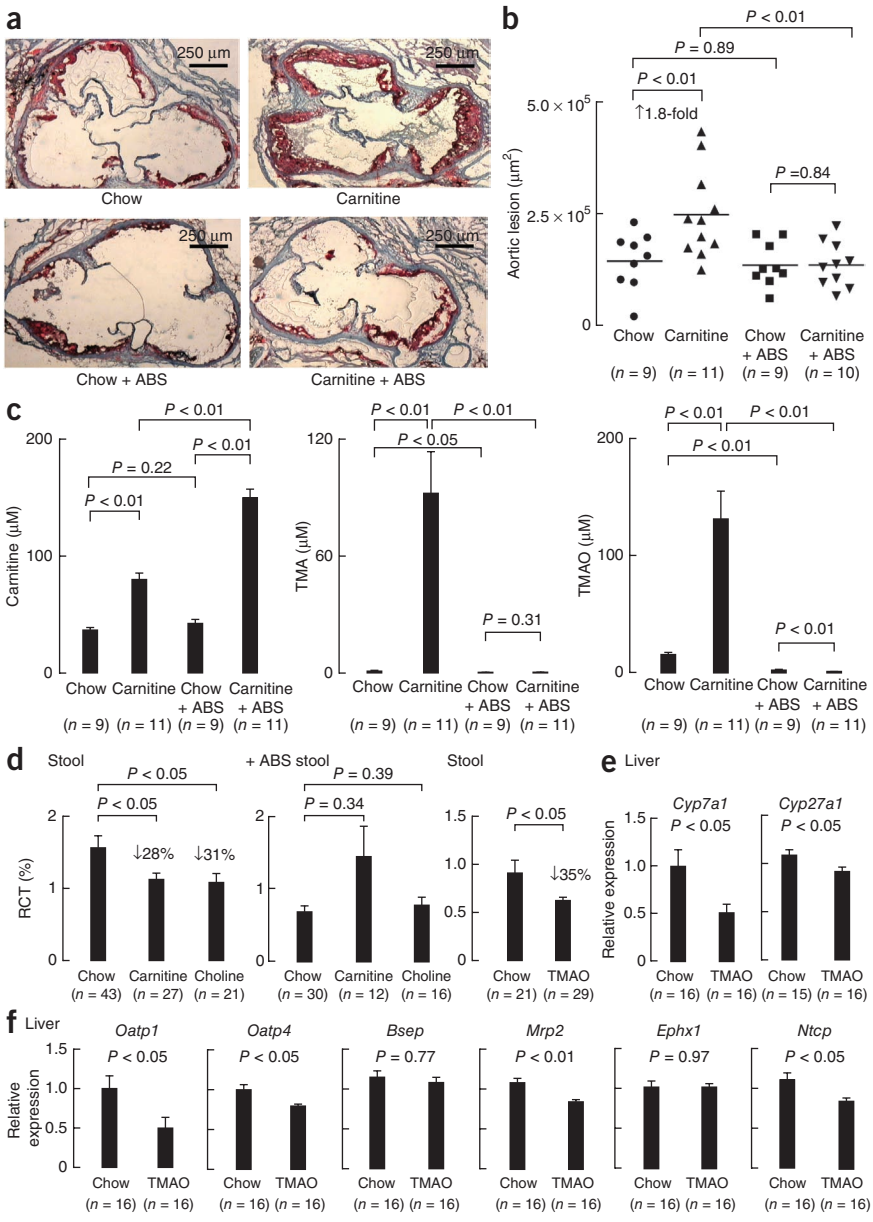

Figure 5 Dietary L-carnitine accelerates atherosclerosis and inhibits reverse cholesterol transport in a microbiota dependent fashion. (**a**) Representative oil red O–stained aortic roots (counterstained with hematoxylin) of 19-week-old *Apoe*$^{-/-}$ female mice on the indicated diets in the presence versus absence of antibiotics (ABS) as described in the Online Methods. (**b**) Quantification of mouse aortic root plaque lesion area. *Apoe*$^{-/-}$ female mice at 19 weeks of age were started on the indicated diets at the time of weaning (4 weeks of age) before killing, and lesion area was quantified as described in the Online Methods. (**c**) Carnitine, TMA and TMAO concentrations as determined using stable-isotope-dilution LC-MS/MS analysis of plasma recovered from mice at the time of killing. (**d**) RCT (72-h stool collection) in adult female (>8 weeks of age) *Apoe*$^{-/-}$ mice on normal chow versus diet supplemented with either L-carnitine or choline, as well as after suppression of microbiota using cocktail of antibiotics (+ ABS). Also shown are RCT (72-h stool collection) results in adult female (>8 weeks of age) *Apoe*$^{-/-}$ mice on normal chow versus diet supplemented with TMAO. (**e**,**f**) Relative mRNA levels (to *Actb*) of mouse liver candidate genes involved in bile acid synthesis or transport. Data are expressed as means ± s.e.m.

revealed by diagnostic cardiac catheterization (Kruskal-Wallis $P < 0.001$) (**Fig. 4d**).

We also examined the relationship between fasting plasma concentrations of carnitine and incident (3-year) risk for major adverse cardiac events (MACE: composite of death, myocardial infarction, stroke and revascularization). Elevated carnitine (4th quartile) concentration was an independent predictor of MACE, even after adjustments for traditional CVD risk factors (**Fig. 4e**). After further adjustment for both plasma TMAO concentration and a larger number of comorbidities that might be known at time of presentation (for example, extent of CAD, ejection fraction, medications and estimated renal function), the significant relationship between carnitine and MACE risk was completely abolished (**Fig. 4e**). Notably, we observed a significant association between carnitine concentration and incident cardiovascular event risks in Cox regression models after multivariate adjustment, but only among those subjects with concurrent high plasma TMAO concentrations ($P < 0.001$) (**Fig. 4f**). Thus, although plasma concentrations of carnitine seem to be associated with both prevalent and incident cardiovascular risks, these results suggest that TMAO, rather than carnitine, is the primary driver of the association of carnitine with cardiovascular risks.

Dietary L-carnitine promotes microbiota-dependent atherosclerosis

We next investigated whether dietary L-carnitine has an impact on the extent of atherosclerosis in the presence or absence of TMAO formation. We fed *Apoe*$^{-/-}$ mice from the time of weaning a normal chow diet versus the same diet supplemented with L-carnitine. Aortic root atherosclerotic plaque quantification revealed approximately a doubling of disease burden in L-carnitine supplemented mice compared to normal chow–fed mice (**Fig. 5a**,**b**). Parallel studies in mice placed on an oral antibiotic cocktail to suppress intestinal microbiota showed marked reductions in plasma TMA and TMAO concentrations (**Fig. 5c**) and complete inhibition of the dietary L-carnitine–dependent increase in atherosclerosis (**Fig. 5b**). Of note, the increase in atherosclerotic plaque burden with dietary L-carnitine occurred in the absence of proatherogenic changes in plasma lipid, lipoprotein, glucose or insulin levels; moreover, both biochemical and histological analyses of livers from any group of the mice failed to show evidence of steatosis (**Supplementary Tables 3** and **4** and **Supplementary Fig. 12**).

Plasma concentrations of carnitine were significantly higher in L-carnitine–fed mice compared to normal chow–fed controls ($P < 0.05$) (**Fig. 5c**). Plasma carnitine concentrations were even higher in mice supplemented with L-carnitine in the antibiotic arm of the study (**Fig. 5c**), presumably as a result of the reduced capacity of microbiota to catabolize L-carnitine. However, as the

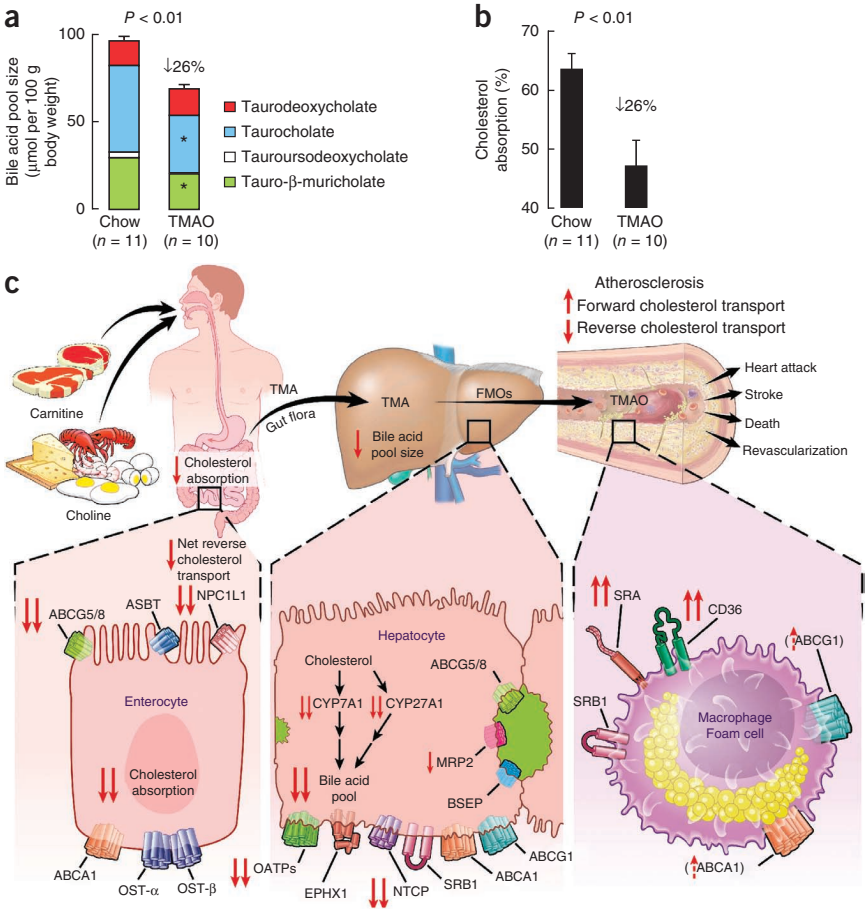

Figure 6 Effect of TMAO on cholesterol and sterol metabolism. (**a**,**b**) Measurement of total bile acid pool size and composition (**a**) and cholesterol absorption (**b**) in adult female (>8 weeks of age) *Apoe*$^{-/-}$ mice on normal chow diet versus diet supplemented with TMAO for 4 weeks. Data are expressed as means ± s.e.m. (**c**) Summary scheme outlining the proposed pathway by which microbiota participate in atherosclerosis. The microbiota metabolizes dietary L-carnitine and choline to form TMA and TMAO. TMAO affects cholesterol and sterol metabolism in macrophages, liver and intestine.

L-carnitine–supplemented mice that received antibiotics did not show enhanced atherosclerosis, these results are consistent with the notion that it is a downstream microbiota-dependent metabolite, not L-carnitine itself, that promotes atherosclerosis.

TMAO inhibits RCT

To identify additional mechanisms by which TMAO might promote atherosclerosis, we first noted that TMAO and its trimethylamine nutrient precursors are all cationic quaternary amines that could potentially compete with arginine, thereby limiting its bioavailability and reducing nitric oxide synthesis. However, a direct test of this hypothesis with competition studies using [^{14}C]arginine and TMAO in bovine aortic endothelial cells demonstrated no decrease in [^{14}C]arginine transport (**Supplementary Fig. 13**).

In recent studies we showed that TMAO can promote macrophage cholesterol accumulation in a microbiota-dependent manner by increasing cell surface expression of two proatherogenic scavenger receptors, CD36 and scavenger receptor A (SRA)[9,22,23]. We envisioned three non-exclusive mechanisms through which cholesterol can accumulate within cells of the artery wall: enhancing the rate of influx (as noted above), enhancing synthesis or diminishing the rate of efflux. To test whether TMAO might alter the canonical regulation of cholesterol biosynthesis genes[24], we loaded macrophages with cholesterol in the presence or absence of physiologically relevant TMAO concentrations. However, TMAO failed to alter mRNA levels of the low-density lipoprotein (LDL) receptor or cholesterol synthesis genes (**Supplementary Fig. 14**). Parallel studies examining macrophage inflammatory gene expression[25] and desmosterol levels in the culture medium also failed to show any effect of TMAO (**Supplementary Figs. 14** and **15**).

We next examined whether TMAO might inhibit cholesterol removal from peripheral macrophages by testing whether dietary sources of TMAO (choline or L-carnitine) inhibit RCT *in vivo* using an adaptation of an established model system[26]. Mice on either choline (1.3% choline chloride by mass)- or L-carnitine–supplemented diets showed significantly less (~30%, $P < 0.05$) RCT compared to normal chow–fed controls (**Fig. 5d**). Furthermore, suppression of intestinal microbiota (and plasma TMAO concentrations) with oral broad-spectrum antibiotics completely blocked the diet-dependent (for both choline and L-carnitine) suppression of RCT (**Fig. 5d**), suggesting that a microbiota-generated product (for example, TMAO) inhibits RCT (**Supplementary Fig. 16**). To further test this hypothesis, we placed mice on a TMAO-containing diet. They showed a 35% decrease in RCT relative to mice on a normal chow diet (**Fig. 5d**, $P < 0.05$). Further examination of plasma, liver and bile showed significantly less [^{14}C]cholesterol recovered from plasma of TMAO-fed compared to chow-fed mice (16% lower, $P < 0.05$) but no changes in counts recovered from liver or bile (**Supplementary Fig. 17**).

TMAO alters sterol metabolism *in vivo*

To better understand the molecular mechanisms through which TMAO suppresses RCT, we examined candidate genes and biological processes in compartments (macrophages, plasma, liver and intestine) known to participate in cholesterol and sterol metabolism and RCT. We exposed peritoneal macrophages recovered from wild-type C57BL/6J mice to TMAO *in vitro* and quantified mRNA levels of the cholesterol transporters Abca1, Srb1 and Abcg1. TMAO treatment led to modest but statistically significant increases in expression of Abca1 and Abcg1 ($P < 0.05$; **Supplementary Fig. 18**). Parallel studies showed corresponding modest TMAO-dependent increases in Abca1-dependent cholesterol efflux to apoA1 as cholesterol acceptor in RAW 264.7 macrophages ($P < 0.01$; **Supplementary Fig. 19**). Collectively, these results suggest that the observed global reduction in RCT *in vivo* induced by TMAO is unlikely to be accounted for by changes in the expression of these transporters. Parallel examination of plasma recovered from mice in the RCT experiments showed no differences in total cholesterol and high-density lipoprotein cholesterol concentrations (**Supplementary Table 5**).

In parallel studies, we examined the mRNA levels of known cholesterol transporters (Srb1, Abca1, Abcg1, Abcg5, Abcg8 and Shp) in mouse liver but found only a modest difference for Srb1 expression (**Supplementary Fig. 20**). Western blot analysis of liver from TMAO-supplemented mice, however, showed no change in the abundance of Srb1 protein compared to chow (control) mouse livers (**Supplementary Fig. 21**). In contrast, mRNA levels in the liver of the key bile acid synthetic enzymes Cyp7a1 and Cyp27a1 were significantly lower in mice supplemented with dietary TMAO, with no change in expression of the upstream regulator Shp ($P < 0.05$ for each; **Fig. 5e** and **Supplementary Fig. 20**). Multiple bile acid transporters in the liver (Oatp1, Oatp4, Mrp2, and Ntcp) also showed significant dietary TMAO–induced decreases in expression ($P < 0.05$ each); however, Bsep and Ephx1 did not (**Fig. 5f**). In contrast to the liver, TMAO-induced changes in bile acid transporter gene expression were not observed in the gut (**Supplementary Fig. 22**). Taken together, these data show that the gut microbiota–dependent metabolite TMAO affects a major pathway for cholesterol elimination from the body, the bile acid synthetic pathway, at multiple levels.

Consistent with the effects of TMAO on bile acid transporter gene expression, mice supplemented with TMAO had a significantly smaller total bile acid pool size compared to normal chow–fed mice ($P < 0.01$) (**Fig. 6a**). Dietary supplementation with TMAO also markedly lowered mRNA expression of both types of intestinal cholesterol transporters: Npc1L1, which transports cholesterol into enterocyte from the gut lumen[27], and Abcg5-Abcg8, which transport cholesterol out of enterocytes into the gut lumen[27] (**Supplementary Fig. 23**). Previous studies using either *Cyp7a1*- or *Cyp27a1*-null mice demonstrated a reduction in cholesterol absorption[28,29]. In separate studies, dietary TMAO supplementation compared to normal chow similarly promoted a decrease (26% reduced compared to normal chow–fed mice, $P < 0.01$) in total cholesterol absorption (**Fig. 6b**).

DISCUSSION

The dietary nutrient L-carnitine has been studied for over a century[30]. Although eukaryotes can endogenously produce L-carnitine, only prokaryotic organisms can catabolize it[11]. A role for intestinal microbiota in TMAO production from dietary L-carnitine was first suggested by studies in rats[31]. Although TMAO production from alternative dietary trimethylamines has been suggested in humans, a role for the microbiota in the production of TMAO from dietary L-carnitine in humans has not previously been demonstrated[31–33]. The present studies reveal an obligatory role of gut microbiota in the production of TMAO from ingested L-carnitine in humans. They also suggest a new nutritional pathway in CVD pathogenesis that involves dietary L-carnitine, the intestinal microbial community and production of the proatherosclerotic metabolite TMAO. Finally, these studies show that TMAO modulates cholesterol and sterol metabolism at multiple anatomic sites and processes *in vivo*, with a net effect of increasing atherosclerosis.

Our results also suggest a previously unknown mechanism for the observed relationship between dietary red meat ingestion and accelerated atherosclerosis. Consuming foods rich in L-carnitine (predominantly red meat) can increase fasting human L-carnitine concentrations in the plasma[34]. Meats and full-fat dairy products are abundant components of the Western diet and are commonly implicated in CVD. Together, L-carnitine and choline-containing lipids can constitute up to 2% of a Western diet[14,15,35]. Numerous studies have suggested a decrease in atherosclerotic disease risk in vegan and vegetarian individuals compared to omnivores; reduced levels of dietary cholesterol and saturated fat have been suggested as the mechanism explaining this decreased risk[36,37]. Notably, a recent 4.8-year randomized dietary study showed a 30% reduction in cardiovascular events in subjects consuming a Mediterranean diet (with specific avoidance of red meat) compared to subjects consuming a control diet[38]. The present studies suggest that the reduced ingestion of L-carnitine and total choline by vegans and vegetarians, with attendant reductions in TMAO levels, may contribute to their observed cardiovascular health benefits. Conversely, an increased capacity for microbiota-dependent production of TMAO from L-carnitine may contribute to atherosclerosis, particularly in omnivores who consume high amounts of L-carnitine.

One proatherosclerotic mechanism observed for TMAO in the current studies is suppression of RCT (**Fig. 6c**). Dietary L-carnitine and choline each suppressed RCT ($P < 0.05$), but only in mice with intact intestinal microbiota and increased TMA and TMAO concentrations. Suppression of the intestinal microbiota completely eliminated choline- and L-carnitine-dependent suppression of RCT. Moreover, direct dietary supplementation with TMAO promoted a similar suppression of RCT. These results are consistent with a gut microbiota–dependent mechanism whereby generation of TMAO impairs RCT, potentially contributing to the observed proatherosclerotic phenotype of these interventions. Another mechanism by which TMAO may promote atherosclerosis is through increasing macrophage SRA and CD36 surface expression and foam cell formation[9] (**Fig. 6c**). Within macrophages, TMAO does not seem to alter known cholesterol biosynthetic and uptake pathways[24,39] or the more recently described regulatory role of desmosterol in integrating macrophage lipid metabolism and inflammatory gene responses[25]. In the liver, TMAO decreased the bile acid pool size and lowered the expression of key bile acid synthesis and transport proteins (**Fig. 6c**). However, it is unclear whether these changes contribute to the impairment of RCT. Of note, TMAO lowered expression of Cyp7a1, the major bile acid synthetic enzyme and rate-limiting step in the catabolism of cholesterol. The effect of TMAO is thus consistent with reports of human *Cyp7a1* gene variants that are associated with reduced expression of Cyp7a1, leading to decreased bile acid synthesis, decreased bile acid secretion and enhanced atherosclerosis[40–42]. Furthermore, upregulation (as opposed to downregulation) of Cyp7a1 has been reported to lead to expansion of the bile acid pool, increased RCT and reduced atherosclerotic plaque area in susceptible mice[43–45]. Within the intestine, we found that TMAO concentration was also associated with changes in cholesterol metabolism. However, the reduction in cholesterol absorption observed, although consistent with the reduction in intestinal Npc1L1 expression[46] (as well as hepatic Cyp7a1 and Cyp27a1 expression[28,29]), cannot explain the suppression of RCT observed after dietary supplementation with TMAO.

Thus, the molecular mechanisms through which gut microbiota formation of TMAO leads to inhibition of RCT are not entirely clear. It is also not known whether TMAO interacts directly with a specific receptor or whether it acts to alter signaling pathways indirectly by altering protein conformation (that is, via allosteric effects). Whereas TMA has been reported to influence signal transduction by direct interaction with a family of G protein–coupled receptors[47,48], TMAO, a small quaternary amine with aliphatic character, is reportedly capable of directly inducing conformational changes in proteins, stabilizing protein folding and acting as a small-molecule protein chaperone[49,50]. It is thus conceivable that TMAO may alter many signaling pathways without directly acting at a 'TMAO receptor'.

A noteworthy finding is the magnitude with which long-term dietary habits affect TMAO synthetic capacity in both humans (vegans and

vegetarians versus omnivores) and mice (normal chow versus chronic L-carnitine supplementation). Analyses of microbial composition in human feces and mice cecal contents revealed specific taxa that segregate with both dietary status and plasma TMAO concentrations. Recent studies have shown that changes in enterotype are associated with long-term dietary patterns[19]. We observed that plasma TMAO concentration varied significantly ($P < 0.05$) according to previously reported enterotypes. We also showed an obligatory role for gut microbiota in TMAO formation from dietary L-carnitine in mice and humans. The differences observed in TMAO production after an L-carnitine challenge in omnivore versus vegan subjects is striking, and is consistent with the observed differences in microbial community composition. Recent reports have shown differences in microbial communities among vegetarians and vegans versus omnivores[51]. Of note, we observed an increase in baseline plasma TMAO concentrations in what has historically been called enterotype 2 (*Prevotella*), a relatively rare enterotype that in one study was associated with low animal-fat and protein consumption[19]. In our study, three of the four individuals classified into enterotype 2 are self-identified omnivores, suggesting more complexity in the human gut microbiome than anticipated. Indeed, other studies have demonstrated variable results in associating human bacterial genera, including *Bacteroides* and *Prevotella*, to omnivorous and vegetarian eating habits[18,52]. This complexity is no doubt in part attributable to the fact that there are many species within any genus, and distinct species within the same genus may have different capacities to use L-carnitine as a fuel and form TMA. Indeed, prior studies have suggested that multiple bacterial strains can metabolize L-carnitine in culture[53], and species within the genus *Clostridium* differ in their ability to use choline as the sole source of carbon and nitrogen in culture[54]. Our results suggest that multiple 'proatherogenic' (that is, TMA- and TMAO-producing) species probably exist. Consistent with this supposition, others have reported that several bacterial phylotypes are associated with a history of atherosclerosis and that human microbiota biodiversity may in part be influenced by carnivorous eating habits[16,19,55].

The association between plasma carnitine concentrations and cardiovascular risks further supports the potential pathophysiological importance of a carnitine → gut microbiota → TMA/TMAO → atherosclerosis pathway (**Fig. 6c**). The association between high plasma carnitine concentration and CVD risk disappeared after TMAO levels were added to the statistical model. These observations are consistent with a proposed mechanism whereby oral L-carnitine ingestion contributes to atherosclerotic CVD risk via the microbiota metabolite TMAO. There are only a few reports of specific intestinal anaerobic and aerobic bacterial species that can use L-carnitine as a carbon nitrogen source[10,11,56].

L-carnitine is essential for the import of activated long-chain fatty acids from the cytoplasm into mitochondria for β-oxidation, and dietary supplementation with L-carnitine has been widely studied. Some case reports of L-carnitine supplementation have reported beneficial effects in individuals with inherited primary and acquired secondary carnitine deficiency syndromes[13]. L-Carnitine supplementation studies in chronic disease states have reported both positive and negative results[57,58]. Oral L-carnitine supplementation in subjects on hemodialysis raises plasma L-carnitine concentrations to normal levels but also substantially increases TMAO levels[57]. A broader potential therapeutic scope for L-carnitine and two related metabolites, acetyl-L-carnitine and propionyl-L-carnitine, has also been explored for the treatment of acute ischemic events and cardiometabolic disorders (reviewed in ref. 58). Here too, both positive and negative results have been reported. Potential explanations for the discrepant findings of various L-carnitine intervention studies are differences in the duration of dosing or in the route of administration. In many studies, L-carnitine or a related molecule is administered over a short interval or via the parenteral route, thereby bypassing gut microbiota (and hence TMAO formation).

Discovery of a link between L-carnitine ingestion, gut microbiota metabolism and CVD risk has broad health-related implications. Our studies reveal a new pathway potentially linking dietary red meat ingestion with atherosclerosis pathogenesis. The role of gut microbiota in this pathway suggests new potential therapeutic targets for preventing CVD. Furthermore, our studies have public health relevance, as L-carnitine is a common over-the-counter dietary supplement. Our results suggest that the safety of chronic L-carnitine supplementation should be examined, as high amounts of orally ingested L-carnitine may under some conditions foster growth of gut microbiota with an enhanced capacity to produce TMAO and potentially advance atherosclerosis.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.A.K. participated in laboratory, mouse and human studies, assisted in statistical analyses, helped design the experiments and drafted the manuscript. Z.W. performed the initial metabolomics study and assisted with mouse and mass spectrometry analyses. B.S.L. synthesized d3- and d9-carnitine for studies, assisted with mass spectrometry analyses and helped draft the manuscript. E.B.B. and X.F. assisted in performance of mass spectrometry analyses of the large human clinical cohort study. Y.W. and L.L. performed the statistical analyses and critically reviewed the manuscript. J.D.S. helped with aortic root atherosclerosis analyses and critical review of the manuscript. J.A.D. assisted in experimental design. J.A.B. and B.T.S. assisted in laboratory and mouse experiments. E.O. and A.J.L. performed and helped interpret mouse cecal microbiota analyses. J.C., F.D.B., H.L., G.D.W., J.D.L. and R.M.K. assisted in human subject microbiota analyses and helped interpret human microbiota data. M.W. and J.M.B. assisted with measurement of bile acid pool size and helped with critical review of the manuscript. W.H.W.T. helped with human studies and critical review of the manuscript. S.L.H. conceived of the idea, helped design the experiments, provided the funding for the study and helped draft and critically revise the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mice and general procedures. Breeders of all conventional mice (C57BL/6J and *Apoe*$^{-/-}$ mice on a C57BL/6J background) were obtained from Jackson Laboratories. All animal studies were performed under approval of the Animal Research Committee of the Cleveland Clinic. Liver cholesterol was quantified by gas chromatography MS, and liver triglyceride was measured using glycerol phosphate oxidase reagent as described in **Supplementary Methods**. Mouse plasma lipids and glucose and human fasting lipid profile, C-reactive protein (CRP) and glucose were measured using the Abbott ARCHITECT platform, Model ci8200 (Abbott Diagnostics). Mouse HDL was isolated using density ultracentrifugation, and insulin levels were quantified by enzyme-linked immunosorbent assay as described in **Supplementary Methods**. Human plasma myeloperoxidase was measured using US Food and Drug Administration–cleared CardioMPO assay (Cleveland Heart Lab).

Research subjects. All research subjects gave written informed consent. All protocols were approved by the Cleveland Clinic Institutional Review Board. Two cohorts of subjects were used in the present studies. The first group of volunteers (n = 30 omnivores and n = 23 vegetarians or vegans) had extensive dietary questioning and stool, plasma and urine collection. A subset of subjects with stool collected also underwent oral L-carnitine challenge (n = 5 omnivores and n = 5 vegans), consisting of d3(methyl)-carnitine (250 mg within a veggie capsule (Wonder Laboratories)). Where indicated, additional omnivores and one vegan also underwent L-carnitine challenge testing with combined ingestion of the synthetic d3-(methyl)- L-carnitine capsule (250 mg) and an 8-ounce beef steak (consumed within 10 min). Male and female volunteers were at least 18 years of age. Volunteers participating in the L-carnitine challenge tests were excluded if they were pregnant, had chronic illness (including a known history of heart failure, renal failure, pulmonary disease, gastrointestinal disorders or hematologic diseases), had an active infection, had received antibiotics within 2 months of study enrollment, had used any over-the-counter or prescriptive probiotic or bowel cleansing preparation within the past 2 months, had ingested yogurt within the past 7 d, or had undergone bariatric or other intestinal (for example, gallbladder removal, bowel resection) surgery. All other research subjects were derived from GeneBank, a large longitudinal tissue repository with connecting clinical database from sequential consenting stable subjects undergoing elective cardiac evaluation. Further description of the GeneBank cohort can be found in **Supplementary Methods**.

Human L-carnitine challenge test. Consenting adult men and women fasted overnight (12 h) before performing the L-carnitine challenge test, which involved baseline blood and spot urine collection, and then oral ingestion (t = 0 at time of initial ingestion) of a veggie capsule (size O) (Wonder Laboratories) containing 250 mg of a stable isotope–labeled d3-L-(methyl)-carnitine (under an Investigational New Drug exemption from the US Food and Drug Administration). Where indicated, for a subset of subjects, the L-carnitine challenge also included a natural source of L-carnitine (a cooked 8-ounce sirloin steak) eaten over a 10-min period concurrent with taking the capsule containing the d3-L-(methyl)-carnitine. After combined ingestion of the steak and d3-L-(methyl)-carnitine, a series of sequential venous blood draws were performed at the indicated time points, and a 24-h urine collection was performed. An ensuing 1-week treatment period of oral antibiotics (metronidazole 500 mg and ciprofloxacin 500 mg twice daily) was given to suppress intestinal microbiota that use carnitine to form TMA and TMAO; the L-carnitine challenge was then repeated. After at least 3 weeks off of all antibiotics to allow reacquisition of intestinal microbiota, a third and final L-carnitine challenge test was performed. Dietary habits (vegan versus ominivore) were determined using a questionnaire assessment of dietary L-carnitine intake, similar to that conducted by the Atherosclerotic Risk in Community study[59]. d3-L-(methyl)-carnitine was prepared by taking sodium L-norcarnitine dissolved in methanol and reacting it with d3-methyl iodide (Cambridge Isotope) in the presence of potassium hydrogen carbonate to give d3- L-(methyl)-carnitine. Further details regarding d3- L-(methyl)-carnitine synthesis, purification and characterization are described in **Supplementary Methods**.

Metabolomics study. We previously reported results from a metabolomics study where small-molecule analytes were sought that associated with cardiovascular risks[9]. The metabolomics study had a two-stage screening strategy. In the first phase, unbiased metabolomics studies were performed on randomly selected plasma samples from a learning cohort generated from Genebank subjects who had experienced a major adverse cardiovascular event (defined as nonfatal myocardial infarction, stroke or death) (n = 50) in the 3-year period following enrollment versus age- and gender-matched controls (n = 50) who had not experienced an event. A second phase (validation cohort) of unbiased metabolomics analyses was then performed on a nonoverlapping second cohort of cases (n = 25) and age- and gender-matched controls (n = 25) using identical inclusion and exclusion criteria. Further details regarding the unbiased metabolomic approach can be found in **Supplementary Methods**.

Identification of L-carnitine and quantification of TMAO, TMA and L-carnitine. Matching collision-induced dissociation (CID) spectra of an unknown plasma metabolite with identical retention time and mass-to-charge ratio (m/z) as authentic L-carnitine (m/z = 162) were obtained as described in **Supplementary Methods**. Concentrations of carnitine, TMA and TMAO isotopologues in mouse and human plasma samples were determined by stable-isotope-dilution LC-MS/MS in positive multiple reaction monitoring (MRM) mode using deuterated internal standards on an AB Sciex API 5000 triple quadrupole mass spectrometer (Applied Biosystems) as described in **Supplementary Methods**. In studies quantifying endogenous carnitine and ingested d3-L-(methyl)-carnitine, d9-carnitine was used as internal standard. d9-carnitine was prepared by dissolving 3-hydroxy-4-aminobutyric acid (Chem-Impex Intl.) in methanol and exhaustive reaction with d3-methyl iodide (Cambridge Isotope Labs) in the presence of potassium hydrogen carbonate. Further details regarding synthesis, purification and characterization of d9-carnitine can be found in **Supplementary Methods**.

Human microbiota analyses. Stool samples were stored at −80 °C, and DNA for the gene encoding 16S rRNA was isolated using the MoBio PowerSoil kit according to the manufacturer's instructions. DNA samples were amplified using V1-V2 region primers targeting bacterial 16S genes and sequenced using 454/Roche Titanium technology. Sequence reads from this study are available from the Sequence Read Archive (controlled feeding experiment: SRX037803, SRX021237, SRX021236, SRX020772, SRX020771, SRX020588, SRX020587, SRX020379, SRX020378 (metagenomic); cross-sectional study of diet and stool microbiome: SRX020773, SRX020770). The overall association between TMAO measurements and microbiome compositions was assessed using PermanovaG[60] by combining both the weighted and unweighted UniFrac distances. Associations between TMAO measurements and individual taxa proportions were assessed by Spearman's rank correlation test. False discovery rate (FDR) control based on the Benjamini-Hochberg procedure was used to account for multiple comparisons when evaluating these associations. Each of the samples was assigned to an enterotype category on the basis of their microbiome distances (Jensen-Shannon distance) to the medoids of the enterotype clusters as defined in the COMBO data[19]. Association between enterotypes and plasma TMAO concentration was assessed by Wilcoxon rank-sum test. Student's t-test was used to test the difference in means of TMAO concentration between omnivores and vegans. A robust Hotelling T^2 test was used to examine the association between both the proportion of specific bacterial taxa and TMAO concentrations in groups using R software version 2.15 (ref. 61).

Mouse microbiota analysis. Microbial community composition was assessed by pyrosequencing 16S rRNA genes derived from ceca of mice fed a normal chow (n = 11) or L-carnitine (n = 13) diet. DNA was isolated using the MoBio PowerSoil DNA Isolation Kit. The V4 region of the 16S ribosomal DNA gene was amplified using bar-coded fusion primers (F515/R806) with the 454 A Titanium sequencing adaptor as further described in **Supplementary Methods**. The relative abundances of bacteria at each taxonomic level were computed for each mouse, a single representative sequence for each OTU was aligned using PyNAST and a phylogenetic tree was built using FastTree as further described in **Supplementary Methods**. Spearman correlations were calculated to assess correlations between relative abundance of gut microbiota and mouse plasma TMA and TMAO concentrations. False discovery rates (FDR) of the multiple comparisons were estimated for each taxon based on the P values resulted from correlation estimates, as further described in **Supplementary Methods**.

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A robust Hotelling T^2 test was used to examine the association between both the proportion of specific bacterial taxa and mouse plasma TMA and TMAO concentrations in groups using R software version 2.15 (ref. 61).

Aortic root lesion quantification. Apolipoprotein E–knockout mice on a C57BL/6J background (*Apoe*$^{-/-}$) were weaned at 28 d of age and placed on a standard chow control diet (Teklad 2018). L-Carnitine was introduced into the diet by supplementing mouse drinking water with 1.3% L-carnitine (Chem-Impex Intl.), 1.3% L-carnitine and antibiotics, or antibiotics alone. The antibiotic cocktail dissolved in mouse drinking water has previously been shown to suppress commensal gut microbiota and included 0.1% ampicillin sodium salt (Fisher Scientific), 0.1% metronidazole, 0.05% vancomycin (Chem Impex Intl.) and 0.1% neomycin sulfate (Gibco)[20]. Mice were anaesthetized with ketamine and xylazine before terminal bleeding by cardiac puncture to collect blood. Mouse hearts were fixed and stored in 10% neutral-buffered formalin before being frozen in optimal cutting temperature medium for sectioning. Aortic root slides were stained with oil red O and counterstained with hematoxylin. The aortic root atherosclerotic lesion area was quantified as the mean of sequential sections of 6 microns approximately 100 microns apart[9].

Germ-free mice and conventionalization studies. 10-week-old female Swiss Webster germ-free mice (SWGF) were obtained from the University of North Carolina Gnotobiotics Core Facility. Germ-free mice underwent gastric gavage with the indicated isotopologues of L-carnitine (see below for details of L-carnitine challenge) immediately following removal from the germ-free microisolator shipper. After the L-carnitine challenge, germ-free mice were conventionalized by being housed in cages with nonsterile C57BL/6J female mice. Approximately 4 weeks later, the L-carnitine challenge was repeated. Quantification of natural abundance and isotope-labeled L-carnitine, TMA and TMAO in mouse plasma was performed using stable-isotope-dilution LC/MS/MS as described above.

Mouse L-carnitine challenge studies. C57BL/6J female or *Apoe*$^{-/-}$ female mice were given synthetic d3-L-carnitine (150 µl of a 150 mM stock) dissolved in water via gastric gavage using a 1.5-inch 20-gauge intubation needle. Plasma was collected from the saphenous vein at baseline and at the indicated time points. *Apoe*$^{-/-}$ female mice were used in the study examining the inducibility of microbiota to generate TMA and TMAO following carnitine feeding. For these studies, mice were placed on an L-carnitine–supplemented diet (1.3% L-carnitine in drinking water) for 10 weeks. Quantification of the abundance of native and isotope-labeled forms of carnitine, TMA and TMAO in mouse plasma was performed using stable-isotope-dilution LC-MS/MS as described above.

Mouse reverse cholesterol transport, cholesterol absorption and bile acid pool size studies. Adult female (>8 weeks of age) *Apoe*$^{-/-}$ mice were placed on either a chow diet or an L-carnitine, choline- or TMAO-supplemented diet for 4 weeks before performance of reverse cholesterol transport, cholesterol absorption or bile acid pool size/composition studies as described in **Supplementary Methods**. In some RCT experiments, mice were treated with a cocktail of oral antibiotics (as in atherosclerosis studies described above) for 4 weeks before enrollment. RCT studies were performed using subcutaneous (in the back) injection of [^{14}C]cholesterol-labeled bone marrow–derived macrophages, as further detailed in **Supplementary Methods**. Feces were collected and analyzed as described in **Supplementary Methods**. For cholesterol absorption experiments, mice were fasted 4 h before gavage with olive oil supplemented with [^{14}C]cholesterol and [^{3}H]β-sitostanol. Feces were collected over a 24-h period and analyzed as described in **Supplementary Methods**. Total bile acid pool size and composition were determined in female *Apoe*$^{-/-}$ mice, with analysis of the combined small intestine, gallbladder, and liver, which were extracted together in ethanol with nor-deoxycholate (Steraloids) added as an internal standard. The extracts were filtered (Whatman paper #2), dried and resuspended in water. The samples were then passed through a C18 column (Sigma) and eluted with methanol. The eluted samples were again dried down and resuspended in methanol. A portion of the sample was subjected to HPLC using Waters Symmetry C18 column (4.6 × 250 mm no. WAT054275, Waters Corp.) and a mobile phase consisting of methanol:acetonitrile:water (53:23:24) with 30 mM ammonium acetate, pH 4.91, at a flow rate of 0.7 ml min^{-1}. Bile acids were detected by an evaporative light spray detector (Alltech ELSD 800, nitrogen at 3 bar, drift tube temperature 40 °C) and identified by comparing their respective retention times to those of standards (taurocholate and tauro-β-muricholate from Steraloids; taurodeoxycholate and taurochenodeoxycholate from Sigma; tauroursodeoxycholate from Calbiochem). For quantification, peak areas were integrated using Chromperfect Spirit (Justice laboratory software) and bile acid pool size was expressed as µmol per 100 g body weight after correcting for procedural losses based on the nor-deoxycholate internal standard.

Effects of TMAO on macrophage cholesterol biosynthesis, cholesterol efflux, inflammatory genes and desmosterol levels. The effects of cholesterol loading on the expression of macrophage cholesterol biosynthetic and inflammatory genes, macrophage LDL receptor gene expression and macrophage desmosterol abundance were analyzed as previously described[25]. Thioglycollate-elicited mouse peritoneal macrophages (MPMs) were harvested and cultured in RPMI 1640 supplemented with 10% FCS and penicillin plus streptomycin. MPMs were then lipoprotein-starved further in culture for 18 h in the absence versus presence of increasing concentrations of cholesterol, acetylated LDL or vehicle with or without 300 µM TMAO dehydrate (Sigma). Desmosterol in the cholesterol-loading studies was quantified by stable-isotope-dilution GC/MS analysis. Further details of these studies and cholesterol efflux studies are described in **Supplementary Methods**.

RNA preparation and real-time PCR analysis. RNA was purified from tissue (macrophage, liver or gut) using the animal tissue protocol from the Qiagen RNeasy mini kit. Small bowel used for RNA purification was sectioned sequentially in five equal segments from the duodenum to illeum before RNA preparation. Purified total RNA and random primers were used to synthesize first-strand cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) reverse transcription protocol. Quantitative real-time PCR was performed using Taqman quantitative RT-PCR probes (Applied Biosystems, Foster City, CA) and normalized to tissue β-actin by the $\Delta\Delta C_T$ method using StepOne Software v2.1 (Applied Biosystems, Foster City, CA).

Statistical analyses. Student's *t*-test or a Wilcoxon nonparametric test were used to compare group means as deemed appropriate. The analysis of variance (ANOVA, if normally distributed) or Kruskal-Wallis test (if not normally distributed) was used for multiple group comparisons of continuous variables, and a Chi-square test was used for categorical variables. Odds ratios for various cardiac phenotypes (CAD, PAD and CVD) and corresponding 95% confidence intervals were calculated using logistic regression models. Kaplan-Meier analysis with Cox proportional hazards regression was used for time-to-event analysis to determine hazard ratio and 95% confidence intervals for adverse cardiac events (death, myocardial infarction, stroke and revascularization). Adjustments were made for individual traditional cardiac risk factors (age, gender, diabetes mellitus, systolic blood pressure, former or current cigarette smoking, LDL cholesterol, HDL cholesterol), extent of CAD, left ventricular ejection fraction, history of myocardial infarction, baseline medications (aspirin, statins, beta blockers and angiotensin-converting-enzyme (ACE) inhibitors) and renal function by estimated creatinine clearance. Kruskal-Wallis test was used to assess the effect of the degree of coronary vessel disease on L-carnitine levels. A robust Hotelling T^2 test was used to examine the difference in the proportion of specific bacterial genera along with subject TMAO levels between the different dietary groups[61]. All data were analyzed using R software version 2.15 and Prism (Graphpad Software).

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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