Trimethylamine N-Oxide From Gut Microbiota in Chronic Kidney Disease Patients: Focus on Diet

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Low-protein diet is the recommended nutritional intervention for nondialysis chronic kidney disease (CKD) patients because excess protein intake can damage kidney function and produce uremic toxins. Some of these toxins are generated from amino acids breakdown by gut microbiota as p-cresyl sulfate and indoxyl sulfate that have been clearly associated with cardiovascular mortality in CKD patients. Another uremic toxin, trimethylamine N-oxide (TMAO), a degradation product of choline and L-carnitine (which come mainly from animal protein such as red meat and eggs) is now considered as a proatherogenic metabolite. In the present review, we will highlight the relationship between TMAO, diet and cardiovascular aspects, and the potential concerns about TMAO in nondialysis CKD patients.

Introduction

CARDIOVASCULAR DISEASE (CVD) is a major problem in chronic kidney disease (CKD) patients.[1,2] In addition to traditional cardiovascular risk factors, the nontraditional ones are gaining attention in scientific community like the possible imbalance of gut microbiota. In fact, recent studies have identified the gut microbiota imbalance as a new factor that may contribute to inflammation and oxidative stress leading to CVD.[3,4]

To date, little information is available about the imbalance of gut microbiota in CKD patients. Vaziri et al.[5] showed by microarray data that hemodialysis (HD) patients presented similar mean relative richness (the number of bacterial taxa in a sample) when compared with healthy individuals; however, there was a significant difference in the relative abundances of bacterial groups within the subfamilies.

In another study from our group, nondialysis patients also presented similar average number of bands evaluated by denaturing gradient gel electrophoresis technique, but data suggest possible differences in the gut microbiota between nondialysis CKD patients and healthy individuals.[3]

Although there are few studies on the composition of gut microbiota in CKD patients,[3,5–7] it is well known that the uremic toxins such as p-cresyl sulfate (PCS) and indoxyl sulfate (IS) both derived from breakdown of amino acids by gut microbiota are accumulated in these patients. These uremic toxins have been associated with cardiovascular mortality[8,9] and metabolic disturbances.[10] More recently, trimethylamine N-oxide (TMAO), also produced by gut microbiota, has been recognized as a proatherogenic metabolite.[11]

The high consumption of some animal protein sources, especially red meat and eggs may contribute to high TMAO levels because these foods contain high amount of its precursors, choline, and L-carnitine.[11–13] TMAO is excreted in the urine via kidney and can be removed in a HD session.[13,14] However, the hypothesis is that, in nondialysis CKD patients, TMAO levels would be increased. This review provides an overview of the studies focusing on diet and TMAO and future perspectives for nutritional therapy in nondialysis CKD patients.

TMAO and the Diet

The dietary carnitine and choline (derived from lecithin-phosphatidylcholine) when reaching the gut are metabolized by microbiota and produce an intermediate compound known as trimethylamine that is oxidized in the liver by enzyme flavin-containing monooxygenase 3 (FMO_3) in TMAO.[11,13]

Some varieties of normal gut bacteria, such as species of *Acinetobacter* convert dietary carnitine and lecithin into TMAO.[15] Analysis of bacterial 16S RNA sequences in fecal specimens revealed that subjects with enriched bacteria of

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Financial Disclosure and Support: See Acknowledgments on page 464.
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1051-2276/$36.00
http://dx.doi.org/10.1053/j.jrn.2015.06.004

the genus *Prevotella*, which is increased by high-fat diets, presented higher TMAO levels than subjects with an enrichment of the genus *Bacteroides*.[11] In the same study, the authors grouped volunteers by dietary status as either vegan or vegetarian ($n = 23$) or omnivore ($n = 30$) and performed a challenge that consisted of ingestion of a steak. The authors observed that the amount of both urinary and plasma TMAO increased in omnivore individuals, but not in vegans or vegetarians.[11]

In a very elegant study, Hartiala et al.[16] demonstrated in mice and humans by comparative genome-wide association studies that the levels of TMAO were only slightly predisposed by genes suggesting that the diet is an important axis for controlling TMAO levels in humans.

The greatest dietary sources of choline (lecithin) are eggs, liver, beef and pork, and red meat contains a great amount of L-carnitine that is endogenously converted into TMAO.[11,15,17]

The Nutrition Board of the Institute of Medicine[18] estimated an adequate intake of choline to be 550 mg per day for men and 425 mg per day for women. According to United States Department of Agriculture database for the choline content of common foods, the richest food in choline is egg (yolk; ~250 mg of total choline/100 g), followed by meat and fish (~75 mg of total choline/100 g), whole grains (<75 mg of total choline/100 g), breakfast cereals (~50 mg of total choline/100 g), vegetables and fruits (~30 mg of total choline/100 g), milk (~10 mg of total choline/100 g), and fat and oils (~5 mg of total choline/100 g).[19]

L-carnitine, the second precursor of TMAO, is a nutrient considered as a conditionally essential nutrient that transports long-chain fatty acids through the interior of mitochondrial membranes to produce energy.[20] According to the Annals of the New York Academy of Sciences,[21] the supplementation of L-carnitine is not necessary for adults and healthy children because carnitine is not considered an essential nutrient. Dietary reference intakes and recommended dietary allowance for carnitine was not established.[22] In individuals with normal renal function, excess of carnitine is excreted via kidneys.[13]

According to Tang et al.,[13] an excessive intake of food containing phosphatidylcholine and choline must be avoided because these metabolites lead to increased production of TMAO. In addition, a vegetarian diet or a diet with high intake of fibers could reduce total choline intake. However, it is important to recall that choline is a semi essential nutrient; and therefore, food containing choline should not be totally excluded from the diet.[19]

Lenz et al.[23] compared metabolomics urinary profile of British and Swedish population and showed higher urinary excretion of TMAO in Swedish population due to fish-based food, which was not seen in British population who were asked to avoid fish intake 24 hours before urine collection. One of the International Study of Macro/Micronutrients and Blood Pressure study publications[24] compared metabolomics profiles of different populations (Japan, China, and North America) and the Japanese population, regularly following a diet rich in fish, presented higher urinary excretion of TMAO. Lloyd et al.[25] compared dietary intake of different groups of food in a randomized trial and observed association between salmon intake and urinary TMAO excretion. Taken together, these data would suggest that fish intake would contribute to increased production of TMAO. However, a study showed that TMAO was associated with urinary nitrogen excretion for both meat and fish intake.[26] Taken together these data suggest that the composition of the diet is of upmost importance, as it was showed in an experimental study that a high-fat and high-caloric diet increased serum TMAO levels.[27]

Bennet et al.[28] examined dietary, genetic, and hormonal factors regulating TMAO levels in mice and human. The experimental study showed that FMO_3 (FMO family member with highest specificity activity) was reduced in males due to downregulation by androgens as compared with females. In humans, they also demonstrated the higher expression of FOM_3 in women as compared with men. The authors demonstrated that the supplementation of the control diet with choline (1%) did not affect the expression of hepatic FMO_3 in either male or female mice. Thus, compared to a chow diet, choline supplementation in both male and female mice increased plasma TMAO levels, which were markedly increased in females. In addition, TMAO-fed and choline-fed mice experienced increased kidney injury marker.[29]

Koeth et al.[11] performed an interesting experimental and clinical study on TMAO and showed that the reduced ingestion of L-carnitine and total choline by vegans and vegetarians (humans) was associated with decreased TMAO levels. On the other hand, gut microbiota of omnivores produced higher levels of TMAO because of the increased ingestion of L-carnitine mainly from red meat. Similar data have been previously shown by Xu et al.[30] as lactovegetarians presented decreased urinary concentration of TMAO. Stella et al.[31] reported high urinary TMAO excretion in response to meat intake. Intake of food rich in choline such as egg may be also implicated with higher TMAO production. In fact, Miller et al.[12] showed that egg intake (≥2 eggs/day) was associated with high levels of plasma and urine TMAO levels.

It is important to highlight that the urinary clearance of TMAO is supported by the high correlation between urine and plasma levels. Therefore, an efficient excretion mechanism is needed to counteract accumulation of TMAO.[13] In addition, studies have shown that the consumption of food containing high levels of TMAO precursors (L-carnitine and choline) such as red meat and eggs is implicated with atherosclerosis.[11,15] Recently, TMAO has been associated to CVD in general population.[8,13]

TMAO and Cardiovascular Disease

In humans, TMAO is associated with CVD even after adjustment for traditional cardiovascular risk factors,[13,15] and studies have investigated the link between cardiovascular aspects and TMAO (Table 1). A follow-up study that enrolled 4,007 adults who were undergoing elective diagnostic cardiac catheterization showed that the TMAO plasma levels of those who presented cardiovascular events during 3 years thereafter were significantly higher when compared to those without cardiovascular events.[13] Conversely, patients receiving HD presented higher levels of TMAO when compared with controls; however, there was no significant association concerning TMAO concentrations and cardiovascular death.[32] Recent data[29] from a cohort of stable patients undergoing elective diagnostic coronary evaluation showed that those with underlying CKD Stage 3+ ($n = 521$) presented higher levels of fasting plasma TMAO than those with regular renal function ($n = 3,166$; $P < .01$).

In animal models, administration of TMAO was strongly correlated with plaque size.[15] In patients undergoing cardiac evaluation, high L-carnitine levels concurrently with high TMAO levels predicted an increased risk for both prevalent CVD and major incident cardiac events (myocardial infarction, stroke, or death).[11]

The cardiovascular implication of TMAO seems to be due to the downregulation of reverse cholesterol transport (RCT). Koeth et al.[11] demonstrated that dietary L-carnitine and choline suppressed RCT in animals with intact intestinal microbiota. In addition, these authors showed that suppression of intestinal microbiota completely eliminated choline and L-carnitine-dependent suppression of RCT. Furthermore; a macrophage cholesterol accumulation can be produced by TMAO in a microbiota-dependent way, which increases the expression of CD36 and scavenger receptor A.[15,33,34]

It is important to remember that intrinsically, choline and L-carnitine are not implicated with negative physiological effects, and in fact, both do present positive clinical aspects. Carnitine possesses essential functions such as skeletal support and cardiac muscle function, and supplementation with carnitine may improve left ventricle ejection fraction during heart failure.[35] Furthermore, carnitine may help to reduce mortality after acute myocardial infarction; thus, for this indication, L-carnitine supplementation can be recommended.[36] The problematic pathway is that some gut flora metabolizes l-carnitine into TMAO. In individuals with normal renal function, excess of carnitine is excreted via the kidneys.[13] Patients on HD who received oral L-carnitine supplementation presented higher L-carnitine levels and substantially increased TMAO levels[37] contrasting with data showing that TMAO is supposedly removed by HD.[14] Actually, it was recently shown that patients undergoing HD and peritoneal dialysis presented higher serum levels of TMAO than controls.[32,38] Kaysen et al.[32] highlight that one explanation for no association between TMAO and cardiovascular outcomes in HD patients is that the damage to the vascular endothelium in this population is in a stage where further exposure can no longer affect outcome. Actually, both LDL and TMAO have a slighter or no influence on outcomes in patients undergoing HD, and may be the effects of these molecules are related with the level of renal function.[32,39]

Although Bain et al.[14] showed that TMAO was significantly higher in HD patients and significantly decreased after a HD session, there is a possibility for TMAO levels to be overestimated due to methodological issues of the assays applied in the study.

In any case, reducing the conversion of choline or L-carnitine into TMAO could be considered as a beneficial intervention,[40] especially in nondialysis CKD patients. Reduction of dietary L-carnitine may be easier to achieve by simple reduction of red meat intake, already a key recommendation for patients with CKD.

Low-Protein Diet for Nondialysis CKD Patients and TMAO

High protein intake can lead to glomerular hyperfiltration, proteinuria, glomerular sclerosis, and loss of kidney function.[41,42] In contrast, low-protein diet (LPD) became widespread to improve metabolic alterations and possibly delay the progression of CKD by improving lipid profile, reducing the loss of remaining nephrons, controlling insulin resistance, oxidative stress, and proteinuria, controlling hyperparathyroidism, and decreasing albuminuria, the generation of nitrogenous wastes and inorganic ions and uremic symptoms.[43] LPD is considered an important nutritional intervention as several worldwide studies have reported many beneficial results for nondialysis CKD patients. In fact, a Cochrane systematic review demonstrated that the nutritional intervention with LPD extends renal survival in nondiabetic CKD patients.[44] Collectively with the LPD treatment, the source of protein intake is an aspect that should be reconsidered for CKD patients because studies have shown that the high intake from animal protein is related with albuminuria and the progression of CKD.[45,46]

Guidelines about nutrition and CKD recommend the adherence of an LPD (0.6 g/kg/day) for nondialysis CKD patients from stage 3 to 5 (glomerular filtration rate < 60 mL/min/1.73 m^2). In addition, it is suggested that patients with less reduced levels of glomerular filtration rate, e.g., 50 mL/min/1.73 m^2, a planed low protein diet can delay the progression of renal failure.[47,48]

The control of the amount of protein intake is not only useful to limit uremia but also helps controlling CVD.[1] The cardiovascular mortality in CKD patients is much higher when compared with general population.[2] Another

Table 1. Trimethylamine N-Oxide Levels and Its Relationship With Diet and Cardiovascular Disease

Authors	Study	Results	Technique to detect TMAO
Kaysen et al. (2015)	TMAO levels of patients ($n = 235$) receiving HD	TMAO levels of CKD patients were higher than controls/No correlation of TMAO with cardiovascular outcomes	Liquid chromatography and online tandem mass spectrometry
Tang et al. (2015)	5-year follow-up, CKD patients ($n = 521$) and controls ($n = 3,166$)/ experimental study	CKD patients presented higher TMAO levels; mice fed with choline or TMAO presented progressive renal tubule interstitial fibrosis and dysfunction	Stable isotope dilution assay and high-performance liquid chromatography with online electrospray ionization tandem mass spectrometry
Gao et al. (2014)	Experimental/randomized; 24 male C57BL/6 mice received diet rich in TMAO	↑ HOMA-IR and proinflammatory cytokine	LC/MS/MS
Miller et al. (2014)	RCT—$n = 6$ eggs (yolk) challenges	≥2 eggs ↑ TMAO levels	LC/MS/MS
Koeth et al. (2013)	L-carnitine plasma levels and CVD risk in 2,595 individuals	↑ TMAO levels and carnitine levels associated with incident cardiovascular event risk	LC/MS/MS
Tang et al. (2013)	Prospective study on 4,007 patients	↑ TMAO levels predicted an increased CVD risk	Stable isotope dilution assay and high-performance liquid chromatography with online electrospray ionization tandem mass spectrometry
Wang et al. (2011)	Dietary choline or TMAO supplementation in mice	↑ Atherosclerosis plaque size	LC/MS
Bennet et al. (2013)	Experimental (mice), choline-enriched diet versus chow diet	Choline supplementation resulted in ↑plasma TMAO levels	Stable isotope dilution HPLC with online electrospray ionization tandem mass spectrometry
Koeth et al. (2013)	L-carnitine challenge ($n = 5$ omnivores and $n = 5$ vegans)	TMAO production was higher in omnivore subjects	LC/MS/MS
Tang et al. (2013)	Phosphatidylcholine challenge randomized ($n = 40$) healthy volunteers; 6 volunteers were given antibiotics for a week and retested	↑ TMAO after challenge; TMAO levels reduced after antibiotics and reappeared after withdrawal of antibiotics	Stable isotope dilution assay and high-performance liquid chromatography with online electrospray ionization tandem mass spectrometry
Li et al. (2012)	Experimental (*Macaca mulatta)*, high-fat and high-cholesterol diet	↑ Serum TMAO	^{1}H NMR spectroscopy
Rasmussen et al. (2012)	Randomized ($n = 77$); overweight, nondiabetic subjects; 8-week low-calorie diet and after randomly assigned to a high or low protein diet for 6 months	TMAO was correlated to urinary nitrogen A tendency toward an increased urinary TMAO with the high protein diet	^{1}H NMR spectroscopy
Lloyd et al. (2011)[25]	Randomized ($n = 24$); 4 test foods: oily fish, cruciferous vegetable; berry fruit or a whole-grain wheat cereal with semi skimmed milk	A combination of trimethylamine N-oxide and 1-methylhistidine was associated with oily fish intake	Gas chromatography–mass spectrometry and flow injection electrospray mass spectrometry
Xu et al. (2010)[29]	Metabolic urinary profiles from 4 groups: lactovegetarian and omnivorous	↓ Urinary concentrations of creatinine, taurine, and TMAO were in lactovegetarians	^{1}H NMR spectroscopy
Dumas et al. (2006)	INTERMAP study: urine metabolite profiles of different populations: Aito Town, Japan ($n = 259$); Chicago, Illinois ($n = 315$); and Guangxi, China ($n = 278$)	↑ Urinary TMAO levels were particularly dominant in Aito Town population, consistent with the high dietary intake of fish	^{1}H NMR spectroscopy
Stella et al. (2006)	Randomized ($n = 12$, healthy male) Intervention with washout; 3 diets: vegetarian, low meat, and high meat	↑ Urinary levels of TMAO in high meat intervention	^{1}H NMR spectroscopy
Lenz et al. (2004)	Assessment of urinary TMAO of 2 populations: Swedish and British subjects (male and female)	Fish diet of Swedish subjects could be related with the higher excretion of urinary TMAO	^{1}H NMR spectroscopy

CKD, chronic kidney disease; CVD, cardiovascular disease; 1H NMR, proton nuclear magnetic resonance; HD, hemodialysis; HOMA-IR, homeostatic model assessment-insulin resistance; HPLC, high performance liquid chromatrography; INTERMAP, The International Study of Macro/Micronutrients and Blood Pressure; LC/MS, liquid chromatography with online mass spectrometry; LC/MS/MS; liquid chromatography with tandem mass spectrometry; RCT, randomized control trial; TMAO, trimethylamine N-oxide.

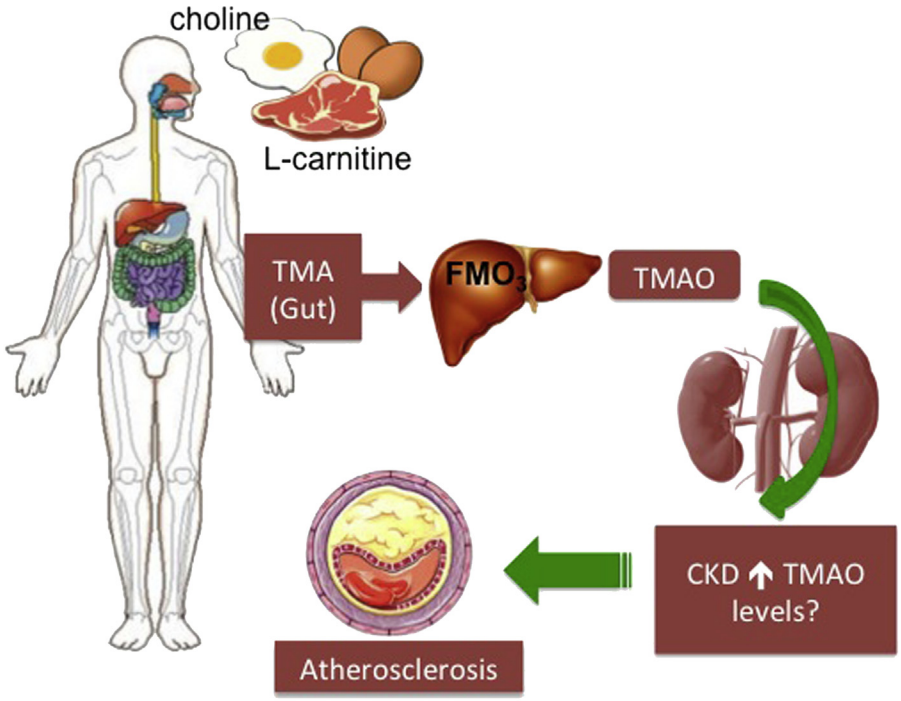

Figure 1. Atherosclerosis process by increased levels of trimethylamine n-oxide from gut microbiota in chronic kidney disease patients.

important aspect regarding CVD is that the traditional risk factors such as dyslipidemia, diabetes, and hypertension do not completely elucidate the increased risk for CVD among kidney disease patients.[49] Nontraditional cardiovascular risk factors include decreased hemoglobin levels and high concentrations of proinflammatory and prothrombotic factors such as C-reactive protein, fibrinogen, interleukin-6, factor VIII, and high levels of lipoprotein (a; Lp[a]).[50] More recently, novel nontraditional cardiovascular risk factors such as uremic toxins, that is, PCS and IS have emerged because of the retention of these toxins while the loss of kidney function progresses.[9]

A high protein intake leads to increased levels of uremic toxins, which contributes to dysregulation of saccharolytic bacteria and increased amount of proteolytic bacteria. Consequently, the protein metabolism stimulates generation of uremic toxins such as PCS, IS, and TMAO.[13,51] Both uremic toxins (PCS and IS) derive from colonic bacterial fermentation of dietary protein,[52] and the diminished protein absorption by the small intestine further decreased clearance by the kidneys and lead to PCS and IS accumulation in CKD patients. The higher levels of PCS and IS are known to contribute to the progression of CKD and complications of CVD,[5,53] and a study with healthy individuals showed that vegetarian diet is associated with reduced production of PCS and IS.[54]

Taken together, PCS, IS, and TMAO are all produced by gut microbiota from ingestion of protein food sources. However, to the best of our knowledge, there is no study that investigated the relationship of food intake and TMAO in nondialysis CKD patients. A recent published review concluded that avoiding some amino acids through an LPD could contribute to reduce the production of uremic toxins in nondialysis CKD patients. Dietary sources of these compounds are present mainly in animal protein foods such as eggs, dairy products, and red meat, which in a gut-microbiota dependent way would increase the production of uremic toxins.[4]

Currently, there is no study investigating the effects of a diet designed to control the protein intake quantitatively and qualitatively and to measure its effects on urinary and plasma levels of TMAO. Because TMAO is a uremic toxin that requires renal function integrity to be eliminated,[13] a treatment for nondialysis CKD patients that limit the consumption of food rich in TMAO and its precursors appears relevant to reduce uremic complications and cardiovascular risk. A possible way to reduce TMAO levels in nondialysis CKD patients is first to control the consumption of L-carnitine that can be achieved by a diet low in red meat[11] and second to reduce choline through a diet low in red meat and egg.[12,19]

Practical Application

Future perspectives regarding the LPD for nondialysis CKD patients should be reconsidered focusing on the management of the intake of animal protein sources, and studies

should be performed to measure the effects of such strategies and uremic toxins from the gut microbiota (Fig. 1).

Acknowledgments

The authors declare that they have no relevant financial interests.

The authors would like to acknowledge CNPq (National Counsel of Technological and Scientific Development) and FAPERJ (Foundation Carlos Chagas Filho of Support of Research of the Rio de Janeiro State) and the Higher Education Personnel Improvement Coordination/ French Evaluation Committee for University Cooperation with Brazil.

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