



# L-Carnitine intake and high trimethylamine N-oxide plasma levels correlate with low aortic lesions in ApoE<sup>-/-</sup> transgenic mice expressing CETP



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## ABSTRACT

**Objective:** Dietary L-carnitine can be metabolized by intestinal microbiota to trimethylamine, which is absorbed by the gut and further oxidized to trimethylamine N-oxide (TMAO) in the liver. TMAO plasma levels have been associated with atherosclerosis development in ApoE<sup>-/-</sup> mice. To better understand the mechanisms behind this association, we conducted *in vitro* and *in vivo* studies looking at the effect of TMAO on different steps of atherosclerotic disease progression.

**Methods:** J774 mouse macrophage cells were used to evaluate the effect of TMAO on foam cell formation. Male ApoE<sup>-/-</sup> mice transfected with human cholesteryl ester transfer protein (hCETP) were fed L-carnitine and/or methimazole, a flavin monooxygenase 3 (FMO3) inhibitor that prevents the formation of TMAO. Following 12 week treatment, L-carnitine and TMAO plasma levels, aortic lesion development, and lipid profiles were determined.

**Results:** TMAO at concentrations up to 10-fold the Cmax reported in humans did not affect *in vitro* foam cell formation. In ApoE<sup>-/-</sup> mice expressing hCETP, high doses of L-carnitine resulted in a significant increase in plasma TMAO levels. Surprisingly, and independently from treatment group, TMAO levels inversely correlated with aortic lesion size in both aortic root and thoracic aorta. High TMAO levels were found to significantly correlate with smaller aortic lesion area. Plasma lipid and lipoprotein levels did not change with treatment nor with TMAO levels, suggesting that the observed effects on lesion area were independent from lipid changes.

**Conclusion:** These findings suggest that TMAO slows aortic lesion formation in this mouse model and may have a protective effect against atherosclerosis development in humans.

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## 1. Introduction

Cardiovascular diseases (CVDs) are the major cause of deaths worldwide [1]. It is estimated that 23.3 million people will die from heart disease and stroke by 2030 [2]. Atherosclerosis, one of the leading causes of CVDs is affected by dyslipidemia, genetic background, environmental factors, stress, inflammatory status, low physical activities, nutrition, diabetes and hypertension [3–6]. Interestingly, recent studies have reported the contribution of gut microbiota on the progression of atherosclerosis [7–11].

Intestinal microbiota is comprised of more than 100 trillion typically non-pathogenic commensal microorganisms, and it is estimated that more than 90% of the DNA in a human body is actually microbial DNA [12–14]. Intestinal microbiota aids in the digestion, absorption and metabolism of nutrients [15,16]. However, more recently, microbiota was reported to contribute to increased risk of cardiovascular disease, obesity, insulin resistance, type II diabetes and asthma [17–22].

L-carnitine is a quaternary ammonium compound involved in the energy metabolism that is produced in liver and kidney from lysine and methionine or taken up with the diet. It is required for fatty acid transport to mitochondria and  $\beta$ -oxidation [23]. Clinical studies suggested a beneficial role of L-carnitine on atherosclerosis

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and CVD [7,19,24,25]. A systematic review and meta-analysis of 13 controlled trials, including 3629 patients, showed that L-carnitine supplementation was associated with a 27% reduction in all-cause mortality, 65% reduction in ventricular arrhythmias and 40% reduction in the development of angina and infarct size [25]. However, recent clinical studies in humans reported an association between plasma levels of trimethylamine N-oxide (TMAO), a metabolite that can be generated from L-carnitine, choline or betaine, and atherosclerosis development [8,22,26,27]. When not absorbed by the gut, these precursors can be metabolized to trimethylamine (TMA). TMA is then absorbed by the gut into the circulation and is oxidized by a family of liver flavin-containing mono-oxygenases (FMO) [28]. The resulting metabolite, TMAO is then excreted as it is in urea [29]. Recent studies linked TMAO plasma levels to atherosclerotic disease progression. The cause of this association, however, is still unclear [7,19,25].

In an attempt to provide a mechanistic explanation for this association, Koeth and colleagues used a common model of atherosclerosis, the ApoE<sup>-/-</sup> mouse [8]. Mice were fed large amounts of L-carnitine (2000 mg/kg/day) resulting in elevated plasma TMAO levels. TMAO was correlated with increased atherosclerotic lesion size, possibly resulting from a 35% decrease in reverse cholesterol transport (RCT) compared to control [8]. Although ApoE<sup>-/-</sup> mice have been used extensively as an atherosclerosis model, they lack cholesteryl-ester transfer protein (CETP), a key enzyme in the RCT, which transfers cholesterol ester from high density lipoprotein (HDL) to low density lipoprotein cholesterol (LDL) [30]. In addition, the small numbers of animals that showed larger aortic lesions (only in four out of 11 animals) and the lack of dose–response (a single TMAO dose has been assessed) made it difficult to understand the nature of the relationship between TMAO plasma levels and effects on lesion size [8]. More recently, the same authors reported that diet-induced  $\gamma$ -butyrobetaine had similar effects on the aortic lesion size [31]. These effects are resolved with antibiotic treatment, which reduce the conversion of L-carnitine to TMA [8,31].

Several mouse models expressing human CETP have been developed [32,33], and used to explore CETP inhibition as a therapeutic target for atherosclerosis treatment [34,35]. In the current study, using ApoE<sup>-/-</sup> mice expressing hCETP treated with two doses of L-carnitine and/or methimazole, an inhibitor of FMO, we show for the first time that high TMAO plasma levels were significantly associated with reduced aortic lesion area. Data presented here strongly suggest that TMAO reduces aortic lesion progression in this model. The observed effects on lesion size were independent from plasma lipid and lipoprotein levels. Furthermore, TMAO at concentrations 10-fold higher than the reported human plasma maximum concentration did not affect foam cell formation in mouse macrophages *in vitro*. The potential role of TMAO in the prevention of atherosclerosis disease progression in humans is discussed.

## 2. Material and methods

### 2.1. Cells and reagents

J774 cells and acetylated LDL (AcLDL) were a kind gift from George Rothblat (Children's Hospital of Philadelphia). L-carnitine was provided by Lonza Inc. (Allendale, NJ) and methimazole and TMAO were purchased from Sigma–Aldrich (Milwaukee, WI).

### 2.2. Cholesterol efflux assay

Cholesterol efflux studies were conducted as previously described with minor modifications [36]. Labeling medium included acetylated LDL (12 or 50  $\mu$ g/ml) and varying doses of

TMAO (ranging from 0 to 100  $\mu$ M). Efflux media containing 20  $\mu$ g/ml human ApoA-I, 20  $\mu$ g/ml human HDL3 or media alone were added to untreated cells (–cAMP). In addition, 20  $\mu$ g/ml human ApoA-I or medium alone were added to cells treated with cpt-cAMP. Cellular free cholesterol (FC) and cholesteryl ester (CE) separated using thin-layer chromatography were quantitated by liquid scintillation counting. Release of [<sup>3</sup>H]-cholesterol from cells during 4 h efflux was expressed as a percentage of [<sup>3</sup>H]-cholesterol present as FC within cells at time zero. Comparison of cholesterol efflux to ApoA-I from cells both with and without cAMP treatment was measured to verify the contribution of the ABCA1 cholesterol efflux pathway. All cholesterol efflux values are expressed as average  $\pm$  standard deviation of triplicate measurements.

### 2.3. Cholesterol mass assay

Effects of TMAO on cholesterol loading of macrophages were conducted without radiolabeled cholesterol in parallel with cholesterol efflux assays. Lipids were extracted from cells, and total cholesterol mass was determined using Cholesterol E kit (Wako Diagnostics, USA). The fraction of cholesterol mass present as free cholesterol (FC) and esterified cholesterol (CE) in cells was calculated by multiplying the total cholesterol mass by the fraction of cellular [<sup>3</sup>H]-cholesterol present as FC and CE. Cellular protein was determined using Markwell-modified Lowry assay [37]. All cholesterol mass determinations ( $\mu$ g/mg cell protein) are expressed as average  $\pm$  standard deviation of triplicate measurements.

### 2.4. Animals

Male ApoE KO mice (ApoE<sup>-/-</sup>) purchased from Jackson Laboratories (Bar Harbor, ME) were transfected with an adeno-associated viral vector containing the human CETP gene (AAV2/8-hCETP, obtained from ReGenX Biosciences, Rockville, MD) as described by Tanigawa et al. [38]. CETP expression was verified at 2 and 14 weeks following CETP-AAV transfection using a commercially available ELISA kit from ALPCO Diagnostics (Salem, NH). Two weeks post-transfection, mice were switched to Western Diet TD.88137 from Harlan Laboratories (Indianapolis, IN) containing 21% fat (wt/wt) and 0.15% cholesterol for 12 weeks of treatment. Mice were then sacrificed and tissues removed for analysis. All treatments were prepared fresh weekly, aliquoted into daily dosing volumes and stored at –20 °C. Aliquots were thawed at room temperature for 1 h prior to dosing. L-carnitine and methimazole were dissolved in sterile dH<sub>2</sub>O and administered once daily by oral gavage at specified doses based on body weight at a volume of 1 ml per 100 g of body weight. Blood was collected at 0, 6 and 12 weeks of treatment via the orbital plexus under isoflurane anesthesia (3%). All animals were weighed weekly to monitor health. Animal housing and experimental methods were approved by Vascular Strategies Institutional Animal Care and Use Committee.

### 2.5. Plasma analysis of L-carnitine and trimethylamine N-oxide

Plasma levels of L-carnitine and trimethylamine N-oxide (TMAO) in mice were analyzed using liquid chromatography-mass spectrometry (LC-MS) as previously described by Koeth et al., 2013 [8]. In brief, 50  $\mu$ L of the mouse plasma samples were pipetted into a 2 mL centrifuge tube, where 50  $\mu$ L of HPLC grade acetonitrile and 100  $\mu$ L of internal standard solution were then added. All samples were targeted to contain 1  $\mu$ g/mL synthetic d9 - L-carnitine hydrochloride (Sigma Aldrich) and 0.1  $\mu$ g/mL d9-TMAO (Cambridge Isotope Laboratories, Tewksbury, MA). The mixture was vortexed for 2 min, followed by centrifugation. The upper supernatant was taken for HPLC-MS/MS analysis, using an Agilent 1200 HPLC and

6410 triple quadrupole MS system. The elution was carried out on a Phenomenex Synergi 4  $\mu$ m Polar-RP column (150  $\times$  4.6 mm, 4  $\mu$ m, part No. 00F-4336-E0). All analyses were performed at a column temperature of  $30 \pm 1$  °C with a mobile phase of 10 mM ammonium formate and methanol. Detection and quantification of L-carnitine and TMAO levels (ppm) was achieved by ESI-MS/MS operating in the positive ion mode and the developed method was validated as per the requirements for the ICH guidelines.

## 2.6. Lipoprotein plasma profiling

Total cholesterol, triglycerides, phospholipids and free cholesterol in mouse plasma were determined using colorimetric kits from Wako Diagnostics (Richmond, VA). Cholesterol Ester was determined by subtraction of free cholesterol from total cholesterol. HDL-C, LDL-C and VLDL-C were determined by gel electrophoresis using SPIFE3000 and Cholesterol-Vis kit from Helena Laboratories (Beaumont, TX).

## 2.7. Assessment of atherosclerosis and histochemistry

After 12 weeks of treatment, animals were sacrificed and thoracic aorta isolated, trimmed of fat and fixed in formalin for 48–72 h for en face morphometric analysis. In addition, a small segment of the ascending aorta with heart attached was reserved for aortic root analysis. For en face analysis, aortas were laid out, pinned on black matrix and stained with Sudan IV as described elsewhere [39]. Images were taken using a Nikon Digital Sight DS-Fi1 camera connected to a SMZ45T stereoscope (Nikon, Inc, Melville, NY). Following staining, morphometric analysis was performed using NIS Elements software (Nikon, Inc, Melville, NY) to determine the lesion area. After en face analysis, lipid was extracted from aortas using the Bligh and Dyer method [40] and total cholesterol, free cholesterol and cholesterol ester concentrations (as  $\mu$ g lipid per mg protein) were determined as described above for cellular cholesterol mass analysis.

For aortic root analysis, the heart with approximately 5 mm of the ascending aorta was cut away from the aortic arch. The apex of the heart was removed, and remaining heart with the attached aortic segment placed in Tissue-Tek OCT (Optimal Cutting Temperature) embedding medium (Sakura Finetek USA, Inc, Torrance, CA), and frozen in a dry ice/2-methylbutane bath. 10 mm thick sections were cut from the ascending aorta through the entire aortic sinus until the ventricular chamber was reached. Sections were stained with hematoxylin-eosin, and imaged using a Nikon Digital Sight DS-Fi1 camera connected to an Eclipse 50i microscope. Lesion areas were quantitated using NIS Elements software (Nikon, Inc, Melville, NY).

## 2.8. Statistical analysis

Student's *t* test analysis performed in MS Excel was used where appropriate to determine differences between treatments and vehicle groups for both *in vitro* and *in vivo* studies. A  $p < 0.05$  was considered statistically significant. *In vitro* data are expressed as means  $\pm$  standard deviation (SD) of triplicate measurements. *In vivo* data are expressed as means  $\pm$  standard error of the mean (SEM) of each treatment group.

# 3. Results

## 3.1. TMAO effect on foam cell formation

One of the first steps in atherosclerosis development is foam cell formation, characterized by engorgement of arterial

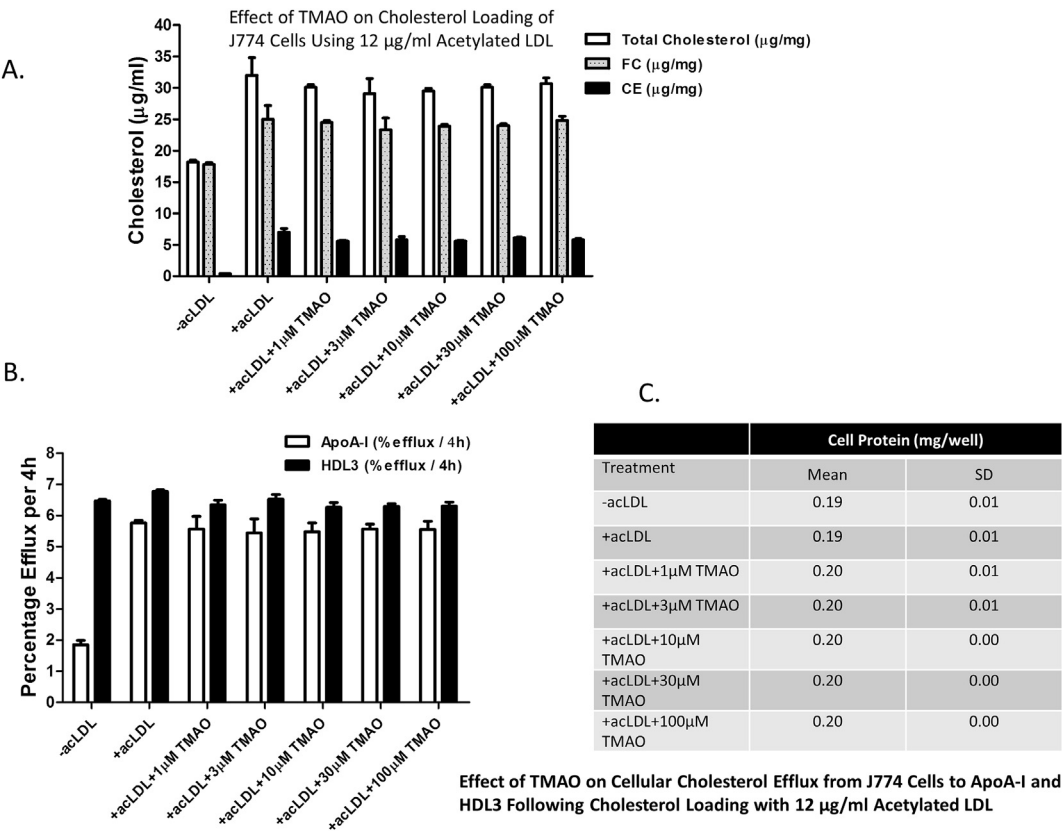
macrophages by oxidized LDL cholesterol [41]. Foam cell formation initiates development of plaque lesions [42–44]. We evaluated the effects of TMAO on foam cell formation and cholesterol efflux. Foam cell formation was assessed by measuring cholesterol loading of mouse macrophages with acLDL in the presence of increasing concentrations of TMAO (1, 3, 10, 30 and 100  $\mu$ M). Two different concentrations of acetylated LDL cholesterol (12 and 50  $\mu$ g/ml) have been identified in the linear range of the receptor response (data not shown). Fig. 1A shows that total, free and esterified cholesterol content of J774 macrophages at 12  $\mu$ g/ml acetylated LDL were not affected by any of the TMAO concentrations. In parallel, cholesterol efflux from acLDL loaded J774 cells to ApoA-I and HDL3 were also measured with increasing TMAO concentrations. Fig. 1B shows that cholesterol efflux from J774 macrophages using ApoAI as an acceptor (which assesses the ABCA1-mediated cholesterol efflux) and HDL3 (which accesses passive transport as well as SR-BI and ABCG1-mediated cholesterol efflux) were not affected by different concentrations of TMAO (10, 30 and 100  $\mu$ M). These TMAO concentrations were not toxic to the cells as assessed by total protein content (Fig. 1C). No differences were seen in cholesterol uptake or efflux between control and TMAO-treated macrophages, suggesting that foam cell formation was not affected by TMAO. No TMAO effect was observed when macrophages were loaded with a higher concentration of 50  $\mu$ g/ml acetylated cholesterol (data not shown).

## 3.2. In vivo plasma levels of L-carnitine and TMAO

ApoE<sup>−/−</sup> mice expressing hCETP were assigned to 5 different treatment groups (15 animals per group) according to their total cholesterol levels and fed a western diet. Treatment conditions included vehicle, 87 mg/kg and 352 mg/kg, equivalent to a low and a high equivalent human dose (500 mg and 2000 mg L-carnitine per day). In addition, to block formation of TMAO, two additional treatment groups given methimazole at 15 mg/kg [45], alone or in combination with 352 mg/kg L-carnitine were included. CETP expression levels were assessed before the start of the treatment and at study end, and were found to be stable (55–70  $\mu$ g/ml, data not shown). None of the treatments affected the weight of the mice (Fig. 2A). Plasma L-carnitine and TMAO levels were evaluated. A significant dose dependent increase of L-carnitine for treated mice compared to the controls was observed (Fig. 2B). Interestingly, methimazole by itself led to a significant decrease in L-carnitine plasma levels. This may be due to non-specific inhibition of L-carnitine transport in the gut by methimazole [46]. Fig. 2C shows a significant increase of plasma TMAO levels upon administration of L-carnitine at 352 mg/kg. However, the lower dose did not produce a significant change compared to control. As expected, the addition of methimazole led to decreased TMAO levels, but this decrease did not reach the control level, suggesting that this dose of methimazole is not sufficient to completely suppress TMAO formation (Fig. 2C).

## 3.3. TMAO effect on aortic lesions

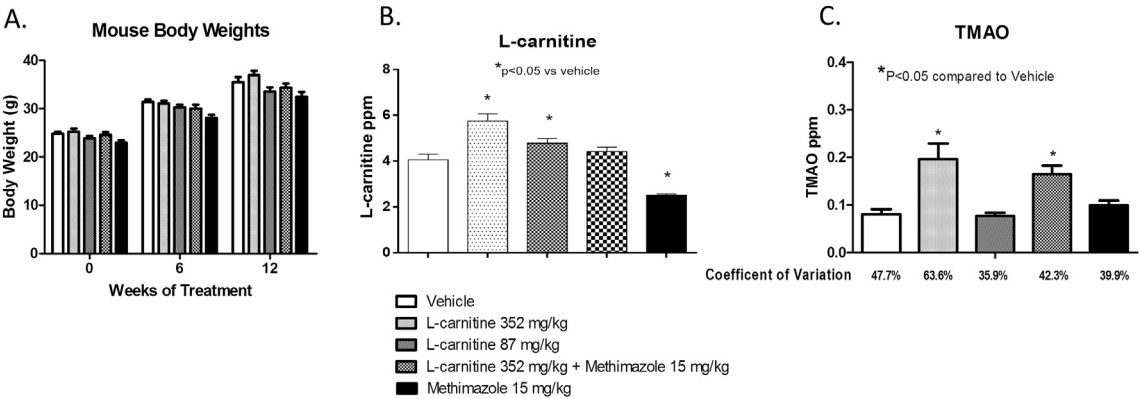
After 12 weeks of treatment, animals were sacrificed for aortic lesion assessment. Fig. 3A–E and Supplemental Fig. 1 show results of lesion analysis for two sites where atherosclerotic lesions commonly develop in mice, the aortic root and the thoracic aorta (including the aortic arch, although in humans the abdominal aorta is the most common). Aortic root analysis (area in  $\mu$ m<sup>2</sup>) showed a very small but significant decrease in lesion size in the L-carnitine treated groups compared to control group (Fig. 3A). Surprisingly, methimazole treatment also led to decreased aortic root lesions. This may be the result of the anti-oxidant and anti-inflammatory effects reported for methimazole [47,48]. Analysis of lesion area



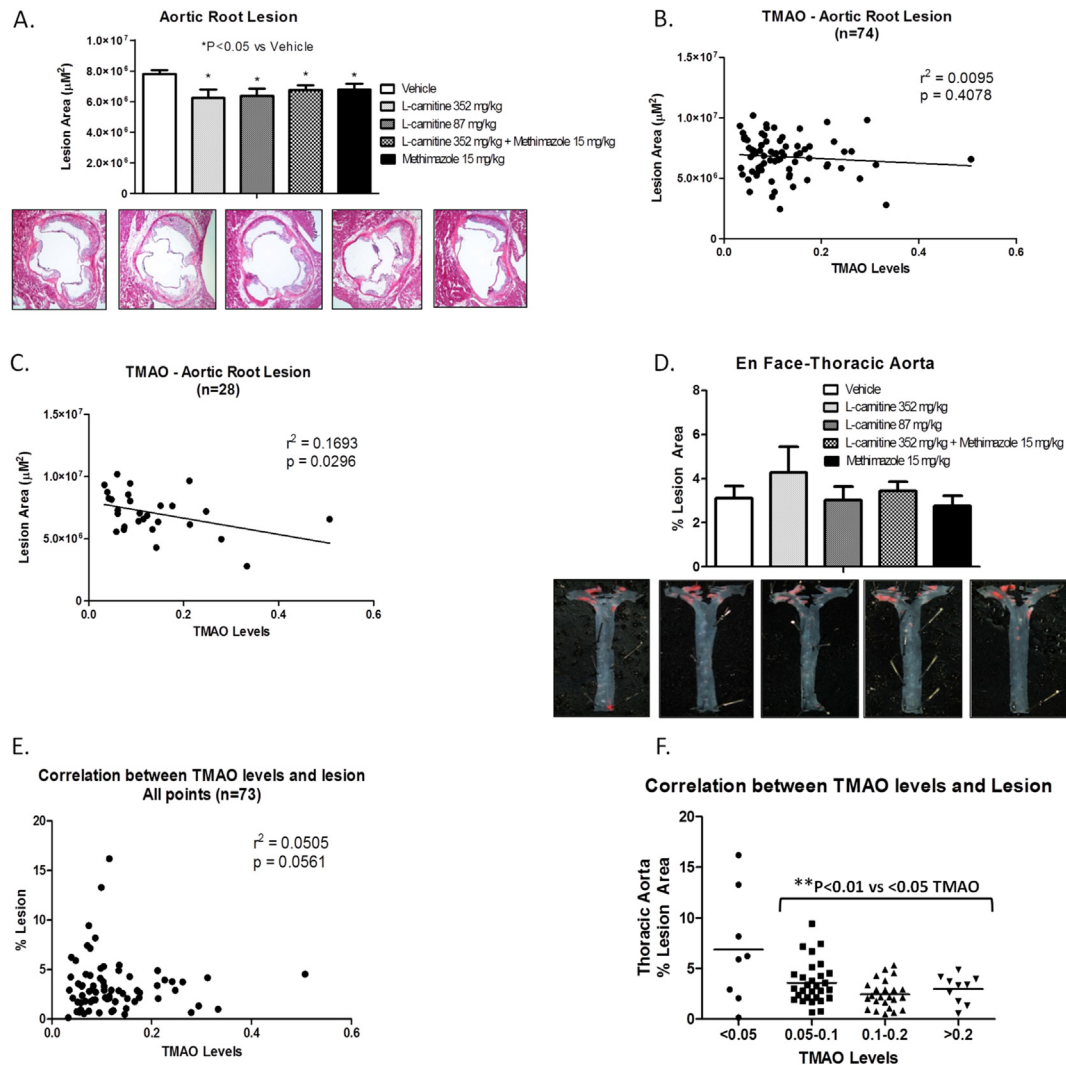
**Fig. 1.** Effects of TMAO on macrophage cholesterol loading and efflux. A) Total, free, and esterified cholesterol content was evaluated in J774 mouse macrophage cells treated with the indicated concentrations of TMAO. Cells were incubated with acetylated LDL (12  $\mu\text{g/ml}$ ), and increasing concentrations of TMAO for 24 h. Following treatments, cholesterol mass (free cholesterol, FC or cholesteryl ester, CE) concentrations ( $\mu\text{g/mg}$  cell protein) were determined. B) Cholesterol efflux was evaluated using ApoA-I and HDL3 as acceptors. J774 cells were treated with the indicated concentrations of TMAO and efflux to media containing either 20  $\mu\text{g/ml}$  human apoA-I or 20  $\mu\text{g/ml}$  human HDL3, for 4 h was measured. C) The mean  $\pm$  SD protein content (mg/well) for each group is also depicted in the table to assess the toxicity of each treatment. TMAO; trimethylamine N-oxide.

as a function of TMAO concentrations over all animals did not result in a significant correlation (Fig. 3B). However, when only the high L-carnitine treatment group (352 mg/kg) is considered compared to vehicle-treated mice, a significant inverse correlation was observed (Fig. 3C,  $p = 0.0296$ ). On the other hand, en face morphometric analysis showed no significant differences in thoracic aorta lesion area among the different treatment groups (Fig. 3D). However, when thoracic aortic lesion area was assessed as a function of TMAO plasma levels (and independently from treatment group) for all mice, a negative correlation was obtained

(Fig. 3E,  $p = 0.0561$ ). This correlation was not significant and tended to have two slopes. One at TMAO concentrations higher than 0.1 ppm and another at levels lower than 0.1 ppm. When mice were divided into different subgroups based on their TMAO levels ( $<0.05$  ppm, 0.05–0.1 ppm, 0.1–0.2 ppm, and  $>0.2$  ppm), significantly lower levels of lesion were found in the elevated TMAO level groups ( $>0.05$  ppm) compared to the lowest TMAO level ( $<0.05$  ppm) ( $p = 0.0148$ , Fig. 3F). All together, these results show that high TMAO levels significantly correlated with lower lesion incidence.



**Fig. 2.** Plasma levels of L-carnitine and TMAO *in vivo*. ApoE KO mice were transfected with a CETP-AAV at 7–8 weeks of age prior to drug treatments. Animals were assigned to the indicated treatment groups for 12 weeks. A) Mouse body weights for all treatments were evaluated to monitor overall health. Plasma levels of B) L-carnitine and C) TMAO were measured following the 12 weeks of treatment. TMAO; trimethylamine N-oxide. The coefficient of variation for TMAO production is shown for each treatment group.



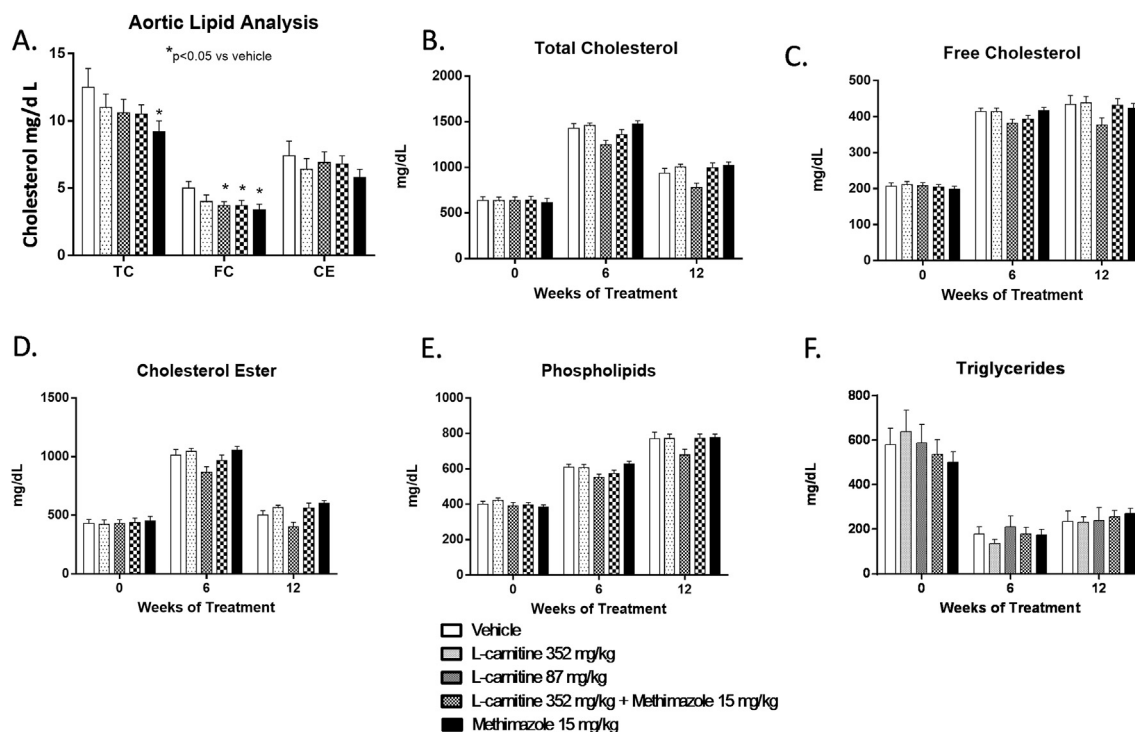
**Fig. 3.** Effect of TMAO production on aortic lesions *in vivo*. Following 12 weeks of treatment, animals were sacrificed for aortic lesion assessments. A) Sections (~5 mm) of the aortic root were cut, followed by hematoxylin-eosin staining and subsequent Nikon imaging. The lesion area ( $\mu\text{m}^2$ ) for each treatment group was then plotted as the mean  $\pm$  SD from 15 animals. B) A correlation analysis for all treatments between TMAO and aortic root lesion area was conducted ( $n = 74$  animals). C) A correlation analysis between L-carnitine (352 mg/kg)-induced TMAO levels and aortic root lesion area versus vehicle-treated mice was conducted ( $n = 28$  mice). D) Thoracic aortas were isolated, formalin fixed, and stained with Sudan IV for en face analysis using a Nikon computerized imaging system. The % lesion area in thoracic aortas for each treatment group was then expressed as the mean  $\pm$  SEM from 14 animals. E) A correlation analysis between % lesion area in thoracic aorta and TMAO concentration was conducted for all treatment points ( $n = 73$ ). F) A correlation analysis between the thoracic aorta % lesion area and very low (<0.05 ppm), low (<0.1 ppm), moderate (0.1–0.2 ppm), and high (>0.2 ppm) TMAO levels was conducted. The results are expressed as the mean  $\pm$  SEM from 73 animals. TMAO; trimethylamine N-oxide.

### 3.4. Effect of TMAO on aortic and plasma lipid profile

Lipid mass analysis of extracts from the thoracic aortas of the treatment mice did not show any differences in lipid content (Fig. 4A). Moreover, the total, free and esterified cholesterol, as well as phospholipids and triglycerides, were assessed at 0, 6 and 12 weeks of treatment (Fig. 4B–F). No significant changes in cholesterol levels across treatments, relative to their controls, were observed (Fig. 4B–D). Phospholipid and triglyceride levels were not changed (Fig. 4E–F). No significant change in VLDL-C, LDL-C, or HDL-C among the different treatment groups compared to the control was observed (Fig. 5A–C). The trend of an increase in HDL-C in the high dose L-carnitine group compared to the control group is in agreement with previous reports where L-carnitine treatment increased HDL-C [49,50]. All together, the data suggest that the effects of TMAO observed on lesion development are independent from plasma lipid changes in this mouse model.

### 4. Discussion

L-carnitine was first isolated from meat extract over a century ago by two Russian scientists [51], who named the compound after the Latin word *carnis*, meaning meat since it is found mainly in meat products [52]. Supplementation with L-carnitine has long been associated with enhanced energy generation by stimulating fatty acid  $\beta$ -oxidation, decreasing recovery time following muscle stress, and promoting hematopoiesis [53]. L-carnitine has routinely been added to infant formula for 30 years, because the endogenous biosynthesis in infants is too low to meet their requirements. In addition, L-carnitine has also been linked to cardio-protection due to its ability to attenuate fatty acid ester buildup and its antioxidant effects [25,54]. However, recent investigation into L-carnitine metabolites generated by gut microbiota has challenged the potential benefit of dietary L-carnitine against cardiovascular diseases. Koeth and colleagues [8,31] demonstrated an obligatory role



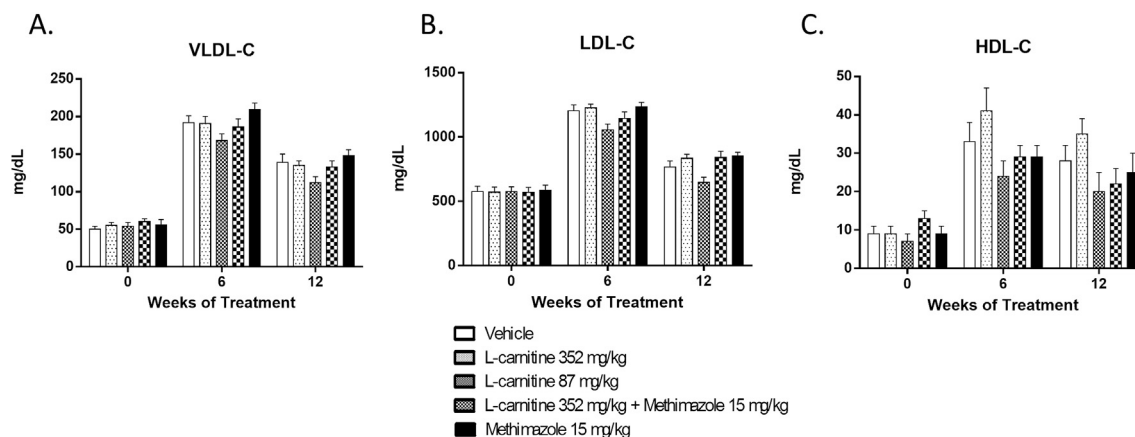
**Fig. 4.** Effects of TMAO on aortic and plasma lipid levels *in vivo*. A) Aortic lipid content (total cholesterol, TC; free cholesterol, FC; and cholesteryl ester, CE) was measured following 12 weeks of the indicated treatments. Blood from each animal was collected at 0, 6, and 12 weeks of treatment for plasma lipid analysis: B) Total cholesterol, C) free cholesterol, D) cholesterol ester, and E) phospholipids were analyzed and expressed as the mean  $\pm$  SEM from 15 animals. F) Triglycerides were determined using colorimetric kits from Wako Diagnostics (Richmond, VA). The results are expressed as the mean  $\pm$  SEM from 15 animals.

of intestinal microbiota in production of TMAO and other metabolites from ingested L-carnitine in mammals, which has been associated with the pathogenesis of atherosclerosis [8,31].

In an attempt to address the direct role of TMAO on atherosclerosis and understand the nature of the reported association [8], we conducted a series of studies assessing two stages of atherosclerosis development as described by Libby [43], i) *in vitro* foam cell formation and function, and ii) *in vivo* aortic lesion progression in relation to plasma TMAO levels. We first examined the effect of TMAO on cholesterol loading and efflux from mouse macrophages (Fig. 1A–B) as indication of foam cell formation and function. Irrespective of the dose used, TMAO treatment had no effect on acetylated LDL cholesterol loading or cholesterol efflux, indicating for

the first time that TMAO does not impact foam cell formation [55].

We then evaluated the plasma levels of TMAO in ApoE<sup>-/-</sup> mice expressing hCETP following 12 weeks of treatment with high (352 mg/kg) or low (87 mg/kg) L-carnitine administered by oral gavage, in the presence or absence of the FMO inhibitor methimazole. If TMAO was causative in lesion progression, suppressing formation of TMAO with methimazole in mice should reduce aortic lesion size, whereas no change compared to high L-carnitine treatment would be expected if atherosclerosis was independent of TMAO. Conversely, if TMAO was preventative, suppression with methimazole would result in increased lesion size. Both the high and low dose of L-carnitine used in this study was sufficient to increase its plasma levels, but only the high dose led to a significant



**Fig. 5.** Effects of TMAO on lipoprotein particle distribution and triglycerides *in vivo*. Blood was collected at 0, 6, and 12 weeks of treatment. Plasma lipoproteins A) VLDL-C, B) LDL-C, and C) HDL-C were determined by gel electrophoresis using SPIFE3000 and Cholesterol-Vis kit. The results are expressed as the mean  $\pm$  SEM from 15 animals.

increase in TMAO levels (about 2-fold) (Fig. 2B–C). The low dose of L-carnitine did not increase plasma TMAO, possibly because the Western diet itself contains significant amounts of L-carnitine. Interestingly, methimazole, which inhibits the conversion of TMA to TMAO, also reduced the plasma levels of L-carnitine (Fig. 2B). This effect may be the result of non-specific inhibition of L-carnitine transport in the gut, mediated by OCTN1/2, a family of transporters shown to be inhibited by methimazole in human hepatic cells [46]. Increased plasma TMAO levels with high dose L-carnitine was partially attenuated by methimazole (Fig. 2B). The difference in response to the two L-carnitine doses and the resulting TMAO plasma levels may be the result of variations in the gut microbiota between the individual mice, as reflected in the variance (CV % changes, Fig. 2C) shown between treatment groups. Gut microbiota plays a major role in L-carnitine metabolism in the digestive tract, and may be rate limiting in TMAO production. Indeed, we didn't observe an association between TMAO levels and thoracic lesion area within each treatment group compared to the control (Fig. 3B). But when the TMAO levels were considered independently from the treatments, a significant reduction of aortic lesion formation at high TMAO levels (>0.05 ppm) was observed. These results indicate that TMAO may play a protective role in aortic lesion formation (Fig. 3). Since TMAO did not affect the two first steps in atherosclerotic disease progression (endothelial cell dysfunction based on Koeth et al., 2013 and foam cell formation based on this study), it is possible that during disease progression, up-regulation of TMAO may modulate lesion development. TMAO has been reported to function as a chemical chaperone [56,57], possibly facilitating protein folding and tissue remodeling, a step required in the lesion regression. In addition, TMAO has been reported to mitigate stress and reduce apoptosis in epithelial cells challenged with abnormal protein aggregation [58]. Thus, the potential increase in TMAO as a result of L-carnitine supplementation may be a compensatory mechanism to slow lesion progression, rather than the cause on increased lesions as previously reported [8,31].

Given the difference in atherosclerotic lesion development in response to L-carnitine between that reported by Koeth [8] and the present study, it is important to note several differences in how the studies were conducted. There is a significant difference in the dietary composition between the two studies. The current study uses doses of L-carnitine (87 or 352 mg/kg) that are in the range used as dietary supplements and an atherogenic diet to assess lesion development. Whereas Koeth [8] used an L-carnitine dose much higher, approximately 1700 mg/kg/day based on average mouse consumption of 1.3% L-carnitine as part of a normal chow diet. The difference in normal chow vs atherogenic diet alone can be seen in the level of aortic root lesion. Koeth observed lesion sizes of  $1\text{--}5 \times 10^5 \mu\text{m}^2$ , which is low level of lesion development. The current study shows lesion sizes of  $0.6\text{--}1 \times 10^7 \mu\text{m}^2$ , a more advanced stage of lesion development. While both studies used apoE knockout mice, Koeth used female mice [8] and the current study uses male mice that express human CETP. For the current study, a model with CETP expression was chosen because it plays a significant role in reverse cholesterol transport and atherosclerosis progression and is more relevant to the human disease development. Furthermore, in the Koeth study, increased lesions above the baseline were observed only in 3 out of 11 animals, making the difference significant. It is worthy to note that no dose–response was evaluated [8]. Male mice were chosen in our study because they are commonly used as an atherosclerotic model and to avoid variability and high expression levels of FMO isoforms reported in females [59]. FMO isoforms have been shown to be regulated by hormonal factors, leading to much higher TMAO plasma levels in females vs males (about 10-fold higher) [60], making it harder to detect potential effects of TMAO and inhibition of TMAO by

methimazole more challenging. Indeed, using the male ApoE<sup>−/−</sup> model which have lower plasma levels of TMAO and the maximum non-toxic dose of methimazole, TMAO decrease was only partially achieved. By the same virtue, it would be challenging to see significant increases in TMAO levels following L-carnitine supplementation if the basal levels of TMAO were already elevated as seen in female mice.

Koeth and colleagues reported that the lesion development was resolved by antibiotic addition [8]. It is possible that this effect can be the result of the antibiotic effect on the lesions themselves. Various antibiotics, including doxycycline, have been reported to reduce inflammatory markers in ApoE<sup>−/−</sup> mice that contribute to atherosclerosis [61,62] and thereby may confound the interpretation of the Koeth et al. data on the TMAO effects only. It is possible that the up-regulation of TMAO took place after the lesion formation started in these animals. When several TMAO plasma levels were considered and correlated to the lesion occurrence (present study), a significant inverse correlation with the lesion size was observed (Fig. 3F). In addition, our findings agree with previous reports showing that clinically relevant doses of L-carnitine attenuated the progression of atherosclerotic lesions in rabbits [63]. Also, it has been reported that loss of function mutations in the FMO gene, involved in the formation of TMAO, lead to increased risk of ischemic stroke among hypertensive individuals [64]. A recent study by Fukami and colleagues done in 31 hemodialysis patients confirmed that L-carnitine supplementation (900 mg/d for 6 months) resulted in increased TMA and TMAO levels, but decreased markers of vascular injury and oxidative stress. Hemodialysis patients typically have lower than normal plasma L-carnitine levels, which has been related to accelerated atherosclerosis [65].

L-carnitine has been shown to reduce cholesterol and triglycerides, as well as increase high density lipoproteins (HDL) [23]. To that end, we explored whether L-carnitine or its metabolite, TMAO, could modulate cholesterol and lipoprotein levels in ApoE<sup>−/−</sup> mice expressing hCETP, and potentially to affect atherosclerosis development in this model. Our results showed no significant changes in aortic or plasma cholesterol and triglyceride levels (Fig. 4), or distribution of cholesterol between the major lipoprotein classes (VLDL-C, LDL-C, and HDL-C, Fig. 5) between treated mice and controls at 0, 6, and 12 weeks of treatment. It should be noted that, irrespective of treatment received, decreases in total cholesterol, cholesterol ester, LDL-C, VLDL-C and triglyceride were observed between 6 and 12 weeks of treatments. The observed lipid changes may be due to altered response to the Western diet in mice expressing CETP. Taken together, these data suggest a potential role for TMAO in reduced thoracic and aortic root lesion development and atherosclerosis prevention, an effect independent from plasma lipid changes.

In conclusion, the link between gut microbiota metabolism and CVD risk is fairly recent, and has generated much debate over the utility of supplemental intake of L-carnitine or meat intake. Our studies support the previously reported association between TMAO plasma levels and lesion formation and further clarify the nature of this association. Indeed, TMAO may likely provide a protective and not a causative effect on atherosclerosis development.

## Conflict of interest

None.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2015.10.108>.

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