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## Changes in Gut-Microbiota-Related Metabolites and Long-Term Improvements in Lipoprotein Subspecies in Overweight and Obese Adults: The POUNDS Lost Trial

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### Abstract

**Background/Objectives:** Alterations in gut microbiota have been linked to obesity and impaired lipid metabolism. Lipoproteins are heterogeneous, and lipoprotein subspecies containing apolipoprotein C-III (apoCIII) have adverse associations with obesity and related cardiometabolic abnormalities. We investigated associations of weight-loss diet-induced decreases in atherogenic gut-microbial metabolites, trimethylamine N-oxide (TMAO) and L-carnitine, with improvements in atherogenic lipoproteins containing apoCIII among patients with obesity.

**Subjects/Methods:** This study included overweight and obese adults who participated in a 2-year weight-loss dietary intervention, the POUNDS Lost trial. Blood levels of TMAO and L-carnitine were measured at baseline and 6 months after the intervention; 6-month changes in the metabolites were calculated. We evaluated 2-year changes in lipid profiles (n=395) and cholesterol [Chol] in lipoprotein (very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL)) subfractions defined by the presence or absence of apoCIII (n=277).

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**Author Contributions:** YH contributed to the study concept and design, analysis and interpretation of data, drafting and revising the manuscript, statistical analysis, and study supervision. TZ and HH contributed analysis and interpretation of data, and revising the manuscript. JAD contributed to measurements, interpretation of data, and revising the manuscript. GB and FS contributed to acquisition of data, interpretation of data, and drafting and revising the manuscript. LQ contributed to the study concept and design, acquisition of data, analysis and interpretation of data, drafting and revising the manuscript, statistical analysis, and funding and study supervision. All authors were involved in the writing of the manuscript and approved the final version of this article. LQ and YH had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis.

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**Results:** The initial (6-month) decrease in L-carnitine was significantly associated with long-term (2-year) reductions in non-HDL-Chol and LDL-Chol ( $p < 0.05$ ). Also, the decrease in L-carnitine was significantly related to decreases in Chol in LDL with apoCIII ( $p=0.034$ ) and Chol in [LDL + VLDL] with apoCIII ( $p=0.018$ ). We found significant interactions between dietary fat and TMAO on changes in LDL-Chol ( $P_{interaction}=0.013$ ) and Chol in [LDL + VLDL] with apoCIII ( $P_{interaction}=0.0048$ ); a greater increase in TMAO was related to lesser improvements in the lipoprotein outcomes if participants consumed a high-fat compared to a low-fat diet.

**Conclusions:** Changes in TMAO and L-carnitine induced by weight-loss diets were associated with long-term improvements in atherogenic lipoproteins containing apoCIII, implicating that these metabolic changes might be predictive of an individual's response to the dietary treatment to modify the unfavorable lipid profiles in obese patients. Dietary fat intake might modify associations of TMAO changes with long-term improvements of atherogenic cholesterol metabolism in overweight and obese adults.

## Introduction

Alterations of gut microbiota have been linked to the development of obesity and related cardiometabolic abnormalities including impaired lipoprotein metabolism.<sup>1-4</sup> Compelling evidence has implicated gut microbial metabolites, trimethylamine N-oxide (TMAO) and its precursors, as risk factors for major adverse cardiovascular events.<sup>5-9</sup> TMAO is derived from nutrient precursors, such as L-carnitine and choline,<sup>5,6</sup> by activities of gut microbial enzymes to produce trimethylamine (TMA), and TMA is further oxidized to TMAO by the hepatic flavin-containing monooxygenases (FMOs), predominantly the FMO3.<sup>10,11</sup>

Previous studies suggest that the TMAO-generating enzyme FMO3 is a central regulator of hepatic cholesterol and triacylglycerol metabolism<sup>12-14</sup>; and gut microbial metabolism of L-carnitine to TMA-TMAO pathway may be related to cholesterol metabolism in animal models.<sup>6,13</sup> In several human studies, elevated levels of TMAO or L-carnitine were associated with unfavorable levels of cholesterol, such as low-density lipoprotein (LDL) cholesterol (LDL-Chol) and high-density lipoprotein (HDL) cholesterol (HDL-Chol)<sup>15-18</sup>; however, results are not consistent across prior studies.<sup>17-21</sup> The inconsistent findings may be in part due to the heterogeneity of lipoprotein subspecies that were not considered in the previous studies. Lipoproteins are complex particles consisting of cholesterol, phospholipids, and triglycerides, as well as apolipoproteins; lipid and apolipoprotein content in each lipoprotein fraction can be quite different across patients, which differently affects their risks of cardiovascular events.<sup>22</sup> While most clinical studies only examine standard lipoprotein classes, biological functions of lipoproteins are found to vary according to the presence or absence of apolipoprotein C-III (apoCIII). ApoCIII regulates lipoprotein metabolism mainly by impairing apolipoprotein E-mediated clearance of very-low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) from the circulation.<sup>23</sup> Therefore, lipoprotein subspecies with apoCIII, rather than those without apoCIII, have been suggested as better treatment targets for patients.<sup>24-27</sup> LDL, as the most abundant atherogenic plasma lipoprotein, is the key deliverer of cholesterol to the artery wall.<sup>28</sup> Patients with obesity are characterized as having elevated levels of plasma apoCIII and lipoprotein subfractions (such as VLDL and HDL) containing apoCIII.<sup>29,30</sup> It has also been

reported that the risk of CHD contributed by LDL appeared to be driven by LDL with apoCIII.<sup>26</sup> Recent studies found a strong association of plasma apoCIII itself with incident CVD.<sup>27,31</sup>

To our knowledge, no prospective study has determined whether changes in atherogenic microbial metabolites of TMAO and L-carnitine are related to long-term changes in lipids and lipoprotein subspecies defined by the presence of apoCIII in humans. Further, diet is among the principal regulators of TMAO levels,<sup>5,6,8,32,33</sup> as well as cholesterol and lipoprotein metabolism.<sup>23</sup> In the present study, we investigated associations of diet-induced changes in circulating levels of TMAO and L-carnitine with long-term improvements in atherogenic lipids and lipoprotein subspecies defined by apoCIII in a 2-year dietary intervention trial for weight loss, the POUNDS Lost trial. The POUNDS Lost trial is one of the largest and longest randomized clinical trials using energy-reduced diets with different compositions of macronutrients among overweight and obese adults.<sup>34</sup> We also investigated whether macronutrient intakes (such as dietary fat and protein intakes) might modify the associations.

## Materials/Subjects and Method

### Study participants

The POUNDS Lost trial ([ClinicalTrials.gov Identifier: NCT00072995](https://clinicaltrials.gov/ct2/show/study/NCT00072995)) was conducted from October 2004 through December 2007 at two sites: Harvard T.H. Chan School of Public Health and Brigham and Women's Hospital in Boston, MA, and the Pennington Biomedical Research Center of Louisiana State University System, in Baton Rouge, LA.<sup>34</sup> A total of 811 adults who were overweight or obese were randomly assigned to 1 of 4 energy-reduced diets with different macronutrient intake goals in the four diets 1) low-fat and average-protein: 20% fat, 15% protein, 65% carbohydrate, 2) low-fat and high-protein: 20% fat, 25% protein, 55% carbohydrate, 3) high-fat and average-protein: 40% fat, 15% protein, 45% carbohydrate, and 4) high-fat and high-protein: 40% fat, 25% protein, 35% carbohydrate. Major exclusion criteria included the presence of diabetes or unstable cardiovascular disease, the use of medications that affect body weight, and insufficient motivation.<sup>34</sup> A total of 80% of the participants completed the 2-year study. The participants were informed that the study would be comparing diets with different fat, protein, and carbohydrate contents and that they would be assigned a diet at random; randomization assignments to one of 4 diets were generated by the data manager at the coordinating center.<sup>34</sup> Blinding was maintained by naming each diet with colors and using similar foods for each diet. Investigators and staff who measured outcomes were unaware of the diet assignment of the participants. More details of the trial have been presented elsewhere.<sup>34</sup> The study was approved by the human subjects committee at each institution and by data and safety monitoring board appointed by the National Heart, Lung, and Blood Institute. All participants gave written informed consent.

Of the 811 individuals, 510 participants were eligible based on the availability of blood samples and measurements of TMAO and L-carnitine both at the baseline and at 6 months after the intervention. As previously described, median (25th, 75th) values of TMAO and L-carnitine at baseline were within healthy ranges (2.7 [1.8, 3.8]  $\mu\text{M}$  and 34.5 [30.0, 39.3]



were log-transformed before calculating changes to improve the data normality. The primary outcomes were 2-year changes in cholesterol in lipoproteins subspecies defined by apoCIII after the intervention. We investigated whether the initial (i.e., 6-month) changes in TMAO or L-carnitine after the intervention were associated with long-term (i.e., 2-year) improvements in the primary outcomes. The secondary outcomes were changes in common lipids and lipoproteins (triglycerides, HDL-Chol, non-HDL-Chol, and LDL-Chol) at 6 months and 2 years after the intervention. Associations of TMAO changes ( TMAO) or L-carnitine changes ( L-carnitine) with the outcome measurements were examined using the general linear model. We calculated  $\beta$  and (SE) per 1 SD diet-induced decrease in TMAO or L-carnitine with adjusting for covariates of age, sex, ethnicity, diet groups, lipid-lowering medication use, baseline BMI, baseline levels of the respective outcome, and baseline levels of the respective metabolite (baseline TMAO or L-carnitine levels). We also performed a model further adjusting for concurrent weight changes. Mean changes in the outcomes were compared across tertile categories of L-carnitine or TMAO; the lowest tertile category included people with the largest reduction of the diet-induced changes in metabolite levels from baseline to 6 months. To investigate whether different macronutrient intakes (fat or protein) might modify associations, we tested interactions between TMAO or L-carnitine and diet groups (high-/low-fat diet, or high-/low-protein diet) for the outcomes. We also tested interactions between sex and TMAO or L-carnitine to examine whether the main associations were different between men and women. The  $p$  values were 2-sided;  $p$  value  $<0.05$  was considered statistically significant. Statistical analyses were performed with SAS version 9.4 (SAS Institute).

## Results

Characteristics of the study participants are shown in STable 1. At baseline, higher levels of L-carnitine were significantly associated with higher levels of triglycerides ( $p=0.009$ ), total-Chol ( $p=0.037$ ), non-HDL-Chol ( $p=0.003$ ), and LDL-Chol ( $p=0.036$ ), as well as with lower levels of HDL-Chol ( $p=0.023$ ) after adjusting for covariates of age, sex, ethnicity, baseline BMI, and use of lipid-lowering medication (Table 1). Higher L-carnitine levels at baseline were also related to higher levels of Chol in VLDL with apoCIII ( $p=0.027$ ) and Chol in LDL with apoCIII ( $p=0.006$ ) at baseline. There was no significant relation between L-carnitine and Chol in LDL without apoCIII at baseline. Baseline TMAO was not significantly associated with any of the lipid parameters (Table 1;  $p>0.05$  for all).

When we examined associations of changes in TMAO ( TMAO) or changes in L-carnitine ( L-carnitine) from baseline to 6 months after the intervention with improvements in common lipid profiles over 2 years (Table 2), we observed that more decrease in L-carnitine, but not TMAO, was significantly related to greater decreases in non-HDL-Chol ( $\beta$  [SE] per 1 SD decrease of L-carnitine:  $-2.51$  [1.1];  $p=0.023$ ) and LDL-Chol ( $\beta$  [SE]  $-2.26$  [1.03];  $p=0.028$ ) at 6 months. Further, the initial (6-month) decrease in L-carnitine was significantly associated with 2-year improvements in non-HDL-Chol (per 1 SD decrease of L-carnitine:  $\beta$  [SE]  $-3.32$  [1.42],  $p=0.02$ ) and LDL-Chol ( $\beta$  [SE]  $-2.82$  [1.28],  $p=0.028$ ). Mean changes in LDL-Chol and non-HDL-Chol levels across the tertiles of L-carnitine are shown in SFigure 1; participants in the highest tertile (T3) category of L-carnitine (median [25<sup>th</sup>, 75<sup>th</sup>],  $4.7$  [3.2, 6.7]  $\mu\text{M}$ ) had lesser 2-year improvements in LDL-Chol and non-HDL-Chol,

as compared to those in lower two tertiles (median [25<sup>th</sup>, 75<sup>th</sup>] L-carnitine: 0.2 [-0.9, 1.1]  $\mu\text{M}$  in T2 category; -5.0 [-7.5, -3.4]  $\mu\text{M}$  in T1 category). When we performed sensitivity analyses controlling for concurrent weight changes in the multivariate-adjusted model, L-carnitine was still significantly related to 2-year improvements in non-HDL-Chol and LDL-Chol levels, regardless of concurrent weight changes (STable 2).

Next, we examined associations of L-carnitine with 2-year changes in Chol in lipoprotein subfractions defined by the presence or absence of apoCIII (Table 3). In multivariate-adjusted model (model 1), the initial (i.e., 6-month) decrease in L-carnitine was particularly associated with 2-year decreases in Chol in LDL with apoCIII ( $p=0.034$ ), Chol in VLDL with apoCIII ( $p=0.018$ ), and Chol in [LDL + VLDL] with apoCIII ( $p=0.018$ ), but not with Chol in these lipoprotein subfractions without apoCIII ( $p>0.05$  for all). The trend was not appreciably altered after further adjusting for concurrent weight changes in the multivariate-adjusted model (model 2);  $\beta$  (SE) -0.57 (0.24) mg/dl per 1 SD decrease of L-carnitine for changes in Chol in [VLDL + LDL] with apoCIII ( $p=0.035$ ). L-carnitine did not show significant associations for changes in Chol in HDL with apoCIII. Figure 1 shows changes in Chol in LDL and VLDL subfractions that contain apoCIII according to the tertile categories of L-carnitine. We observed that Chol in LDL and VLDL subfractions with apoCIII were elevated at 2 years among participants in the highest tertile (T3) category of L-carnitine. The T3 group did not show increases in Chol in LDL and VLDL subfractions that lack apoCIII (SFigure 2).

We performed sensitivity analyses to examine whether the associations on the primary outcomes (2-year changes in Chol in lipoproteins subspecies defined by apoCIII) were significantly different between men and women by testing interactions between sex and TMAO or L-carnitine. There were also no significant interactions between sex and TMAO or L-carnitine on changes in Chol in lipoprotein subfractions with/without apoCIII ( $P$  for interaction  $>0.05$  for all). Similarly, there were no significant interactions between sex and TMAO or L-carnitine on 2-year changes in triglycerides, HDL-Chol, non-HDL-Chol, and LDL-Chol ( $P$  for interaction  $>0.05$  for all).

We did not find significant associations of TMAO with 2-year changes in Chol in lipoprotein subfractions defined by apoCIII in the study population (STable 3). However, when we investigated whether associations of TMAO with changes in lipid profiles were modified by assigned dietary macronutrient intakes, we found significant interactions between TMAO and dietary fat intake on changes in LDL-Chol ( $P_{interaction}=0.013$ ) and Chol in LDL with apoCIII ( $P_{interaction}=0.005$ ), as well as Chol in [LDL + VLDL] with apoCIII ( $P_{interaction}=0.0048$ ) (Figure 2). In response to a high-fat diet, individuals with an increase of TMAO (in T3 group: median [25<sup>th</sup>, 75<sup>th</sup>], 1.9 [1.3, 4.0]  $\mu\text{M}$ ) had increases in LDL-Chol (panel A) and Chol in LDL with apoCIII (panel B), and Chol in [LDL + VLDL] with apoCIII (panel C). Also, individuals with a decrease of TMAO (in the T1 group: median [25<sup>th</sup>, 75<sup>th</sup>], -2.0 [-3.5, -1.2]  $\mu\text{M}$ ) showed reductions in these lipids markers in response to the high-fat diet. Conversely, among individuals who consumed a low-fat diet, those with greater increases in TMAO had larger reductions in these outcomes (panels A, B, and C in Figure 2). We confirmed that there were significant TMAO-dietary fat interactions on changes in LDL-Chol ( $P_{interaction}=0.012$ ), Chol in LDL with apoCIII ( $P_{interaction}=0.009$ ),

and Chol in [VLDL + LDL] with apoCIII ( $P_{interaction}=0.01$ ) when we further adjusted for concurrent body weight changes in the model. We did not find significant interactions between dietary protein and TMAO or L-carnitine on changes in lipid profiles. There were also no significant interactions between fat intake and L-carnitine on these outcomes.

## Discussion

Our study showed that diet-induced decrease in gut-microbial metabolite L-carnitine was significantly associated with long-term improvements in atherogenic lipid profiles, such as cholesterol in LDL and VLDL subfractions containing apoCIII. Further, dietary fat intake significantly modified associations of TMAO changes with improvements in cholesterol content in these lipoprotein subfractions; we found that the increase in TMAO was associated with long-term increases in blood levels of cholesterol in LDL and VLDL that contain apoCIII among participants who consumed a high-fat diet.

At baseline before the dietary intervention, elevated levels of L-carnitine, but not TMAO, were significantly associated with unfavorable common lipoproteins (LDL, HDL, and non-HDL) in the study population. Similar to our findings, L-carnitine rather than TMAO tended to show a significant association with lipid metabolism in other studies<sup>17,20</sup>; prior observational studies failed to find significant associations of TMAO with LDL,<sup>17–21</sup> HDL,<sup>17,20,21</sup> or triglycerides.<sup>17,20,21</sup> We also observed that higher baseline L-carnitine levels were associated with unfavorable atherogenic lipid profiles at baseline, such as cholesterol in LDL and VLDL subfractions containing apoCIII. Further, our study is novel in that we evaluated lipoprotein subtypes based on apoCIII, and we showed that 2-year improvements in cholesterol in LDL and VLDL subfractions that contained apoCIII were significantly predicted by L-carnitine changes, independently of weight changes. Changes in L-carnitine were assessed during the initial 6 months after the intervention to predict 2-year improvements of these outcomes, which indicates that the initial L-carnitine might predict an individual's response to the dietary treatment to modify unfavorable lipid profiles associated with CVD.

ApoCIII is produced in both the liver and intestine<sup>38</sup> and present on triglyceride-rich lipoproteins (such as VLDL and IDL) and LDL in plasma.<sup>39</sup> ApoCIII also regulates triglyceride-rich VLDL metabolism.<sup>23,26</sup> Existing evidence has also shown a significant association of cardiovascular events with blood apoCIII levels in total plasma or in VLDL and LDL.<sup>27,31</sup> Also, apoCIII is involved in the formation of small dense LDL<sup>39,40</sup> via increased flux of the overproduced apoCIII containing VLDL through lipolytic pathways to dense LDL.<sup>40</sup> A previous study reported that increases in apoCIII levels following a high carb diet were associated with decreases in LDL size as well as that decreases in apoCIII levels following weight loss were associated with increases in LDL peak particle diameter.<sup>41</sup> Also, the heterogeneous metabolic effects of lipoprotein subtypes based on the presence or absence of apoCIII may explain their increased risk of CVD.<sup>24–26</sup> LDL with apoCIII, rather than LDL without apoCIII, strongly predicts coronary events independently of other lipids.<sup>25,26</sup> Further, TMAO and L-carnitine have been considered as novel risk factors for cardiovascular events independently of traditional risk factors<sup>6–9</sup> and emerge as new potential therapeutic targets,<sup>42</sup> although detailed mechanisms have not been fully

clarified in humans. Our study shows that alterations in lipoprotein subtypes associated with CVD might be one of the potential biological mechanisms for the cardiovascular adverse effects of TMAO and L-carnitine in patients with obesity. Also, our study suggests, for the first time, that weight-loss diet-induced decreases L-carnitine may be predictive of long-term improvements in atherogenic lipid and lipoprotein subtypes among patients who are overweight or obese, independently of concurrent weight changes. In addition, it has been debated about how to predict the long-term changes in metabolic outcomes based on the initial changes in response to diet and lifestyle interventions. Although further investigations are necessary to explain the detailed mechanisms, we speculate that several pathways might be involved, such as that the initial decreases in L-carnitine might be related to persistent long-term changes in lipid-metabolism-related gut microbiota<sup>43,44</sup> and gastrointestinal hormonal adaptations<sup>45,46</sup> to the weight-loss dietary interventions.

The observed associations of L-carnitine with unfavorable lipid profiles are also supported by previous studies showing that the microbial metabolism of L-carnitine promoted atherosclerosis<sup>6,47</sup> through the subsequent formation of TMA and its oxidation to TMAO by the host hepatic enzyme.<sup>10,11</sup> The gut microbiota-driven TMA/FMO3/TMAO pathway has been suggested as a regulator of lipid metabolism;<sup>13</sup> the TMAO-generating enzyme FMO3 may regulate plasma lipoproteins and hepatic lipid metabolism.<sup>12–14</sup> For example, one study shows that knockdown of FMO3 in cholesterol-fed mice altered biliary lipid secretion, blunted intestinal cholesterol absorption, and increased fecal cholesterol loss, and reduced the production of hepatic cholesteryl esters.<sup>13</sup> Also, dietary TMAO precursors may suppress reverse cholesterol transport through gut microbiota-dependent mechanisms.<sup>6</sup> A recent study of European adults suggests that gut microbiota composition may be associated with VLDL particles of various sizes.<sup>2</sup> Also, the intestinal microbiota may regulate the circulating cholesterol levels, hepatic cholesterol synthesis, and enterohepatic circulation.<sup>48</sup> On the other hand, a few studies of human subjects did not observe clear correlations of the hepatic *FMO3* expression and liver fat content or steatohepatitis<sup>15,49</sup>, and further studies are warranted to examine details of biological processes, such as including hepatic *FMO3* expression, hepatic lipogenesis, and alterations of microbiota itself and related metabolites, in relation to apolipoprotein/lipoprotein metabolism.

Our study suggests that atherogenic lipoprotein profiles may be regulated by the interplay of dietary fat intake and changes in TMAO, suggesting the importance of precision dietary interventions in improving metabolic abnormalities among patients with obesity. In participants who consumed a high-fat diet, increases in TMAO were associated with increases in LDL and cholesterol in LDL and VLDL subfractions containing apoCIII. These results are consistent with our previous study of the POUNDS Lost participants in which we found that dietary fat intake modified the associations of TMAO changes with insulin sensitivity.<sup>36</sup> The interaction between dietary fat and TMAO metabolism in regulating lipoproteins is biologically plausible. For example, diets high in fat and saturated fatty acids may negatively affect gut microbiota richness and diversity,<sup>50</sup> and an animal study suggests that small bowel microbiota regulates host dietary fat digestion and absorption through multiple mechanisms.<sup>51</sup> In a study of non-obese adults, a high-fat diet increased postprandial plasma TMAO levels whereas postprandial plasma L-carnitine levels did not change after eating the high-fat diet.<sup>32</sup> Also, a study of normal-weight individuals showed



that a 4-week high-fat diet significantly increased plasma TMAO concentrations, although plasma L-carnitine concentrations did not increase.<sup>33</sup> A recent study (n=14) also observed that a 4-week high-fat diet was associated with an increase in circulating TMAO but not carnitine, as compared to baseline.<sup>52</sup> Further investigations are warranted on whether the TMAO-atherogenic lipid and lipoprotein associations differed significantly by intakes of specific food items and types of dietary fat.

Our study has several strengths. We assessed the changes in TMAO and L-carnitine in one of the largest and longest dietary intervention trials, rather than using measurements of metabolites at a single time point. We evaluated the 2-year changes in lipoprotein subspecies in a relatively large number of study participants. Also, patients with diabetes or unstable CVD were not included in this trial, which minimized the effect of pre-existing adverse disease on gut-microbial metabolites as well as lipoprotein measurements. The findings on the interactions of dietary fat and TMAO were consistent for different lipid outcomes, which indicates the robustness of our conclusion. Nonetheless, our study also has several potential limitations. Our study could not determine whether changes in L-carnitine and TMAO causally affected intestinal cholesterol metabolism; further studies are necessary to understand detailed mechanisms considering the microbiota itself and cholesterol absorption in the intestine. Also, TMAO and L-carnitine may be affected by other factors than diet, such as unmeasured endogenous factors. Our study participants were adults who were overweight or obese, and the majority white and mainly well-educated. Further research would be necessary to confirm our findings, especially in populations that are more representative of the diverse US population as well as populations globally.

In conclusion, our study indicated that diet-induced decrease in the circulating metabolite L-carnitine was significantly associated with the long-term improvements in atherogenic lipid profiles, such as LDL and VLDL subfractions containing apoCIII. Dietary fat intake might modify associations of TMAO changes with long-term improvements of atherogenic cholesterol metabolism in overweight and obese adults.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data availability:

Dataset analyzed during the present study is available from the corresponding author on reasonable request. The data supporting the conclusions of this work are included in the manuscript.

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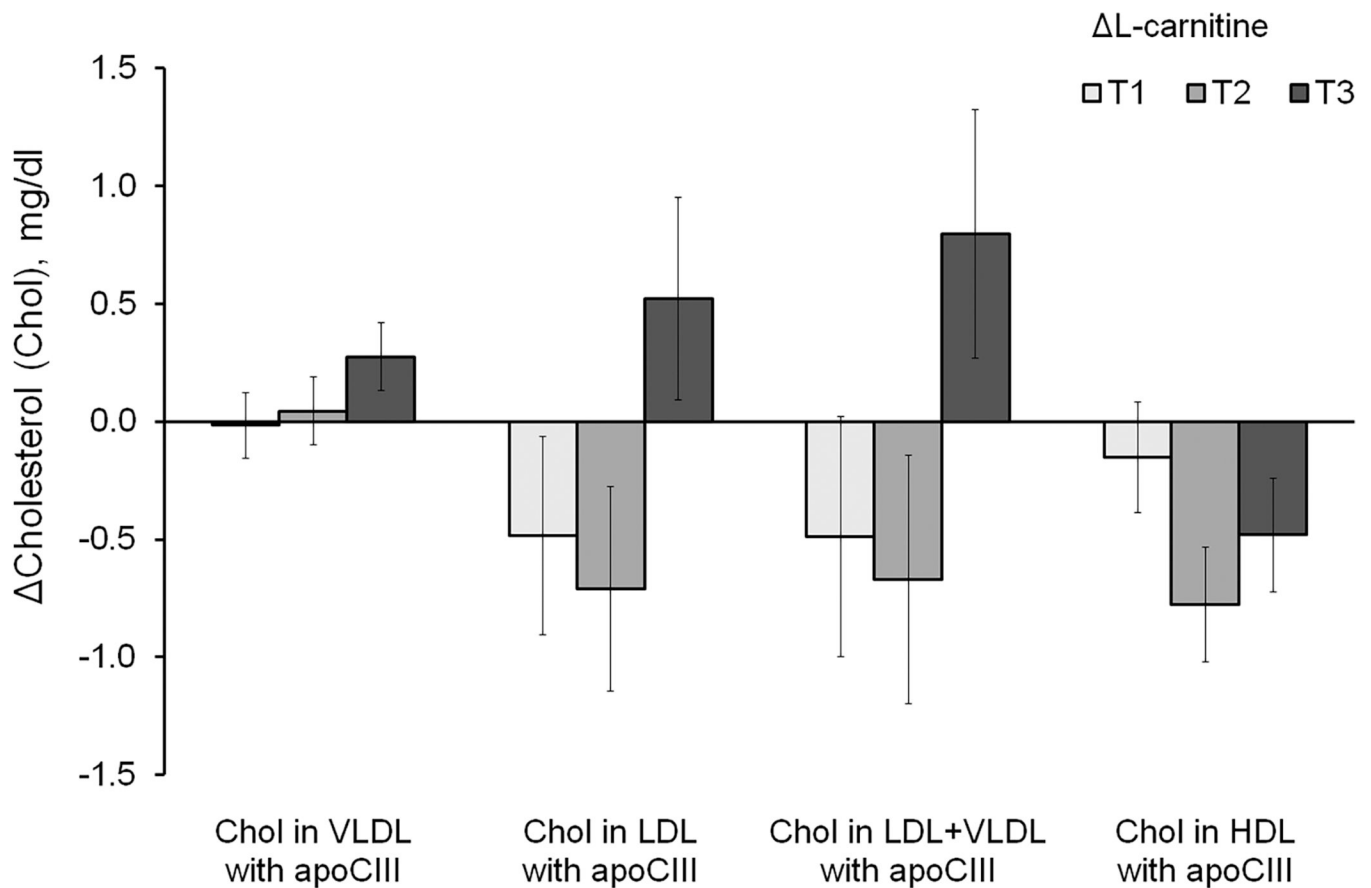
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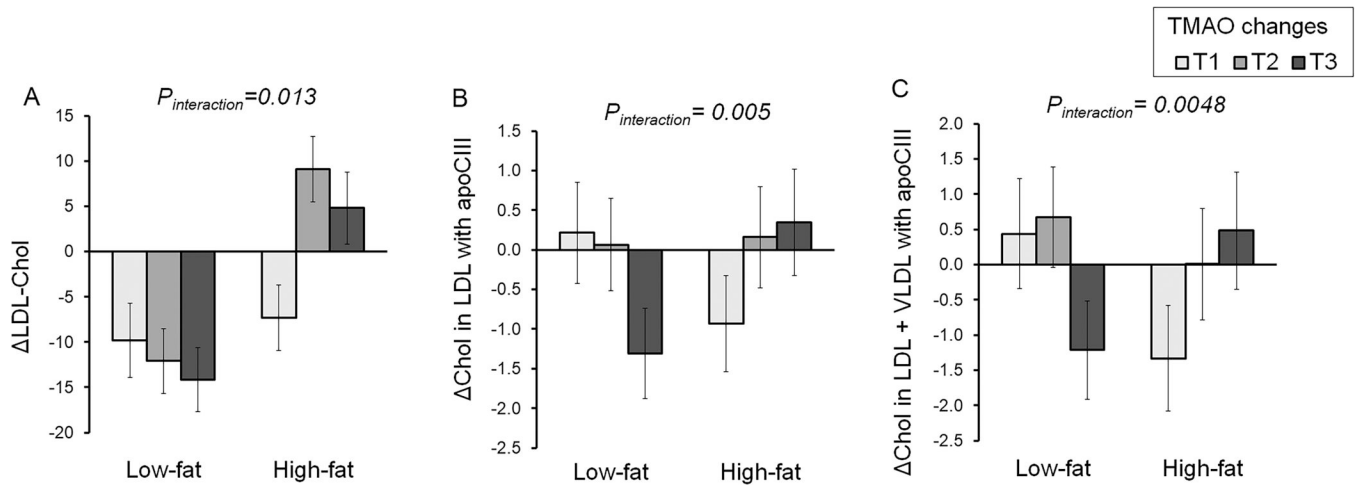
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**Figure 1:** Two-year changes in cholesterol in lipoproteins containing apolipoprotein (apo) C-III according to tertile (T) categories of L-carnitine changes. Data are mean and SE in the general linear model after adjusting for age, sex, ethnicity, diet groups, use of lipid-lowering medication, baseline BMI, baseline levels of the respective outcome, and baseline L-carnitine. For L-carnitine, median (25th, 75th) values were T1: -5.0 (-7.5, -3.4)  $\mu$ M, T2: 0.2 (-0.9, 1.1)  $\mu$ M, and T3: 4.7 (3.2, 6.7)  $\mu$ M, respectively.



**Figure 2:**

Changes in LDL cholesterol and cholesterol in LDL and VLDL containing apolipoprotein C-III (apoCIII) at 2 years according to tertile (T) categories of TMAO changes in low-fat or high-fat diet group.

$P_{interaction}$  between dietary fat and TMAO changes were for each outcome. Data are mean and SE in the general linear model after adjusting for age, sex, ethnicity, diet groups, use of lipid-lowering medication, baseline BMI, baseline levels of the respective outcome, and baseline TMAO. For TMAO, median (25th, 75th) values were T1:  $-2.0$  ( $-3.5, -1.2$ )  $\mu\text{M}$ , T2:  $0$  ( $-0.3, 0.3$ )  $\mu\text{M}$ , and T3:  $1.9$  ( $1.3, 4.0$ )  $\mu\text{M}$ , respectively among the total participants.

Associations of TMAO or L-carnitine with lipid parameters at baseline before the dietary intervention

**Table 1:**

Outcome lipid variables	TMAO-baseline		L-carnitine-baseline	
	N	$\beta$ (SE)	P	$\beta$ (SE)
Triglycerides	510	-0.04 (0.02)	0.06	0.06 (0.02)
Total cholesterol (Chol)	510	-0.66 (1.56)	0.67	3.38 (1.62)
Non-HDL Chol	510	-1.64 (1.50)	0.27	4.62 (1.55)
LDL-Chol	510	-0.02 (1.37)	0.99	2.99 (1.42)
HDL-Chol	510	0.98 (0.53)	0.06	-1.24 (0.55)
<i>Chol in each lipoprotein subfraction by apolipoprotein C-III (apoCIII)</i>				
Chol in VLDL with apoCIII	277	0.03 (0.07)	0.7	0.17 (0.08)
Chol in VLDL without apoCIII	277	-0.48 (0.61)	0.43	2.17 (0.63)
Chol in LDL with apoCIII	277	0.20 (0.26)	0.45	0.75 (0.27)
Chol in LDL without apoCIII	277	-0.52 (2.0)	0.79	2.17 (2.1)
Chol in HDL with apoCIII	276	0.06 (0.27)	0.81	-0.18 (0.28)
Chol in HDL without apoCIII	277	-0.33 (1.03)	0.75	-1.63 (1.08)

$\beta$  (SE) for each outcome per 1 SD increment of log-transformed TMAO or L-carnitine after controlling for age, sex, ethnicity, use of lipid-lowering medication, and baseline BMI. Triglyceride value was log-transformed before analysis.

Abbreviations: TMAO, trimethylamine N-oxide; Cho, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; apoCIII, apolipoprotein C-III.



**Table 2:** Associations of the initial (6-month) changes in TMAO ( TMAO) or L-carnitine ( L-carnitine) with changes in lipid levels

Follow-up time point Outcomes	Mean, outcome changes	N	TMAO, baseline to 6 months		L-carnitine, baseline to 6 months	
			$\beta$ (SE)	P	$\beta$ (SE)	P
<b>At 6 months</b>						
Log-triglycerides	-0.24	510	0.05 (0.02)	0.014	-0.02 (0.02)	0.38
Non-HDL cholesterol	-11.3	510	2.28 (1.44)	0.11	-2.51 (1.1)	0.023
LDL cholesterol	-5.6	510	1.36 (1.34)	0.31	-2.26 (1.03)	0.028
HDL cholesterol	1.8	510	-0.6 (0.4)	0.13	0.25 (0.31)	0.42
<b>At 2 years</b>						
Log-triglycerides	-0.22	395	0.03 (0.03)	0.24	-0.03 (0.02)	0.12
Non-HDL cholesterol	-10.7	395	0.18 (1.87)	0.92	-3.32 (1.42)	0.02
LDL cholesterol	-5.4	395	-0.47 (1.68)	0.78	-2.82 (1.28)	0.028
HDL cholesterol	4.5	395	-0.19 (0.51)	0.71	0.38 (0.39)	0.33

Effect size,  $\beta$  (SE) indicates per 1 SD decrease in each metabolite for the outcomes after controlling for age, sex, ethnicity, diet groups, use of lipid-lowering medication, baseline BMI, baseline levels of the respective outcome, and the respective metabolite at baseline (TMAO at baseline or L-carnitine at baseline).

Associations of diet-induced changes ( ) in L-carnitine with 2-year changes in cholesterol in different lipoprotein subfractions by the presence or absence of apolipoprotein C-III (apoCIII)

**Table 3:**

Lipoprotein subfractions Outcomes	Model 1		Model 2	
	$\beta$ (SE)	P	$\beta$ (SE)	P
<i>Lipoproteins with apoCIII</i>				
Chol in LDL with apoCIII	-0.42 (0.2)	0.034	-0.37 (0.19)	0.059
Chol in VLDL with apoCIII	-0.15 (0.06)	0.018	-0.13 (0.06)	0.039
Chol in [LDL + VLDL] with apoCIII	-0.57 (0.24)	0.018	-0.5 (0.23)	0.035
Chol in HDL with apoCIII	0.02 (0.11)	0.84	0.02 (0.11)	0.84
<i>Lipoproteins without apoCIII</i>				
Chol in LDL without apoCIII	-1.11 (1.4)	0.43	-1.23 (1.41)	0.38
Chol in VLDL without apoCIII	-0.32 (0.35)	0.36	-0.18 (0.34)	0.61
Chol in [LDL + VLDL] without apoCIII	-1.21 (1.45)	0.4	-1.21 (1.46)	0.41
Chol in HDL without apoCIII	-0.32 (0.53)	0.55	-0.50 (0.53)	0.35

$\beta$  (SE) per 1 SD decrease of L-carnitine at 6 months after the intervention for each outcome. Model 1 included covariates of age, sex, ethnicity, diet groups, use of lipid-lowering medication, baseline BMI, baseline levels of the respective outcome, and baseline L-carnitine. Model 2 included covariates in model 1 and concurrent weight changes.