Trymethylamine-N-oxide, a gut-derived metabolite, induces myofibroblastic activation of valvular interstitial cells through endoplasmic reticulum stress

Short title: TMAO induces activation of valve interstitial cells

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ABSTRACT

Calcific aortic valve disease currently lacks effective treatments beyond surgical valve replacement, due to an incomplete understanding of its pathogenesis. Emerging evidence suggests that the gut microbiome influences cardiovascular health through the production of metabolites derived from dietary components. Among them, trimethylamine-N-oxide (TMAO) has been identified as a potential causal factor for several cardiovascular conditions. However, its role in the development of aortic valve disease remains poorly understood. This study sought to investigate the impact of TMAO on valvular interstitial cells (VICs), the most abundant cell type in the aortic valve. Here, we demonstrate that TMAO activates VICs towards a myofibroblastic profibrotic phenotype. Using an *in vitro* protocol to generate quiescent VICs, we found that TMAO induces the upregulation of myofibroblastic markers in a sex-independent manner. These quiescent VICs were more sensitive to TMAO than conventionally cultured VICs. Treatment with TMAO also elevated extracellular matrix production and oxidative stress, phenotypic hallmarks of an activated profibrotic state. Finally, inhibition of the endoplasmic reticulum stress kinase prior to TMAO treatment blocked all effects of this metabolite. These findings suggest that TMAO contributes to the early stages of valve disease by promoting VIC activation through endoplasmic reticulum stress mechanisms. Understanding the role of TMAO and other gut-derived metabolites in the pathogenesis of valve disease could inform the development of novel preventive or therapeutic strategies to modify or delay disease progression. Furthermore, these insights underscore the importance of host-microbiome interactions and highlight the potential for targeted dietary interventions to mitigate cardiovascular disease risk.

Key words: TMAO, metabolites, gut microbiome, valvular interstitial cells, aortic valve disease, ER stress

INTRODUCTION

Despite the quadrupling of the global prevalence of calcific aortic valve disease (CAVD) over the last three decades [1], surgical valve replacement remains the sole treatment option [2,3]. The development of novel pharmacological treatments depends on the identification of the cellular and molecular mechanisms involved in the pathogenesis of valvular disease. Accumulating evidence indicates the gut microbiome influences cardiovascular health, partly through the production of metabolites derived from dietary components [4–6]. While these microbial metabolites have been identified as key mediators in the pathogenesis of various cardiovascular conditions [7,8], their potential roles in the progression of CAVD remain incompletely understood.

Trimethylamine N-oxide (TMAO) has emerged as a gut-derived metabolite of particular interest due to its association with multiple cardiovascular diseases [9–12]. TMAO is produced through a two-step process. First, gut bacteria metabolize dietary micronutrients present in high-fat foods, such as choline and carnitine, generating trimethylamine [10,13,14]. This intermediate is then transported to the liver, where hepatic flavin monooxygenases oxidize it to TMAO, subsequently entering the systemic circulation [15–17]. In clinical studies, high circulating TMAO levels have been associated with increased risk of major adverse cardiovascular events [18], coronary heart disease [19], cardiometabolic disease [20], hypertension [21], and atherosclerosis [22]. Furthermore, animal and *in vitro* studies implicate TMAO as a causal factor in the development of cardiovascular disease through mechanisms involving endothelial dysfunction [23–25] , inflammation [26], foam cell formation [27], and fibrosis [28].

Increasing evidence suggests that TMAO may be involved in the progression of aortic valve sclerosis and calcification [29–32]. In a retrospective clinical study, patients with severe aortic stenosis had higher serum levels of TMAO compared to control subjects, even after adjusting for baseline characteristics [32]. Increased TMAO was also associated with poor adverse outcomes after transcatheter aortic valve replacement [32]. A similar cohort study found elevated TMAO to be a predictor of CAVD [31]. Moreover, in recent *in vivo* mouse studies, supplementation with dietary choline led to elevated TMAO levels, thickened aortic valves, and valvular fibrosis [30,31]. Treatment with 3,3-dimethyl-1-butanol, an inhibitor of TMA formation, prevented these effects [30,31]. Collectively, these clinical and animal data suggest a causal connection between TMAO and CAVD, with further research necessary to precisely define the contributions of this gut metabolite to the initiation of valve disease.

One of the early events in the development of CAVD is the activation of valvular interstitial cells (VICs), which maintain the structural integrity and function of the valve [33]. In a healthy state, these fibroblast-like cells remain quiescent [34]. However, during the progression of CAVD, qVICs undergo phenotypic changes, transitioning first into activated myofibroblasts and later into osteoblastic-like cells [35]. This myofibroblastic transformation marks a crucial initial step in the cascade of pathological events during the early stages of valve disease [36,37]. Others have shown that TMAO can modulate VIC phenotype *in vitro* towards fibrotic [30] and osteoblastic [31] behavior. Additionally, Li *et al.* demonstrated that TMAO can induce cardiac fibroblast activation marked by increases in proliferation, migration, and collagen deposition [28]. Hence, we hypothesized that

45 TMAO may contribute to the early stages of valve disease by activating quiescent VICs
46 (qVICs).

47 Testing this hypothesis through conventional cell culture techniques is challenging
48 because VICs spontaneously activate when cultured on traditional tissue culture plastic
49 [36,37]. As a result, these *in vitro* culture approaches that predominantly exhibit the
50 activated (aVIC) phenotype may not reliably represent the process of VIC activation or
51 the characteristics of a healthy valve. To address this issue, we have previously
52 developed a protocol to generate qVICs in 2D *in vitro* culture [38]. Using this protocol, we
53 sought to assess the impact of TMAO on healthy quiescent VICs. Here, we demonstrate
54 that TMAO triggers qVIC activation towards a profibrotic myofibroblastic phenotype
55 independent of cellular sex. Furthermore, we show that qVICs are more sensitive than
56 aVICs to this gut metabolite. Finally, we determined that TMAO induces VIC activation by
57 promoting endoplasmic reticulum (ER) stress mediated by the endoplasmic reticulum
58 stress kinase (PERK) pathway.

59

60 **MATERIALS AND METHODS**

61 **VIC Isolation, qVIC Generation, and TMAO Treatment**

62 Aortic valve leaflets were harvested from male and female (6 to 9 months) porcine
63 hearts (Animal Technologies, Tyler, Texas). Male and female cells were cultured
64 separately throughout the expansion and all experiments. Aortic valve leaflets were
65 excised and thoroughly washed in a heart wash solution containing deionized water,
66 M199 powder, 2% penicillin/streptomycin, and 1% L-glutamine. The leaflets were then
67 incubated at 37°C for 30 minutes in a collagenase II solution (Worthington Biochemical

68 Corporation). Following incubation, the valves were vortexed for 30 seconds to dislodge
69 the valvular endothelial cells. After aspirating the solution, the remaining undigested
70 tissue was removed, and placed in fresh collagenase II solution at 37°C. After a 2 hour
71 incubation, the leaflets were vortexed for 2 minutes to dislodge the VICs, which were then
72 passed through a 100μm cell filter for filtration. The VIC suspension was centrifuged at
73 500 RCF, and the resulting cell pellet was resuspended, and plated on tissue culture
74 flasks in low glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.
75 Upon reaching confluency, qVICs were generated following our previously
76 established protocol [38]. Briefly, VICs were passaged onto collagen-coated (2 μg/cm^2;
77 Human Collagen Solution, Type III, Advanced Biomatrix) tissue culture polystyrene
78 (TCPS) and cultured in low glucose DMEM supplemented with 2% FBS, 1%
79 penicillin/streptomycin, 5.25 μg/mL insulin and 10 ng/mL fibroblast growth factor (FGF;
80 Peprotech) for 10 days. To generate aVICs, the cells were cultured on uncoated TCPS in
81 low glucose DMEM supplemented with 10% FBS.
82 For all experiments, qVICs and aVICs were seeded at a density of 10,000 cells/cm^2
83 and cultured in DMEM supplemented with 2% FBS. Twenty-four hours after seeding, VICs
84 were treated with TMAO (Thermo Scientific) at concentrations ranging from 25 μM to 150
85 μM. VICs were re-fed and treated again with TMAO every 48 hours. Untreated VICs
86 served as the negative control, while treatment with transforming growth factor beta (TGF-
87 β_1; 0.5 ng/mL for qVICs and 5 ng/mL for aVICs) served as the positive control. VIC
88 phenotype was analyzed after 3 and 5 days of treatment.
89
90

91 **Immunocytochemistry for Myofibroblastic Markers**

92 The expression of proteins associated with the myofibroblastic phenotype was
93 analyzed via immunocytochemistry. VICs were washed twice with phosphate-buffered
94 saline (PBS) and fixed with 10% formalin for 10 minutes. After formalin removal, the cells
95 were washed with PBS and permeabilized with 0.5% Triton X for 5 minutes before
96 blocking with 3% bovine serum albumin (BSA) for 1 hour. Following blocking, the VICs
97 were incubated with anti-αSMA primary antibody (monoclonal, clone 1A4; Sigma Aldrich)
98 diluted 1:500 in 1% BSA or anti-smooth muscle protein 22-α (SM22; Abcam) diluted 1:500
99 in for 90 minutes at room temperature. The primary antibody was then removed, and the
100 cells were washed twice with PBS. Next, the samples were incubated with an Alexa Fluor
101 555 goat anti-mouse secondary antibody (Invitrogen) in 1% BSA (1:1000) for 60 minutes
102 at room temperature. After three washes with PBS, DAPI staining was applied for 5
103 minutes to label the nuclei, followed by an additional three PBS washes. Fluorescent
104 images were captured using a BZ-800 Keyence microscope. Integrated fluorescent
105 intensity per field of view was quantified with BZ analysis software and normalized to the
106 corresponding DAPI-stained nuclei count.

107

108 **Gene Expression Analysis with qRT-PCR**

109 To evaluate gene expression levels, quantitative reverse transcription–polymerase
110 chain reaction (qRT-PCR) was employed. VIC RNA was extracted using the RNEasy Mini
111 Kit (Qiagen) and converted into cDNA using the High-Capacity cDNA Reverse
112 Transcription Kit (Applied Biosystems). qRT-PCR was then performed with TaqMan Gene
113 Expression Assays (Applied Biosystems) for myofibroblastic markers (αSMA [ACTA2] and

114 Transgelin [SM22]), extracellular matrix proteins (fibronectin [FN] and collagen type I
115 [COL1A1]), and osteoblastic markers (alkaline phosphatase [ALP] and bone
116 morphogenetic protein [BMP]). The comparative CT (ΔΔCT) method was applied, with
117 gene expression for each experimental condition normalized to the endogenous control
118 GAPDH and then compared to the untreated control condition. In comparing male and
119 female VICs, the gene expression levels were compared to those of the untreateted
120 female control.
121
122 **Quantification of Proliferation and Apoptosis**
123 Cell proliferation was analyzed using the Click-iT EdU Alexa Fluor 488 Cell
124 Proliferation Imaging Kit (Invitrogen). VICs were treated with EdU for 8 hours, fixed,
125 permeabilized, and labeled with Alexa Fluor 488 per the kit's instructions. Cell nuclei were
126 stained with DAPI, and fluorescent images were acquired using a Keyence BZ-800
127 microscope. Positive EdU-labeled cells were quantified using the Keyence BZ analyzer
128 software and normalized to the total number of cells labeled with DAPI to obtain the
129 percentage of proliferating cells.
130 Apoptosis was measured with the SenosoLyte Homogeneous AFC Caspase 3/7
131 Assay Kit (AnaSpec). In brief, VICs were incubated with Caspase-3/7 substrate for 60
132 minutes at 37°C. End-point fluorescence intensity readings were acquired with a
133 SpectraMax microplate reader (Molecular Devices) at an excitation wavelength at 500nm
134 and normalized to the average DAPI cell count per well for the corresponding condition.
135
136

137 **Quantifying collagen and fibronectin secretion and deposition through ELISAs**

138 Fibronectin deposition was analyzed semi-quantitatively using an *in situ* ELISA.
139 After 5 days of TMAO treatment, VICs were fixed with 10% formalin for 10 minutes
140 followed by treatment with hydrogen peroxide (0.3% v/v in methanol) for 1 hour to quench
141 endogenous peroxidase activity. Subsequently, samples were blocked with 3% BSA
142 overnight and stained on the next day with a mouse anti-fibronectin antibody (1:500;
143 monoclonal IgG1; Santacruz) in 1% BSA for 2 hours at room temperature. After removing
144 the primary antibody, the cells were washed 3 times with PBS. The primary antibody was
145 then labeled with horseradish peroxidase–linked goat secondary antibody (Goat anti-
146 Mouse IgG Secondary Antibody; Thermo Fisher Scientific) diluted 1:1000 in 1% BSA for
147 40 minutes. After additional washing with PBS, 1-Step™ Turbo TMB-ELISA substrate
148 solution (Thermo Scientific) was added for 5 minutes, and the reaction was stopped with
149 2N sulfuric acid**.** Absorbance at the 450 nm wavelength was measured using a
150 SpectraMax microplate reader. To account for background signal, 2 wells per condition
151 were incubated with no primary antibody. The absorbance from these background
152 controls was subtracted from the total absorbance for each condition. Finally, the
153 absorbance values were normalized based on the previously calculated average DAPI
154 cell count for each condition.

155 We also quantified fibronectin and collagen secretion in the culture supernatant via
156 DuoSet ELISAs (R&D Systems). VIC culture supernatant was collected 3 days post-
157 treatment, and the ELISAs were performed according to the guidelines provided by the
158 manufacturer. Absorbance was measured (λ = 450 nm) using a SpectraMax plate reader.

159 The calculated concentration of fibronectin or collagen in each condition was normalized
160 to the average DAPI cell count for that condition.
161
162 **Reactive Oxygen Species Assessment**
163 VIC generation of reactive oxygen species was quantified using the ROS-Glo™
164 H_2O_2 Assay kit (Promega). Briefly, the cells were incubated with H_2O_2 substrate for 6
165 hours at 37°C. Later, a luciferin-based detection solution was added and incubated for 20
166 minutes. Luminescence was measured using a SpectraMax plate reader and normalized
167 to average DAPI cell counts. ROS levels were also assessed using the Cellular ROS
168 Assay Kit (Abcam) VICs were incubated with the cell-permeant reagent, 2',7'-
169 dichlorofluorescin diacetate, for 45 minutes. This substrate fluoresces in the presence of
170 reactive oxygen species. Fluorescence images were captured with a Keyence BZ
171 microscope. Integrated fluorescent intensity per field of view was quantified with BZ
172 analysis software and normalized to the corresponding DAPI-stained nuclei count.
173
174 **Evaluation of Endoplasmic Reticulum Stress**
175 Endoplasmic Reticulum (ER) stress was analyzed using the ER-ID® Red assay kit
176 (Enzo Life Sciences). Briefly, cells were washed twice with PBS and fixed in 10% formalin
177 for 10 minutes. Following fixation, the cells were washed again with PBS and
178 permeabilized with 0.5% Triton X-100 for 5 minutes. The cells were then incubated with
179 ER-ID® Red and Hoechst 33342 nuclear stain for 30 minutes at room temperature. After
180 incubation, the cells were washed three times with the provided assay buffer. Fluorescent
181 images were captured using a BZ-800 Keyence microscope. The integrated fluorescent

182 intensity per field of view was quantified using BZ analysis software and normalized to
183 the Hoechst-stained nuclei count.
184
185 **Inhibition of the PERK Pathway and TGF-β_1 Receptor**
186 qVICs were seeded at a density of 10,000 cells/cm^2 in low glucose DMEM
187 containing 2% FBS. After 24 hours, the cells were treated with GSK2656157 (1μM; Santa
188 Cruz), an inhibitor of the endoplasmic reticulum stress kinase (PERK) pathway, or
189 SB431542 (Selleckchem), an inhibitor of the TGF- β type I receptor, for 1 hour at 37°C.
190 Following this initial treatment, the cells were treated with 150 μM TMAO for 3 days. At
191 the end of the experiment, cell proliferation, the expression of myofibroblastic markers,
192 ROS production, and ECM deposition were assessed.
193
194 **Statistical Analysis**
195 Each experiment was performed using VICs isolated and pooled from 3 to 4
196 individual porcine hearts separated by sex with n=4-6 technical replicates per condition.
197 One-way ANOVA followed by Tukey's multiple comparisons test was used for all analyses,
198 except where comparisons involved the effects of the PERK inhibitor or sex-based
199 responses to treatment. In those cases, a two-way ANOVA was applied. All the statistical
200 analyses were conducted using GraphPad Prism software, and the data are presented
201 as mean ± standard deviation.
202
203
204

205 **RESULTS**

206 **TMAO activates quiescent valvular interstitial cells**

207 First, we generated quiescent VICs (qVICs) to test the hypothesis that TMAO
208 triggers VIC activation. Primary female porcine VICs were cultured on collagen-coated
209 plates (2 μg/cm^2) in media supplemented with 2% fetal bovine serum (FBS), insulin (5.25
210 μg/mL), and fibroblast growth factor (10 ng/mL) for 10 days as previously reported [38].
211 The generated qVICs were then treated with varying concentrations of TMAO (25 to 150
212 μM) for 3 days (Fig 1A). For these experiments, two positive controls were included –
213 VICs treated with TGF-β_1 (1 ng/mL), a known stimulus of myofibroblastic differentiation
214 [39], and activated VICs (aVICs) generated through standard culture on uncoated TCPS
215 in media supplemented with 10% FBS.

216 Treatment with TMAO at concentrations equal to or above 75 μM led to significant
217 upregulation of *ACTA2* (Fig 1B) and *SM22* (Fig 1C), known gene markers for the activated
218 myofibroblastic phenotype [40]. Significant increases in the expression levels of the
219 corresponding proteins, αSMA (Fig 1D-E) and transgelin (Fig 1F-G) were also observed
220 via immunocytochemistry, confirming the phenotypic shift toward a myofibroblastic state
221 after TMAO treatment. Additionally, TMAO-treated qVICs exhibited α-SMA and transgelin
222 expression levels comparable to those observed after treatment with TGF-β_1 (Fig 1A-G).
223 qVICs treated with 150 μM TMAO also displayed increased proliferation rates, equivalent
224 to those of both activation controls (Fig 2H). No significant differences in apoptosis were
225 observed across any treatment groups (S1 Fig). The increase in both the expression of
226 myofibroblastic markers and proliferation indicates that TMAO indeed promotes the
227 transition of qVICs to an activated state.

228

229 **Treatment with TMAO induces extracellular matrix (ECM) production**

230 Because the activation of qVICs into myofibroblastic-like cells is often associated
231 with increased ECM deposition [35], we also evaluated the impact of TMAO treatment on
232 the production of two ECM proteins: collagen I and fibronectin. After 3 days of treatment,
233 TMAO significantly upregulated the gene expression of *COL1A1* (Fig 2A) and *FN* (Fig 2B)
234 in qVICs, reaching levels comparable to those observed after treatment with TGF-β_1. The
235 upregulation of *COL1A1* was observed only at the highest concentration of 150 μM, while
236 *FN* was upregulated at 75 μM and higher concentrations. To account for potential post-
237 transcriptional modifications and protease degradation [41], we also assessed the
238 production of these components at the protein level. No statistically significant differences
239 were observed in collagen secretion (Fig 2C) or deposition (S2A Fig) following TMAO
240 treatment. In contrast, TMAO treatment significantly increased fibronectin secretion at all
241 concentrations (Fig 2D) and deposition at concentrations ³≥ 75 μM (S2B Fig).

242 We next sought to examine the effects of TMAO on conventionally cultured
243 activated VICs (Fig 3A). For these experiments, the control consisted of untreated aVICs.
244 aVICs treated with TGF-β_1 (10 ng/mL) and untreated qVICs served as additional positive
245 and negative controls, respectively. Initially, female aVICs were treated with the same
246 concentrations used for qVICs (25-150 μM). However, TMAO had no effect on α-SMA
247 expression (S3A-B Fig) or proliferation (S3C Fig) at these concentrations. Thus, we
248 proceeded to treat the aVICs with higher TMAO concentrations (300 μM and 600 μM; Fig
249 3A). After 3 days of treatment, we observed a significant upregulation of the *ACTA2* gene
250 at 600 μM (Fig 3B), while the *SM22* gene was upregulated at concentrations of 300 μM

251 and 600 μM (Fig 3C). However, this increase in myofibroblastic marker expression was
252 not reflected at the protein level for either α-SMA (Fig 3D-E) or transgelin (S4A-B Fig). At
253 these higher concentrations, TMAO led to a small decrease in cell proliferation for all
254 concentrations (S4C-D Fig).

255 Finally, we evaluated the production of ECM proteins by aVICs treated with TMAO.
256 Treatment with high concentrations of TMAO resulted in statistically significant increases
257 in *COL1A1* (Fig 3F) and *FN* expression (Fig 3G) in aVICs compared to the untreated
258 control. While the upregulation of *COL1A1* was observed at all three concentrations, the
259 *FN* gene was only upregulated at the highest concentration - 600 μM. Notably, when both
260 myofibroblastic (Fig 3B-C) and ECM (Fig 3F-G) genes were significantly upregulated, the
261 gene expression levels surpassed those induced by treatment with TGF-β_1, the most
262 widely characterized profibrotic cytokine in the aortic valve [40,42] .

263 In summary, these findings suggest that TMAO promotes profibrotic behavior in
264 both qVICs and aVICs, driving increased ECM production, a critical hallmark in the early
265 progression of valve disease [43].

266

267 **qVIC activation by TMAO is not sex-dependent**

268 CAVD exhibits significant sex-based differences in its pathophysiology [43–45]
269 partially driven by differences in the response to disease stimuli between males and
270 females at the cellular level [46–48]. Thus, we investigated whether sex influenced the
271 qVIC response to TMAO. Male and female qVICs were treated with TMAO (25 – 150 μM)
272 and TGF-β_1 (1 ng/mL). Treatment with TMAO at concentrations ranging from 75 μM to
273 150 μM led to a significant upregulation of the *ACTA2* (S5A Fig) and *SM22* (S5B Fig)

genes in both male and female qVICs. Corresponding protein-level increases in α-SMA (Fig. 4A-B) and transgelin (S5C Fig) were also observed with TMAO treatment at concentrations of 75 μM to 150 μM for both sexes.

For most treatment conditions, no sex-related differences were observed in the gene expression levels of these markers. However, for qVICs treated with 75 μM TMAO, there was a significantly higher upregulation of the *ACTA2* gene in the female cells compared to male VICs (S5A Fig), with the opposite effect for *SM22* (S5B Fig). No sex-based differences were detected in the expression of either α-SMA (Fig 4A-B) or transgelin (S5C Fig) at the protein level. Similar results were observed for proliferation (S5D Fig), were both male and female VICs proliferated at statistically higher rates after exposure to TMAO compared to the control, with no sex-based differences observed for any treatment condition.

As observed previously for female qVICs, male qVICs treated with TMAO concentrations equal or higher than 75 μM resulted in the upregulation of the ECM-related genes *FN* and *COL1A1* (S4E-F Fig). We also observed increased fibronectin deposition by qVICs of both sexes at these concentrations (Fig 4C). Consistent with our prior data, TMAO had no effect on collagen deposition (S4G Fig). No statistically significant differences in the response to TMAO were observed between male and female qVICs for any of these ECM-related end points.

These results demonstrate that TMAO treatment drives myofibroblastic activation and ECM production in both female and male qVICs, with minimal sex-based differences in the cellular response to this metabolite under the tested experimental conditions.

297

298 **TMAO triggers oxidative and ER stress in qVICs**

299 Next, we explored the effects of TMAO on qVIC metabolism. Reactive oxygen
300 species (ROS) have been implicated in both the progression of CAVD [49] and the cellular
301 response to TMAO in other cardiovascular contexts [50]. Therefore, we assessed the
302 production of ROS after treating female qVICs with TMAO for 3 days. TMAO led to an
303 increase in intracellular ROS production at 75 μM - 150 μM concentrations compared to
304 the untreated control (Fig 5A). These elevated ROS levels were also observed in the cell
305 culture media (Fig 5B). Similar increases in ROS were observed in aVICs and after
306 treatment with TGF-$ß_1$.

307 Because TMAO has also been reported to drive endoplasmic reticulum (ER) stress
308 [51], we stained TMAO-treated qVICs with an ER-ID® Dye (Enzo Life Sciences). The
309 detected intensity of this dye is proportional to the levels of ER stress in the stained cells
310 [52]. Treatment with TMAO (75 μM) led to a statistically significant increase in ER stress
311 compared to the untreated control (Fig 5C-D). No increase in ER stress was observed in
312 qVICs treated with TGF-β_1 or in the aVICs controls, suggesting this effect is unique to
313 TMAO. These results establish that TMAO leads to metabolic dysfunction in qVICs
314 through both oxidative and ER stress.

315

316 **TMAO drives qVIC activation through the PERK pathway**

317 Finally, we sought to identify the specific molecular mechanisms through which
318 TMAO activates qVICs. Chen *et al.* have previously demonstrated that TMAO drives ER
319 stress and metabolic dysfunction in hepatocytes by directly binding and activating the

320 endoplasmic reticulum stress kinase (PERK) [53]. Hence, we explored the role of the
321 PERK pathway in the context of qVIC activation by TMAO. Female qVICs were pre-
322 treated with a PERK inhibitor, GSK2656157, for 1 hour prior to treatment with either
323 TMAO (150 μM) or TGF-β_1 (1 ng/ml). GSK2656157 is a selective competitive inhibitor of
324 PERK that blocks its activity by binding to the PERK kinase domain, thereby inhibiting its
325 phosphorylation [54]. PERK inhibition successfully blocked the increase in ER stress
326 previously observed after treatment with TMAO, while having no effect on the untreated
327 or TGF- β controls (Fig 6A-B). Similarly, qVICs treated with the inhibitor did not exhibit an
328 increase in ROS production after exposure to TMAO (Fig 6C). Inhibition of the PERK
329 pathway had no effect on ROS production for qVICs treated with TGF-β_1 (Fig 6C),
330 suggesting that these two stimuli induce oxidative stress through distinct molecular
331 pathways.

332 Having established that the GSK2656157 inhibitor successfully prevented the
333 metabolic dysfunction induced by TMAO, we explored whether it also blocked the qVIC
334 transition to an activated myofibroblastic phenotype. PERK inhibition significantly reduced
335 the expression of both α-SMA (Fig 6D-E) and transgelin (S6A-B Fig) at the gene (S6C-
336 D) and protein levels in qVICs treated with TMAO, effectively suppressing the transition
337 to a myofibroblastic phenotype. Similar effects were observed for proliferation rates (Fig
338 6F). Treatment with the inhibitor had no significant effect on apoptosis (S6E Fig).

339 We also evaluated the role of PERK in regulating the pro-fibrotic response to
340 TMAO. qVICs pre-treated with the PERK inhibitor did not upregulate the expression of
341 *COL1A1* (Fig 6G) and *FN* (Fig 6H) upon treatment with TMAO. For all phenotypic
342 outcomes, TGF-β_1-mediated qVIC activation remained unaffected by PERK inhibition (Fig

343 6C-H). Moreover, blocking of the TGF-β_1 receptor with the SB431542 inhibitor, did not
344 interfere with the effects of TMAO on qVICs (SFig 7). Overall, these findings support the
345 hypothesis that TMAO induces qVIC activation through ER stress and signaling via the
346 PERK pathway.
347

348 **DISCUSSION**

349 The gut microbiome is increasingly recognized as an important contributor to heart
350 health [55–58]. More specifically, microbiome-derived metabolites like TMAO have been
351 implicated in the initiation and progression of multiple cardiovascular diseases [59–63].
352 Patients with aortic valve stenosis exhibit significantly elevated serum TMAO levels
353 compared to healthy controls, suggesting a relationship between this metabolite and
354 valve disease [32]. However, the specific mechanisms by which TMAO contributes to
355 CAVD are not fully understood. In the current study, we demonstrate that treatment with
356 TMAO induces VIC activation towards a profibrotic myofibroblastic phenotype through
357 molecular pathways specific to endoplasmic reticulum stress. Because VIC activation is
358 a critical early step in the progression of disease [64–66], these results suggest that
359 TMAO may play an important role in the initiation of CAVD.

360 Prior research demonstrated that TMAO stimulates osteogenic differentiation [31]
361 and increased αSMA expression [30] in conventionally cultured human aVICs. Here, we
362 expand upon this work by focusing on VICs of a quiescent phenotype and the critical early
363 myofibroblastic transformation that precedes the osteogenic differentiation characteristic
364 of later disease stages [43,64]. We found that treatment of porcine qVICs with TMAO at
365 concentrations greater than 75μM led to an upregulation of myofibroblastic markers and

366 increased proliferation, indicating the transition to an activated myofibroblast-like
367 phenotype. Li *et al.* had previously observed that treatment of human aVICs with higher
368 concentrations (200μM) of TMAO leads to the upregulation of osteoblastic markers like
369 alkaline phosphatase and bone morphogenic protein 4 [31]. It is therefore possible that
370 TMAO may exert differential effects on VICs depending on both phenotype and
371 concentration.

372 In support of this hypothesis, quiescent VICs responded to TMAO treatment at
373 concentrations 4 to 10-fold lower than activated VICs in our experiments. This result
374 aligns with prior work demonstrating that qVICs are significantly more sensitive than
375 aVICs to treatment with TGF-β_1 [38]. Clinically, a mean difference of 2.2 μM TMAO
376 separates patients with stroke from the control group, indicating that even modest
377 differences in TMAO levels can have pathological significance [67]. The differences in the
378 response to profibrotic stimuli between qVICs and aVICs underscore the importance of
379 designing *in vitro* models that include cells with a physiological phenotype appropriate to
380 the research question. In this study, the intentional use of VICs in a quiescent state
381 provided valuable insights into the early cellular responses to TMAO.

382 In the kidney, heart, and liver, TMAO has been implicated in fibrosis by driving not
383 only fibroblast activation but also ECM deposition [68–71]. This appears to also be the
384 case in the aortic valve. Recently, Xiong *et al.* demonstrated that exposing conventionally
385 cultured human VICs to TMAO increases collagen deposition *in vitro* and *in vivo* [30]. Our
386 study builds upon these findings by showing that TMAO upregulates ECM production in
387 both quiescent and activated VICs. More specifically, we observed the upregulation of
388 fibronectin secretion at both the gene and protein level for both VIC phenotypes. On the

389 other hand, collagen I was upregulated at the gene but not the protein level. This
390 difference between *COL1A1* gene expression levels and collagen I deposition may be
391 attributed to several factors, including post-translational modifications, increased matrix
392 metalloproteinase activity, or impaired collagen secretion and assembly [72,73]. It may
393 also be explained by a potential limitation of the *in-situ* ELISA assay, where collagen
394 protein levels may have reached saturation, thus limiting the detection of subtle changes
395 in collagen deposition [74]. Alterations in the content and organization of the ECM
396 significantly impact the mechanical and biological properties of valve leaflets [75,76].
397 Therefore, increases in ECM production driven by TMAO may directly promote the
398 progression of valve disease and contribute to impaired valve function through leaflet
399 thickening, increased stiffness, and the exacerbation of pathological cell behavior [77–
400 82].

401 A key finding of our study is that TMAO induces qVIC activation through molecular
402 mechanisms independent of TGF-β signaling, the most widely studied pathway for VIC
403 activation [42,83,84]. Treatment of qVICs with both TMAO and TGF-β_1 significantly
404 impacted VIC metabolism, with both stimuli resulting in a state of oxidative stress.
405 However, only TMAO led to ER stress. Furthermore, inhibiting the PERK pathway did not
406 affect TGF-β_1-induced activation, nor did inhibition of the TGF-β receptor influence
407 TMAO-driven activation. This indicates that TMAO and TGF-β_1 activate qVICs through
408 distinct molecular mechanisms despite driving similar outcomes—myofibroblastic
409 transition, ECM production, and ROS generation.

410 We are not the first to link TMAO to increased ER stress. This metabolite has been
411 proposed as a biomarker for pathogenic ER stress in the lung [85] and is known to induce

412 ER stress in the kidney [69], liver [86], and heart [87]. ER stress usually activates all three
413 pathways of the unfolded protein response: PERK, inositol-requiring enzyme 1 (IRE1),
414 and activating transcription factor 6 (ATF6) [88]. In 2019, Chen *et al.* identified PERK as
415 a receptor for TMAO, showing that TMAO directly binds to and selectively activates the
416 PERK branch in renal cells [53]. In our study, blocking the PERK receptor effectively
417 inhibited TMAO-induced qVIC myofibroblastic differentiation and ECM production, with
418 previous studies observing similar effects in human VICs [30,31]. Collectively, this
419 evidence indicates that TMAO also exerts its effects in the aortic valve through the PERK
420 pathway. Given the past failures of lipid-lowering approaches [89,90], furthering the
421 understanding of the PERK pathway and other metabolic pathways triggered by TMAO
422 may lead to the identification of novel potential therapeutic or diagnostic markers.

423 Sex is a key factor and biological variable in CAVD, with men exhibiting a higher
424 prevalence of calcification [91–94], and women displaying greater fibrotic remodeling [92].
425 Cellular-scale sex differences in VIC behavior have also been observed *in vitro*
426 [47,95,96]. These sex-specific manifestations suggest that the mechanisms driving CAVD
427 could differ between males and females. Here, we found that TMAO activates both male
428 and female qVICs with no statistically significant differences in the response between
429 sexes, suggesting that sex may not be a major biological variable influencing the
430 response to this metabolite. However, it is important to note that these cells were cultured
431 on a stiff 2D substrate. Prior research has shown that male and female VICs exhibit
432 distinct behaviors when cultured in 3D substrates [48,97,98]. For example,
433 myofibroblastic activation is higher in female than male VICs on soft hydrogels that
434 resemble a healthy valve microenvironment, with an even greater sex difference in

435 activation observed in stiff substrates [48]. Thus, future studies leveraging these 3D
436 culture systems will be necessary to definitively establish whether the VIC response to
437 TMAO varies depending on cellular sex.

438 There are additional important considerations for the interpretation of our results.
439 First, we used porcine VICs for our experiments. This a common practice in the field due
440 to their similarities with human cells [99–101], the absence of a human cell line, and the
441 challenges associated with procuring human tissue [99]. However, species-specific
442 differences may affect cellular responses. For example, human VICs are less prone to
443 spontaneous *in vitro* activation than porcine VICs [38]. Nonetheless, others have
444 demonstrated that TMAO also affects the phenotype of human VICS *in vitro* [28,30],
445 suggesting that our findings are applicable across species. Second, despite the increased
446 sensitivity of qVICs compared to conventionally cultured cells, our study utilized TMAO at
447 concentrations higher than those present physiologically. Serum TMAO levels hover
448 around 1-2μM and 2-7μM for healthy and aortic valve stenosis patients, respectively [32].
449 Considering the chronic nature of valve disease and most cardiovascular conditions, it is
450 possible that prolonged exposure to this metabolite is necessary to observe effects at low
451 TMAO concentrations. Clark-Greuel *et al.* demonstrated that prolonged TGF-β_1 exposure
452 significantly influenced VIC behavior, leading to increased calcium deposition and
453 calcification observed over 14 days compared to shorter exposures of 3 or 7 days [102].
454 This highlights the potential for time-dependent changes in VIC responses to profibrotic
455 stimuli. Future experiments focusing on the impact of long-term exposure to lower,
456 clinically relevant TMAO concentrations may lead to the emergence of additional

457 pathological phenotypes or calcification markers that would not manifest in shorter
458 studies.

459 In summary, our study provides critical insights into the role of TMAO in the early
460 stages of CAVD, demonstrating its ability to activate qVICs towards a profibrotic
461 myofibroblastic phenotype via the PERK pathway, a key regulator of ER stress and
462 oxidative imbalance. By leveraging our culture strategy to maintain VICs in their quiescent
463 phenotype, we were able to identify that quiescent VICs respond to TMAO treatment at
464 much lower concentrations than conventionally cultured activated VICs. This observation
465 emphasizes the importance of developing physiologically relevant *in vitro* models that
466 mimic the conditions seen in healthy and early disease valves. Overall, our results
467 contribute to the growing body of knowledge connecting dietary patterns and the gut
468 microbiome to the regulation of cardiovascular health. Understanding the contributions of
469 TMAO and other gut metabolites to the early stages of valve disease may pave the way
470 for the development of preventative strategies to delay the progression of CAVD or
471 targeted treatment alternatives beyond valve replacement. Moreover, these insights also
472 emphasize the potential for dietary interventions to mitigate cardiovascular and metabolic
473 disease risk.

474

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AUTHOR CONTRIBUTIONS

Conceptualization and methodology: S.S. and A.M.P; investigation: S.S., S.D.S. and T.K.; formal analysis: S.S., S.D.S., and T.K., visualization and writing – original draft: S.S.; writing – review & editing: S.S. and A.M.P.; supervision and funding acquisition: A.M.P.

DISCLOSURES

The authors declare no competing interests.

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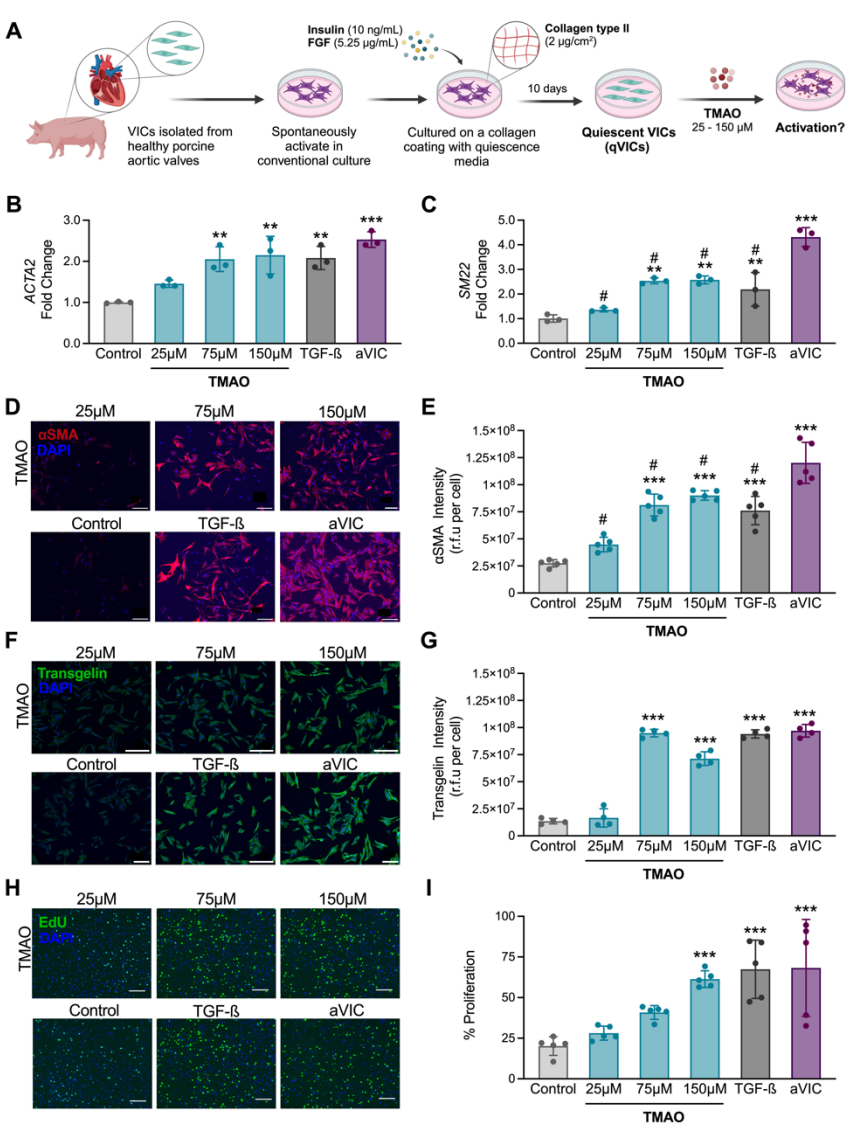

Figure 1. TMAO triggers qVIC activation. (A) Schematic illustration of the experimental design. Primary VICs were isolated from porcine aortic valves and cultured to generate quiescent VICs (qVICs). qVICs were treated with TMAO for 3 days. (B-C) Quantification of myofibroblastic gene expression levels for (B) *ACTA2* and (C) *SM22* via qRT-PCR. (D) Representative images of immunocytochemistry staining for αSMA (red). Cell nuclei are stained in blue. (E) Quantification of αSMA staining intensity in (D). (F) Representative images of immunocytochemistry staining for transgelin (green). Cell nuclei are stained in blue. (G) Quantification of transgelin staining intensity in (E). (H) EdU staining to identify proliferating cells after 8 hours of incubation with EdU. (I) Quantification of the percentage of EdU-positive proliferating cells in (H). Scale bars represent 200 µm. n = 3-5 replicates per condition. One-way ANOVA followed by Tukey's multiple comparisons test. ***p*<0.005, ****p*<0.001 compared with the control. #*p*<0.01 compared with aVICs.

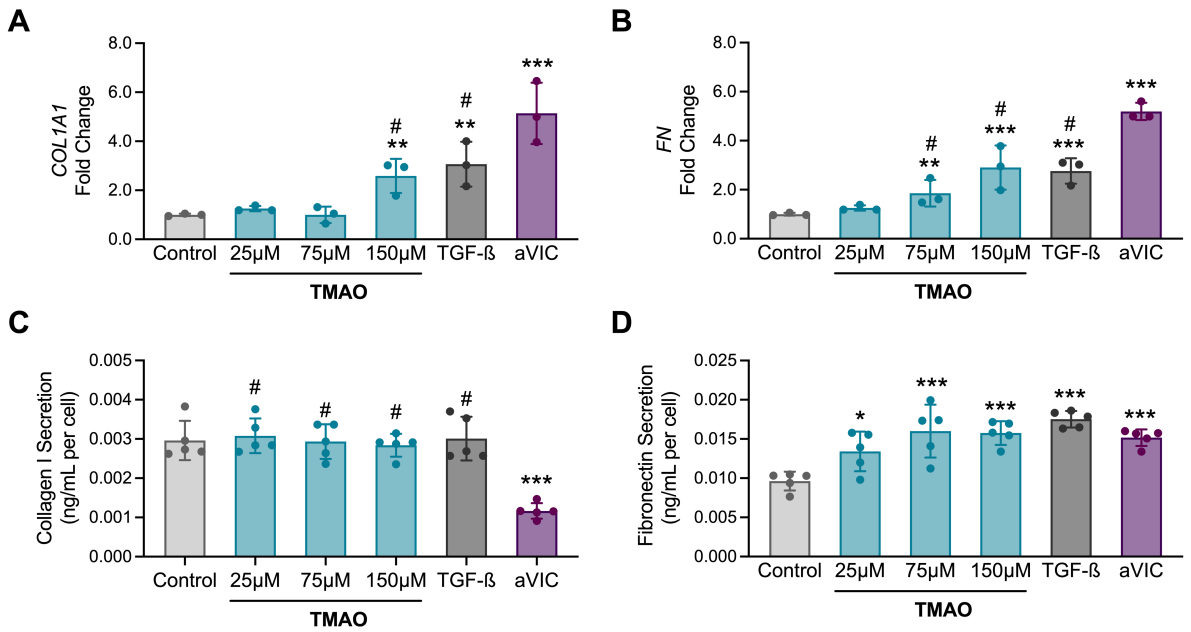

Figure 2. TMAO increases extracellular matrix production by qVICs. (A-B) Gene expression analysis of (A) *COL1A1* and (B) *FN* after 3 days of TMAO treatment via qRT-PCR. (C-D) Quantification of (C) collagen and (D) fibronectin secretion via sandwich ELISAs. n = 3-5 replicates per condition. **$p<0.005$, ***$p<0.001$ compared with the control, #$p<0.05$ compared to aVICs.

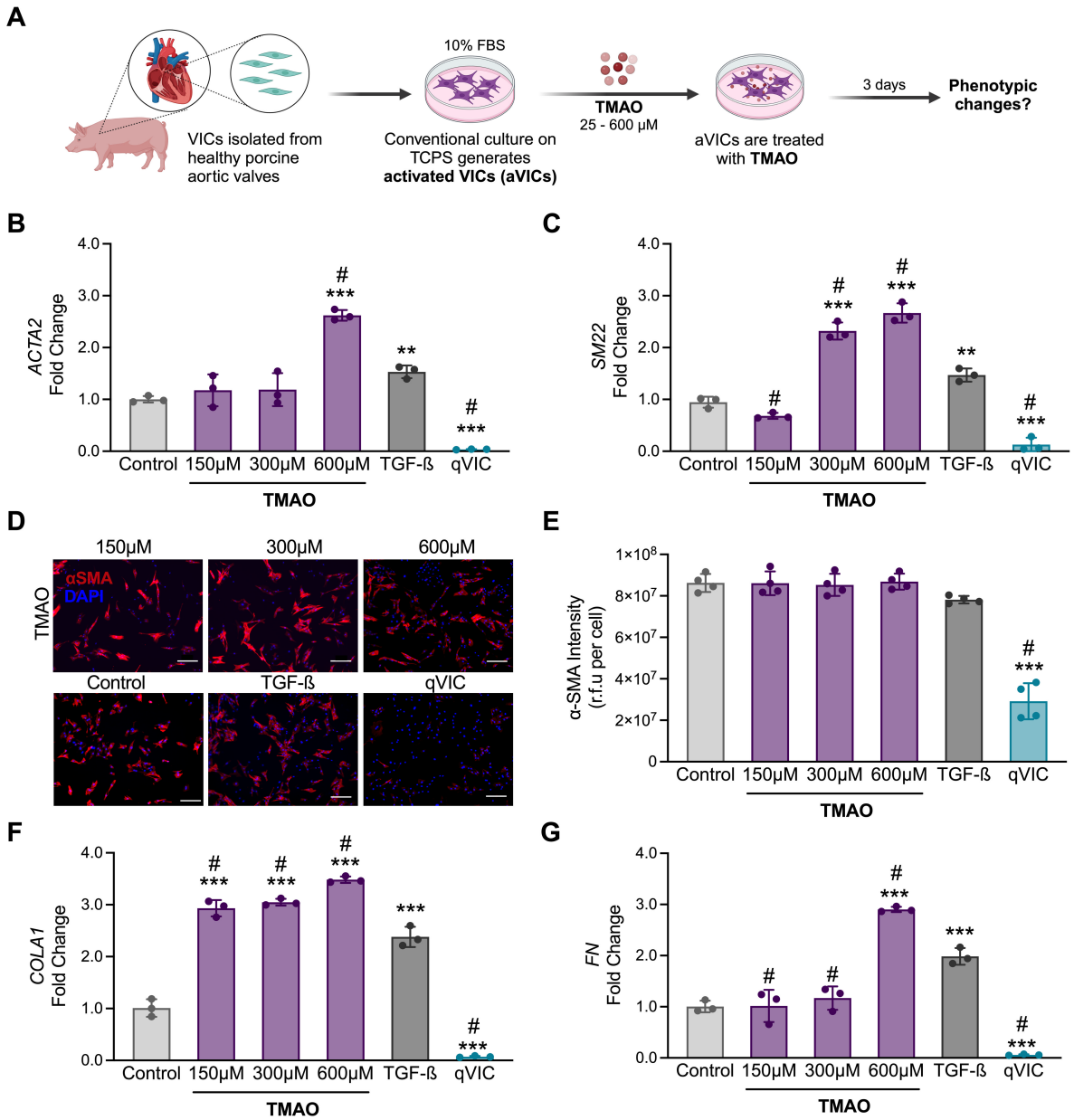

Figure 3. Effects of TMAO on conventionally cultured aVICs. (A) Schematic illustration describing the generation of activated VICs (aVICs) from primary porcine cells through conventional culture. aVIC were treated with TMAO for 3 days. (B-C) Gene expression of (B) *ACT2* (C) *SM22* via qRT-PCR. (D) Immunocytochemistry staining for αSMA. The scale bar represents 200 µm. Cell nuclei are stained blue. (E) Quantification of αSMA staining intensity in (D). (F-G) Analysis of (F) *COL1A1* and (G) *FN* gene expression via qRT-PCR. *n* = 3-5 replicates per condition. One-way ANOVA followed by Tukey's multiple comparisons test. ***p*<0.005, ****p*<0.001 compared with the control. #*p*<0.01 compared to treatment with TGF-ß$_1$.

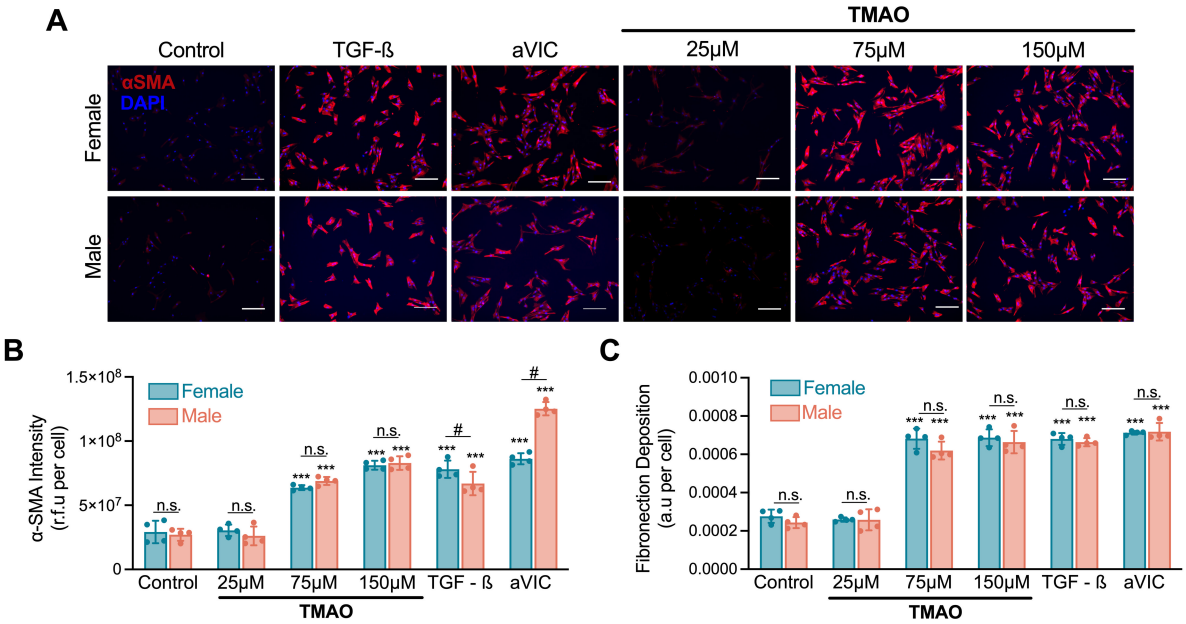

Figure 4. The activation of qVICs by TMAO does not depend on sex. Female and male qVICs were generated and treated with TMAO (25-150μM) for 3 days. (A) Representative images of immunocytochemistry staining for αSMA (red). Cell nuclei are stained blue. Scale bars represent 200 μm. (B) Quantification of αSMA staining intensity in (A). (C) Analysis of fibronectin deposition via *in situ* ELISA. n = 3-5 replicates per condition. Two-way ANOVA was performed, followed by Tukey's multiple comparisons test. **p < 0.005, *p < 0.001 compared to the untreated control for the corresponding sex. n.s. denotes no statistically significant difference between male and female VICs for that condition. #*p*<0.01 for comparison shown.

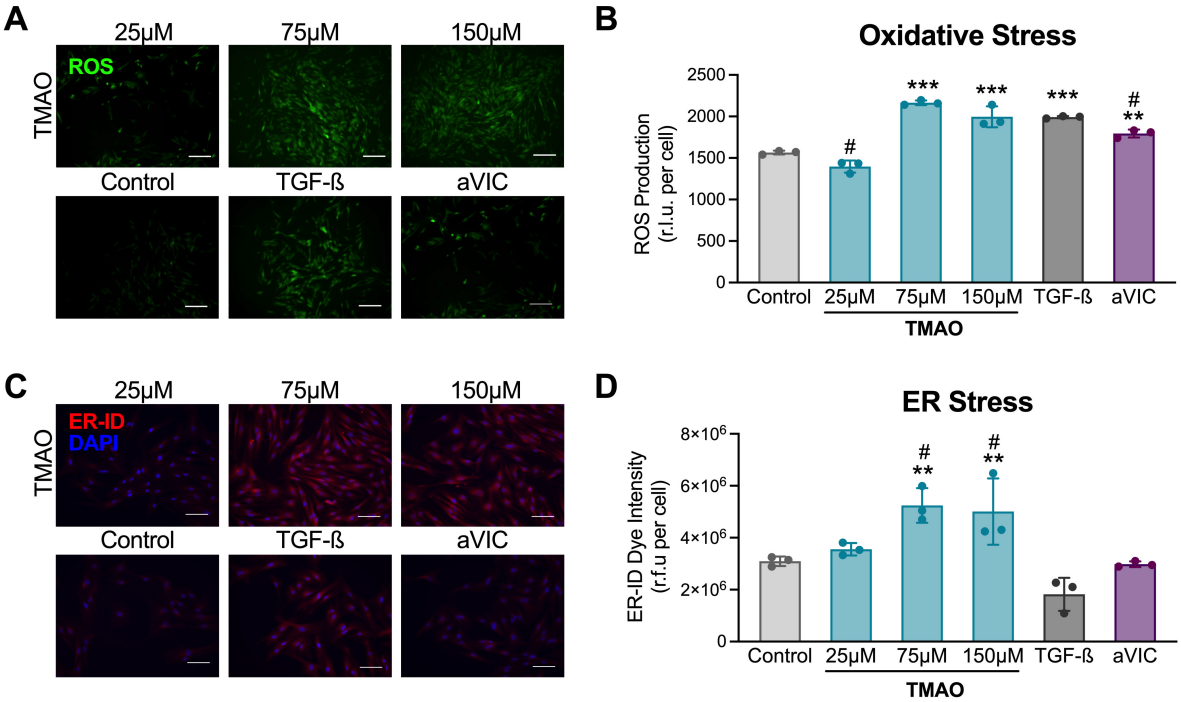

Figure 5. TMAO induces ROS production and ER stress. Female qVICs were treated with TMAO (25-150 μM) for 3 days. (A) Representative fluorescent images of intracellular 2',7'-dichlorofluorescin diacetate (green), indicative of ROS. Scale bars represent 200 μm. (B) Quantification of the ROS-Glo™ luminescent substrate in the culture media normalized to cell number. (C) Representative fluorescence images of the ER-ID Red dye, indicative of ER stress. Cell nuclei are stained blue. Scale bars represent 100 μm. (D) Quantification of fluorescence intensity in (C). n = 3-5 replicates per condition. One-way ANOVA followed by Tukey's multiple comparisons test. $^{**}p<0.005$, $^{***}p<0.001$ compared with the control. $\#p<0.05$ compared to treatment with TGF-$ß_1$.

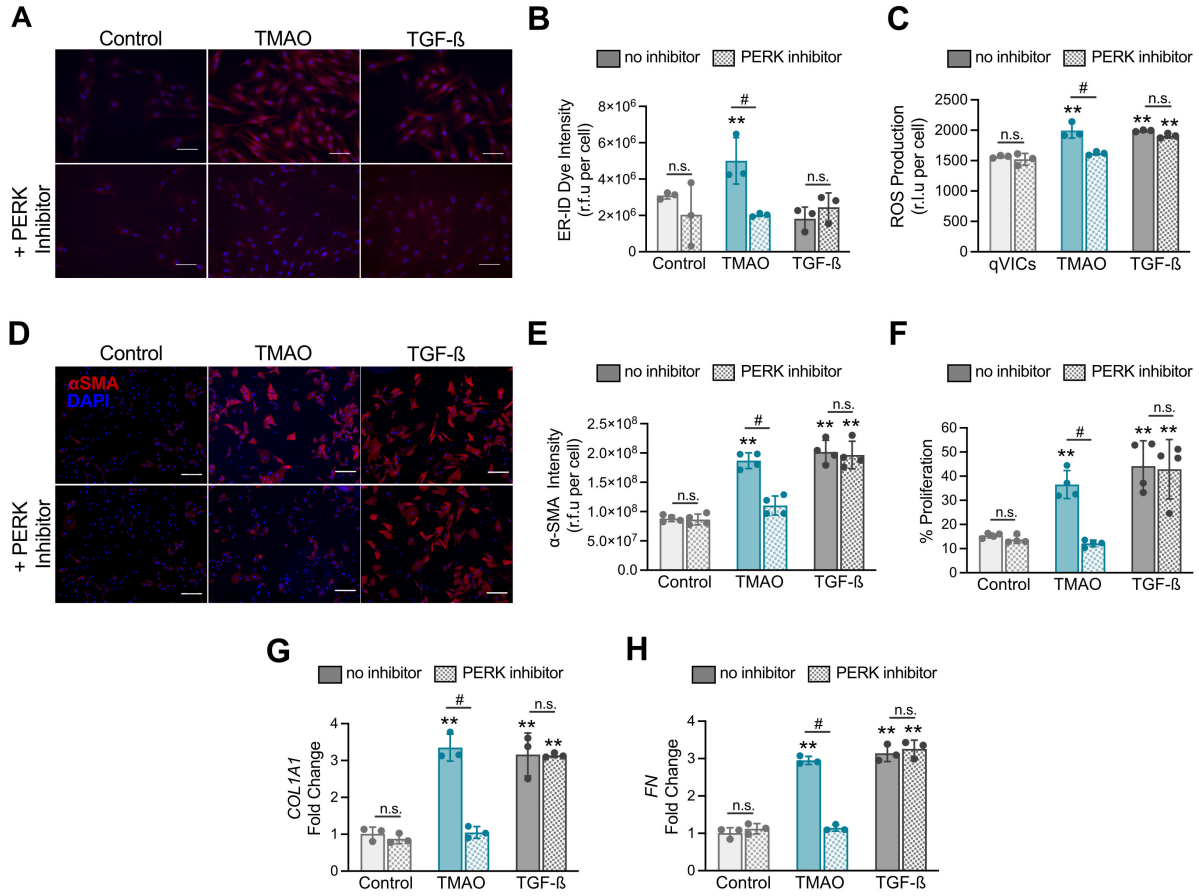

Figure 6. PERK inhibition mitigates the effects of TMAO. Female qVICs were pre-treated with GSK2656157 a PERK inhibitor, for 1 hour prior to treatment with TMAO. (A) Representative fluorescence images of the ER-ID Red dye, indicative of ER stress, at day 3. Cell nuclei are stained blue. Scale bars represent 100 µm. (B) Quantification of fluorescence intensity in (A). (C) Representative images of immunocytochemistry staining for αSMA (red) after 3 days of TMAO treatment. Cell nuclei are stained blue. Scale bars represent 200 µm. (D) Quantification of αSMA staining intensity in (B). (E) Quantification of the percentage of EdU-positive proliferating cells at day 3. (D) Quantification of ROS in the culture media through the ROS-Glo^{TM} luminescent substrate normalized to cell number. (G-H) Gene expression analysis of (G) COL1A1 and (H) *FN.* n = 3-5 replicates per condition. Two-way ANOVA, followed by Tukey's multiple comparisons test. **p<0.005, ***p<0.001 compared with the control. n.s. denotes no statistically significant differences found between samples treated with and without the PERK inhibitor. #*p*<0.001 indicates a significant difference between samples treated with the PERK inhibitor and those without.