

Dietary modification of the microbiome affects risk for cardiovascular disease

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0. Abstract

The incidence of cardiovascular disease (CVD) increases with age and is associated with some syndromes that exhibit aspects of premature aging such as progeria. Various factors are thought to contribute to the progression of CVD including hypertension, hypercholesterolemia, diets rich in saturated and trans fats, etc. Recent reports have uncovered an important connection between diet, the microbiome and CVD. Dietary carnitine (present predominately in red meat) and lecithin (phosphatidyl choline) are shown to be metabolized by gut microbes to trimethylamine (TMA), which in turn is metabolized by liver flavin monooxygenases (especially FMO3 and FMO1) to form trimethylamine-N-oxide (TMAO). High levels of TMAO in the blood strongly correlate with CVD and associated acute clinical events. Plasma TMAO levels may be an important clinical biomarker for CVD. The data suggest that that presence of specific as yet unidentified microorganisms in the gut linked to diet are required for high TMAO levels and TMAO-mediated CVD progression. Development of novel therapeutic approaches to manipulate gut flora may help treat CVD.

1. Introduction

The incidence of cardiovascular disease (CVD) increases with age and is associated with some syndromes that exhibit aspects of premature aging such as progeria. The American Heart Association estimates that as many as 83% of men and 87% of women greater than 80 years of age suffer from some form of cardiovascular disease.

Atherosclerosis is a multifaceted, progressive, inflammatory disease affecting mainly large and medium sized arteries by build-up of so-called “atherosclerotic plaques”. These lesions are comprised of a well-defined structure of lipids, necrotic cores, calcified regions, inflamed smooth muscle cells, endothelial cells, immune cells and foam cells. Macrophages, derived from monocytes, play a pivotal role in the development and progression of atherosclerosis [1]. A key event of atherosclerosis involves the uncontrolled uptake of oxidized low density lipoproteins (oxLDL) in macrophages, which accumulate within the subendothelial space of blood vessel walls [2]. Macrophages characterized by high expression of both mannose and CD163 receptors preferentially traffic to atherosclerotic

lesions at sites of intraplaque hemorrhage [3]. When macrophages fail to restore their cellular cholesterol homeostasis via reverse cholesterol transport (RCT) they transform into foam cells, the main components of fatty streaks. In a complex series of steps still being elucidated, fatty streaks develop into atheromas. Inflammatory processes mediated by cell-cell interactions in atheromas eventually lead to the death of the initial foam cells and macrophages, the recruitment and differentiation of new macrophages and foam cells, and the development of mature atherosclerotic plaques. Atherosclerotic plaques eventually rupture, providing a nidus for platelet activation, thrombus formation and vessel obliteration resulting in myocardial infarction (MI) if the lesions are in coronary circulation.

Various factors are thought to contribute to the progression of CVD including hypertension, hypercholesterolemia, diets rich in saturated and trans fats, etc. However, the discovery of new cofactors may have great benefit for development of a cure or a preventive regimen for CVD.

2. Serum TMAO levels are linked to CVD and result from microbiome-mediated metabolism of dietary carnitine and lecithin.

In an important series of recent papers from Stanley Hazen's laboratory (Cleveland Clinic), dietary carnitine and lecithin (phosphatidyl choline) are shown to be metabolized by gut microbes to trimethylamine (TMA), which in turn is metabolized by liver flavin monooxygenases (especially FMO3 and FMO1) [4] to form trimethylamine-N-oxide (TMAO). High levels of TMAO in the blood strongly correlate with CVD and acute clinical events [5] [6] [7]. These are among the first reports to demonstrate a potential powerful connection between diet, the microbiome and human disease.

The first clue that TMAO levels may play a critical role in CVD came with metabolic MRI and mass spectrometry studies on plasma from 40 samples selected from 2000+ cardiac patients. Those who had experienced a myocardial infarction (MI), stroke or death during a three year period were compared with matched controls. TMAO was identified as the major analyte correlating with CVD [7]. TMAO is an oxidation product of trimethylamine (TMA), which was known to be formed by microorganisms from PC, choline and other trimethylamine-containing species, such as betaine [8]. Fasting levels of 1876 stable subjects showed a correlation of elevated TMAO, choline, and betaine with CVD and CVD phenotypes such as peripheral artery disease, coronary artery disease and MI. [7].

Wang *et al.* showed that gut flora have an obligate role in TMAO formation from dietary choline, using deuterated PC to trace the formation of TMAO in mice pretreated with broad spectrum antibiotics for three weeks versus untreated control animals. TMAO was formed only in the negative control animals, but not the antibiotic-treated mice. As expected, exposing antibiotic-pretreated mice to normal animals for 4 weeks subsequent to antibiotic treatment to allow microbe recolonization reconstituted the ability of the animals to form TMAO. The metabolic pathway from PC to TMAO was shown to be PC → choline → TMA → TMAO (**Fig. 1**) [7].

Dietary choline or TMAO supplementation enhanced atherosclerotic lesion development in atherosclerotic prone C57BL/6J.ApoE^{-/-} mice. Plasma TMAO levels were strongly correlated with plaque size, while blood lipid levels did not increase. Increased plaque formation could be abrogated by co-treatment with antibiotics, consistent with the

need for microbial metabolism. Macrophage scavenger markers CD36 and SR-A1 as well as cholesterol-laden foam cell formation increased in C57BL/6J.ApoE^{-/-} mice fed high choline diets, effects which were dependent on the presence of normal gut flora [7]. These results were not surprising given the increase in plaque formation and the important role macrophages and macrophage-derived foam cells play in atherosclerosis.

To confirm the clinical relevance of the mouse results, two clinical studies in humans were performed. Blood and urine samples were analyzed from 40 human volunteers treated with a mixture of unlabeled and deuterium-labeled PC. Six of the volunteers were given metronidazole and ciprofloxacin for a week and retested. The volunteers were then retested at one month. TMAO and TMA were detected after choline stimulation in the first visit and at one month (three weeks after withdrawal of antibiotics). As expected from the mouse studies, almost no TMAO or TMA was detected after one week of antibiotic treatment, consistent with the obligate role of gut flora in humans in induction of plasma TMAO from choline [5]. Some protection from possible pathological effects of elevated TMAO may be afforded by high levels of TMAO excretion in the urine.

A clinical outcomes study on 4007 patients (average age 63 and 2/3 male) at the Cleveland Clinic revealed that subjects with elevated TMAO (highest quartile) had an increased risk of death (hazard ratio, 3.37; 95% CI, 2.39 to 4.75; P<0.001) and an increased nonfatal myocardial infarction or stroke rate (hazard ratio, 2.13; 95% CI, 1.48 to 3.05; P<0.001) [5]. Overall inclusion of TMAO as a covariate with known risk factors improved risk estimation by 8.6% [5]. These data suggest that determining TMAO levels may prove to an effective biomedical marker for major event risk in CVD.

Given the potential importance of TMAO to the pathogenesis of CVD and the recent meta analysis of prospective cohort studies showing no association of CVD with dietary saturated fat intake [9], Koeth *et al.* [6] examined if L-carnitine, a trimethylamine abundant in red meat with similar structure to choline (**Fig. 1**), also resulted in elevated TMAO levels and increased atherosclerosis in experiments that parallel those done for PC. A reanalysis of the unbiased small-metabolite metabolomic analysis that identified TMAO as a CVD risk revealed that L-carnitine was also associated with CVD. Human volunteers fed an 8 oz. steak and deuterated L-carnitine showed modestly increased TMAO and L-carnitine over time. Five volunteers were treated with broad-spectrum antibiotics for a week, which completely abrogated the increase in plasma TMAO, but not the increase in carnitine. Re-challenge with carnitine several weeks after the cessation of antibiotic treatment, again resulted in elevated TMAO, suggesting that intestinal microbiota are necessary for carnitine to increase TMAO levels, similar to the results for choline challenge [6].

High variation in the response to dietary carnitine and the importance of gut flora suggested that the subjects' dietary habits should be analyzed. Long-term vegans (> 5 years) were compared with omnivores who were frequent red meat eaters after a carnitine challenge. Both fasting TMAO levels and carnitine-induced TMAO levels were markedly reduced in the vegans compared with the meat-eating omnivores. Analysis of bacterial 16S RNA sequences in fecal specimens showed that on average individuals with enriched bacteria of the genus *Prevotella* had higher TMAO levels than those with an enrichment of the genus *Bacteroides* [6]. However, more work needs to be performed to identify the specific microorganisms involved in metabolizing carnitine and PC to TMA.

As expected, mice fed carnitine responded similarly to those challenged with choline (see above): carnitine-induced elevated TMAO that was dependent upon the presence of intact gut flora and promoted atherosclerotic plaque formation in Apoe^{-/-} mice. Interestingly, 16S analysis in mice showed different bacterial taxa associated with high TMAO levels than those seen in humans. This was not entirely surprising given known differences in murine and human flora [10]. These results show both the applicability and limits of mouse models to human pathology involving the microbiome and should inform other studies dependent upon bacterial-mediated pathology in mice.

A more extensive series of studies to elucidate potential mechanisms by which TMAO promotes atherosclerosis showed that 1) TMAO does not compete with arginine to reduce nitric oxide synthesis; 2) TMAO increases the rate of cholesterol influx via increased expression of scavenger receptors CD36 and SRA (see above); 3) TMAO does not increase the rate of cholesterol synthesis in macrophages; 4) TMAO does not increase macrophage inflammatory gene expression or desmosterol; 5) TMAO does inhibit reverse cholesterol transport (RCT), which measures the efficiency of cholesterol removal from macrophages, but expression of many known cholesterol transporters did not change in a way consistent with reduced RCT, leaving the precise mechanism unknown and, 6) TMAO decreased the bile acid pool size and lowered the expression of key bile acid synthesis (Cyp7a1, Cyp27a1) and transport proteins in the liver (Oatp1, Oatp4, Mrp2 and Ntcp). However, it is unclear whether these changes contribute to reduced RCT, which appears to play a critical role in how TMAO affects CVD [6].

With the caveat that these results are from one laboratory and require independent confirmation, the potential significance of TMAO and the obligate role of the microbiome in TMAO's production can not be underestimated as a major factor in promoting CVD, especially in precipitating major events associated with CVD.

3. Medical Relevance

Current recommendations to inhibit the progression of CVD and reduce the risks of major CV events include a healthy diet rich in fruits and vegetables and low in saturated and trans-fats, regular exercise, control of hypertension (if present) by ACE inhibitors or ARBs, and reduction of hypercholesterolemia (if present) by statins.

Recent work suggesting a key role for TMAO in promoting CVD and a critical role for the microbiome in production of TMAO is potentially of great significance. Of particular importance is the potential in using TMAO as a biomarker for CVD risk. Although larger clinical trials are necessary, the size of the reported preliminary trials (>1000) and the magnitude of the reported risk are encouraging.

For those at risk of CVD, which may include most human beings, control of TMAO levels may prove quite prudent. Of immediate relevance is that supplementation with choline or L-carnitine may be unwise in most circumstances, despite possible benefits in cognitive or muscle function respectively, unless TMAO levels are carefully monitored.

On the other hand, it well known that choline is an important nutrient [11]. Low levels of choline can lead to organ dysfunction. Choline is necessary for the production of the neurotransmitter acetylcholine and PC is a critical component of cell membranes. Complete

elimination of choline or PC from one's diet would be unproductive as well as difficult to achieve. However excess choline, such as that found in eggs, may be worth avoiding.

Reduction of dietary L-carnitine may be easier to achieve by simple reduction of red meat intake, already a key recommendation for patients with CVD. However, given that carnitine supports skeletal and cardiac muscle function and reports that carnitine supplements can help restore left ventricle ejection fraction during heart failure [12], there may exist special circumstances where L-carnitine supplementation is recommended. Ironically, a meta-analysis of available clinical data suggests that L-carnitine supplementation is helpful after acute MI in reducing mortality and the occurrence of major events [13].

However, it is important to remember that neither choline nor L-carnitine intrinsically have negative physiological effects and indeed may be of clinical benefit. Rather the problem is that some gut flora metabolize them to TMA, which in turn is metabolized into TMAO. Controlling the conversion of choline or L-carnitine into TMAO may prove the more effective strategy. For example, in the case of L-carnitine, a diet low in red meats may be sufficient to suppress TMAO production even with an occasional meal of red meat. One strategy that is not advised is long-term antibiotic use which proved ineffective in the mouse studies from the Hazen lab.

Reduced TMAO levels may be achieved by blocking the biochemical pathways that convert choline or L-carnitine into TMA with, for example, a new small molecular inhibitor. Alternately, it may be possible to outcompete TMA-producing bacterial species with a probiotic containing more benevolent species that lack the ability to metabolize choline or L-carnitine into TMA. Another possibility would be to engineer or discover bacteria that metabolize TMA into a harmless byproduct before it enters the bloodstream.

Overall, given that vegans appeared to have the lowest basal levels of TMAO, increased vegetable and fruit intake relative to red meat may be the simplest and safest recommendation.

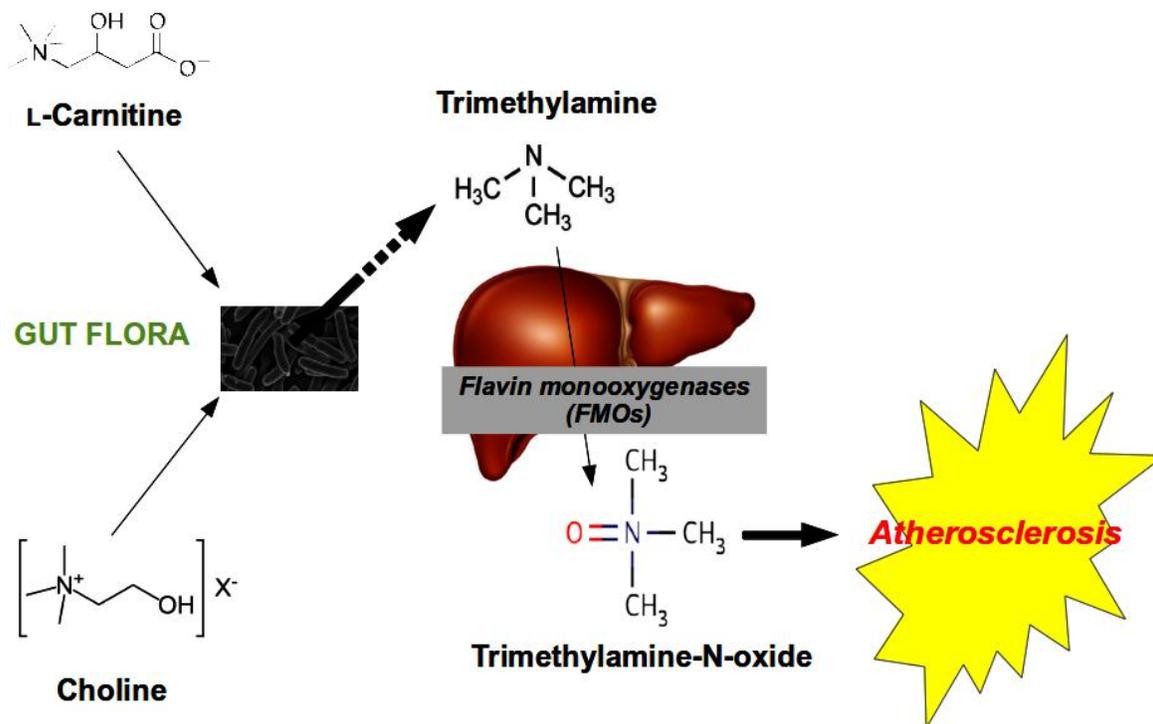
4. Conclusion

The pathogenesis of CVD remains a very active topic of research. Recent work showing that the microbiome contributes to the progression of CVD by converting choline and L-carnitine into TMA and eventually TMAO may lead to new therapies for CVD as well as to establish TMAO as an important biomarker. Reduction of L-carnitine and choline in the diet through decreased red meat consumption is presently the simplest and safest means to control TMAO levels. Development of novel therapeutics based on manipulating the microbiome may lead to improved outcomes in CVD.

5. References

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