Cardiovascular Disease and Red Meat: How TMAO Fooled Us All

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KEYWORDS

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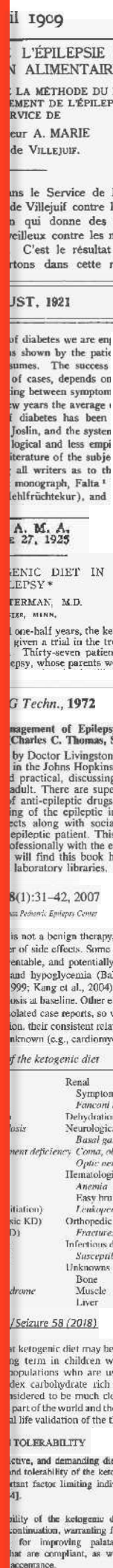

COMMENTARY

Cardiovascular Disease: How TMAO Fooled Us All

Although elevated plasma levels of TMAO have been associated with cardiovascular disease in a seemingly "cut and dry" case against red meat, the causal factors still remain remarkably unclear. Zad Rafi, BSc, Neuroscience, and Biostatistician offers his analysis of how TMAO fooled us all.

In 2011, a group of researchers published a paper in Nature linking the compound trimethylamine N-oxide to cardiovascular disease (CVD). Since then, TMAO's role in the pathology of CVD has accumulated more and more evidence, convincing several skeptics along the way of its importance. For those unfamiliar, TMAO is a metabolite produced by gut microbes from dietary substrates such as choline, betaine, and carnitine, which are all found in animal products. These dietary substrates are converted by certain microbes into the gas trimethylamine (TMA), which is then oxygenated by certain liver enzymes to form TMAO.

Microbes are necessary for this process because experiments have shown that oral antibiotics suppress TMAO production, even in the presence of these dietary substrates [Tang 2013]. Once formed, TMAO is then transported to tissues in the body, where it accumulates, while some of it is cleared by the kidneys.

MECHANISMS OF TMAO

There are several mechanisms by which TMAO is believed to cause cardiovascular disease, but the main one involves higher levels of it increasing scavenger receptors in macrophages [Velasquez 2016]. More scavenger receptors increase the likelihood of these macrophages binding low-density lipoprotein (LDL) and forming foam cells. The creation of foam cells and their localization to fatty deposits in blood vessels can disrupt cholesterol influx, esterification, efflux, and promote inflammation, all increasing the risk of CVD [YU 2013] . This process can be seen in the image below, taken from Tang et al. [Tang 2013] **[Figure 1]**

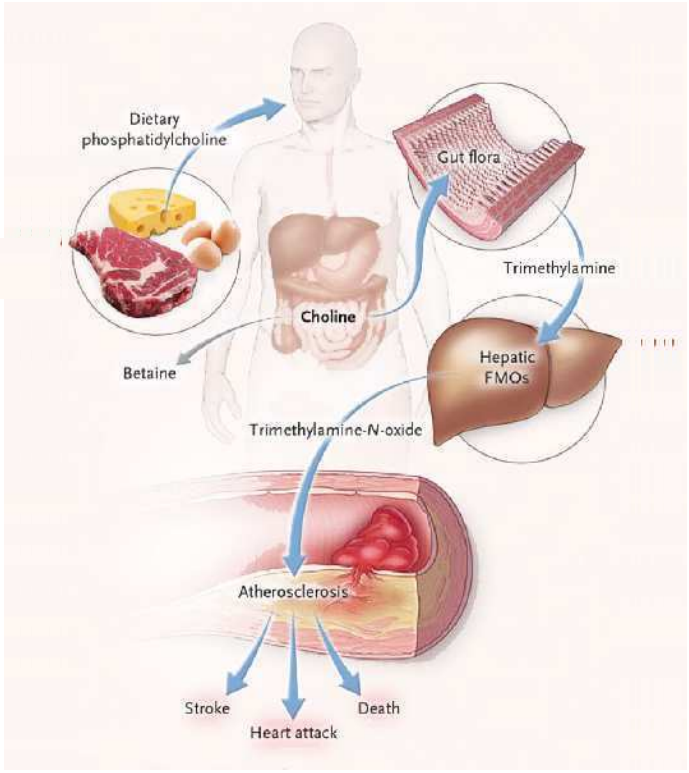

FIGURE 1: Tang et al. 2013. Intestinal Microbial Metabolism of Phosphatidylcholine and Cardiovascular Risk. New England Journal of Medicine.

TMAO AND CARDIOVASCULAR DISEASE

Several observational studies have found associations between TMAO levels and CVD risk, but more importantly, three recent systematic reviews and meta-analyses of cohort studies have also found increases in all-cause mortality and CVD events.[Heianza 2017a; Meyer 2017; Qi 2018; Schiattarella 2017].[Diagram 1]

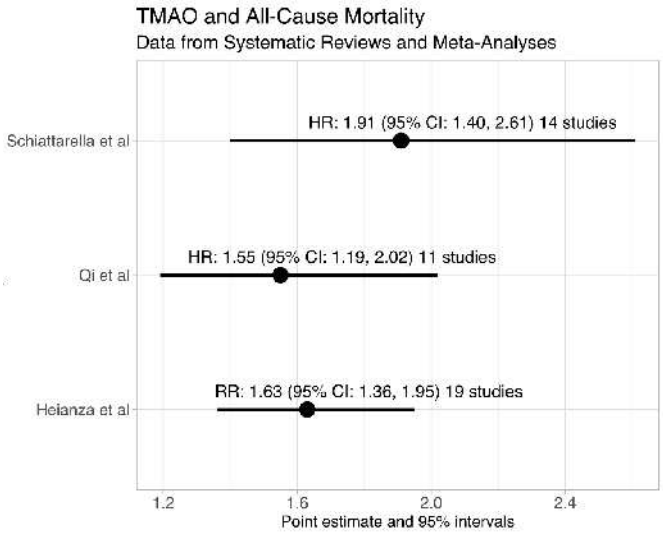

DIAGRAM 1: TMAO and All-Cause Mortality, Data from Systematic Reviews and Meta-Analysis.

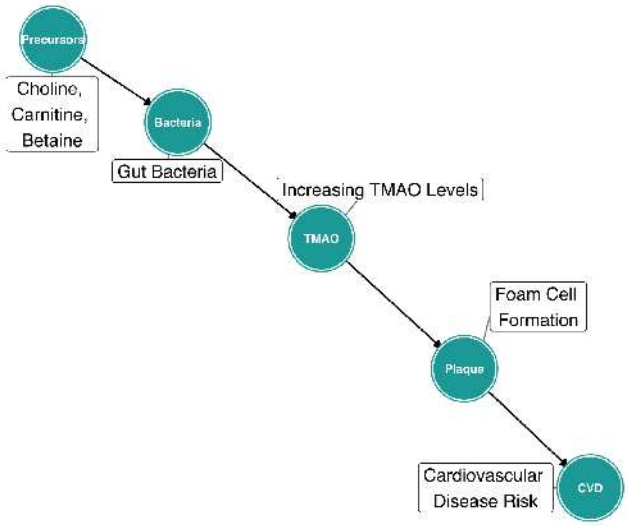

DIAGRAM 2: TMAO and Cardiovascular Disease, a Causal Diagram

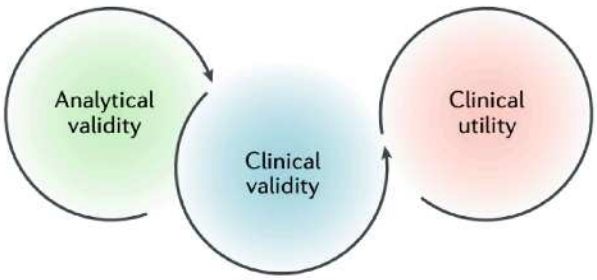

FIGURE 2: Kraus VB. Biomarkers as drug development tools: Discovery, validation, qualification and use. Nature Reviews Rheumatology.

If we assume, for the sake of this discussion, that the biological mechanisms are valid and that the results of the cohort studies are not seriously plagued by stochastic error and bias in nutritional epidemiology, then we may visualize our knowledge with a causal diagram. [Diagram 2]

To recap, certain dietary substrates, found mostly in animal products, get converted into TMA by gut microbes and then into TMAO by liver enzymes, which then contribute to the production of foam cells, which when excessively localized, wreak all sorts of havoc. This evidence has convinced many clinicians and researchers to discourage the consumption of animal products, especially red meat, a source of carnitine. Some cardiologists have even recommended that patients get their blood TMAO levels measured to predict their CVD risk, meaning these health professionals consider TMAO to be a biomarker with clinical validity and utility.

However, In the words of Kraus, [Kraus 2018] clinical validity for a biomarker is: "How well the test measures a clinical feature of a disease, disease outcome or treatment outcome is required to demonstrate the relevance of the test to the clinical condition as a guide to clinical decision-making." [Figure 2]:

Kraus also describes clinical utility as: "How well the test improves patient outcomes, confirms or changes a diagnosis, determines appropriate therapy or identifies individuals at risk of a disease — is required to determine how well a test balances benefits and harms when used in patient management. A process separate from biomarker qualification governs the approval of a biomarker as a medical test." All of these characteristics are more likely to be met if the biomarker is in the causal pathway of the disease [Figure 3].

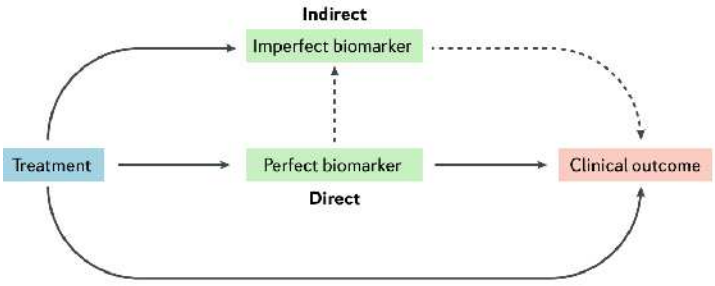

FIGURE 4: Kraus VB. Biomarkers as drug development tools: Discovery, validation, qualification and use. Nature Reviews Rheumatology. 2018;14(6):354. doi: 10.1038/s41584-018-0005-9

So, if we assume that TMAO is in the causal pathway of CVD, then theoretically it should accurately predict CVD risk, and that reducing TMAO levels via certain interventions should also lower the risk of cardiovascular disease. However, the literature on TMAO as a biomarker is preliminary and mixed [Barrea 2018; Chhibber-goel 2017; Fogelman 2015; Janeiro 2018]. Nevertheless, that hasn't stopped researchers from devising strategies to reduce TMAO formation. For example, take a look at some proposed interventions in [Table 1] below [Velasquez 2016].

While many of these may seem like low-cost interventions with minimal side effects, some interventions such as taking antibiotics can have more serious consequences, and should only be considered if TMAO is a valid marker of the causal pathway.

INCONSISTENCIES WITH TMAO

Several characteristics of TMAO conflict with known findings. For example, fish and other seafood contain some of the highest amounts of free TMAO, yet several

Therapy	The Effects	Remarks
Antibiotics [14]	Decreased TMAO plasma levels	Nonspecific, chronic use impossible and emergence of antibiotic resistance is likely
Microbiomes [16,53,54]	Decreased TMA formation in the gut	Safety and engraftment unclear in human
	Reducing TMAO to TMA	
Reduced L-carnitine and choline consumption [3]	Decrease TMAO level	L-carnitine is found to reduce all-cause mortality, cardiac symptoms in patients with myocardial infarction
Resveratrol [55]	Decreased TMA and TMAO plasma levels	It also changed the quantities of microbes
Meldonium [56]	Reduced TMA production by the intestinal microbiota bacteria	Targeting bacterial TMA-production.
3,3-dimethyl-1-butanol [38]	Inhibition of TMA formation through inhibition of microbial TMA lyases	Other inhibitory mechanisms e.g.,: changes in microbial taxa or inhibition of foam cell formation and atherosclerotic lesion
FMO3 enzyme inhibition [57]	Prevented the oxidization of TMA to TMAO	It has other effects on the regulation of both lipid metabolism and inflammation

TABLE 1: Trimethylamine N-Oxide: The Good, the Bad and the Unknown. Toxins. 8. 326. 10.3390/toxins8110326.

prospective studies have found these foods to be associated with positive CVD outcomes [Cho 2017; Zeisel 2003; Zhang 1999]. One possible explanation may be that these observational studies are simply unreliable and suffer from far too much measurement error and analytical flexibility [Keogh 2014; Patel 2015]. However, even randomized controlled crossover trials have shown that lean white fish reduces lipids that are risk factors for cardiovascular disease [Aadland 2015].

Some have argued that the omega-3 fatty acids and other compounds in fish offset the harmful effects of TMAO, however, much of this is speculative.

Those who remain skeptical of TMAO believe that it is a confounder and that it is the presence of certain microbes that contributes to the pathology of CVD, and these microbes also happen to produce TMAO [Cho 2017; Landfald 2017]. We can see this perspective visualized with the causal diagram below [**Diagram 3**].

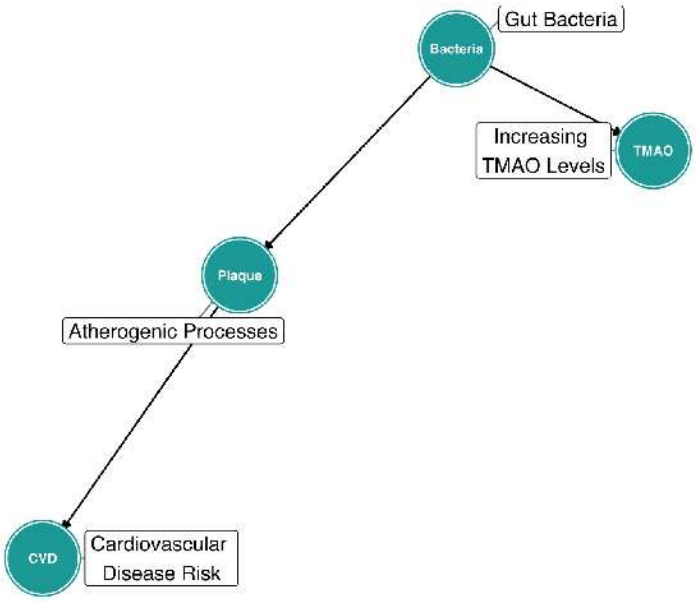

DIAGRAM 3: Microbial Contribution to TMAO Production and Cardiovascular Disease, a Causal Diagram

It is difficult to truly know the role of TMAO in the pathology of CVD without a randomized trial explicitly manipulating TMAO levels and looking at clinical endpoints. Luckily, a recently published study [Jia 2019] with a powerful design offers us the next best thing.

MENDELIAN RANDOMIZATION

In observational studies where the exposure is usually nonrandom, the ability to infer cause and effect tends to be limited by hidden confounders, reverse causality, measurement error, and selection bias, even with the use of complex methods that attempt to make groups as comparable as possible. However, a relatively new study design, called Mendelian randomization, offers a way to address many of these issues [Haycock 2016].

It uses a technique developed in econometrics called instrumental variable analysis, where certain instruments, such as genes associated with the exposure, are used as proxies for exposures such as TMAO, choline, betaine, and carnitine. In observational studies, we cannot randomly sample or assign TMAO and its precursors (practically) and look at clinical endpoints, but we can rely on the first and second laws of Mendel's inheritance because during cellular meiosis, alleles are randomly segregated and assorted, making it unlikely that many inherited genotypes are associated with population-level confounders.

In a Mendelian randomization study, once the instruments (certain genes) are known to be valid, they are tested for associations with the outcomes of interest, such as cardiovascular disease events. This method is powerful since random assortment deals with many of the issues that statistical methods cannot.

A group of researchers recently used this approach to test the associations between TMAO, choline, betaine, and carnitine, and outcomes such as CVD events, type 2 diabetes risk, and chronic kidney disease. The researchers searched published databases of genome-wide

association studies to look for genetic variants that were linked to the exposures (TMAO, choline, betaine, carnitine) in order to produce their instrument. They selected single nucleotide polymorphisms (SNPs) that met the genome-wide association significance level ($p < 0.000005$), since these are more likely to be replicable. These instruments were then tested for associations with the clinical outcomes. Unfortunately, there are no free lunches, even in Mendelian randomization studies. In order for the instrument to be valid, it must meet certain assumptions [Haycock 2016]:

1. The association between the instrument (the genes) and the outcomes must only be through the exposure to which the genes are linked. That means if the genes/SNPs (like those that produce TMAO) affect the outcome (CVD) in other ways, the instrument is invalid.

2. The genes must truly be associated with the exposure of interest. That means the genes used in the instrument must truly explain a portion of the variance in the exposures (such as choline, betaine, carnitine, and TMAO).

3. The genes cannot be associated with unmeasured confounders that are associated with the exposure and the outcome. These assumptions can be seen from an image taken from the original paper [Jia 2019] [**Diagram 4**] .

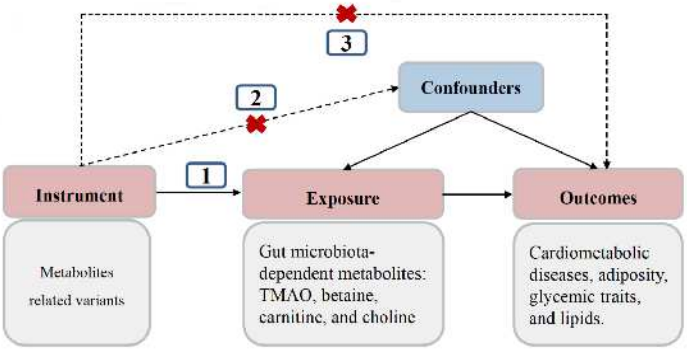

DIAGRAM 4: Jia J, Dou P, Gao M, et al. Assessment of Causal Direction Between Gut Microbiota-Dependent Metabolites and Cardiometabolic Health: Abi-Directional Mendelian Randomisation Analysis. Diabetes. June 2019

The researchers made sure their genetic variants met these assumptions by looking at the strength of the associations between the genetic variants and the metabolites and selected independent SNPs with the strongest associations (lowest P-value) for each variant. The drawback of this approach is that it can also weaken the instruments since there are bound to be SNPs with lower test statistics that can still explain a notable amount of variance. The authors explain how much metabolite variance is explained by their selected SNPs:

"Our genetic analysis showed that 15.4% of betaine, 17.1% of carnitine, 8.0% of choline, and 9.6% of TMAO were explained by its SNPs."

The authors set their alpha level at $P \leq 0.0005$ (0.05/100) after Bonferroni correction for multiple comparisons and took results ≤ 0.05 as suggestive of an association. They conducted an apriori power analysis with an alpha level of 0.05, to detect at least a 30% increase in the odds of cardiometabolic events for the dietary substrates and metabolites. MR power calculations showed that we have 87%, 84%, 78%, 81% power to test significant ($P<0.05$) causal effect (OR=1.3) of betaine, carnitine, choline, and TMAO on cardiometabolic events, respectively.

Statistical power and statistical significance hold less weight in most observational studies because there is no random mechanism. From Greenland (1990), Randomization provides the key link between inferential statistics and causal parameters [Greenland 1990]. Inferential statistics, such as P-values, confidence intervals, and likelihood ratios, have very limited meaning in causal analysis when the mechanism of exposure assignment is largely unknown or is known to be nonrandom. It is my impression that such statistics are often given a weight of authority appropriate only in randomized studies.

However, because there is a random process here (random assortment of alleles during meiosis), the authors may be more justified in testing statistical hypotheses and focusing on power and significance. Of course, this is not an encouragement for making mindless dichotomous decisions without looking at all of the data.

RESULTS

For genetically predicted higher TMAO, none of the associations with cardiometabolic outcomes were statistically significant [**Figure 5**]. Although for chronic kidney disease and myocardial infarction, there were small increases in the odds (8%) and the interval estimates were quite wide, with increases in odds as high as 60% and 99% for myocardial infarction and chronic kidney disease, respectively, being compatible with the data.

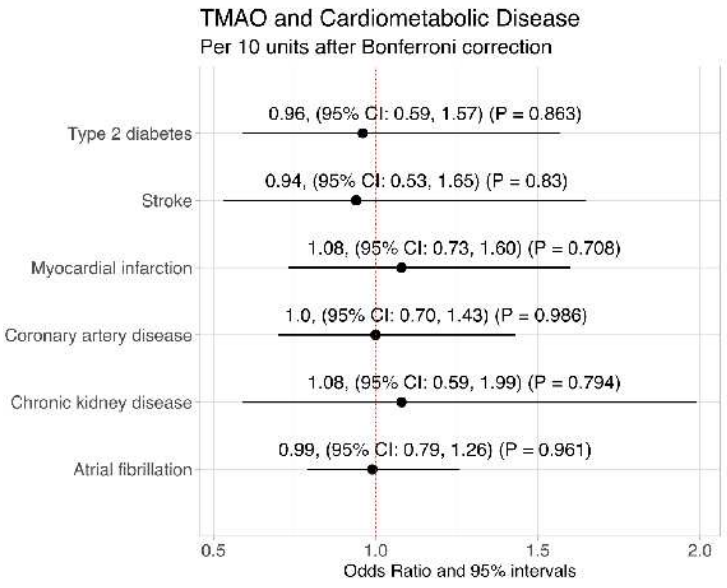

FIGURE 5: Jia J, Dou P, Gao M, et al. Assessment of Causal Direction Between Gut Microbiota-Dependent Metabolites and Cardiometabolic Health: Abi-Directional Mendelian Randomisation Analysis. Diabetes. June 2019

For genetically predicted choline, there was an 84% increase in the odds of type 2 diabetes 1.84, (95% CI: 1.00, 3.42) (P = 0.05) and for betaine, there was a 32% decrease in the odds of type 2 diabetes 0.68 (95% CI: 0.48, 0.95) (P = 0.023).

Because this study was a bidirectional Mendelian randomization study, the authors were able to use instruments for the exposures and the outcomes to assess causal effects in both directions. A diagram of what this generally looks like is shown below, taken from Haycock et al. [Haycock 2016] [**Diagram 5**].

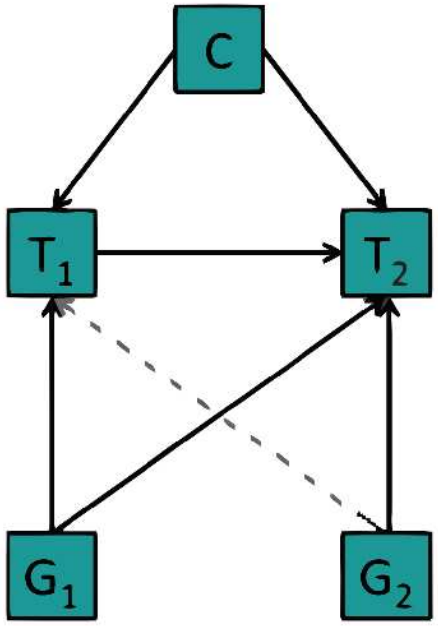

DIAGRAM 5: Bidirectional Mendelian Randomization, Haycock et al 2016

In Bidirectional Mendelian randomization, if a trait (T1) is causally associated with another (T2), then the genetic variant associated with T1 (G1) will be associated with both T1 and T2. However, the reverse (gray dashed line) will not be true and the genetic variant associated with T2 (G2) will not be associated with T1 unless the relation is truly bidirectional [Haycock 2016].

We further examined the causal effects of cardiometabolic diseases on gut-dependent metabolites. We found that T2DM was causally associated with lower betaine (beta: -0.111, SE: 0.035, P=0.002) and higher TMAO levels (0.13±0.036, P<0.0001) per each 1 unit higher log odds. CKD was also causally associated with higher TMAO levels (0.483±0.168, P=0.004) per each 1 unit higher log odds. This makes sense since TMAO levels are maintained by the kidney and chronic kidney disease would lead to higher levels of TMAO in the blood. So, although previous studies often found associations, the direction of the relationship (drawn below) was in the opposite one [**Diagram 6**].

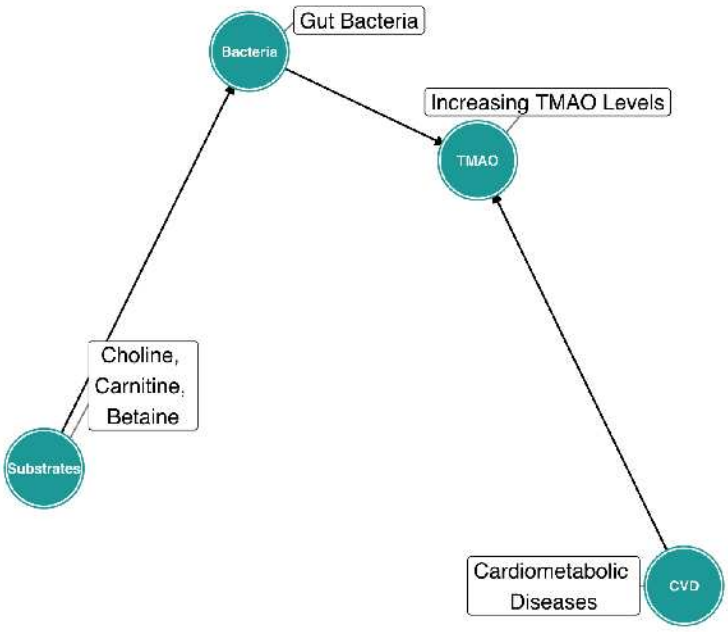

DIAGRAM 6:Causal effects of cardiometabolic diseases on gut-dependent metabolites

The authors conclude by providing the following practical recommendations:

"Genetic instruments used in Mendelian randomization studies should be pre-specified prior to data collection and analysis. The underlying IV assumptions should be tested carefully, as far as possible."

"Sample sizes should be chosen where possible to ensure that the expected value of F is greater than 10. F statistics should be quoted when reporting study results, but F statistics or other measures of instrument strength in the data should not determine the analysis used."

"Where there are potential problems with weak instruments, results should be assessed for bias by sensitivity analyses, using various assumptions in the model for genetic association and methods of IV analysis such as LIML."

DISCUSSION

Although this study provides compelling, contradictory data, its conclusions are also limited by some methodological choices. For example, the authors chose to use the genome-wide significance level ($P < 0.000005$) to choose SNPs that were associated with the exposures, which would have lead to weaker instruments that could've biased the associations towards the null.

Burgess & Thompson, 2011 describe this elegantly in their Statistics in Medicine paper [Burgess 2011.] A 'weak instrument' is defined as an instrument for which the statistical evidence of association with the phenotype (X) is not strong. An instrument can be weak if it explains a small amount of the variation of the phenotype, where the amount defined as 'small' depends on the sample size. The F statistic in the first stage regression of X on G is usually quoted as a measure of the strength of an instrument.

Although IV methods are asymptotically unbiased, they demonstrate systematic finite sample bias. This bias, known as 'weak instrument bias', is in the direction of the confounded observational association between phenotype and outcome, and depends on the strength of the instrument. Weak instruments are also associated with underestimated confidence intervals and poor coverage properties.

A generally quoted criterion is that an instrument is weak if the F statistic in the G–X regression is less than 10. However, using instruments with F>10 only reduces bias to less than a certain level, and problems with weak instrument bias still occur.

Despite these limitations, the study showed that there were very small associations between the exposures of interest and the clinical outcomes, which have often been large in nearly every cohort study, likely due to confounding. Furthermore, the study found that type 2 diabetes and chronic renal disease were causally associated with TMAO levels, addressing the problem of reverse causality encountered by previous cohort studies.

Results like this make it apparent how difficult it is to determine the nature of dietary-health relationships. Although there are valid mechanisms by which TMAO could contribute to cardiometabolic diseases, there are several other confounding mechanisms that may explain away the associations between the exposure and the outcome. In the absence of random mechanisms, it's painstakingly difficult to understand the direction of the effects in such complex situations.

Even though there was support from mechanistic studies, cohort studies, and randomized trials looking at the effects of foods on TMAO levels, the results of this study with a random mechanism strongly suggest that the causal relationship is in the opposite direction. However, this is only one study and a small piece of the puzzle. Future studies with random mechanisms (whether they are mendelian randomization studies or randomized trials) will need to replicate these findings.

AUTHORS NOTE

The results of this study and the history of TMAO remind me of a quote by Steve Goodman in his recent paper in The American Statistician [Goodman 2019].

"A clinical trial that shook the core of cardiology was the Cardiac Arrhythmia Suppression Trial (CAST), completed in 1991 (Echt et al. 1991). The therapies tested in the CAST trial were known to stop the arrhythmias thought responsible for sudden cardiac death, which was then killing more than one person every minute, or about 1200 per day. CAST revealed that these drugs, the most widely prescribed drugs in the United States, were almost quadrupling the sudden death rate, killing more Americans in the preceding decade than had died in most wars."

"This taught the cardiology community the danger of using surrogate endpoints, and the unreliability of mechanistic knowledge more effectively than could a thousand lectures, papers, or causal diagrams. Yet few physicians in other disciplines know of it and have learned that lesson. These other areas have their own stories, yet even the most dramatic, like the Duke "omics" story (Micheel et al. 2012) are little known outside their own domains." ■

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