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L-carnitine in omnivorous diets induces an atherogenic gut microbial pathway in humans

Robert A. Koeth,1,2,3 Betzabe Rachel Lam-Galvez,4 Jennifer Kirsop,1,2 Zeneng Wang,1,2 Bruce S. Levison,1 Xiaodong Gu,1,2 Matthew F. Copeland,4 David Bartlett,1 David B. Cody,4 Hong J. Dai,5 Miranda K. Culley,1 Xinmin S. Li,1,2 Xiaoming Fu,1,2 Yuping Wu,6 Lin Li,1,2 Joseph A. DiDonato,1,2 W.H. Wilson Tang,1,2,3 Jose Carlos Garcia-Garcia,4 and Stanley L. Hazen1,2,3

1Department of Cellular and Molecular Medicine, Lerner Research Institute, 2Center for Microbiome and Human Health, and 3Department of Cardiovascular Medicine, Cleveland Clinic, Cleveland, Ohio, USA.

BACKGROUND. L-carnitine, an abundant nutrient in red meat, accelerates atherosclerosis in mice via gut microbiota-dependent formation of trimethylamine (TMA) and trimethylamine N-oxide (TMAO) via a multistep pathway involving an atherogenic intermediate, γ-butyrobetaine (γBB). The contribution of γBB in gut microbiota-dependent L-carnitine metabolism in humans is unknown.

METHODS. Omnivores and vegans/vegetarians ingested deuterium-labeled L-carnitine (d3-L-carnitine) or γBB (d3-γBB), and both plasma metabolites and fecal polymicrobial transformations were examined at baseline, following oral antibiotics, or following chronic (≥2 months) L-carnitine supplementation. Human fecal commensals capable of performing each step of the L-carnitine→γBB→TMA transformation were identified.

RESULTS. Studies with oral d3-L-carnitine or d3-γBB before versus after antibiotic exposure revealed gut microbiota contribution to the initial 2 steps in a metaorganismal L-carnitine→γBB→TMAO pathway in subjects. Moreover, a striking increase in d3-TMAO generation was observed in omnivores over vegans/vegetarians (≥20-fold; P = 0.001) following oral d3-L-carnitine ingestion, whereas fasting endogenous plasma L-carnitine and γBB levels were similar in vegans/vegetarians (n = 32) versus omnivores (n = 40). Fecal metabolic transformation studies, and oral isotope tracer studies before versus after chronic L-carnitine supplementation, revealed that omnivores and vegans/vegetarians alike rapidly converted carnitine to γBB, whereas the second gut microbial transformation, γBB→TMAO, was diet inducible (L-carnitine, omnivorous). Extensive anaerobic subculturing of human feces identified no single commensal capable of L-carnitine→TMA transformation, multiple community members that converted L-carnitine to γBB, and only 1 Clostridiales bacterium, Emergencia timonensis, that converted γBB to TMA. In coculture, E. timonensis promoted the complete L-carnitine→TMA transformation.

CONCLUSION. In humans, dietary L-carnitine is converted into the atherosclerosis- and thrombosis-promoting metabolite TMAO via 2 sequential gut microbiota-dependent transformations: (a) initial rapid generation of the atherogenic intermediate γBB, followed by (b) transformation into TMAO via low-abundance microbiota in omnivores, and to a markedly lower extent, in vegans/vegetarians. Gut microbiota γBB→TMAO transformation is induced by omnivorous dietary patterns and chronic L-carnitine exposure.

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Conflict of Interest: ZW, BSL, and SLH are named as co-inventors on patents relevant to this work. MFC and HJD are employees of Procter & Gamble, and have the right to receive royalty payment for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland Heartlab, Quest Diagnostics, and Procter & Gamble. SLH also reports having been paid as a consultant by Procter & Gamble. The Procter & Gamble Company, Cincinnati, Ohio, USA. Gamble and are named as co-inventors on patents relevant to this work.

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Introduction

Recent studies identify a mechanistic link between a Western diet, gut microbiota-dependent metabolism, and development of both cardiovascular disease (CVD) and metabolic diseases (1–3). For example, the ingestion of trimethylamine-containing (TMA-containing) dietary nutrients enriched in animal products such as choline, phosphatidylcholine, and L-carnitine can serve as precursor for the ultimate generation of an atherogenic metabolite, trimethylamine N-oxide (TMAO), via a metaorganismalt pathway involving initial gut microbial formation of TMA, followed by host hepatic conversion of TMA into TMAO (4–8). Elevated plas-
ma TMAO concentrations predict incident CVD risks in multiple clinical cohorts (4–7, 9–14), and both animal models with microbiotal transplantation (4, 15) and various studies on mice and humans with raised TMAO have revealed mechanistic and prognostic links between microbial TMAO production and both atherosclerosis and thrombosis, as well as chronic kidney disease and heart failure (4–6, 12, 13, 16–18). Importantly, recent studies aimed at blocking the metaorganismal TMAO pathway through multiple approaches have confirmed the inhibition of diet-dependent atherosclerosis in animal models, either by targeting the initial microbial formation of TMA from choline through use of a nonlethal small-molecule inhibitor (19), or by targeting the second step in the pathway, the major host gene responsible for converting microbe-generated TMA into TMAO, flavin monoxygenase 3 (20, 21). More recently, a family of potent and highly selective mechanism-based suicide substrate inhibitors were developed and shown to promote the irreversible inhibition of gut microbiota–dependent generation of TMA, suppression of host TMAO levels, and, in parallel, inhibition of platelet hyperresponsiveness and thrombosis potential in animal models (22). Moreover, microbial transplantation studies in germ-free mice with human commensals containing a functional (but not a deletion mutant) microbial cutC gene (responsive for choline→TMA transformation) (23) and genetic manipulation (both gain of function and loss of function) of host hepatic flavin monoxygenase 3 have been shown to modulate host TMAO levels, platelet responsiveness, and thrombosis potential in vivo (24). Thus, substantial and mounting evidence supports a direct contributory role of gut microbiota as a participant in CVD pathogenesis, and preclinical studies indicate that the therapeutic targeting of microbial contributors to the TMAO metaorganismal pathway may hold promise as a novel approach for the prevention or treatment of CVD. Accordingly, an improved understanding in humans of the microbial pathways involved in TMA/TMAO formation from distinct nutrients is an essential and necessary step in the development of targeted interventions to interrupt these processes.

L-Carnitine, a nutrient found primarily in red meat, was recently reported to be metabolized by gut microbiota to form TMA via the 2-component Rieske-type l-carnitine oxygenase CntA/B (25, 26), which requires molecular oxygen for activity. Although CntA/B may be responsible for some TMA formation by γ-proteobacteria intimately associated with the intestinal epithelium using oxygen that diffuses into the luminal content, because of its oxygen requirement, it is unlikely to be the primary physiologic source of l-carnitine→TMA activity in the mostly anoxic luminal intestinal environment. Furthermore, CntA/B-encoding genes have been identified only in facultative organisms and not in the many anaerobes that form the bulk of the gut microbiota (27). However, isotope tracer studies confirm that TMA and TMAO are sequentially made from orally ingested l-carnitine in both mice and humans, and l-carnitine supplementation has been shown to accelerate atherosclerosis development in murine models of the disease (6, 26). Parallel clinical studies show that omnivores have higher circulating concentrations of l-carnitine than vegans or vegetarians, and that elevated plasma concentrations of l-carnitine in subjects is associated with incident CVD risks (e.g., heart attack, stroke, and death) independent of traditional CVD risk factors, but only in the presence of elevated TMAO (6). Despite these intriguing findings, is it unclear whether the mechanistic connection between l-carnitine ingestion and gut microbial production of TMA, and its downstream metabolite, TMAO, helps to explain the frequently observed association in epidemiologic studies of a dose-dependent relationship between red meat consumption and CVD risks (28–31). An improved understanding of oral l-carnitine metabolism in mammals and the potential involvement of gut microbiota–dependent processes is thus of considerable interest.

Upon more recent investigation of gut microbial metabolism of l-carnitine in mice, oral l-carnitine was discovered to not be directly converted into TMA via a simple 1-step process (i.e., a microbial l-carnitine TMA lyase activity; l-carnitine→TMA). Rather, the majority of orally ingested l-carnitine in mice produced a previously unappreciated intermediary metabolite, γ-butyrobetaine (γBB), in a gut microbiota-dependent step that occurred at a site anatomically proximal to, and at a rate 1,000-fold higher than the rate of the formation of TMA (26). Historically, γBB was discovered because of its role as the proximate endogenous biosynthetic intermediate in l-carnitine synthesis in mammals, a multistep pathway that generates l-carnitine from lysine (32–34). Dietary supplementation of mice with γBB was shown to produce both TMA and TMAO, and to accelerate atherosclerosis, but only in the presence of gut microbiota (26). Thus, studies in mice have revealed a multistep metaorganismal pathway that is initiated by sequential gut microbiota–dependent conversions of oral l-carnitine into an atherogenic intermediate, γBB, which is then converted into TMA, the immediate precursor for host hepatic transformation into TMAO (i.e., l-carnitine γBB→TMA→TMAO). Whether γBB is a major product of gut microbial catabolism of dietary l-carnitine in humans, and the relationship of chronic dietary patterns (e.g., omnivorous versus vegan/vegetarian) and l-carnitine exposure to γBB metabolism in humans, are unclear. Herein, we explore the role of the gut microbiota in γBB generation in humans, and the impact of preceding dietary history and oral l-carnitine supplementation on both γBB generation and catabolism to TMA/TMAO in subjects. Finally, we also identify and characterize human fecal microbial community members that participate in the conversion of l-carnitine into TMA via the generation and catabolism of γBB.

Results

**Dietary l-carnitine produces γBB in a gut microbiota-dependent manner in humans.** In prior studies we showed gut microbiota–dependent TMA and TMAO generation following oral l-carnitine ingestion in omnivores, but virtually nonexistent TMA/TMAO formation from oral l-carnitine in long-standing (>1 year) vegans and vegetarians (6). However, we did not look for γBB formation in those studies. Therefore, in initial pilot clinical studies (Figure 1), we first explored (in omnivores) whether γBB could be formed from l-carnitine following oral ingestion of heavy isotope–labeled l-carnitine (d 1rown l-carnitine), and the potential participation of gut microbiota in that reaction. At the initial baseline visit, serial venous sampling performed after oral d 1rown l-carnitine challenge revealed rapid increases in plasma concentrations of d 1rown l-carnitine, and subsequent increases in d 1rown γBB and d 1rown TMAO following a lag phase (Figure 1, A–C, filled circles). After the initial baseline challenge, subjects were placed on a week-long oral regimen of a...
cocktail of poorly absorbed antibiotics previously shown to effectively suppress intestinal microbiota (6), and then the d₃-l-carnitine challenge was repeated (Figure 1, open circles). Complete suppression of d₃-TMAO and almost complete suppression of d₃-γBB formation were observed (Figure 1). These results are consistent with results observed in mice (26) and strongly support a role for gut microbiota in γBB generation from dietary l-carnitine in humans. Finally, examination of plasma d₃-l-carnitine concentrations before versus after exposure to the antibiotics cocktail also showed no differences (Figure 1C).

*Ingestion of γBB produces TMAO in a gut microbiota-dependent manner in humans.* We next sought to test whether gut microbiota-formed γBB could produce TMAO in humans. Heavy isotope-labeled γBB (d₉-γBB) was synthesized and used to perform a similar oral “γBB challenge” of subjects (n = 6 omnivores). Following oral ingestion, rapid elevation in plasma concentrations of both d₉-γBB and d₉-l-carnitine was observed, followed by d₉-TMAO generation after a lag phase (Figure 2, A–C, filled circles). Interestingly, after a week-long suppression of the gut microbiota with oral broad-spectrum antibiotics cocktail, a rechallenge with oral d₉-γBB showed complete suppression of d₉-TMAO formation in subjects, confirming gut microbiota-dependent conversion of γBB into TMAO (Figure 2A, open circles). The production of d₉-l-carnitine from oral d₉-γBB, however, was not suppressed with the oral antibiotics; moreover, on closer inspection, while the peak plasma concentration of d₉-γBB (2 hours) after oral ingestion significantly decreased by 8 hours (P < 0.05), the peak blood concentrations of d₉-l-carnitine showed a relative plateau between 2 and 8 hours (Figure 2, B and C). These observations indicate that d₉-l-carnitine production from oral d₉-γBB occurs via a mechanism that is not suppressed by antibiotics, and are consistent with expected results from the endogenous l-carnitine biosynthetic pathway (i.e., host conversion of γBB to l-carnitine during carnitine biosynthesis) (32–34). Finally, examination of plasma d₉-γBB concentrations before versus after exposure to the antibiotics cocktail showed no differences (Figure 2B).

*Omnivores generate significantly more TMAO than vegans/vegetarians from oral L-carnitine because of marked increase in gut microbiota conversion of γBB.* We had previously reported that d₃-l-carnitine challenge in omnivores showed significantly elevated d₃-TMAO generation in comparison with vegans/vegetarians (6); we first recapitulated these findings and confirmed that omnivores generate significantly more TMAO than vegans/vegetarians from oral L-carnitine because of marked increase in gut microbiota conversion of γBB into TMAO.
vores (n = 15) have a much greater capacity to generate d3-TMAO from orally ingested d3-l-carnitine than vegans and vegetarians (n = 9) (Figure 3A, left panel). To determine the origins of this difference, we next explored each step in the multistep reaction. Notably, despite the dramatic difference in d3-TMAO production from oral d3-l-carnitine in omnivores versus vegans/vegetarians, the difference observed in the first step of that gut microbiota-mediated transformation, conversion of d3-l-carnitine to d3-γBB, was not significant, and if anything showed a trend toward a lower conversion rate in the omnivores (P = 0.051; Figure 3A, right panel). Further, plasma concentrations of d3-γBB observed following oral d3-l-carnitine challenge were approximately 20-fold lower in omnivores (n = 15; d3-γBB, n = 12) participating in an oral d3-l-carnitine (250 mg) challenge. The left panel illustrates the marked differences in d3-TMAO generation previously reported in omnivores versus vegans/vegetarians. The right panel shows a small difference in plasma d3-γBB concentration between omnivores and vegans/vegetarians. Data represent mean ± SEM. A Mann-Whitney test was used to compare the AUCs between dietary groups. (B) Box-and-whisker plots of fasting plasma concentrations of γBB from subjects (n = 9) before versus after 1 week of oral broad-spectrum antibiotics to suppress gut microbiota. Boxes represent the 25th, 50th, and 75th percentiles, and whiskers represent the 10th and 90th percentiles. Differences were assessed using a Wilcoxon matched-pairs test. (C) Fasting plasma concentrations of γBB in vegans/vegetarians (n = 32) versus omnivores (n = 40). Boxes represent the 25th, 50th, and 75th percentiles, and whiskers represent the 10th and 90th percentiles. A Mann-Whitney test was used to assess differences between groups. (D) Baseline human fecal metabolite studies in vegans/vegetarians and omnivores (n = 10 each group). Fecal samples were incubated anaerobically with d3-l-carnitine, and d3-TMA and d3-γBB were quantified by LC-MS/MS. Data are expressed as mean ± SEM. A Mann-Whitney test was used to assess differences between groups. (E) Baseline human fecal metabolite studies in vegans/vegetarians (n = 10) versus omnivores (n = 10). Fecal samples were incubated with d3-l-carnitine or d3-γBB as indicated. Metabolites were quantified by LC-MS/MS. Data are expressed as mean ± SEM. A Mann-Whitney test was used to assess differences between groups.

Figure 3. γBB is a major gut microbiota metabolite of l-carnitine, and TMA formation from γBB is influenced by dietary habits. (A) Plasma d3-TMAO and d3-γBB concentrations in vegans/vegetarians (d3-TMAO, n = 9; d3-γBB, n = 9) versus omnivores (d3-TMAO, n = 15; d3-γBB, n = 12) participating in an oral d3-l-carnitine (250 mg) challenge. The left panel illustrates the marked differences in d3-TMAO generation previously reported in omnivores versus vegans/vegetarians. The right panel shows a small difference in plasma d3-γBB concentration between omnivores and vegans/vegetarians. Data represent mean ± SEM. A Mann-Whitney test was used to compare the AUCs between dietary groups. (B) Box-and-whisker plots of fasting plasma concentrations of γBB from subjects (n = 9) before versus after 1 week of oral broad-spectrum antibiotics to suppress gut microbiota. Boxes represent the 25th, 50th, and 75th percentiles, and whiskers represent the 10th and 90th percentiles. Differences were assessed using a Wilcoxon matched-pairs test. (C) Fasting plasma concentrations of γBB in vegans/vegetarians (n = 32) versus omnivores (n = 40). Boxes represent the 25th, 50th, and 75th percentiles, and whiskers represent the 10th and 90th percentiles. A Mann-Whitney test was used to assess differences between groups. (D) Baseline human fecal metabolite studies in vegans/vegetarians and omnivores (n = 10 each group). Fecal samples were incubated anaerobically with d3-l-carnitine, and d3-TMA and d3-γBB were quantified by LC-MS/MS. Data are expressed as mean ± SEM. A Mann-Whitney test was used to assess differences between groups. (E) Baseline human fecal metabolite studies in vegans/vegetarians (n = 10) versus omnivores (n = 10). Fecal samples were incubated with d3-l-carnitine or d3-γBB as indicated. Metabolites were quantified by LC-MS/MS. Data are expressed as mean ± SEM. A Mann-Whitney test was used to assess differences between groups.
and vegan/vegetarian alike ($P < 0.001$; Figure 3D). These results indicate that BB is a major gut microbial metabolite formed from oral L-carnitine in humans (for both omnivores and vegans/vegetarians). Second, fecal microbiota from omnivores (vs. vegans/vegetarians) showed a significantly enhanced ($P = 0.02$) enzymatic capacity to produce TMA from L-carnitine (Figure 3E, left). And yet, third, no difference was observed in the fecal transformation of L-carnitine to BB in vegans/vegetarians compared with omnivores ($P = 0.54$; Figure 3E, middle). Finally, results of the fecal polymicrobial culture studies indicate that the marked increased generation of TMA (and TMAO) from oral L-carnitine in omnivores versus vegans/vegetarians appears to be due to significantly (up to 10-fold) greater gut microbial conversion of BB to TMA in omnivores relative to vegans/vegetarians (i.e., chronic exposure to L-carnitine in an omnivorous diet is associated with an increase in microbial capacity to catalyze the transformation of BB to TMA; $P = 0.04$; Figure 3E). Collectively, these data suggest that in the multistep metabolism of dietary L-carnitine into TMAO (i.e., L-carnitine $\rightarrow$ BB $\rightarrow$ TMA $\rightarrow$ TMAO), gut microbial metabolism of L-carnitine $\rightarrow$ BB occurs rapidly in omnivore and vegan/vegetarian alike, and the latter microbial transformation of BB to TMA is the one markedly enhanced in omnivores over vegans/vegetarians (i.e., influenced by chronic dietary habits; Figure 3E).

**Figure 4. L-carnitine supplementation enhances the synthetic capacity of gut microbiota to form TMAO.** (A) Plasma d3-TMAO concentrations in sequential venous blood draws after oral d3-L-carnitine challenge in vegans ($n = 7$) and omnivores ($n = 8$) at baseline, visit 1 ($V_1$, 1 month), and visit 2 ($V_2$, 2–3 months). Data represent mean ± SEM. A zero-inflated linear mixed-effects model reveals that plasma d3-TMAO is significantly higher after L-carnitine supplementation. (B) Plasma TMAO concentrations in vegans ($n = 7$) and omnivores ($n = 10$) at baseline and following daily L-carnitine supplementation at visit 2 ($V_1$, 1 month), and visit 3 ($V_2$, 2 months). Boxes represent the 25th, 50th, and 75th percentiles, and whiskers represent the 10th and 90th percentiles. A repeated-measures 1-way ANOVA test was used to assess differences between baseline visits. (C) Plasma d3-BB concentrations in sequential venous blood draws after oral d3-L-carnitine challenge in vegans/vegetarians ($n = 7$) and omnivores ($n = 5$) at baseline, visit 1 ($V_1$, 1 month), and visit 2 ($V_2$, 2 months). Data represent mean ± SEM. A zero-inflated linear mixed-effects model reveals that plasma d3-BB production is not significantly higher after L-carnitine supplementation in vegans/vegetarians or omnivores.

Chronic dietary L-carnitine supplementation enhances gut microbiota-dependent generation of TMAO. The significant differences noted in overall metabolism of oral L-carnitine $\rightarrow$ TMA/TMAO in omnivores and, to a lower extent, in vegans/vegetarians are a striking finding. We therefore sought to further explore the impact of chronic daily dietary L-carnitine exposure on these differences. Both vegans/vegetarians ($n = 7$) and omnivores ($n = 10$) gave consent and were instructed to continue with their typical diets but with the addition of supplemental L-carnitine (500 mg L-carnitine tartrate per day, provided in a Vegicap). Subjects were monitored at baseline, at 1 month, and after 2–3 months of continuous daily supplemental L-carnitine ingestion by examination of the rate of plasma appearance of both d3-TMAO (Figure 4A) and d3-BB (Figure 4C) following oral d3-L-carnitine challenge. In addition, fasting plasma levels of endogenous TMAO (Figure 4B) and BB (Supplemental Figure 2) were monitored. At baseline, vegans/vegetarians showed minimal synthetic capacity to produce d3-TMAO following ingestion of d3-L-carnitine, whereas omnivores readily generated d3-TMAO (Figure 4A). After 1 month of daily L-carnitine supplementation, enhancement in the formation of d3-TMAO following d3-L-carnitine ingestion was observed in vegans/vegetarians and omnivores alike (Figure 4A). Continuation of daily L-carnitine supplementation for at least an additional month resulted in no further increase in d3-L-carnitine $\rightarrow$d3-TMAO transformation in omnivores, but continued to increase d3-TMAO generation in vegans/vegetarians following oral d3-L-carnitine challenge (Figure 4A). Examination of individual plots of plasma d3-TMAO production from oral d3-L-carnitine challenges among vegans/vegetarians ($n = 7$) demonstrated that the mean increase observed was driven by only a subset ($n = 3$) of subjects, with over half ($n = 4$) of the vegans/vegetarians demonstrating essentially no metabolic capacity to convert oral d3-L-carnitine into d3-TMAO even after months of L-carnitine supplementation (Supplemental Figure 3). Fasting plasma concentrations of TMAO in both omnivores and vegans/vegetarians increased upon chronic (1 month) L-carnitine supplementation, but did not significantly further increase with another month of supplementation (Figure 4B). Interestingly, in both vegans/vegetarians and omnivores, chronic L-carnitine supplementation...
We next sought to biochemically characterize microbial transformation activities in feces of subjects before versus after L-carnitine supplementation. A subset of subjects (n = 7 omnivores, n = 6 vegans/vegetarians) consented to provide feces at both baseline and after L-carnitine supplementation for analyses (Figure 5). At baseline, comparisons between omnivores and vegans/vegetarians showed no significant differences in any of the fecal microbial metabolic transformations monitored (d9-L-carn to d9-TMA, d9-L-carn to d9-γBB, and d9-γBB to d9-TMA; P = 0.52, P = 0.63, P = 0.35, respectively) (Figure 5, A–C). However, following chronic L-carnitine supplementation, fecal samples recovered from omnivores showed significantly enhanced generation of d9-TMA from either d9-L-carnitine or d9-γBB relative to vegan/vegetarian fecal samples (P < 0.01 and P = 0.01, respectively; Figure 5, A and C). Further, while dietary L-carnitine supplementation induced no differences in fecal transformation of the first step of the L-carnitine→γBB→TMA/TMAO metaorganismal pathway in either omnivores or vegans/vegetarians, dietary L-carnitine provision induced enhanced fecal microbial transformation of the second microbial step in the overall pathway (i.e., d9-γBB→d9-TMA) in omnivores (P = 0.02), but not vegans/vegetarians (P = 0.69) (Figure 5C).

Multiple organisms are involved in the gut microbial production of TMA from L-carnitine. Since L-carnitine catabolism is mediated by microbial enzymes, we sought to identify the organisms involved in anaerobic L-carnitine degradation in fecal samples from a healthy omnivore donor (Figure 6A). In previous studies, single gastrointestinal microbial strains were reported to be unable to catabolize L-carnitine into TMA (35). We therefore hypothesized that multiple organisms are needed for catabolism of L-carnitine to TMA presumably with γBB as an intermediate. Therefore, we isolated not only single microbial colonies from feces but also subcommunities of 2–3 and 4–5 colonies, and tested the ability of each single isolate or subcommunity to produce TMA from L-carnitine. Though no single isolate produced TMA, 2 subcommunities out of 768 did produce TMA, and 1 of those subcommunities was further fractionated using a similar strategy as before (i.e., single, 2–3, and 4–5 colonies; Figure 6A). A noticeable enrichment in the number of TMA-producing subcommunities was observed, but again no individual isolate could perform the L-carnitine→TMA transformation. The subcommunity producing the most TMA was spread across 5 species pools (SP1 through SP5; Supplemental Table 1). Individual pools and combinations were evaluated for L-carnitine→TMA activity using a fractional factorial design (Figure 6B). Absence of either SP2 or, to a lesser extent, SP5 abolished the L-carnitine→TMA activity, whereas maximum production of TMA was attained only when both SP2 and SP5 were present. Each species pool (SP2 and SP5) was further subfractionated into individual colonies, and all members were evaluated alone or in combination with the members of the other pool. Only the combination of SP5-56 or SP5-62 with SP2-71 yielded maximum TMA production (Figure 6C). These data confirm our hypothesis and demonstrate that at least 2 different organisms are required for the anaerobic catabolism of L-carnitine to TMA. Both SP5-56 and SP5-62 were identified as isolates of Eggerthella lenta by 16S-rRNA gene sequencing. Further characterization of SP2-71 indicated that it was not a single strain, but a combination of 4 microbes (Supple-
mental Figure 4A). Of the 4 microbes, 1 had not been reported at the time of isolation, but showed 99% 16S-rRNA gene sequence identity (Supplemental Figure 4B) with an organism recently isolated by others and classified as *Emergencia timonensis* (37). Interestingly, *E. timonensis* (SP2-71.3) produced TMA in the absence of molecular oxygen, suggesting a novel microbial pathway for l-carnitine catabolism independent of the CntA/B oxygenase (25, 26).

Human commensal utilization of l-carnitine is decoupled from TMA production and generates γBB as intermediate. We next studied which of the isolated human commensals were necessary and sufficient to metabolize l-carnitine to TMA. Each of the microbes contained in species pool SP2-71 was evaluated alone or in combination with *E. lenta* (species pool SP5-62; Figure 7A). l-Carnitine was always consumed in the presence of *E. lenta* (SP5-62), regardless of whether other microbes were present. However, TMA production was decoupled from l-carnitine utilization and was only produced when both *E. lenta* (SP5-62) and SP2-71.3 (or the entire SP2-71 pool) were present, which suggests that *E. lenta* (SP5-62) is associated with l-carnitine consumption and SP2-71.3 is responsible for TMA production (Figure 7A). We mined the genome of *E. lenta* (SP5-62) for genes likely to be associated with l-carnitine utilization and noticed genes of the *caiTABCDE* gene operon that encode for a crotonobetaine reductase, l-carnitine-CoA transferase, and l-carnitine-CoA ligase, among other genes (Figure 7B). Reasoning that the enzymes encoded by these genes could be responsible for the utilization of l-carnitine and its conversion to γBB (Figure 7C), we used comparative genomics tools to identify 3 other organisms that contain the *caiTABCDE* gene operon (Supplemental Figure 5), and assessed their ability to consume l-carnitine and produce γBB (Figure 8). Although none of the 3 microbes, *Escherichia fergusonii*, *Edwardsiella tarda*, and *Proteus penneri*, produced TMA, all 3 consumed l-carnitine while producing γBB, suggesting that the utilization of l-carnitine is associated with the presence of the *caiTABCDE* genes. The combination of any of the 3 microbes with *E. timonensis* (SP2-71.3) led to anaerobic, oxygen-independent, TMA production from l-carnitine and consumption of γBB (Figure 8). The *E. timonensis* type strain SN18 (37) performed very similarly to *E. timonensis* SP2-71.3 in this study (data not shown).

During l-carnitine catabolism by human gut commensals and fecal polymicrobial anaerobic cultures, γBB accumulates as an intermediate before it is anaerobically converted to TMA. We then determined the kinetics of consumption of l-carnitine and production of γBB and TMA by *P. penneri* (ATCC 35198), one of the human commensals shown to produce γBB, alone or in combination with *E. timonensis* (SP2-71.3) (Figure 9A). Addition of l-carnitine to an
anaerobic culture of \( P. \ penneri \) alone (in M9 minimal medium) resulted in quantitative transformation of \( L \)-carnitine to \( \gamma \)BB within 4 hours with no TMA production. \( E. \ timonensis \) alone did not consume \( L \)-carnitine or produce TMA from \( L \)-carnitine. However, the combination of \( P. \ penneri \) and \( E. \ timonensis \) led to a transient accumulation of \( \gamma \)BB that was subsequently consumed with concomitant production of TMA (Figure 9A, top). When M9 minimal medium was instead supplemented with \( \gamma \)BB, \( P. \ penneri \) alone was unable to consume \( \gamma \)BB or produce TMA, whereas only \( E. \ timonensis \) was sufficient to produce TMA from \( \gamma \)BB in the presence or absence of \( P. \ penneri \) (Figure 9A, bottom). To our knowledge, this is the first report of a single microbial strain capable of producing TMA from one of the metabolites of the \( L \)-carnitine pathway under anaerobic conditions. Moreover, kinetic analyses reveal that the production of \( \gamma \)BB precedes production of TMA, consistent with \( \gamma \)BB being needed to induce the \( \gamma \)BB→TMA phenotype.

To assess whether the transient buildup of \( \gamma \)BB in vitro with only 2 microbial strains represents the biochemical transforma-

Discussion
Historically, \( \gamma \)BB is known for its role as the last biosynthetic intermediate in a series of endogenous reactions used to synthesize \( L \)-carnitine from lysine (38). Given the important role of \( L \)-carnitine in fatty acid transport and metabolism, early studies of \( \gamma \)BB (sometimes coined “pre-\( L \)-carnitine”) focused on its ability to form \( L \)-carnitine and facilitate fatty acid transport (32, 39). Early studies recognized that mammals: (i) lack the capacity to catabolize \( L \)-carnitine; (ii) gut microbial bacteria have the capacity to degrade \( L \)-carnitine into numerous products; and (iii) \( \gamma \)BB may be a gut microbial product of \( L \)-carnitine after, in one study, trace concentrations of radiolabeled \( \gamma \)BB were discovered in feces following oral ingestion of radiolabeled \( L \)-carnitine (39–41).

However, gut microbial formation and catabolism of \( \gamma \)BB during oral \( L \)-carnitine metabolism in omnivores versus vegans/vegetarians, the human commensals that can utilize \( \gamma \)BB as a nutrient, and the relationship of this metabolite to the metaorganismal TMAO pathway and CVD had not yet been explored.
An overview of dietary L-carnitine metabolism in humans, its relationship to both γBB and CVD risks, and the impact of preceding dietary pattern (omnivorous versus vegan/vegetarian), as well as chronic L-carnitine supplementation, as revealed by the present studies, are summarized in the scheme shown in Figure 10. Also shown are the adverse clinical outcomes associated with elevated systemic TMAO levels in subjects, and enhanced cardiometabolic phenotypes observed with elevated TMAO levels in animal models (4–6, 12, 13, 16–18).

In addition to being a product of endogenous synthesis during L-carnitine generation, the present studies reveal that γBB is a major product of gut microbiota–dependent catabolism of orally ingested L-carnitine in humans, and is an intermediate in TMAO formation from L-carnitine. Moreover, the present studies reveal that the overall conversion of orally ingested L-carnitine to TMAO is a complex multistep process requiring an initial gut microbial L-carnitine—γBB conversion, followed by a second γBB→TMA transformation. Further, each step is carried out by different members of the gut microbial community. In addition, chronic dietary exposure to L-carnitine results in induction of enhanced gut microbiota catabolism of L-carnitine principally mediated by the second step — γBB catabolism producing TMA — resulting in greater TMAO generation in the omnivorous versus the vegan/vegetarian host (Figure 10). Importantly, γBB→TMA occurs in an anaerobic environment, which suggests that it is catalyzed not by the CntA/B oxygenase, but rather by a novel oxygen-independent enzymatic pathway for the conversion of metabolites of the L-carnitine pathway to TMA. It is also notable that the microbial γBB→TMA activity was induced even in some vegans/vegetarians despite continuation of a vegan/vegetarian diet, with L-carnitine supplementation. Despite this, the TMAO production capacity still appeared greater in omnivores compared with vegans/vegetarians. Remarkably, among the vegans/vegetarians examined, roughly half (4 of 7) demonstrated little or no capacity to produce more TMA or TMAO from L-carnitine, despite 2 or more months of the daily L-carnitine supplementation. Vegans/vegetarians unable to produce TMAO from L-carnitine had no antibiotic or probiotic exposure (like all subjects enrolled), were recruited from the same geographic region as the other subjects, and were apparently healthy with no significant known comorbidities. Given the low proportion of culturable human fecal commensals identified in the subculture experiments that were capable of promoting the second microbial transformation reaction (γBB→TMA), we speculate that the vegans/vegetarians unable to produce TMA and thus TMAO from L-carnitine simply lack microbial community members, like E. timonensis, that possess the polypeptide(s) required to promote the γBB→TMA metabolic transformation.

While the present studies reveal important aspects of L-carnitine metabolism in humans and illuminate the intermediary involvement of γBB in TMAO generation from dietary L-carnitine, multiple important questions remain before we can exploit this newfound knowledge to develop therapeutic interventions that target this pathway. In this study, human gut commensals that can metabolize L-carnitine into γBB, and γBB into TMA, are reported for the first time to our knowledge. Although examination of fecal polymicrobial cultures indicates both the presence and dietary induction of fecal microbial γBB→TMA transforming activity, the microbial gene(s) encoding this enzyme activity, an important potential therapeutic target, remain unknown. Early studies with the bacterial isolate Acinetobacter calcoaceticus have reported a link between γBB and TMA production (42). We also recently reported association studies between specific cecal microbial taxa and plasma γBB concentrations in mice (26). Further characterization of the human commensals capable of both reactions (L-carnitine→γBB and γBB→TMA), and discovery of the enzyme(s) responsible for TMA production, are of interest — particularly since this latter microbial step in the overall pathway appears to be the major one induced during chronic dietary red meat exposure (or directly with L-carnitine supplementation) in an omnivorous diet (Figure 10). While speculative, it is a logical extension that induction of the γBB→TMA transformation activity may play a role in the heightened CVD risks observed in omnivores versus vegans/vegetarians, or subjects who eat diets rich in red meat. This hypothesis merits further study.

One of the more surprising findings of the present studies was the relatively small amount of d3-γBB found in plasma compared with d3-TMAO after d3-L-carnitine challenge, especially after ex vivo fecal studies showed that γBB is a major metabolite.
Moreover, in mice we observed that chronic l-carnitine supplementation resulted in markedly elevated plasma γBB levels (more so than l-carnitine or TMAO), and it was the first step of the overall gut microbial catabolic pathway (i.e., l-carnitine → γBB) that was induced in mice following chronic l-carnitine dietary exposure (26). However, the present studies in humans revealed that γBB plasma levels do not increase significantly following chronic dietary exposure to supplemental l-carnitine, and no differences were observed in plasma levels of omnivores versus vegans/vegetarians. While the origins of the observed differences between mice and humans with respect to the l-carnitine → γBB → TMA → TMAO pathway are not clear, the fact that the second reaction in the conversion of l-carnitine into TMA (i.e., γBB → TMA) showed marked induction with dietary exposure in subjects likely plays a major role in the observed differences. Still to be determined is whether this gain of function is the result of changes in the structure or the function of the gastrointestinal microbial community. That there exist interspecies differences in biosynthetic pathways mediated by gut microbiota metabolism between mice and humans is consistent with numerous other examples of how gut microbiomes of mammalian species can differ (44).

Multiple recent meta-analyses have confirmed that plasma TMAO levels are dose-dependently associated with incident CVD and mortality risks across multiple patient populations and geographic areas (45–47). In fact, in one meta-analysis, each 10-μM increase in TMAO was reported to be associated with an absolute 7.6% increased risk of all-cause mortality (45). It is also worth noting that animal model studies have illustrated a mechanistic contribution of microbial TMA and TMAO generation to enhanced development of atherosclerosis, platelet reactivity and thrombosis, and both impairment in renal function and fibrosis, as well as adverse ventricular remodeling and heart failure (refs. 4–6, 13, 16, 17, and Figure 10). From a public health standpoint, the impact of TMAO elevation during l-carnitine supplementation is unknown, but both vegan/vegetarian and omnivore on average showed a greater than 10-μM increase in circulating TMAO levels with l-carnitine supplementation. Owing to the critical physiologic role of l-carnitine in cellular fatty acid metabolism, there has been a belief that oral consumption of l-carnitine is beneficial in energy expenditure, when, in fact, there is equivocal evidence for enhancement in healthy individuals (48–54). Yet use of l-carnitine by the nutritional supplement industry, particularly in some energy drinks, and to a lesser extent in some protein supplements, is generally regarded as safe. Notably, the dose of l-carnitine supplement that subjects received in this study was well within the range of normal dietary exposure, and is comparable to the amount of l-carnitine in a single can of some energy drinks marketed presently, as well as the content of l-carnitine in a typical restaurant-portion steak dinner (55, 56). The present studies demonstrate that frequent dietary l-carnitine, even among some vegans/vegetarians, induces the gut microbiota's capacity to produce TMAO, and may thus foster proatherogenic processes (6). It is also relevant that in recent studies, we have shown that TMAO directly interacts with human platelets (both isolated and in whole blood), altering stimulus-dependent calcium signaling and promoting platelet hyperresponsiveness (4). Moreover, elevated systemic concentrations of TMAO enhanced thrombosis potential in vivo in animal models (4), and in recent clinical intervention studies, elevation in plasma TMAO induced with dietary choline supplementation altered platelet hyperresponsiveness in subjects (omnivores and vegans/vegetarians alike), as monitored by platelet aggregometry (17). The present studies show that chronic exposure to dietary l-carnitine similarly elevates TMAO concentrations. Whether parallel alteration in platelet responsiveness and long-term thrombosis risk occurs in subjects remains to be determined, but as noted above, elevated serum levels of carnitine are associated with enhanced risks of major adverse cardiovascular events (6).
Methods

Materials and general procedures. Human fasting lipid profiles and blood chemistries were measured using the Abbott ARCHITECT platform, model ci8200 (Abbott Diagnostics). Human subject gut microbiota suppression was achieved using a cocktail of oral poorly absorbed antibiotics as previously described (7). All stable isotope–labeled compounds used in the present studies were synthesized as described below. Plasma levels of endogenous and stable isotope–labeled \( l \)-carnitine, \( \gamma \)-BB, and TMAO were determined by stable isotope dilution liquid chromatography–tandem mass spectrometry (LC-MS/MS) in positive multiple reaction monitoring (MRM) mode as previously described (6) using a Shimadzu 8050 triple quadrupole mass spectrometer with ultra-HPLC interface. Laboratory personnel performing MS analyses were blinded to sample group allocation and clinical data during analysis.

Research subjects. Healthy subjects were enrolled in a study named CARNIVALE (NCT01731236; https://clinicaltrials.gov/ct2/show/NCT01731236), which studies gut microbial metabolism of \( l \)-carnitine in subjects. Subjects were divided into vegan/vegetarian or omnivore groups based on extensive dietary questioning. Subjects performed the oral \( l \)-carnitine challenge test by consuming 250 mg of synthetic \( d_3 \)-(methyl)-\( l \)-carnitine within a capsule (Vegicap, Catalent), followed by serial venous plasma draws collected in EDTA vacutainer tubes for

Figure 10. Scheme of \( l \)-carnitine and \( \gamma \)-BB metabolism in humans, links to gut microbial TMAO generation, and adverse cardiometabolic phenotypes.

FMO, flavin-containing monooxygenase.

There remain some limitations to these studies. First, while antibiotic suppression is used to invoke a role for gut microbiota in the generation of the metabolite(s) monitored, antibiotics can also have direct effects on the host, altering normal metabolism (57, 58). Secondly, we did not have access to hepatic tissues in subjects, and cannot definitely rule out that there may be some dietary influence on flavin monooxygenase–dependent conversion of TMA to TMAO. Similarly, we did not explore whether dietary patterns differentially influence renal excretion of TMAO. Finally, the studies used relatively small numbers of individuals, and thus do not allow for investigation of significant differences in sex, race, or other subgroups. We are thus cautious about the generalizability of these studies to greater populations.

In summary, the present studies add to our understanding of gut microbiota involvement in nutrient metabolism relevant to red meat ingestion, a Western diet, and CVD risks. Our studies reveal that \( l \)-carnitine→TMA is a multistep biochemical transformation mediated by multiple members of the gut microbial community via formation of an intermediate, \( \gamma \)-BB, previously shown to be proatherogenic in animal models. These studies also indicate that daily consumption of \( l \)-carnitine, even while maintaining a vegan/vegetarian diet, can induce the gut microbiota–dependent \( \gamma \)-BB→TMA transformation, resulting in heightened formation of the atherogenic and prothrombotic metabolite TMAO. Finally, our studies provide important insights for efforts aimed at development of therapeutic interventions designed to inhibit dietary \( l \)-carnitine conversion into TMAO in humans.
d₃-L-carnitine metabolite quantification. Other subjects underwent a similar γBB challenge test following oral administration of 250 mg of synthetic d₃-(methyl)-γBB (also provided in Vegicap). Dietary habits of subjects were determined using a questionnaire similar to that conducted by the Atherosclerotic Risk in Community (ARIC) study (59). Subjects were excluded from L-carnitine or γBB challenge testing 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, received antibiotics within 2 months of study enrollment, used any over-the-counter or prescription probiotic or bowel-cleansing preparation within the past 2 months, ingested yogurt within the past 7 days, or had undergone bariatric or other intestinal (e.g., bowel resection/reconstruction) surgery. Subjects were recruited by self-selection at Cleveland Clinic. Neither subject participants nor those administering the intervention were blinded, though investigators performing MS analyses were blinded to sample identities other than sample identification codes. Where indicated, a subset of subjects were instructed to continue with their current diet but, in addition, were placed on oral L-carnitine supplement (500 mg L-carnitine tartrate daily) and rechallenged in follow-up visits at 1 month and 2–3 months from baseline. All but 1 omnivorous subject completed all 3 visits. Some subjects had only baseline and 24-hour measurements taken of d₃-L-carnitine metabolites. Others underwent the entire L-carnitine or γBB challenge (i.e., had samples drawn 0, 2, 4, 6, 8, and 24 hours after ingestion of the indicated heavy isotope compound). If subjects experienced side effects of the noted treatment including, but not limited to, nausea, vomiting, diarrhea, or fever, they were instructed to stop treatment. If subjects failed to comply or did not tolerate the intervention, they were removed from the relevant analysis. Subjects were paid for each complete participation visit. A total of 35 subjects were enrolled in the heavy-isotopologue challenge studies, which included either d₉- or d₃-L-carnitine. Subjects were stratified by diet into n = 11 vegans/vegetarians and n = 24 omnivorous subjects. One enrolled vegan/vegetarian subject was later excluded as a result of reported nausea and a low BMI, 1 omnivorous subject declined the challenge after being enrolled and giving consent, 3 subjects enrolled in the antibiotic suppression arms did not tolerate the antibiotics (gastrointestinal distress) and were thus excluded from the study, and 2 subjects admitting noncompliance with daily L-carnitine supplementation were only included in baseline challenge studies. Thus, n = 10 vegans/vegetarians and n = 20 omnivorous subjects were included in the studies (see Supplemental Table 2 for baseline data). Baseline data for 3 omnivorous subjects were unavailable. Six of 7 vegans/vegetarians and 7 of 8 omnivores enrolled in the L-carnitine supplementation and challenge studies were used in the exploratory fecal metabolite studies based on availability of fecal samples. A second group of self-selected healthy volunteers on the Procter & Gamble campus (all omnivores) gave consent and donated fecal samples for microbial culture and biochemical analyses.

Human L-carnitine and d₃-γBB challenge test. Adults who had given consent performed a 12-hour fast overnight before performing the L-carnitine challenge test, which involved a baseline blood sample and then oral consumption (0 at time of initial ingestion) of capsules containing 250 mg d₃-L-carnitine (under investigational new drug exemption). In a second group of subjects, a d₃-γBB challenge was performed with 250 mg d₃-γBB. Following the baseline blood draw and ingestion of the capsule of isotope tracer, sequential venous serial blood draws were performed at noted time points for the determination of d₉-γBB metabolites. Urine was collected as a spot baseline sample and a sample after 24-hour challenge. After completion of the initial L-carnitine or γBB challenge (baseline or visit 1), an ensuing week-long treatment of oral antibiotics (7) was given to suppress intestinal microbiota, and the challenge (d₃-L-carnitine or d₃-γBB) was then repeated (visit 2).

Synthesis of d₃-L-carnitine and d₃-γBB. Synthesis of d₃-L-carnitine was prepared as previously described (6, 60). Briefly, L-norcarnitine (3-hydroxy-4-dimethylaminobutyric acid) was prepared from L-carnitine (Chem Impex International) with triphenol (Sigma-Aldrich) in N,N-dimethylformamide (Sigma-Aldrich) and then was converted to its sodium salt with sodium hydroxide by the method of Colucci et al. (61). Sodium L-norcarnitine was recrystallized from ethanol and ethyl acetate before conversion to d₃-L-carnitine. Some d₃-L-carnitine was synthesized by dissolving sodium L-norcarnitine in methanol and reacting it with d₃-methyl iodide (Cambridge Isotope Laboratories) in the presence of potassium hydrogen carbonate to give d₃-L-carnitine per Chen and Benoiton (62). d₃-L-Carnitine was isolated by silica gel column rinsing with methanol elution. The product was dried via azeotropic distillation of absolute ethanol and subsequently recrystallized from ethanol and acetone. The crystalline product was dried over P₂O₅ in vacuo and stored in a desiccator by refrigeration. Structural analysis of d₃-L-carnitine was confirmed by both high-resolution MS and NMR, and found to be more than 98% pure by LC-MS, NMR, and TLC.

Synthetic d₇-γBB was prepared as a chloride salt from γ-amino butyric acid in methanol with potassium hydrogen carbonate and d₇-methyl iodide, as previously described (26). Briefly, (3-carboxypropyl)trimethyl(d₇)ammonium chloride (d₇-butyrobetaine chloride, d₇-γBB) was prepared from γ-amino butyric acid in methanol with potassium hydrogen carbonate and d₇-methyl iodide (all from Sigma-Aldrich) (63). The reaction lasted 72 hours and was transferred onto a bed of silica gel (pores 60 Å, particles 40–60 μm; Agela Technologies) and equilibrated and washed in methanol. Rotary evaporation was used to yield the crude product, which was dissolved in water and titrated to pH 7.2. Further water was azeotropically removed by rotary evaporation with ethanol. The product was dissolved in absolute ethanol, filtered, concentrated to dryness by rotary evaporation, and dissolved in hydrochloric acid. The product was redissolved in a minimal amount of methanol and treated with 5 volumes of acetone to precipitate crystal formation. The crystals were filtered under suction, and dried under vacuum at 60°C. All reagents were obtained from Sigma-Aldrich unless otherwise indicated. Structural analysis of d₇-γBB was confirmed by both high-resolution MS and multinuclear NMR, and found to be more than 98% pure by LC-MS, NMR, and TLC.

Isolation and characterization of microorganisms involved in L-carnitine catabolism. Fecal samples were collected from volunteer donors who gave informed consent. Subject privacy and confidentiality of identifiable information were protected. Immediately after fecal samples were collected, glycerol stock suspensions were generated and sample aliquots snap-frozen in liquid nitrogen and maintained at −80°C until use. Thawed fecal slurry (200 mg feces) was suspended in 2 ml M9 minimal medium, with vigorous mixing to homogenize sample and break any clumps. The fecal slurry was centrifuged at low speed to eliminate large particulate matter and then at high speed for 2 minutes to harvest cells. Cells were then washed twice with M9, filtered through a 35-μm cell strainer, and diluted 1:100 in enrichment media (brain heart infusion [BHI] media supplemented with 500 μM
l-carnitine). Cultures were grown under anaerobic conditions at 37°C for 12 days, diluting 1:100 in enrichment media every 2 days. After 12 days in culture, enriched communities were spread and plated in BH1 plates supplemented with 500 μM L-carnitine. To reduce the complexity of the microbial community, single colonies, groups of 2-3 colonies, and groups of 4+ colonies totaling 768 cultures were then picked and inoculated into 1 ml of BH1 containing 50 μM d9-l-carnitine. After 24 hours at 37°C under anaerobic conditions, a sample of culture supernatant was collected and quenched by addition of formic acid to a final concentration of 0.1%. The amount of d9-TMA produced was determined by LC-MS/MS. The top d9-TMA–producing well was selected and replated, repeating the community simplification steps as described above. Once the top d9-TMA–producing community was identified, the cells were spread and plated. Single colonies were isolated and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using a Biotyp er System (Bruker Daltonics Inc.) (64). Isolates with similar high-confidence identification were pooled into 4 separate species pools, SP1 through SP4. All isolates with no reliable identification by MS were pooled into a fifth pool, SP5, and subsequently identified by 16S-rRNA sequence analysis (AccuGENX-ID, Accugenix).

Single microbial isolates or pools of isolates were evaluated alone or in combination for their ability to catabolize compounds of the l-carnitine pathway. Starter cultures were grown in BH1 for 24 hours at 37°C under anaerobic conditions. Cells were harvested by centrifugation and washed in M9 minimal medium. After washing, cells were resuspended in M9 supplemented with 50 μM d9-l-carnitine or 50 μM d9-BB as indicated. For kinetic experiments with fecal communities, fecal slurries prepared as described above were used instead of starter cultures. Reactions were allowed to proceed for up to 32 hours at 37°C under anaerobic conditions, as indicated. Samples of culture supernatant quenched by addition of formic acid to a final concentration of 0.1% were analyzed by LC-MS/MS to quantify concentrations of d9-TMA, d9-l-carnitine, and/or d9-BB.

**Human fecal microbial incubation with deuterium-labeled l-carnitine and γBB.** Human fecal samples were collected, snap-frozen in glycerol stock using liquid nitrogen, and maintained at –80°C until use. Feces (50 mg) was incubated with 1 ml reaction solution containing 150 mM d9-l-carnitine, d9-BB, or d9-γBB as indicated within 10 mM HEPES, pH 7.4, in a gas-tight reaction vial under anaerobic (Argon) conditions at 37°C. Reactions were stopped 16 hours later by addition of formic acid to a final concentration of 0.1%. The products were determined by LC-MS/MS with d9-choline and 13C4-d9-N-TMA as added internal standards and normalized to mass of fecal content.

**General statistics.** A Mann-Whitney (Wilcoxon rank sum test) 2-tailed nonparametric test or a Pearson χ2 test was used to compare group means and AUCs. A Wilcoxon matched-pairs test or a zero-inflated linear mixed-effects model was used to assess linked human and baseline treatment effects as deemed appropriate. A repeated-measures 1-way ANOVA test was used to assess differences of baseline plasma concentrations of γBB and TMAO between multiple visits. For all analyses a P value less than 0.05 was considered significant. All data were analyzed using R software version 3.4.3, JMP version 14, or Prism (GraphPad Software).

**Study approval.** All clinical investigations were conducted according to Declaration of Helsinki principles. All research subject gave written information consent prior to inclusion in the study, and all human studies were approved by the Cleveland Clinic institutional review board. Participant samples were identified by code number only.

**Author contributions**

RAK helped conceive, design, perform, and organize many of the experiments, assisted in statistical analyses, and assisted in drafting of the manuscript. BRLG conducted microbial isolation and characterization experiments. ZW assisted with mass spectrometry and human fecal polymicrobial culture enzyme assays. BSL, DB, and XG synthesized and purified all heavy isotope–labeled compounds used in the studies. MFC and DBC performed kinetics polymicrobial studies. JK helped in recruitment and performance of the human l-carnitine and γBB challenge studies. HJD, EBB, XSL, XF, and MKC assisted with mass spectrometry analyses. YW and LL performed statistical analyses. JAD helped with study design. WHWT helped with human studies design and oversight. JCGG conceived and designed microbial isolation and characterization studies and helped draft the manuscript. SLH conceived the idea, helped design experiments, and helped draft the manuscript. All authors participated in critical review of the manuscript.

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Address correspondence to: Stanley L. Hazen, Department of Cellular and Molecular Medicine, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, mail code NC-10, Cleveland, Ohio 44195, USA. Phone: 216.445.9763; Email: hazens@ccf.org.

DB’s present address is: Department of Diagnostic Radiology, Mayo Clinic, Rochester, Minnesota, USA.

MKC’s present address is: Department of Vascular Medicine Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA.

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