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Diet, Microbes and Murine Atherosclerosis--Complexity?

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It is well recognized that the composition of the diet exerts a major influence on atherogenesis in several species[1]. Other than by the induction of hypercholesterolemia, the mechanisms of dietary influence are not always clear. One important modulator of this influence is the gut microbiome[2,3]. Microbes in the intestinal lumen affect the expression of genes in the enterocytes related to immune function and lipid metabolism. Among the small molecules generated or modified by the microbiome are short chain fatty acids, lysophosphatidic acid, bile acid and, of particular relevance to this editorial, choline metabolites. In this issue is an interesting paper from the Bäckhed laboratory studying the differential effect of choline supplementation of chow and Western type diet (WTD) on atherosclerosis in male *Apoe*$^{-/-}$ mice[4].

The relationship between choline metabolites and cardiovascular disease (CVD) stems from the seminal observations made by Wang and colleagues[5]. An unbiased search for small molecules in human plasma that are associated with increased risk for CVD identified choline, betaine and trimethylamine oxide (TMAO). In preclinical studies of female *Apoe*$^{-/-}$ mice fed either a standard low-fat chow diet or one supplemented with 1% choline, aortic root lesions, as calculated as absolute lesion area, was enhanced. TMAO is generated in the liver from trimethylamine (TMA), a gas generated by the metabolism of choline by TMA lyase in intestinal microbes. Dietary choline may be free or derived from the phospholipolysis of lecithin (phosphatidylcholine) present in a normal diet. The hepatic enzymes responsible for converting TMA to TMAO are flavin mono-oxygenases (FMO), particularly FMO3 and to a lesser extent FMO1. The specific activity of FMO3 is much higher in female than in male livers[6]. To establish that the formation of TMA in the intestine is dependent on the gut microbiome, some animals were treated with poorly absorbed broad-spectrum antibiotics to deplete the gut microbiota. Under this protocol, no significant level of TMAO is demonstrable in the plasma and the choline-induction of atherosclerosis is

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None

attenuated. Wang and colleagues performed some experiments with male and female mice, but, as male mice seemed to be less sensitive than female mice to supplementation with dietary choline, so most of their studies report results with female mice.

In the report of Jonsson and colleagues[4] germ-free mice and conventionally raised mice were fed either a chow or a WTD containing standard low levels of choline (0.08%) or enriched in choline (1%−1.2%). As expected no TMAO was detected in the plasma of germ-free mice regardless of choline status. Also, no secondary bile acids or deconjugated bile acids were noted in germ-free mice. Their major findings were threefold. (1) Germ-free mice have larger aortic root lesions when maintained on chow. Conventionally raised mice fed chow had lower total plasma cholesterol than germ free mice. In these chow fed mice, plasma cholesterol level correlated with the size of the lesion. (2) The absence of microbiota had no effect on atherosclerosis in mice fed WTD. (3) Increased levels of plasma TMAO had no effect on aortic root atherosclerosis in conventionally raised male mice. Although most of their studies were performed with male *Apoe*$^{-/-}$ mice, in one experiment they found no effect of choline supplementation on the atherosclerosis of female *Apoe*$^{-/-}$ mice fed either chow or WTD, so they performed essentially all of their other studies on male mice. On the surface, this report appears to contrast with other studies pointing toward an impact of TMAO on atherosclerosis. The remainder of this editorial concerns the possible explanations for these apparently contrasting findings.

It is important to note in considering data on conventionally raised mice that the microbiome may well have different composition in the vivaria of different laboratories[7]. Treatment of male mice, but not female mice, with antibiotics to deplete the gut microbiota showed a trend towards enhanced lesion size[5]. Accelerated atherosclerosis in germ-free mice has been noted[8].The higher level of atherosclerosis in germ-free mice suggests that some component(s) of the conventionally raised mice may afford protection against the development of atherosclerosis. It should be noted that the immune system of germ-free mice is not identical to that in conventionally raised mice[7]. Other than choline and its metabolites, there are other products of microbial metabolism that have been shown to contribute to atherogenesis, such as short chain fatty acids[9]. In the assessment of atherosclerosis in the aortic root, Jonsson and colleagues took the mean of two sections and expressed the results as the proportion of vessel size occupied by lesion and noted no change with choline supplementation. This mode of expression normalizes lesion for the size of the vessel along the vessel extent (however, they did not indicate precisely where they took their sections for analysis) or normalizes for any adaptive change in vessel diameter during lesion development[10]. When they measured absolute lesion size they noted a significant increase with choline supplementation of WTD fed mice. Thus, the way lesion size is expressed may well influence the conclusions. Despite this, there was still no relationship between lesion size and TMAO plasma concentration in male mice. TMAO was measured only in males. A major difference between these studies and those of the Hazen group is the gender of the mice used. The latter predominantly employ female mice while Jonsson and colleagues mostly use male mice. There appears to be quite a strong gender-based difference in choline metabolism. TMAO concentration is almost 10-fold higher in the plasma of females than males[5]. This is correlated with large differences in hepatic FMO3 expression[6]. FMO3 is expressed in the livers of male mice up to the age of 6 weeks, after which its expression is

switched off. This could be significant for the comparison of these two studies. The Hazen group enriched the diet with choline at 4 weeks of age, while the Bäckhed group only added the supplementary choline at 8 weeks of age, by which time FMO3 is no longer being expressed in male livers. FMO3 expression is induced by the bile acid cholic acid in an FXR dependent manner. This may, in part, account for the pro-atherogenic effect of the cholate containing Paigen diet. In males FMO3 is expressed in extrahepatic tissue, including in the aorta. TMAO stimulates endothelial and smooth muscle cell signaling molecules including NF-kB[11]. Taking advantage of the highly resolving hybrid mouse diversity panel (HMDP) crossed into the CETP transgenic, dominant *Apoe* Leiden background to elicit hyperlipidemia Bennett and colleagues observed a large gender bias in TMAO levels[12]. In females TMAO level accounted for 7.8% of the genetic determination of lesion area. There was no contribution of this metabolite to the genetic determination of atherosclerosis in male mice. The gender bias was also clearly observed in the offspring born to hypercholesterolemic $Apoe^{-/-}$ mice[13]. Female mice born to hypercholesterolemic mothers, but not those born to normocholesterolemic mothers, exhibited larger atherosclerotic lesions in the aortic root at 25 weeks of age, but not in the thoracic or abdominal aorta. Female offspring mice also had 5–8 fold higher plasma TMAO levels than males and this correlated with higher expression levels of hepatic FMO3. This phenotype was not observed in male offspring. When account is taken of the marked gender bias, the results of Jonsson and colleagues is not entirely inconsistent with the literature. The most distinct finding that is poorly explicable is the absence of an influence of choline supplementation in the female mice examined in one experiment by Jonsson and colleagues. Parenthetically, it is noteworthy that the gut microbiome contributes to the gender bias in autoimmune disease[14].

While most of this discussion deals with the role of the choline-TMA-TMAO axis on atherogenesis, it is clear from recent work that this axis plays an important role, in glucose and lipid metabolism as well as cholesterol homeostasis [6, 15, 16]. Indeed, this work may provide some insight into the correlation of cholesterol levels with aortic root atherosclerosis between germ free mice and conventionally raised mice observed by Jonsson et al.

The study of Jonsson and colleagues and related murine studies have some limitations. First, the fact that only aortic root atherosclerosis responds to manipulation of dietary choline suggests that the mouse is not a perfect preclinical model for the role of choline as risk factor for human CVD, especially if one bears in mind that in patients TMAO levels correlate with vascular disease in various sites including coronary artery and peripheral vascular[5]. Although hepatic FMO3 expression is lower in men than women, the difference is not as dramatic as it is for mice[6]. So far there is no indication of a gender bias in the role of dietary choline on human cardiovascular disease. Second, is the fact that plasma cytokine levels do not all show the expected correlations with lesions. Plasma cytokine levels are reflective of production by innate and adaptive immune cells, though their level in the plasma may not fully reflect the state of activation of these cells in lesions and the draining lymphoid tissues[17].

In summary, we have compared the response of $Apoe{-/-}$ mice to diets enriched in dietary choline in the laboratories of Bäckhed[4] and Hazen[5].Their results are not concordant. The Bäckhed laboratory did not observe any effects of choline enrichment on aortic root

atherosclerosis in either male or female mice, though they focused mostly on males. On the other hand, the Hazen laboratory studied mostly female mice which were shown to be more sensitive the effect of choline enrichment on atherogenesis. Both groups measured plasma TMAO after addition of choline to the diet and observed that TMAO production is dependent on the presence of intestinal bacteria. But Jonsson and colleagues found no relation between TMAO levels and lesion size in their male mice, while Wang et al found a statistically significant correlation of TMAO levels and lesion size, particularly in female mice. A part from differences in gender, there are other experimental differences, most notably the timing of the introduction of choline into the diet and duration of choline feeding that may have impacted on the effect of choline supplementation and TMAO on atherosclerosis. In addition, it is well known that mice raised in different specific pathogen-free vivaria have different microbiomes. Furthermore, it appears that the choline-TMA-TMAO axis has complex effects on several metabolic pathways and how each of these factors may contribute to the impact of this axis on atherogenesis remains to be more fully explored. It is clear that these studies have uncovered unexpected, complex metabolic interactions that will require careful future investigations controlling for various factors including gender, age of initiation of diet and microbiome content to fully understand the impact of this axis on atherogenesis. Not the least of these future studies is the consolidation of the observation that the choline content of the normal human diet represents a significant factor for the development of cardiovascular disease.

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