Gut Microbiota-Derived Trimethylamine N-Oxide Contributes to Abdominal Aortic Aneurysm Through Inflammatory and Apoptotic Mechanisms

Tyler W. Benson
University of Cincinnati College of Medicine

Kelsey A. Conrad
University of Cincinnati College of Medicine, kconrad@smith.edu

Xinmin S. Li
Cleveland Clinic Foundation

Zeneng Wang
Cleveland Clinic Foundation

Robert N. Helsley
Cleveland Clinic Foundation

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Gut microbiota-derived trimethylamine N-oxide contributes to abdominal aortic aneurysm through inflammatory and apoptotic mechanisms

Tyler W. Benson, Ph.D.1,3,* Kelsey A. Conrad, Ph.D.1,3,5,* Xinmin S. Li, Ph.D.6,7, Zeneng Wang, Ph.D.6,7, Robert N. Helsley, Ph.D.6, Rebecca C. Schugar, Ph.D.6, Taylor M. Coughlin, B.S.1,3,5, Caris Wadding-Lee, B.S.1,3,5, Salma Fleifil, B.S.1,3, Hannah M. Russell, Ph.D.1,3,5, Timothy Stone, Ph.D.2,4, Michael Brooks, B.S.1,3, Jennifer A. Buffa, M.S.6,7, Kevin Mani, M.D., Ph.D.8, Martin Björck, M.D., Ph.D.8, Anders Wanhainen, M.D., Ph.D.8, Naseer Sangwan, Ph.D.6,7, Sudha Biddinger, M.D.9, Rohan Bhandari, M.D.6,10, Akiirayi Ademoya, B.S.6, Crystal Pascual, B.S.6, W.H. Wilson Tang, M.D.6,7,10, Michael Tranter, Ph.D.1,3,5, Scott J. Cameron, M.D., Ph.D.6,10, J. Mark Brown, Ph.D.6,7, Stanley L. Hazen, M.D., Ph.D.6,7,10, A. Phillip Owens III, Ph.D.1,3,5

1Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0542, USA
2Department of Environmental Health, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0542, USA
3Division of Cardiovascular Health & Disease, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0542, USA
4Division of Biostatistics and Bioinformatics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0542, USA
5Pathobiology and Molecular Medicine Graduate Program, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0542, USA

Address for Correspondence: A. Phillip Owens III, PhD, University of Cincinnati, 231 Albert Sabin Way ML: 0542, Cincinnati, OH 45267-0542, Telephone: (513) 558 3428, Fax: (513) 558 4545, phillip.owens@uc.edu.
*Shared first authorship; equal contributions
Disclosures
S.L.H., Z.W. report being named as co-inventors on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics, and being eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland HeartLab, a wholly owned subsidiary of Quest Diagnostics, Procter & Gamble and Zehna Therapeutics. S.L.H. reports being a paid consultant for Procter & Gamble and Zehna Therapeutics, and having received research funds from Procter & Gamble, Zehna Therapeutics and Roche Diagnostics. J.M.B. reports being named as co-inventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular and metabolic disease therapeutics, and being eligible to receive royalty payments for inventions or discoveries related to cardiometabolic therapeutics from Zehna Therapeutics. The other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

List of Supplemental Materials
Complete Materials and Methods
Tables S1 – S6
Figures S1 – S8
Full unedited gels for Figure 7E
References only cited in the Supplemental Material: 59 – 71
Abstract

**BACKGROUND:** Large-scale human and mechanistic mouse studies indicate a strong relationship between the microbiome-dependent metabolite trimethylamine N-oxide (TMAO) and several cardiometabolic diseases. This study aims to investigate TMAO's role in the pathogenesis of AAA and targeting its parent microbes as a potential pharmacologic intervention.

**METHODS:** TMAO and choline metabolites were examined in plasma samples, with associated clinical data, from two independent patient cohorts (n=2,129 total). Mice were fed high choline diet and underwent 2 murine AAA models, angiotensin II (AngII) infusion in low-density lipoprotein receptor deficient (Ldlr−/−) mice or topical porcine pancreatic elastase in C57BL/6J mice. Gut-microbial-production of TMAO was inhibited via broad-spectrum antibiotics, targeted inhibition of the gut microbial enzymatic dyad CutC/D with fluoromethylcholine (FMC), or utilizing mice genetically deficient in FMO3 (Fmo3−/−). Finally, RNAseq of in vitro human VSMCs and in vivo mouse aortas were used to investigate how TMAO impacts AAA.

**RESULTS:** Elevated TMAO was associated with increased AAA incidence and growth in both patient cohorts studied. Dietary choline supplementation augmented plasma TMAO and aortic diameter in both mouse models of AAA, which was suppressed with poorly-absorbed oral broad-spectrum antibiotics. Treatment with FMC ablated TMAO production, attenuated choline-augmented aneurysm initiation, and halted progression of an established aneurysm model. Additionally, Fmo3−/− mice had reduced plasma TMAO, aortic diameters, and were protected from AAA rupture compared to WT mice. RNA sequencing and functional analyses revealed choline supplementation in mice or TMAO treatment of human VSMCs augmented gene pathways associated with the endoplasmic reticulum (ER)-stress response, specifically, the ER stress kinase Perk.

**CONCLUSIONS:** These results define a role for gut microbiota-generated TMAO in AAA formation via upregulation of ER-stress related pathways in the aortic wall. Additionally, inhibition of microbiome-derived TMAO may serve as a novel therapeutic approach for AAA treatment, where none currently exist.

**Keywords**
Abdominal aortic aneurysm; trimethylamine N-oxide; gut microbiome; ER Stress; PERK
INTRODUCTION

Abdominal aortic aneurysms (AAAs) are focal dilations of the abdominal aorta, often occurring in the segment of the aorta below the kidneys and are clinically defined as an infrarenal aortic diameter of \( \geq 3.0 \) cm.\(^1\) AAAs affect 5 – 10\% of men and 1\% of women over the age of 65 and are now the thirteenth leading cause of death in the United States (estimated 15,000 deaths annually).\(^2,3\) Likely prompted by a diverse array of initial injuries, the progression of AAAs are characterized by increases in inflammatory mediators, reactive oxygen species (ROS), matrix metalloproteinase (MMP) activity, and vascular smooth muscle cell (VSMC) apoptosis, leading to progressive dilation and weakening of the aortic wall. Left untreated, progressive AAA dilatation often results in fatal rupture.\(^4,5\) Currently, the only treatment option for AAA patients is surgical intervention, pursued only when the risk of aneurysm rupture outweighs the risk of surgery.\(^3\) Though many drugs have been investigated as potential therapeutics, none have consistently shown to attenuate AAA progression or rupture in humans.\(^6-9\)

Until recently, dietary constituents were assumed to be absorbed in our intestine and metabolized by our resident human cells. In fact, the gut microbiome, a community of trillions of commensal organisms acting collectively as a metabolically active endocrine-like organ, is now known to contribute to host physiology via the digestion of macronutrients, vitamin synthesis, and the generation of biologically active metabolites.\(^10\) Importantly, while the majority of the microbiome functions to propagate homeostasis, recent studies have demonstrated specific microbiota-derived metabolites can contribute to several cardiometabolic diseases.\(^10,11\) Specifically, the microbiota-derived metabolite trimethylamine N-oxide (TMAO) has repeatedly been associated and mechanistically linked with multiple cardiovascular diseases, such as atherosclerosis and concomitant myocardial infarction (MI), stroke, and heart failure.\(^12-14\) TMAO has also been linked to metabolic diseases such as insulin resistance and type II diabetes, chronic kidney disease, obesity, and alcohol-associated hepatitis liver injury in both human and animal studies. While the mechanism(s) of TMAO-induced increases in these disease states are still being investigated, this meta-organismal metabolite is known to induce ROS, cytokine production, and pro-fibrotic/inflammatory signaling pathways, in conjunction with the activation of platelets, endothelial cells, and vascular smooth muscle cells.\(^12,13,15-21\) Despite extensive investigation of the gut microbiota (specifically TMAO) in cardiometabolic diseases, a role for the TMAO meta-organismal pathway in AAA is vastly understudied.

Results from several recent publications suggest possible links of the gut microbiome to aneurysmal disease. For instance, TMAO levels predict long-term adverse event risk and mortality in patients with peripheral artery disease (PAD).\(^22\) Additionally, TMAO was associated with acute aortic dissection (AAD) in a recent small case-control study using metabolomics to compare AAD patients versus healthy controls.\(^23\) Moreover, the gut microbiome facilitates angiotensin II (AngII)-induced vascular ROS and hypertension.\(^24,25\) Importantly, the overall renin-angiotensin system (RAS), which is intimately linked to aneurysm disease pathology, has also emerged as a potential mediator of several microbiota-related effects via the local gastrointestinal RAS.\(^26\) As such, a recent study found the gut microbiome is markedly different when analyzed in mice with AAA versus controls.\(^27\)
further examine the link between the gut microbiome and AAA, we demonstrate that plasma TMAO concentrations correlate with risk for AAA in two independent human cohorts, increased plasma TMAO exacerbates AAA formation in multiple murine models of AAA, and TMAO driven AAA is likely mediated by enhanced activation of endoplasmic reticulum (ER) stress-related signaling.

METHODS

Additional (Full) detailed methods are presented in the Supplemental Material. All raw data and analytical methods are available from the corresponding author upon appropriate request.

Mouse Study approvals

All studies were performed under protocol #15–01–29–01 and continued with protocol #20–11–05–02 with approval and in accordance within the guidelines of the University of Cincinnati Institutional Animal Care and Use.

Human study approvals

This study complies with the Declaration of Helsinki. All European research was approved by the Research Ethics Review Board (EPN) of the Uppsala/Örebro region (Authorization/protocol number: Dnr 2007/052). All North American research was approved by the Cleveland Clinic IRB board, protocol #15834–2015. All participants (European and North American) gave written informed consent prior to their participation and sample collection. All patients and controls were of similar ethnic origin (European and Caucasian) and socioeconomic backgrounds.

Statistical analyses

All statistical analyses were performed with SigmaPlot v14.5 (SPSS, Chicago, IL) or RStudio-R version 4.1.2. (2021–11-01) (Vienna, Austria). Values of P < 0.05 were considered statistically significant. Data are represented as mean ± SEM. Data normality was assessed using a Shapiro Wilk test. Two-group comparisons were performed with Student’s t test (parametric) or Mann-Whitney Rank Sum test (non-parametric). Multiple groups were compared using a One-Way ANOVA with Holm-Sidak post hoc analysis or, when normality could not be assumed, a Kruskal-Wallis test with a Dunn post-hoc analysis using Bonferroni multiple testing correction. To compare multiple groups with two independent variables, a Two-Way ANOVA with Holm-Sidak post hoc analysis was used. Two-sided t-tests were used to determine significance of regression coefficients. Multiple Fisher’s Exact tests were performed to compare each group pairwise for categorical values such as aneurysm incidence. When 3 or more independent treatments were present, aortic diameter data was fit to a linear regression model with each individual treatment handled as a binary indicator to assess overall effect of each applied treatment. To compare survival estimates, a log-rank test was performed with multiple comparisons by a post-hoc Holm-Sidak test. The Wilcoxon rank sum test or Welch two sample t-test for continuous variables and chi-square test or Fisher’s exact test for categorical variables were used to examine the difference between the groups, Dunn’s test was used for pairwise multiple comparisons of
the ranked data, Jonckheere-Terpstra test was used to test independent samples against ordered alternatives. Odds ratio (OR) for binary AAA and corresponding 95% confidence intervals (95% CI) were calculated using both univariable (unadjusted) and multivariable (adjusted) logistic regression models. Logistic regression model was adjusted for traditional cardiac risk factors.

RESULTS

Elevated Plasma TMAO is associated with increased abdominal aortic aneurysm in both European and USA cohorts

We examined the clinical significance of TMAO levels with AAA risk in two independent AAA case/control cohorts of stable subjects undergoing cardiovascular evaluations. The European (n=352; Uppsala, Sweden) and USA (n=1777; Cleveland, OH) Cohorts collectively examined comprised a total of 2,129 participants with baseline clinical characteristics in each cohort summarized in Tables 1 and 2. Participants who had AAA at enrollment in either the European Cohort (learning cohort) or the USA Cohort (validation cohort) were older, had a greater prevalence of hypertension, used statins more frequently, and were more likely to be active smokers. In each cohort, we observed a significant association between higher TMAO concentrations and larger infrarenal abdominal aortic diameters (Kruskal-Wallis and Jonckheere-Terpstra test for increasing trend P<0.001, each). Further, plasma concentrations of TMAO were significantly elevated in participants with AAA (defined as a baseline aortic diameter ≥3.0 cm) versus controls (diameter < 3.0 cm) in each cohort (P<0.001) (Figures 1A – 1B). As compared with participants in the lowest quartile of TMAO levels, subjects in the highest quartile (Q4) demonstrated a significantly increased odds for AAA both the European Cohort (Q4 vs Q1 OR 22.5, 95%CI, 10.6–51.3, P<0.001) and the USA Cohort (Q4 vs Q1 OR 2.1, 95%CI, 1.4–3.4, P<0.01). Following multivariable logistic regression modeling to adjust for cardiovascular risk factors including age, sex, smoking, hypertension, prevalent CVD or CAD, medications and indices of renal function, elevated TMAO levels remained independently associated with risk for AAA both the European cohort (OR, 25.1, 95% CI 10.7–63.4, P<0.001) and the USA Cohort (OR, 2.4, 95% CI 1.1–5.1, P<0.05) (Figures 1C – 1D). Subgroup analysis of TMAO with AAA found elevated TMAO levels were significantly associated with increased odds of AAA in men, but not women, with no statistical significance in the association of TMAO with AAA between men and women (e.g., unadjusted Q4 vs Q1, P interaction for men vs women = 0.51; Figure S1). Collectively, these data demonstrated circulating TMAO is associated with abdominal aortic aneurysm in humans.

Dietary choline supplementation raises TMAO and augments AAA in a gut-microbiota dependent manner in mice

To examine the potential of the meta-organismal TMAO pathway in aneurysm formation, we first utilized the angiotensin II (AngII) mouse model of AAA. To suppress intestinal microbiota, a cocktail of poorly absorbed broad-spectrum antibiotics (ABX) were administered in the water ad libitum for one week prior to and throughout the study in select mice compared to untreated water. Dietary choline markedly augmented plasma levels of the gut microbial metabolites TMA and TMAO, while gut microbiota suppression via
ABX diminished plasma TMA and TMAO levels; however, no effect on circulating choline concentrations was observed (Figure S2A – S2C). Conversely, total plasma cholesterol was increased in mice given ABX (Figure S2D). Supplemental dietary choline increased AngII-induced abdominal aortic diameter and aneurysm incidence (increase of ≥50% baseline (or ≥1.2 mm) when compared to mice fed a control diet, whereas ABX suppression of TMAO production eliminated choline-induced increases in these parameters (Figures 2A – 2C). Moreover, linear regression analysis using dummy variables to isolate individual treatment effects demonstrates that ABX significantly decreased aortic diameter while supplemental TMA or TMAO resulted in significant increase (Table S5). Additionally, there was a trend toward increased rupture induced death in mice fed dietary choline compared to control fed and FMC treated mice (Figure 2D). Notably, dietary choline supplementation (and TMAO elevation) also resulted in more robust thrombus formation and increased macrophage infiltration; moreover, all of these changes were significantly reduced by ABX treatment (Figure 2E – 2G). Importantly, ABX-induced suppression of plasma TMA and TMAO levels, as well as aneurysm diameter, incidence, and rupture were completely reversed by add-back experiments where mice were concomitantly placed on ABX to suppress gut microbiota, and provided either TMA (100 mM) or TMAO (75 mM) in the drinking water, bypassing either the gut microbiota, or the entire metaorganismal TMAO pathway (Figures 2A – 2D). While choline diet increased plasma TMA and TMAO in female mice, AAA diameter and incidence were not significantly different in AngII-infused female mice (Figure S3). Together, these results suggested a direct role for gut microbiome-derived TMA/TMAO in the development of AngII-induced AAA.

**Small molecule inhibition of gut microbial TMA lyase activity decreases circulating TMAO and attenuates AAA in mice**

The glycyl radical enzyme CutC and its activating protein CutD function together as the primary choline TMA lyase responsible for the conversion of choline to TMA in gut microbes. Unlike broad spectrum antibiotics, recently developed non-lethal (microbe-friendly) TMA lyase inhibitors were able to significantly decrease plasma TMAO levels in choline-fed mice without affecting other host systems. Mice were provided either the TMA lyase inhibitor fluoromethylcholine (FMC; 0.06 g/L, 0.006% w/v) or regular water, ad libitum, 1 week prior to feeding either a choline-enriched or control diet. Mice receiving FMC had drastically reduced levels of circulating plasma TMA and TMAO (Figure S4A – S4B). In addition, provision of FMC significantly reduced choline-augmented aortic dilation, aneurysm incidence, and rupture-induced death when compared to placebo (Figure 3A – 3C, Table S6). Aortas of mice treated with FMC had significant increases in Type I collagen (Figure 3D and Figure S4E) and attenuated accumulation of macrophage infiltration when compared to placebo-treated mice (Figure 3D and Figure S4F). A causal role for TMAO in the pathology was further supported by observing that FMC-induced suppression of plasma TMAO levels, as well as aneurysm diameter, incidence, and rupture, were each completely restored by providing supplemental TMAO (75 mM) in the drinking water (Figures 3A – 3D and Figure S4A – S4D).

To further assess the potential translation value of these findings, we examined the role of choline supplementation and FMC inhibition in an alternative experimental mouse model.
of AAA. Male *C57BL/6J* mice (8 – 10 weeks) were given FMC/water or plain water, as described above, and underwent the topical elastase model of aneurysm. Similar to the AngII model, supplementation with choline augmented plasma TMA and TMAO, as well as aortic diameters, in the topical elastase model; moreover, all AAA related phenotypes were completely suppressed by the addition of FMC to the water (Figure S5). Together, these data support our hypothesis that gut microbiome-derived TMAO directly promotes AAA in vivo (at least two distinct murine models), and that selectively targeting and inhibiting gut microbial TMA lyase activity effectively protects against TMAO generation and AAA formation in mice.

We next examined the gut microbiome in this critical FMC treatment study to determine potential differences in microbial taxa amongst the groups. We noted that differences in diet, AngII infusion, FMC treatment, and TMAO supplementation can all contribute to significant differences in cecal microbiome communities, demonstrating the sensitivity of this unique system (Figure 3E). Principal coordinates analysis (PCA) of microbial taxa revealed distinct clusters in each independent group, with emphasis on the choline + AngII group to the choline + AngII + FMC group demonstrating the largest shift in microbial taxa (Figure 3F). The analysis of relative microbial taxa abundances showed that choline feeding in the AngII model was associated with enhanced *Parabacteroides* compared to control diet, which is suppressed by FMC treatment (i.e. FMC reversed many supplemental choline diet-induced changes; Figures 3G – 3H). In addition, FMC treatment (versus choline diet alone) was associated with significant enrichment in the genre *Ligilactobacillus* and *Staphylococcus*. In general, these data show that AngII infusion with choline supplementation promotes the reorganization of gut microbial communities, with choline-supplemented diet-induced changes partially reversed by FMC treatment.

**The microbial enzyme inhibitor FMC blocks progression and rupture of established AAA in mice**

Despite decades of progress in therapy and pharmacologic interventions for cardiovascular disease there are still no viable pharmacologic approach to limit AAA growth and progression. Given the effectiveness of FMC to prevent AAA formation described above, FMC was next given to mice with established AAAs to determine its potential as a possible interventional therapeutic. *Ldlr<sup>−/−</sup>* mice (n = 25) were fed a choline-enriched cholesterol diet for 1 week prior to, and throughout AngII infusion for 42 days. At day 28, mice were stratified into two groups with equivalent aneurysmal aortic diameters (aorta > 1.2mm), with one group received untreated control drinking water (n = 9) and the other receiving drinking water supplemented with FMC (0.06 g/L, 0.006% w/t, n = 9) for an additional 14 days (Figure 4A). Time-dependent increases in the aortic diameter were significantly blunted in choline-fed/FMC treated mice versus controls at day 42 (Figures 4B – 4C). Additionally, rupture-induced mortality was attenuated with FMC treatment (Figure 4D; P = 0.065). Plasma TMA/TMAO levels were dramatically reduced (Figure 4E – 4F) at day 42 in mice receiving FMC while plasma choline levels were unaffected (Figure 4G). These data demonstrate that remarkably finding that FMC virtually eliminates TMAO levels and confers protection against growth and rupture of established AAA in mice. Further, this work suggests that using a small molecule inhibitor to selectively inhibit a gut microbial
enzyme (cutC catalyzed choline TMA lyase activity) may serve as a therapeutic option to prevent the progression and rupture of AAA in newly diagnosed AAA patients.

Depletion of FMO3 activity reduces plasma TMAO and blunts AAA formation in mice

After production in the gut flora, TMA is up taken to the portal circulation and transported to the liver where it is converted to TMAO by hepatic FMOs, predominantly FMO3.32 To target the host side of the meta-organismal pathway leading the production of TMAO, mice genetically deficient in FMO3 were obtained and crossed to the Ldlr−/− background.33 As expected, Ldlr−/−/Fmo3−/− fed a choline diet had significantly increased plasma TMA (Figure S6A) while plasma TMAO concentrations were significantly decreased (Figure S6B) and plasma choline was unaffected (Figure S6C). AngII infused Fmo3 deficient mice had significantly reduced aortic diameter, AAA incidence, and rupture-induced death compared to proficient controls (Figure 5A – 5C). Additional characterization of AAA pathology by either picrosirius red or CD68 revealed significantly more type I collagen (Figure 5E) and attenuated macrophage accumulation (Figure 5F) in Fmo3−/− mice as compared to control, respectively.

As a second approach to inhibit FMO3 production of TMAO, high choline and cholesterol diet was additionally supplemented with 3,3′-diindolylmethane (DIM), a reported inhibitor of FMOs34 or cellulose filler as a placebo control. Similar to Fmo3−/− mice, provision of DIM significantly increased plasma TMA levels (Figure S6E), while plasma TMAO levels were significantly diminished (Figure S6F). Likewise, mice infused with AngII and fed the DIM supplemented diet also exhibited reduced aortic diameter (Figure 5G) and AAA incidence (Figure 5H) when compared to control fed mice.

Dietary choline leads to an upregulation of genes associated with ER-stress and apoptosis

VSMCs undergo significant phenotypic changes in AAA pathology and are critically important to both the initiation and progression of aneurysm.35 To explore the underlying mechanisms by which TMAO may contribute to AAA, we assessed the effect of TMAO on human aortic VSMCs (HAVSMCs). HAVSMCs were treated with TMAO (100 μM) or placebo control (saline) for 5 hours and next generation RNA sequencing was performed. TMAO and saline groups demonstrated spatial separation via principle component analysis (Figure 6A) and heatmap clustering (Figure 6B). We identified 792 differentially expressed genes (DEGs), including 497 upregulated and 295 downregulated DEGs (Figure 6C). Gene ontology analysis revealed that DEGs in TMAO-treated HAVSMCs were enriched in ‘response to endoplasmic reticulum (ER)-stress’ and PERK-mediated unfolded protein response (UPR)’ (Figure 6C and Figure 7A). Specifically, protein kinase R-like endoplasmic reticulum kinase (EIF2AK3, also known as PERK), ATF4, ATF5, C/EBP homologous protein (CHOP, also known as DDIT3), growth arrest and DNA damage-inducible protein (GADD34, also known as PPP1R15A), and TRIB3 were markedly upregulated with TMAO treatment (Figure 6D). As these pathways are most associated with cellular apoptosis, we examined the effects TMAO-treated HAVSMCs on programmed cell death. Here, we demonstrate that TMAO treatment results in augmented total and cleaved caspase 3, as well as significantly reduced cell viability with augmented early and late apoptosis in HAVSMCs (Figures 6E – 6H). Additionally, protein levels of MMP-2 and
active MMP-2 were significantly increased in aortic VSMCs treated with TMAO compared to vehicle control (Figure S7B and S7C).

To verify that these effects occurred in vivo, male Ldlr<sup>−/−</sup> mice were fed either a low choline and cholesterol-rich diet (control) or a high choline cholesterol-rich diet for one week, were infused with AngII for 3 days, were sacrificed, aortas collected, and RNA sequencing performed on the suprarenal abdominal aorta (Figure 7A). Similar to HAVSMCs, choline feeding, in combination with AngII, augmented genes associated with apoptosis, ER stress, and the UPR (Figure 7B). Among the most up-regulated genes was Eif2ak3 (Perk), which was increased exponentially over saline chow (256-fold) and AngII chow (120-fold; Figure 7C). Several other genes upregulated in our analysis encode proteins of the Perk arm of the UPR, included Atf4 and Chop (Figure 7C). These results were confirmed by qRT-PCR for Perk and Atf4 (Figures S7D and S7E). Caspase 3 (Casp3), a cysteine protease that plays an essential role in apoptosis, was also significantly up-regulated in the aortas of choline-fed mice. Autophagy related 5 (Atg5) and beclin 1 (Becn1), two autophagy-related proteins, were among the genes most significantly down-regulated in the aortas of choline-fed mice (Figure 6B).

Recent studies report that TMAO binds to PERK and selectively activates the PERK branch of the UPR. Our data demonstrates that TMAO treatment of HA VSMCs and high choline diet also augments PERK. To determine if choline-induced increases in apoptosis and UPR are due to PERK, we repeated our analysis of the aortic transcriptome with the addition of a PERK inhibitor (Perki) fed concomitantly with high choline and cholesterol-rich diet and during AngII infusion (Figure 7A). Inhibition of Perk resulted in a complete ablation of choline-induced UPR activation and caspase-induced genes (Figure 7C). Interestingly, Perk inhibition reversed effects seen with autophagy, resulting in augmented levels of Atg5 and Beclin1. Principle component analysis demonstrated Perk inhibition shifts the gene clusters toward control chow-fed mice (Figure 7D). In contrast to a previous study suggesting TMAO-induced aneurysm formation was due to upregulated VSMC senescence, we found senescent genes CDKN1A (p21), CDKN2C (p14/p16), and GLB (β-galactosidase) were decreased in our human VSMCs treated with TMAO (Figure S8A) and our in vivo model (Figure S8B). Overall, these data confirm a relationship between TMAO and PERK-mediated UPR and, together with our in vivo data, suggest that TMAO mechanistically contributes to AAA through activation of PERK-mediated UPR and VSMC apoptosis.

**DISCUSSION**

Despite significant advancements in pharmacologic treatment of other CVDs, no effective druggable targets have been identified as effective in reducing AAA – rather, the preferred treatment remains surgical repair. Several placebo-controlled studies aimed at potential pharmacologic therapies to treat AAA, including the use of statins, ACEII inhibitors, and antiplatelet agents, have failed to show a decrease in AAA burden, highlighting the need for novel therapeutic targets. Our studies indicate targeting of the gut microbial TMAO pathway reduces aneurysm severity and mortality in vivo. We demonstrate that ABX proved to be an effective way to suppress microbiome-derived TMAO aneurysm formation in the AngII mouse model. However, ABX treatment – alone – also modestly
inhibited aortic diameter increases in our model. This may be due to broad-spectrum MMP inhibition, which has been demonstrated most prominently with doxycycline. While our studies demonstrate this effect can be reversed with exogenous TMAO supplementation, highlighting the importance of TMAO, the overall use of ABX does not represent a viable therapeutic option in aneurysm patients. Human trials administering doxycycline to patients with AAA have been largely unsuccessful in reducing aneurysm growth. This may be due to antibiotics creating a negative selection pressure on gut microbes leading to unpredictable changes in the microbial taxa, development of antibiotic resistance, overgrowth of opportunistic organisms following cessation of antibiotics, and ultimately increases in inflammatory profiles and gut dysbiosis. Therefore, we employed a suicide substrate inhibitor, FMC, which is non-lethal to microbes and irreversibly modifies the enzymatic dyad Cut C/D in microbes expressing this gene cluster. FMC inhibition of TMAO inhibited choline diet-induced aneurysm formation in two distinct mouse models, and excitingly, almost completely blunted aortic diameter growth and death in a model of pre-existing AAA disease, halting progression. FMC treatment was also characterized by reversing some choline-rich diet induced changes in microbial community structure, and also induced an increase in species diversity that is generally associated with a healthier microbial community. More importantly, our work suggests that gut microbial inhibition of TMAO generation may be a potentially safe and novel therapeutic target for the prevention of AAA progression and rupture in humans.

Inflammation has long been recognized as a hallmark of AAA progression. Increased cytokine expression and cell adhesion markers recruit infiltrating immune cells to the vessel wall, which act to further enhance the inflammatory milieu. TMAO has also been intimately linked with inflammation in various cell types. For instance, prolonged choline feeding of hypercholesterolemic Ldlr−/− mice have no appreciable changes in lipid and glucose parameters, but markedly elevated choline-induced TMAO is correlated with increased levels of macrophage Cd68, Cox-2, and Tnfa expression in the aorta. Subsequent treatment of HAVSMCs demonstrates this TMAO induction is due to the activation of p-38 MAP kinase and NF-κB. Here, we demonstrate that choline feeding increases macrophage CD68 infiltration in choline-fed AngII-infused Ldlr−/− mice, and likewise TMAO treatment of HAVSMCs also increases COX2 (4.72 fold increased) and TNFα (6.07 fold increased) over saline treated cells (data not shown). Importantly, increased levels of COX2 and TNFα are also linked to aneurysm formation and progression in several studies. Moreover, TMAO has been found to induce both the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome in the aortas of an apolipoprotein E deficient (apoE−/−) mouse model through its effectors IL-1β, caspase 1, and IL-18, where the inflammasome and these mediators are also linked to AAA formation. However, neither our TMAO treated HAVSMCs or our choline fed Ldlr−/− mice have any increases in NLRP3, IL-1β, CASP1, or IL-18 expression (data not shown). While the results of the current manuscript indicate a mechanism for TMAO driven through increased PERK mediated UPR signaling, other known pro-inflammatory/thrombotic activity of TMAO could also contribute to the worsening of AAA.

While AAAs can occur in both sexes, the incidence and mortality rate is greater in men. Previous studies demonstrate these effects are most likely due to sex hormones, with
testosterone being one of the main agonists of aneurysm severity and ultimate mortality.\textsuperscript{44,45} Despite the limitations of mouse models of AAA compared to human pathophysiology, the Ang II model has a well-established biology compatible with human AAA ruptures and sex-related differences.\textsuperscript{46} Regardless of the profound effect we observe in our studies with the gut microbial-derived TMAO pathway in male mice, we did not observe increases in aneurysm incidence or diameter in choline-fed AngII infused female mice even with similarly increased levels of TMAO. Moreover, in the US cohort, 592 women were enrolled, but only 37 AAA cases were included putting the overall prevalence of AAA in our American cohort lower in women (6.3\%) than that in men (12.5\%). Similar to our mouse studies, we found no significant relationship with elevated TMAO and the odds of AAA in women. However, it should be noted that we did not have any female patients in our European cohort and the overall relationship between TMAO levels and AAA in women warrants further investigation in a larger independent validation study. While multiple U.S. medical societies have clear age-specific guidelines for screening men with certain risk factors for infrarenal AAA, no such guideline exists for women and our numbers are, therefore, reflective of real-life clinical data.\textsuperscript{3}

The unfolded protein response (UPR) is a homeostatic monitoring system for protein biosynthesis, which can detect ER stress from unfolded and misfolded proteins resulting in activation and downstream signaling to either restore ER protein homeostasis or mitigate apoptosis and cell death.\textsuperscript{47} Our data demonstrates that choline diet with AngII infusion results in a marked upregulation of only the PERK-eIF2-ATF4 arm of the UPR signaling pathway, with exponential increases in both PERK (256 fold increased) and ATF4 (15 fold increased). We demonstrate that CHOP is likewise increased (75-fold increase) over AngII infusion alone, suggesting choline-induced TMAO-specific amplification of these pathways. Previous studies have shown that both calcium chloride and AngII induction of aneurysms in mice results in increased UPR signaling, which are similarly upregulated in human AAA specimens.\textsuperscript{48,49} Furthermore, a recent study demonstrated that TMAO selectively activates the PERK branch of the UPR at physiologically relevant concentrations.\textsuperscript{34} Importantly, when mice are given a PERK inhibitor, choline and AngII-induced activation of the PERK arm of the UPR are completely attenuated. We speculated that TMAO was targeting the VSMC region of the aorta, as depletion of this cell population is recognized as a key mediator in the degeneration and dilation of the aorta characteristic of AAA formation.\textsuperscript{50} Indeed, treatment of HAVSMCs resulted in the similar upregulation of PERK and CHOP found in our in vivo studies. Moreover, one of the highest upregulated gene in TMAO-treated VSMCs was ATF5 (40 fold increased over saline control), which serves to potentiate CHOP-dependent apoptosis. Similarly, GADD34 and TRB3 are also increased 10-fold over control, which represents another pro-apoptotic mechanism of prolonged CHOP expression by inducing translation recovery and inhibition of the ERK pro-survival pathway, respectively.\textsuperscript{51} Alternative to apoptosis, the UPR also regulates autophagy, which is critical for vascular integrity during aneurysms. While our data shows ER stress and UPR signaling are augmented, autophagy-related genes autophagy related 5 (Atg5) and beclin-1 (Becn1) are downregulated with choline supplementation, in vivo. Therefore, our data indicates that increased circulating TMAO induces the PERK arm of the UPR resulting in increased VSMC apoptosis and decreased autophagy resulting in the augmented progression of AAA.
Future studies will examine the mechanistic link of the TMAO – ER-stress – UPR – Apoptosis – Autophagy axis in aneurysm formation and progression.

Several studies have advanced the field of small molecule non-lethal bacterial enzyme inhibitors as a novel therapeutic option to impact the microbial production of inflammatory mediators, such as TMAO. Specifically, the gut microbe-targeted choline TMA lyase inhibitor FMC significantly remodels the cecal microbiome in mice, which reverses choline-induced changes with no evidence of toxicity. While it is important to note our analyses were conducted at the genus level, our study demonstrates the CutC/D microbial taxa Escherichia was altered by both choline diet and FMC treatment in our model. The lack of changes in other CutC/D microbial taxa may in part be due to the low abundance of these microbes, making detection of specific and meaningful changes difficult to assess. Importantly, previous studies by our group of researchers have demonstrated that fecal microbiome composition does not predict dietary production of TMAO, so our lack of changes in CutC/D taxa are not overtly concerning. Moreover, the overall changes in the gut microbial endocrine organ are exquisitely sensitive to environmental and dietary factors and so the impact of these newly developed bacterial enzyme inhibitors on the total microbial landscape (not just the CutC/D taxa) are critical to the relation of microbes to cardiovascular disease outcomes. For instance, FMC treatment significantly decreased the prevalence of Parabacteroides, a taxa containing 15 known subspecies that have been implicated as both pro and anti-inflammatory mediators in mouse models of inflammatory bowel disease. Moreover, we found that the abundance of Lactobacillus was significantly increased in choline fed and FMC treated mice. Supplemental Lactobacillus rhamnosus GR-1 has been used as a probiotic for the treatment of myocardial infarction, resulting improvement of systolic and diastolic left ventricular in rodent models. While the impact of specific gut microbes on AAA progression remains unclear, the link between the gut microbiome and cardiometabolic diseases is evident. It will be beneficial to understand how therapies modify gut microbiome to find optimal druggable targets and maximize therapeutic potential. A previous study demonstrated delivery of TMAO to apolipoprotein E deficient (apoE−/−) mice infused with AngII or C57BL/6J mice exposed to calcium chloride resulted in augmented aneurysm formation, which was attributed to VSMC senescence and reactive oxygen species. TMAO delivery increased the senescent markers p16 and p21 and augmented senescent-associated β-galactosidase expression in both aneurysm models, as well as in ex vivo VSMCs treated with TMAO. Our results are in contrast to this study as we found no clear association with senescent programs in either our in vivo mouse model or our ex vivo VSMC cultures via the examination of RNA-sequencing. It is possible this discrepancy may be because of different experimental designs. Previous studies examined advanced aneurysms after 28 days of exogenous TMAO administration for immunohistochemistry and Western blotting, while our results were examined at an early time-point of 3 days post-aneurysm initiation utilizing choline-induced TMAO production and examination by RNA expression. It should be noted that while these effects are confirmed in VSMCs, the majority of the staining appears to be extravascular, in the adventitial regions of the aortic tissue. Further, our results are confirmatory of previous studies demonstrating TMAO activates a PERK-mediated pathway of ER-stress, which is most often associated with apoptotic signaling. While apoptosis and senescence
are considered two of the major outcomes upon irreversible cellular damage, these two events are not related and have distinctive biological pathways.57 Moreover, senescent cells are shown to be resistant to apoptosis, which is in contrast to our mechanistic findings and previous literature demonstrating the critical role of apoptosis in aneurysm pathophysiology.57,58

In conclusion, this work demonstrates a relationship between plasma TMAO and AAA in both humans and mice and establishes a causal role for the gut microbiota in AAA disease. It also indicates that TMAO directly participates in AAA pathogenesis specifically through the activation of the PERK arm of the UPR. Further, this work indicates that the gut microbiota may be selectively targeted to prevent the generation of TMAO and the development, progression, and rupture of AAA. These findings suggest that selectively targeting the gut microbiota may be a feasible therapeutic option for the treatment of AAA in humans. With treatment options limited for AAA patients, this work has the potential to drastically shift clinical care and patient outcomes in this disease population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

TWB, KAC, JMB, SLH, and APOIII conceived and designed the study. TWB, KAC, HMR, TMC, CWL, SF, MB were responsible for animal care. SJC, RNH, RNS, JAB, KM, MB, AW, ZW, JMB, SLH, and APOIII were responsible for phlebotomy, clinical data management, and regulation. APOIII, TWB, KAC, XSL, RNH, RNS, and ZW conducted laboratory testing. TWB, KAC, XSL, RNH, RNS, NS, ZW, MT, SJC, SLH, and APOIII contributed to the data processing. TWB, KAC, and APOIII supervised all aspects of the study. TWB, KAC, and APOIII contributed to initial data interpretation and wrote the manuscript. All authors contributed to final data interpretation and critical revision of the manuscript, and approved the final version of the manuscript.

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Non-standard Abbreviations and Acronyms

AAA Abdominal aortic aneurysm
AAD Acute aortic dissection
ABX Poorly absorbed broad-spectrum antibiotics
AngII Angiotensin II
CHOP C/EBP homologous protein
DEG Differentially expressed genes
DIM 3,3’-diindolylmethane
ER Endoplasmic reticulum
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40. Chen ML, Zhu XH, Ran L, Lang HD, Yi L, Mi MT. Trimethylamine-N-Oxide Induces Vascular Inflammation by Activating the NLRP3 Inflammasome Through the SIRT3-SOD2-mtROS Signaling Pathway. J Am Heart Assoc. 2017;6. doi: 10.1161/jaha.117.006347


Clinical Perspectives

What is New?

- Here, we demonstrate disruption of the meta-organismal production of TMAO by targeting either the gut-microbiome or the liver enzyme FMO3 of the host organism decreases circulating TMAO and protects mice from abdominal aortic aneurysm (AAA) development in two independent mouse models.

- TMAO, not TMA, drives AAA pathogenesis as demonstrated by FMO3 KO mice, which are protected from AAA, and have significantly increased TMA but TMAO production is inhibited.

- TMAO augments the unfolded protein response evident by increase expression of PERK, ATF5, and CHOP while also increasing Caspase 3 activation and apoptosis in VSMCs.

What are the Clinical Implications?

- Our work introduces the gut microbiome and associated metabolites as novel mediators of abdominal aortic aneurysm.

- TMAO and associated analytes may serve as predictive biomarkers for both the incidence and growth rate of abdominal aortic aneurysms.

- Intervention with targeted non-lethal inhibitors of the gut-microbiome provide us with new options for pharmacological treatment of aortic aneurysm.
Figure 1. The association of TMAO levels with AAA in the European and USA AAA case/control cohorts.

Box–Whisker plots of TMAO levels stratified by baseline abdominal aortic diameter and AAA status (A, B). Data are represented as boxplots: middle line is the median, the lower and upper boundaries to the boxes represent 25th and 75th percentiles, and the whiskers represent 10th and 90th percentile; P values were calculated using Kruskal-Wallis (K.W.) test with a Dunn post-hoc analysis, Jonckheere-Terpstra test of increasing trend and Wilcoxon rank sum test; Forest plots indicating the odds of AAA according to the quartiles of TMAO levels, multivariable logistic regression model for odds ratio included adjustments.
for age, sex, current smoking, hypertension, diabetes mellitus, CVD, statins, aspirin and renal insufficiency in the European cohort (C), and for age, sex, HDL, LDL, current smoking, hypertension, diabetes mellitus, CAD, C-reactive protein, statins, aspirin and CKD in the USA cohort (D), symbols represent odds ratios and the 5–95% confidence interval is indicated by line length. The number of AAA cases and total number of participants in each quartile were included.
Figure 2. Dietary choline increases plasma TMAO and augments AAA formation in a gut microbiota/TMAO-dependent manner.

(A) Aortic diameters from mice fed the indicated diets ± ABX and TMA/TMAO supplemented in the drinking water measured ex vivo 28 days after AngII-infusion (n = 10 – 28/group). Plasma TMAO levels displayed as mean ± SEM for each group (n = 9 – 16/group). Significance determined using a Kruskal-Wallis test and Dunn post-hoc analysis. (B) AAA incidence in mice from all groups with an aneurysm defined as an aortic diameter ≥1.2 mm or rupture. Total number of aneurysms observed in each group reported underneath. Significance determined by multiple pairwise Fisher’s exact tests.
(C) Representative images of aortas ex vivo from the indicated groups after 28 days of AngII infusion. (D) Kaplan Meier curve representing survival and aortic rupture-induced deaths post AngII-infusion. Significance determined by log-rank test. (E) Representative aortic sections from the indicated groups stained with Picrosirius Red for type I and type III collagen imaged by brightfield (left) and polarized light (center). Representative aortic sections stained for CD68 (right) and imaged by fluorescent microscopy. (F) Quantification as percent of total section area of type I collagen in Picrosirius Red stained sections and visualized under polarized light (n=9–12/group). Significance determined by two-way ANOVA. (G) Quantification as percent of total section area of CD68 (n = 5 – 10/group). Significance determined by two-way ANOVA. Data represented as data points and mean ± SEM.
Figure 3. FMC inhibits gut microbial production of TMA and TMAO from dietary choline and attenuates AAA in choline-fed mice.

(A) Abdominal aortic diameters from mice fed the indicated diets ± FMC and TMAO supplemented in the drinking water measured ex vivo 28 days after AngII-infusion represented individual data point and mean ± SEM (n = 4 – 36/group). Plasma TMAO levels are shown as mean ± SEM for each group (n = 4 – 16/group). Significance determined using a Kruskal-Wallis test and Dunn post-hoc analysis. (B) AAA incidence in mice from all groups with aneurysm defined as abdominal aortic diameter ≥1.2 mm or rupture. Total number of aneurysms observed in each group reported underneath. Significance determined
by multiple pairwise Fisher's exact tests. (C) Kaplan Meier curve representing survival and aortic rupture-induced deaths post AngII-infusion. Significance determined by log-rank test. (D) Representative aortic sections from the indicated groups stained with Picosirius Red for type I and type III collagen imaged by brightfield (left) and polarized light (center). Representative aortic sections stained for CD68 (right) and imaged by fluorescent microscopy. E) Alpha diversity (Shannon Index) analysis and (F) Principal component analysis (PCA) comparing the 5 groups in bacterial diversity of fecal 16S rRNA profiles (n = 6 – 15/group). (G) Stacked bar chart representing genera-level relative abundance of the 5 groups (n = 6 – 15/group). (H) Bar graph of relative abundance patterns for differentially abundant taxa (Random forest, p<0.05) representative genera-level taxa of the 5 groups (n = 6 – 15/group).
Figure 4. FMC is protective against growth and rupture of established AAAs in choline-fed mice. (A) Study schematic. Mice were started on choline diet 1 week prior to initial AngII-infusion. After 4 weeks, ultrasound was performed to identify mice with developed AAAs (aortic diameter > 1.2mm). Mice were then randomly assigned to either the FMC or placebo treatment groups and subsequently underwent a second round of AngII-infusion. Final aortic diameters were measured 2 weeks post the second round of AngII-infusion. (B) Abdominal aortic diameter of mice with established AAA receiving choline diet (n = 9) or choline diet + FMC (n = 9) at 28 days and 42 days. Significance determined by two-way ANOVA. (C) Aneurysm growth as determined by 28 day and 42 day ultrasound, significance determined by Mann Whitney rank sum test. (D) Kaplan Meier curve representing survival and rupture-induced deaths after randomization. Significance tested by log-rank test. Plasma TMA (E), TMAO (F), and Choline (G) measured at end of study by mass spectrometry in the indicated intervention groups (n = 6 – 9/group). Significance determined by Student’s t-test. Data represented as data points and mean ± SEM.
Figure 5. Disruption of FMO3 production of TMAO protects mice from AngII-induced AAA. 
(A) Aortic diameters in Ldlr<sup>−/−</sup>/Fmo3<sup>−/−</sup> vs. Ldlr<sup>−/−</sup>/Fmo3<sup>+/+</sup> mice measured ex Vivo 28 days after AngII-infusion (n = 9 – 15/group). Plasma TMAO levels displayed as mean ± SEM for each group (n = 8/group). Significance determined by Students t-test. (B) AAA incidence with an aneurysm defined as aortic diameter ≥1.2 mm or rupture. Total number of aneurysms observed in each group reported underneath. Significance determined by multiple pairwise Fisher’s exact tests. (C) Kaplan Meier curve representing survival and aortic rupture-induced deaths post AngII-infusion. Significance determined by log-rank test. (D) Representative aortic sections from the indicated genotypes stained with Picrosirius Red for type I and type III collagen imaged by brightfield (left) and polarized light (center). Representative aortic sections stained for CD68 (right) and imaged by

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fluorescent microscopy. (E) Quantification as percent of total section area of type I collagen in Picrosirius Red stained sections and visualized under polarized light. Significance determined by Student’s t-test (n = 3 – 5/group). (F) Quantification of CD68 staining as percent of total section area of CD68 positive (n = 8/group). Significance determined by Student’s t-test. (G) Aortic diameters measured ex Vivo 28 days after AngII-infusion from mice given dietary DIM compared to placebo treatment (n = 10 – 12/group). Plasma TMAO levels displayed as mean ± SEM for each group (n = 10 – 14/group). Significance determined by Students t-test. (H) AAA incidence with an aneurysm defined as aortic diameter ≥1.2 mm or rupture. Total number of aneurysms observed in each group reported underneath. Significance determined by Fisher’s exact test. (I) Kaplan Meier curve representing survival and aortic rupture-induced deaths post AngII-infusion. Significance determined by log-rank test. Data represented as data points and mean ± SEM.
Figure 6. RNaseq demonstrates increased apoptosis and ER stress and decreased autophagy with TMAO treatment in Vitro.

HAVMCs were prepared with three independent samples, conducted in triplicate, treated with either sterile saline or TMAO (100μM) for 5 hours, where RNA was processed and RNA sequencing was performed. (A) Principle component analysis and (B) heatmap clustering of RNA sequencing data comparing TMAO (100 μM) to saline control. (C) Volcano plot of differentially expressed genes between TMAO and saline treated HVSMCs and (D) fold change of UPR pathway related genes. (E) Representative western blot and (F) quantification of caspase 3 and activated caspase 3 protein levels in TMAO and saline-
treated HVSMCs (saline n = 4, TMAO n = 4). Significance determined by Student’s t test.
(G) Representative and (H) quantified results of flow cytometry analyses in for apoptotic
cells identified by Annexin V and 7-AAD using the indicated gating strategy (n = 5/group).
Circles represent individual data points, while diamonds are mean ± SEM. P values listed
on figure, or *P < 0.01. Relative quantification and gated cells were analyzed using a
Kruskal-Wallis test and Dunn post-hoc analysis. Significance is defined as fold change ≥ 1.5
and FDR P ≤0.05 with Cuffdiff. FDR, false discovery rate.
Figure 7. RNAseq demonstrates short term choline feeding in the AngII AAA mouse model increased apoptosis and ER stress while decreasing autophagy.

(A) Experimental schematic for RNA sequencing analysis from whole abdominal aortic sections from the indicated groups for (B) pathway analysis and (C) gene expression changes in the abdominal aorta isolated from Ldlr−/− mice fed either a control diet with saline infusion (n = 4), control diet with AngII infusion (n = 6), choline diet with AngII infusion (n = 5), or choline diet with AngII infusion plus Perk inhibitor (n = 5). (D) Spatial separation between indicated groups examined using principle component analysis.
Table 1
Baseline characteristics of participants stratified by abdominal aortic aneurysm (AAA) status in the European AAA Case/control cohort (N=352)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All participants (N=352)</th>
<th>Participants without AAA (N=183)</th>
<th>Participants with AAA (N=169)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, years</td>
<td>68.4±5.4</td>
<td>67.2±3.2</td>
<td>69.7±6.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>326(92.6)</td>
<td>177(96.7)</td>
<td>149(88.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>80(22.9)</td>
<td>30(16.6)</td>
<td>50(29.8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>202(58.0)</td>
<td>91(50.8)</td>
<td>111(65.7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>51(14.6)</td>
<td>26(14.4)</td>
<td>25(14.9)</td>
<td>0.99</td>
</tr>
<tr>
<td>CVD, n (%)</td>
<td>40(11.5)</td>
<td>11(6.1)</td>
<td>29(17.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Renal insufficiency, n (%)</td>
<td>12(3.4)</td>
<td>3(1.7)</td>
<td>9(5.3)</td>
<td>0.078</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>138(39.5)</td>
<td>61(33.3)</td>
<td>77(46.4)</td>
<td>0.017</td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>125(35.8)</td>
<td>41(22.4)</td>
<td>84(50.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Baseline TMAO, median/IQR, µmol/L</td>
<td>4.70(3.00-8.03)</td>
<td>3.60(2.50-5.10)</td>
<td>7.40(4.10–13.80)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AAA defined as a baseline abdominal aortic diameter of 3.0 cm or greater.

The Wilcoxon–rank sum test or Welch two sample t-test for continuous variables and the χ² test or Fisher’s exact test for categorical variables were used to determine significant difference between groups.
Table 2
Baseline Characteristics of Participants Stratified by Abdominal Aortic Aneurysm (AAA) Status in USA AAA Case/control cohort (N=1777)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All participants (N=1777)</th>
<th>Participants without AAA (N=1592)</th>
<th>Participants with AAA (N=185)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, years</td>
<td>64.7±11.5</td>
<td>63.9±11.5</td>
<td>71.1±9.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>1185(66.7)</td>
<td>1037(65.1)</td>
<td>148(80.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>225(12.9)</td>
<td>176(11.3)</td>
<td>49(26.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>1254(73.1)</td>
<td>1093(71.4)</td>
<td>161(87.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>578(32.6)</td>
<td>526(33.1)</td>
<td>52(28.1)</td>
<td>0.2</td>
</tr>
<tr>
<td>HDL, median(IQR), mg/dL</td>
<td>35.3(28.5–43.9)</td>
<td>34.9(28.2–42.6)</td>
<td>45.0(36.0–53.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL, median(IQR), mg/dL</td>
<td>92.0(74.0–111.0)</td>
<td>92.0(75.0–112.0)</td>
<td>85.5(69.0–125.8)</td>
<td>0.57</td>
</tr>
<tr>
<td>CAD, n (%)</td>
<td>1277(73.3)</td>
<td>1153(74.1)</td>
<td>124(67.4)</td>
<td>0.049</td>
</tr>
<tr>
<td>CKD, n (%)</td>
<td>336(19.4)</td>
<td>281(18.1)</td>
<td>55(29.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP, median(IQR), mg/L</td>
<td>2.44(1.08–6.25)</td>
<td>2.37(1.06–6.19)</td>
<td>3.10(1.53–6.98)</td>
<td>0.048</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>1100(62.0)</td>
<td>961(60.5)</td>
<td>139(75.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>1180(66.4)</td>
<td>1058(66.3)</td>
<td>122(66.2)</td>
<td>0.79</td>
</tr>
<tr>
<td>Baseline TMAO, median(IQR), µmol/L</td>
<td>4.41(2.70–7.24)</td>
<td>4.31(2.63–7.01)</td>
<td>5.22(3.26–9.17)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AAA was defined as a baseline abdominal aortic diameter of 3.0 cm or greater.

The Wilcoxon–rank sum test or Welch two sample t-test for continuous variables and the χ² test for categorical variables were used to determine significant difference between groups.