



OPEN TMAO enhances TNF- α mediated fibrosis and release of inflammatory mediators from renal fibroblasts

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Trimethylamine-N-oxide (TMAO) is a gut microbiota-derived metabolite and TNF- α is proinflammatory cytokine, both known to be associated with renal inflammation, fibrosis and chronic kidney disease. However, today there are no data showing the combined effect of TMAO and TNF- α on renal fibrosis and inflammation. The aim of this study was to investigate whether TMAO can enhance the inflammatory and fibrotic effects of TNF- α on renal fibroblasts. We found that the combination of TNF- α and TMAO synergistically increased fibronectin release and total collagen production from renal fibroblasts. The combination of TMAO and TNF- α also promoted increased cell proliferation. Both renal proliferation and collagen production were mediated through Akt/mTOR/ERK signaling. We also found that TMAO enhanced TNF- α mediated renal inflammation by inducing the release of several cytokines (IL-6, LAP TGF- β -1), chemokines (CXCL-6, MCP-3), inflammatory and growth mediators (VEGFA, CD40, HGF) from renal fibroblasts. In conclusion, we showed that TMAO can enhance TNF- α mediated renal fibrosis and release of inflammatory mediators from renal fibroblasts in vitro. Our results can promote further research evaluating the combined effect of TMAO and inflammatory mediators on the development of kidney disease.

Keywords TMAO, TNF- α , Renal fibroblasts, Fibrosis, Inflammation

Trimethylamine-N-oxide (TMAO) is the product of oxidation of trimethylamine (TMA). This takes place in the liver by the enzyme flavin-containing monooxygenase 3 and by the gut microbiota^{1,2}. Organic diet compounds such as choline, betaine, L-carnitine existing in dairy products, eggs, red meat and fish are precursors of TMA and TMAO¹⁻³. The kidney is the organ that is primarily responsible for the excretion of TMAO. As a result, kidney function and diet affect the levels of TMAO in blood¹. TMAO has a physiological role in facilitating the homeostasis of organisms. TMAO acts as a protein stabilizer, a natural osmolyte and an electron acceptor. In the kidneys, TMAO protects urine-concentrating medullary cells from dying due to intracellular accumulation of urea^{4,5}.

There is a known association between TMAO and chronic kidney disease (CKD). CKD patients have higher blood levels of TMAO in comparison to non-CKD patients⁶. This is due to decreased glomerular filtration rate (GFR) but also due to increased metabolism of TMA by an altered gut microbiota^{7,8}. In CKD patients, gut microbiome alterations occur due to urea accumulation in the gut⁹. Moreover, TMAO is associated with increased cardiovascular risk and all-cause mortality in CKD patients¹⁰⁻¹².

The renal tubulointerstitium contributes to CKD progression¹³. It consists of tubular cells, fibroblasts, pericytes, immune cells, peritubular endothelium and extracellular matrix¹⁴. Each of these cell types has a role in initiation and progression of tubulointerstitial inflammation and fibrosis. The process of tubulointerstitial fibrosis includes inflammatory cell infiltration, activation of fibroblasts, production and deposition of extracellular matrix, tubular atrophy and rarefaction of microvasculature¹⁵. Following kidney injury, inflammatory cells especially lymphocytes, dendritic cells and macrophages, infiltrate the renal interstitium. They become activated and produce reactive oxygen species, fibrogenic growth factors and cytokines¹⁵. These profibrotic cytokines affect the local microenvironment and induce the phenotypic transition and activation of fibroblasts, tubular cells, endothelial cells and pericytes into myofibroblasts. The majority of the myofibroblasts are derived from interstitial

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fibroblasts¹⁵. During the transition of renal fibroblasts to myofibroblasts, a variety of cytokines, chemokines and growth factors are secreted¹⁶. Tubulointerstitial inflammation and fibrosis lead to oxidative stress, chronic hypoxia, nephron loss, declined renal function and terminal CKD^{13,17}.

TNF- α is an inflammatory mediator released from tubular epithelial cells during kidney injury¹⁸. It is known that TNF- α contributes to renal fibrosis^{18,19}. The main signaling pathways activated by TNF- α are those centered around NF- κ B and MAPK^{18,20}. High renal and serum levels of TNF- α have been found in patients and experimental mouse models with CKD¹⁸. There is evidence of beneficial effect of anti-TNF- α therapy on renal inflammation and renal function in patients with CKD, especially in those with co-existing rheumatic disease²⁰.

There is evidence that TMAO aggravates tubulointerstitial fibrosis and renal inflammation in CKD^{6,21–23}. More specifically, TMAO increased tubulointerstitial fibrosis and deposition of collagen in the kidneys of mice^{6,21}. Moreover, we have recently shown that TMAO promotes renal fibroblast activation and proliferation *in vitro*²². We found that TMAO increases total collagen production and that the effects were mediated by PERK/Akt/mTOR pathway, NLRP3 and caspase-1²². Furthermore, TMAO is known to promote renal inflammation and fibrosis in diabetic kidney disease rats²³. Notably, TMAO contributes to a variety of chronic inflammatory diseases and is associated with vascular inflammation^{24,25}. However, to our knowledge, there are currently no studies that have elucidated if TMAO in combination with the fibrotic cytokine TNF- α can enhance renal fibrosis and inflammation. The aim of this study was to investigate whether TMAO can enhance the inflammatory and fibrotic effects of TNF- α on renal fibroblasts.

Materials and methods

Culture of human fibroblasts

The human renal medullary fibroblast cell line TK173 was used during all experiments (a kind gift from Professor Anton Jan van Zonneveld, Leiden University, Leiden, The Netherlands)²⁶. The renal fibroblasts were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 2 mM L-glutamine, 1 mM non-essential amino acids and 10% fetal bovine serum (FBS) (all from Thermo fisher Scientific, Massachusetts, USA) at 37°C in a 5% CO₂ atmosphere. The fibroblasts were serum starved overnight in DMEM supplemented with 2 mM L-glutamine and 1 mM non-essential amino acids prior to stimulation. During stimulation, DMEM supplemented with 1% FBS, 2 mM L-glutamine and 1 mM non-essential amino acids was used.

Stimulation of renal fibroblasts

Renal fibroblasts were stimulated with TMAO (300 μ M, Sigma-Aldrich, St. Louis, MO, USA), TNF- α (1, 10 or 50 ng/ml, Sigma-Aldrich) or the combination of both for 24–96 h, depending on the experimental setup, at 37°C in 5% CO₂. Renal fibroblasts were pre-stimulated with TMAO for 2 h prior to TNF- α stimulation during the combination treatments. The renal fibroblasts were also pre-incubated with DMSO (vehicle), mTOR inhibitor ridaforolimus (1 μ M, Selleckchem, TX, USA), Akt inhibitor MK-2206 (1 μ M, Selleckchem), PI3K inhibitor wortmannin (1 μ M, Selleckchem) or ERK inhibitor PD98059 (10 μ M, Santa Cruz Biotechnology Inc., Heidelberg, Germany) for 1 h prior to TMAO or TNF- α stimulation. Supernatants were collected and stored at -80°C until further investigation.

Measurement of fibronectin release and cell viability

Fibronectin release from renal fibroblasts was analyzed using the human fibronectin kit (Duo set, ELISA, R&D Systems, Minneapolis, USA) after 24 h. Cell viability was investigated using the Pierce lactate dehydrogenase (LDH) cytotoxicity assay (Thermo Fisher Scientific) following the manufacturer's instructions. The Cytation 3 plate reader was used to evaluate the optic density of all assays.

Proliferation assay

Renal fibroblasts were stimulated with TMAO (300 μ M), TNF- α (1, 10 or 50 ng/ml) or the combination of both for 48 h and incubated at 37°C with 5% CO₂. Renal fibroblasts were pre-stimulated with TMAO for 2 h prior to TNF- α stimulation during the combination treatments. After stimulation, the renal fibroblasts were washed once with PBS. 0.1% Crystal violet (diluted in 20% methanol, Sigma-Aldrich) was then added to the fibroblasts for 10 min at room temperature and the cells were then washed twice with tap water. The renal fibroblasts were then destained with 1% sodium dodecyl sulfate on a shaker at 500 rpm for 5 min and evaluated at 570 nm using the Cytation 3 plate reader.

Total collagen production

Renal fibroblasts were stimulated with TMAO (300 μ M), TNF- α (1, 10 or 50 ng/ml) or the combination of both in the presence of 50 μ g/ml sodium ascorbic acid (Thermo fisher Scientific) for 96 h incubated at 37°C with 5% CO₂. Renal fibroblasts were pre-stimulated with TMAO for 2 h prior to TNF- α stimulation during the combination treatments. After stimulation, total collagen production was evaluated using Sirius red staining (Thermo fisher Scientific). The renal fibroblasts were incubated with 1 mg/ml Sirius red (diluted in picric acid) for 30 min at room temperature. The fibroblasts were then washed with PBS and destained with NaOH 0.1 M on a shaker at 700 rpm for 15 min. The destaining solutions were transferred to a new 96-well plate and evaluated at 540 nm using the Cytation 3 plate reader.

Targeted protein analysis

The renal fibroblasts were stimulated with TMAO (300 μ M), TNF- α (1, 10 or 50 ng/ml) or the combination of both for 24 h and incubated at 37°C with 5% CO₂. Renal fibroblasts were pre-stimulated with TMAO for 2 h prior

to TNF- α stimulation during the combination treatments. The renal fibroblast supernatants were analysed using the proximity extension assay (PEA) technology. In short, a set of antibodies labelled with oligonucleotides are allowed to target proteins. When these antibodies come close to each other, they create a PCR target sequence, which is detected and quantified using standard real-time PCR. The Olink inflammation panel (Olink Bioscience AB, Uppsala, Sweden) utilizes PEA technology, allowing the examination of 92 inflammation-related proteins. The recorded protein values are presented as linearized normalized protein expression levels (NPX). Proteins with signals lower than the limit of detection (LOD) were excluded from further analysis.

Data analysis

All data are expressed as mean \pm SEM. The differences between the groups were analyzed by one-way ANOVA followed by Bonferroni multiple testing correction. Differences were considered statistically significant at $p < 0.05$.

Results

TMAO and TNF- α synergistically increase fibronectin release from renal fibroblasts

We started by investigating whether the fibrotic effect of TNF- α on renal fibroblasts can be enhanced by TMAO. In accordance with our previous findings²², we found no significant difference in fibronectin release from renal fibroblasts stimulated with TMAO 300 μ M compared to unstimulated cells after 24 h (Fig. 1A). Similarly, TNF- α stimulation caused no change in fibronectin release compared to unstimulated cells (Fig. 1A). Interestingly, the combination treatment of TNF- α 1 ng/ml and TMAO synergistically increased fibronectin release in comparison to TNF- α 1 ng/ml alone (Fig. 1A). Renal fibroblasts exhibited no decreased cell viability (LDH release) after 48 h treatment with TMAO or TNF- α alone or in combination (Fig. 1B). Our results suggest that the combined exposure of TMAO and TNF- α 1 ng/ml can increase fibronectin release from renal fibroblasts, independent of cell death.

TMAO and TNF- α promote renal fibroblast proliferation via Akt/mTOR and ERK pathways

Next, we wanted to elucidate whether the combination of TMAO and TNF- α could increase renal fibroblast proliferation. We found that TMAO 300 μ M and TNF- α 1 ng/ml alone or in combination, significantly increased renal fibroblast proliferation compared to unstimulated cells after 48 h (Fig. 2A). Notably, the combination of TNF- α 1 ng/ml and TMAO caused a significant increased proliferation compared to TNF- α 1 ng/ml alone (Fig. 2A). However, this increase was not synergistic. Next, we investigated which signaling pathways mediate the proliferative effect of TMAO and TNF- α on renal fibroblasts. We found that inhibition of Akt (MK-2206), mTOR (Ridaforolimus), ERK (PD98059), but not PI3K (Wortmannin), resulted in reduced cell proliferation after stimulation with TMAO and TNF- α alone or in combination compared to DMSO treated cells after 48 h (Fig. 2B). Taken together, our results show that TMAO and TNF- α mediate their proliferative effect on renal fibroblast using the Akt/mTOR and ERK signaling pathways.

TMAO and TNF- α synergistically increases total collagen production via Akt/mTOR and ERK pathways

We continued to investigate whether TMAO could enhance TNF- α mediated total collagen production from renal fibroblasts. We found that TMAO 300 μ M and TNF- α alone or in combination increased total collagen production compared to unstimulated cells (Fig. 3A). We also found that the combination treatments of TNF- α 1 ng/ml and TMAO significantly induced a synergistic increase in total collagen production compared to TNF- α 1 ng/ml alone (Fig. 3A). Moreover, we found that inhibition of Akt (MK-2206), mTOR (Ridaforolimus) and ERK (PD98059), but not PI3K (Wortmannin), resulted in reduced total collagen production after stimulation with

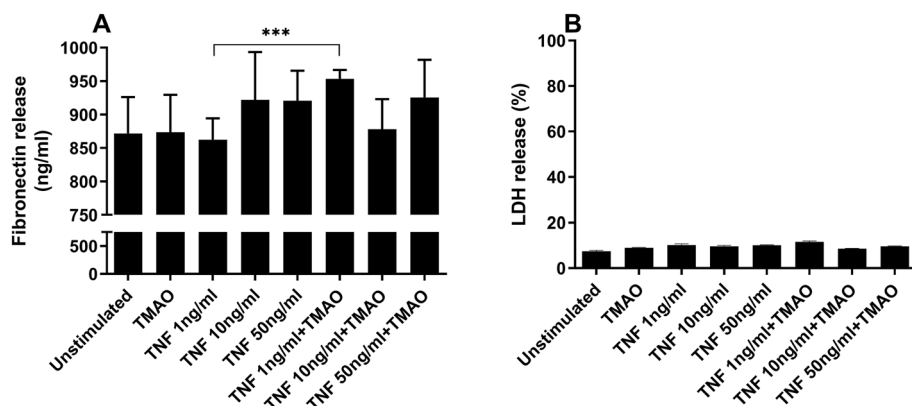


Figure 1. TNF- α and TMAO have a synergistic effect on fibronectin release. Renal fibroblasts were stimulated with TMAO 300 μ M and TNF- α (1, 10, 50 ng/ml) alone or in combination for 24 h (A) or 48 h (B) and fibronectin release (A) and cell viability was evaluated (B). LDH release is presented as % of total LDH. Data are presented as mean \pm SEM ($n = 4$ independent experiments). Asterisks denote statistical significance compared to unstimulated cells ($*p < 0.05$, $***p < 0.001$).

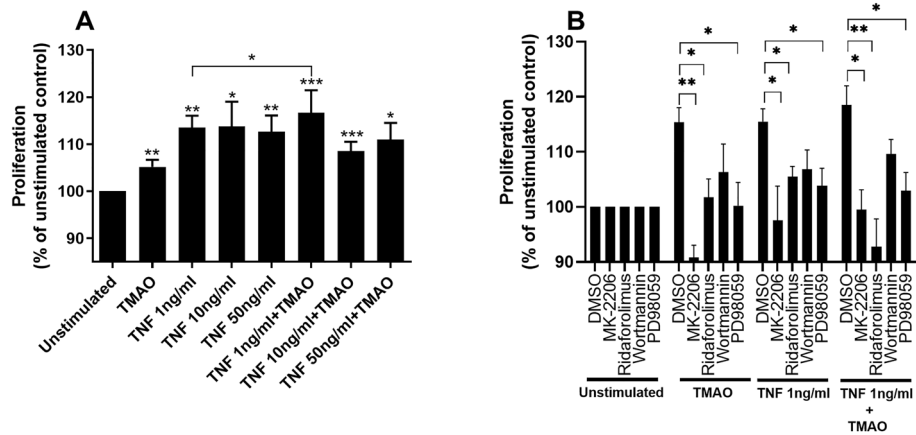


Figure 2. TNF- α and TMAO increase renal fibroblast proliferation. Renal fibroblasts were stimulated with TMAO 300 μ M and TNF- α (1, 10, 50 ng/ml) alone or in combination for 48 h and proliferation was evaluated (A). Renal fibroblasts were also pre-incubated with DMSO (vehicle), Akt inhibitor MK-2206 (1 μ M), mTOR inhibitor ridaforolimus (1 μ M), PI3K inhibitor wortmannin (1 μ M) or ERK inhibitor PD98059 (1 μ M) for 1 h prior to stimulation for 48 h (B) followed by proliferation evaluation. Proliferation is presented as % of unstimulated control. Data are presented as mean \pm SEM ($n = 8-10$ independent experiments). Asterisks denote statistical significance compared to unstimulated cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

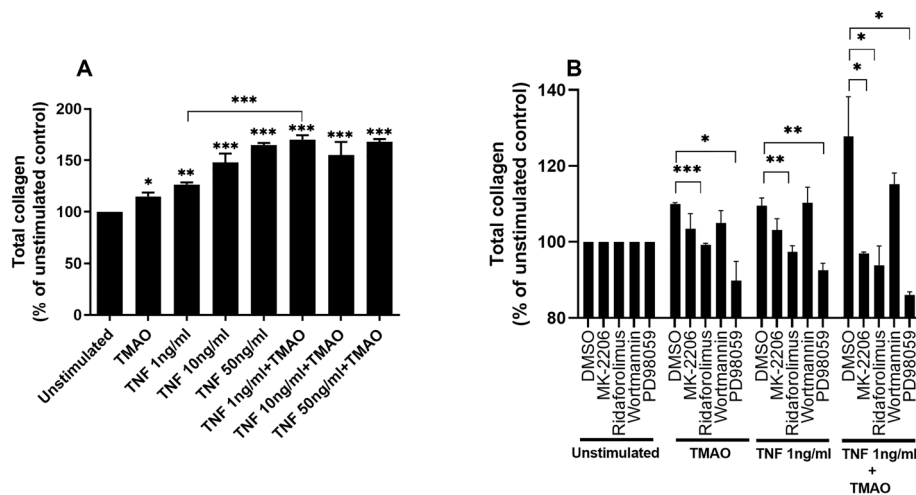


Figure 3. TNF- α and TMAO have a synergistic effect on total collagen production. Renal fibroblasts were stimulated with TMAO 300 μ M and TNF- α (1, 10, 50 ng/ml) alone or in combination for 96 h and total collagen production was evaluated (A). Renal fibroblasts were also pre-incubated with DMSO (vehicle), Akt inhibitor MK-2206 (1 μ M), mTOR inhibitor ridaforolimus (1 μ M), PI3K inhibitor wortmannin (1 μ M) or ERK inhibitor PD98059 (1 μ M) for 1 h prior to stimulation for 48 h (B) followed by total collagen evaluation. Total collagen is presented as % of unstimulated control. Data are presented as mean \pm SEM ($n = 4$ independent experiments). Asterisks denote statistical significance compared to unstimulated cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

TMAO and TNF- α 1 ng/ml compared to DMSO treated cells after 96 h (Fig. 3B). Our findings suggest that the combination of TNF- α 1 ng/ml and TMAO synergistically increases total collagen production via Akt/mTOR and ERK pathways.

TNF- α and TMAO synergistically increase the release of cytokines from renal fibroblasts

Next, we continued to evaluate whether TMAO could enhance TNF- α mediated cytokine /cytokine receptor release from renal fibroblasts using a targeted protein analysis. We found that TMAO 300 μ M and TNF- α alone increased the release of IL-6 compared to unstimulated cells after 24 h (Fig. 4). TNF- α was also found to increase LAP TGF- β -1 release compared to unstimulated cells (Fig. 4). We found that the combination of TNF- α 50 ng/ml and TMAO significantly enhanced the release of LAP TGF- β -1, IL-6, SCF, LIF, CSF-1, IL-10RB and IL-18R1 compared to TNF- α 50 ng/ml alone (Fig. 4). LAP TGF- β -1 release was also significantly increased after TNF- α

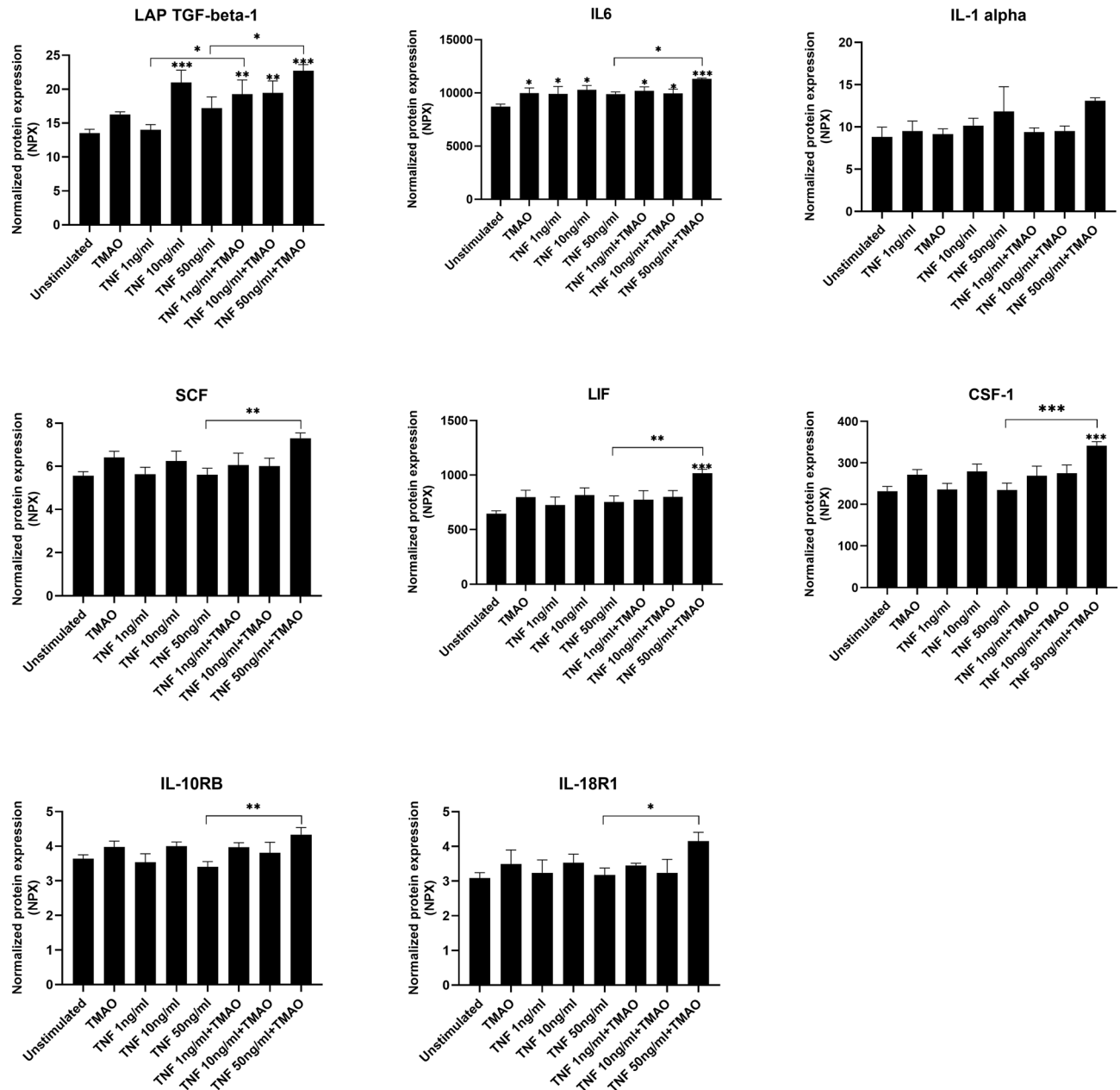


Figure 4. Secretion of cytokines from renal fibroblasts. Renal fibroblasts were stimulated with TMAO 300 µM and TNF-α (1, 10, 50 ng/ml) alone or in combination for 24 h followed by Olink targeted protein analysis. The data are presented as linearized NPX values. Data are presented as mean ± SEM ($n = 4$ independent experiments). Asterisks denote statistical significance compared to unstimulated cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

1 ng/ml and TMAO stimulation compared to TNF-α 1 ng/ml alone (Fig. 4). Taken together, our results suggest that the combination of TNF-α and TMAO can enhance the release of cytokines from renal fibroblasts.

TMAO and TNF-α synergistically increase the secretion of chemokines

In the next step we also wanted to evaluate whether TMAO could enhance TNF-α mediated chemokine release from renal fibroblasts. We found that TMAO 300 µM alone increased the release of IL-8 compared to unstimulated cells after 24 h (Fig. 5). TNF-α was found to increase IL-8, CXCL-1, CXCL-6, MCP-1, MCP-3 and CCL20 release compared to unstimulated cells (Fig. 5). In addition, we found that the combination of TNF-α 50 ng/ml and TMAO significantly enhanced the release of MCP-1, MCP-3 and MCP-2 compared to TNF-α 50 ng/ml alone (Fig. 5). Moreover, the combination of TNF-α 10 ng/ml and TMAO, significantly increased the secretion of MCP-2 compared to TNF-α 10 ng/ml alone. The combination of TNF-α 1 ng/ml and TMAO also increased the release of CXCL-6, MCP-3 and CCL20 compared to TNF-α 1 ng/ml alone (Fig. 5). Hence, our results suggest that the combination of TNF-α and TMAO can enhance the release of several chemokines from renal fibroblasts.

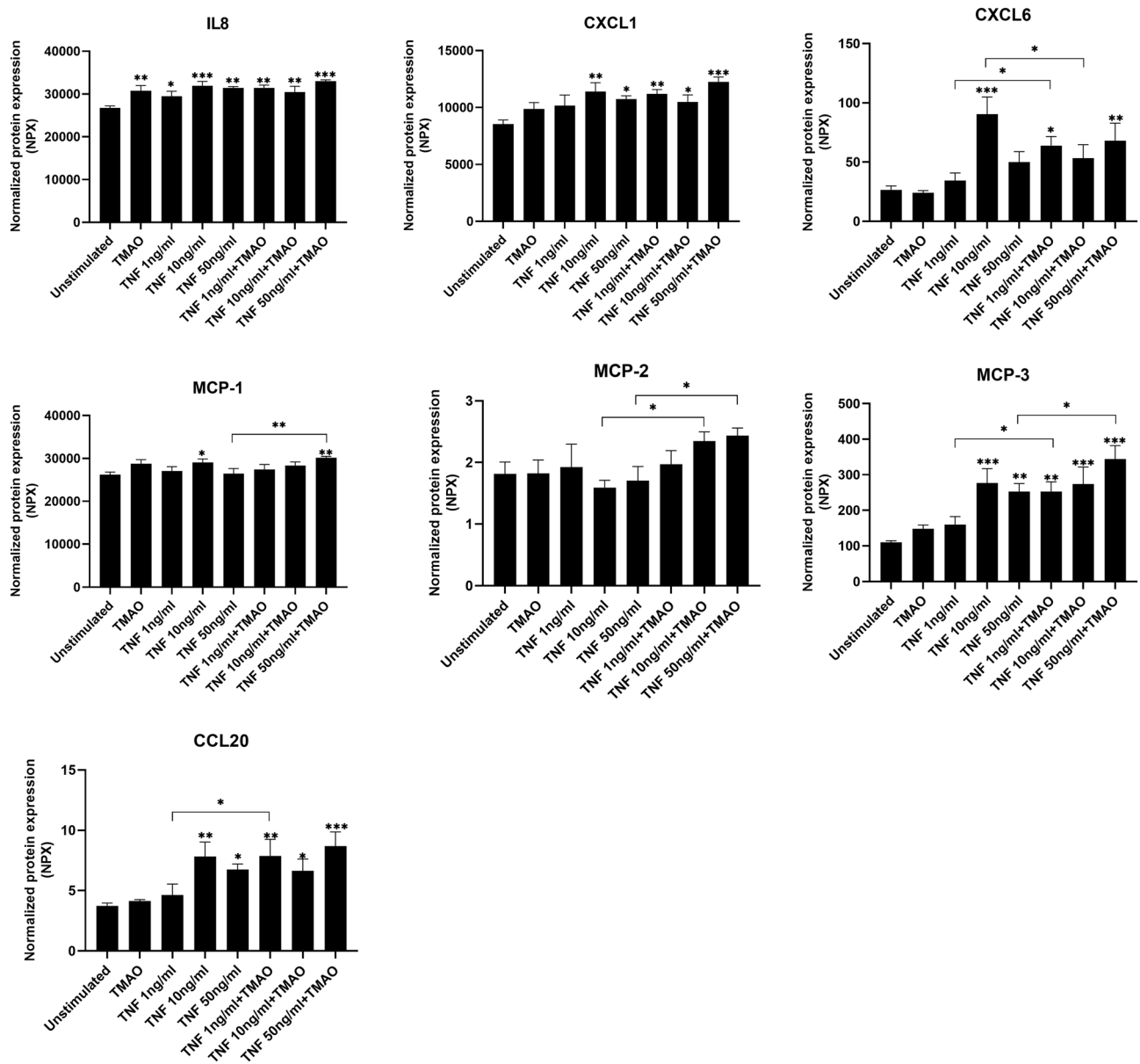


Figure 5. Chemokine release from renal fibroblasts. Renal fibroblasts were stimulated with TMAO 300 μ M and TNF- α (1, 10, 50 ng/ml) alone or in combination for 24 h followed by Olink targeted protein analysis. The data are presented as linearized NPX values. Data are presented as mean \pm SEM ($n=4$ independent experiments). Asterisks denote statistical significance compared to unstimulated cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

TMAO enhances TNF- α mediated release of inflammatory-and growth mediators

Next, we continued to investigate whether TMAO could enhance TNF- α mediated release of additional inflammatory-and growth mediators from renal fibroblasts. We found that TNF- α alone, but not TMAO, significantly increased the release of VEGFA, GDNF, CDCP1, OPG, AXIN1, FGF-5, HGF, 4E-BP1, CD40, CASP-8, TNFRSF9, NT-3, STAMBP compared to unstimulated cells (Fig. 6). We also found that the release of VEGFA, GDNF, CDCP1, OPG, uPA, AXIN1, FGF-5, MMP-1, MMP-10, PD-L1, HGF, Flt3L, 4E-BP1, DNER, CD40, CASP-8, ADA, TNFRSF9, NT-3, TWEAK and STAMBP were enhanced by the combination of TNF- α 50 ng/ml and TMAO compared to TNF- α 50 ng/ml alone (Fig. 6). Furthermore, the release of GDNF, FGF-5, HGF, 4E-BP1, CD40, NT-3, TWEAK and STAMBP were enhanced by the combination of TNF- α 1 ng/ml and TMAO compared to TNF- α 1 ng/ml alone (Fig. 6). Taken together, our results suggest that TNF- α and TMAO enhances the release of several inflammatory-and growth mediators from renal fibroblasts.

Discussion

Several studies indicate that TMAO exacerbates tubulointerstitial fibrosis and renal inflammation in CKD^{6,21–23}. Furthermore, there is substantial evidence demonstrating that TNF- α plays a significant role in contributing to renal fibrosis^{18,19}. However, the additive or synergistic contribution of TMAO and TNF- α on renal inflammation

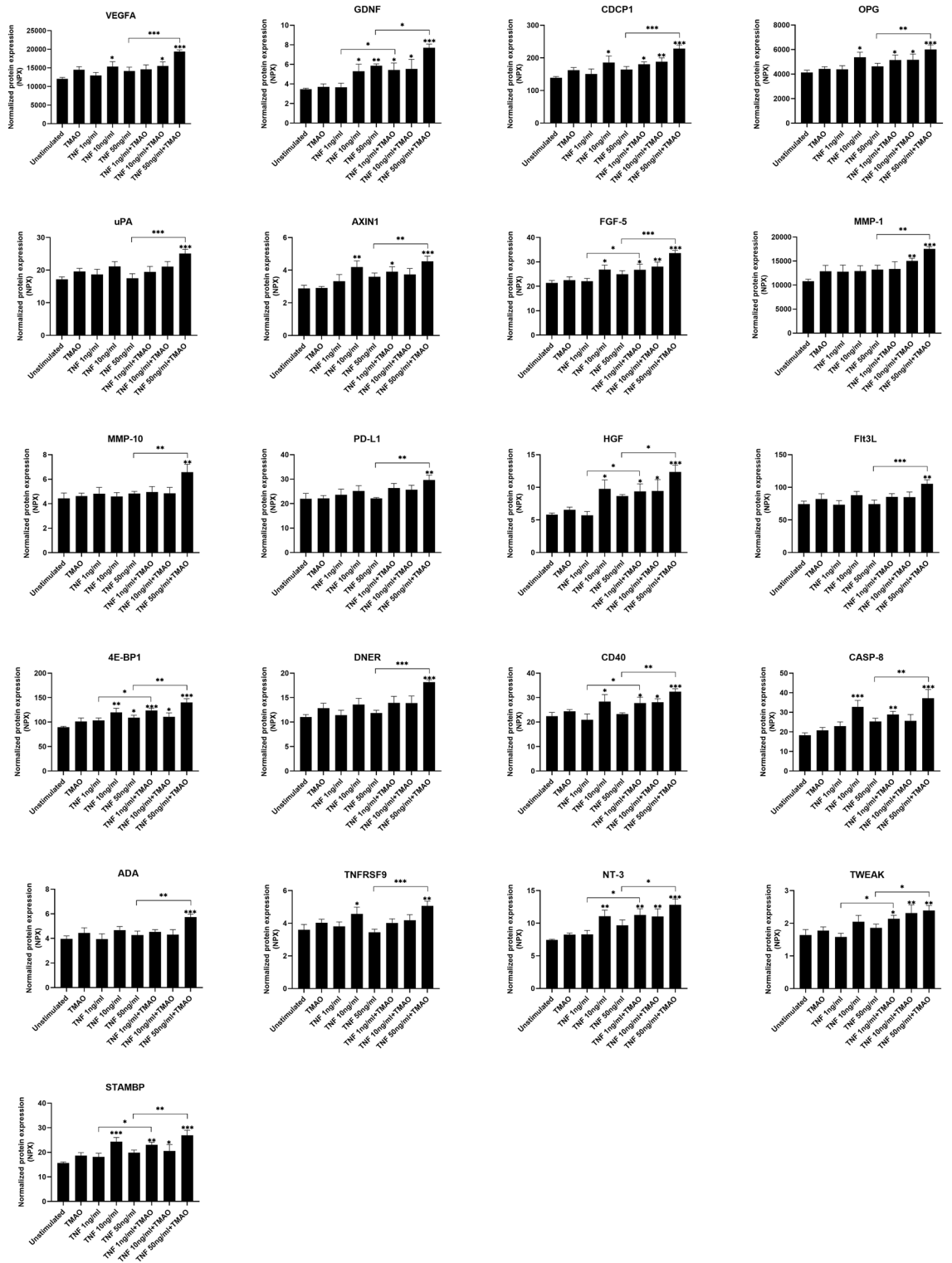


Figure 6. Secretion of inflammatory- and growth mediators from renal fibroblasts. Renal fibroblasts were stimulated with TMAO 300 μ M and TNF- α (1, 10, 50 ng/ml) alone or in combination for 24 h followed by Olink targeted protein analysis. The data are presented as linearized NPX values. Data are presented as mean \pm SEM ($n = 4$ independent experiments). Asterisks denote statistical significance compared to unstimulated cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

and fibrosis is not well understood. Our aim was therefore to investigate if TMAO can enhance the inflammatory and fibrotic effects of TNF- α on renal fibroblast and to elucidate the molecular pathways involved.

We started by evaluating the effect of TMAO and TNF- α on fibronectin release from renal fibroblasts. We found that neither TMAO nor TNF- α increased fibronectin release from renal fibroblasts. This is in accordance with our previous findings²². However, we found that the combination of TMAO and TNF- α can increase fibronectin release compared to TNF- α alone. The observed fibronectin release was independent of fibroblast cell death. We have previously shown that TMAO is unable to induce increased fibronectin release from renal fibroblast up to 96 h posttreatment²². TMAO and TNF- α , independent of concentration, did not mediate increased fibroblast cell death. Fibronectin, a high molecular weight glycoprotein with adhesive properties, holds a pivotal function in both wound-healing processes and the formation of extracellular matrix²⁷. Under pathophysiological conditions, fibronectin expression levels are dramatically increased in the renal tubulointerstitium which contributes to renal fibrosis. Taken together, our findings indicate that the combined exposure of TMAO and TNF- α can increase fibronectin release from renal fibroblasts.

We next evaluated the effect of TMAO and TNF- α on renal fibroblast proliferation and collagen production. We found that both TNF- α and TMAO induced increased fibroblast proliferation and that the combination of TNF- α 1 ng/ml and TMAO induced increased cell proliferation compared to TNF- α alone. However, this increase was more additive than synergistic. Furthermore, the overall difference between the treatment groups was very small, which strengthens the notion of an additive effect. We also found that TMAO and TNF- α alone or in combination increased total collagen production. The combination treatments of TNF- α 1 ng/ml and TMAO synergistically increased total collagen production compared to TNF- α alone. Next, we evaluated which signaling pathways TMAO and TNF- α activate to induce proliferation and collagen production. This was done to evaluate the mechanism behind the observed synergistic effects of TMAO and TNF- α . Our findings showed that both TMAO and TNF- α mediate their proliferative and collagen inducing effects on renal fibroblast via Akt, mTOR and ERK, but not PI3K. We have previously shown that TMAO induced increased renal fibroblast proliferation and collagen production via Akt and mTOR, but not via PI3K²². We have also shown that TMAO can reduce megalin expression in proximal tubular cells via PI3K and ERK²⁸. In addition, TMAO has been linked to promote vascular inflammation via ERK activation²⁹. TNF- α is also known to activate Akt, mTOR, and ERK in different cells^{20,30,31}. Hence, the synergistic effects of TMAO and TNF- α could be explained by their ability to activate the same pathways. Taken together, our findings indicate that Akt, mTOR and ERK, but not PI3K, mediates the effect of TMAO and TNF- α on fibroblast proliferation and collagen production.

We continued to investigate whether TMAO could enhance TNF- α mediated release of inflammatory mediators from renal fibroblasts using Olink multiplex assay including 92 proteins. We found that TMAO could enhance TNF- α mediated release of a variety of cytokines/cytokine receptors LAP TGF β -1, IL-6 SCF, LIF, CSF-1, IL-10RB and IL-18R1 from renal fibroblasts compared TNF- α alone. Each of these cytokines have been shown to play a role in the pathophysiology of kidney disease^{32–38}. TGF- β 1 is known to promote myofibroblast activation and induce the expression of fibronectin and collagen³². IL-6 has also been found to induce tubular atrophy, increase collagen production and accelerate tubulointerstitial fibrosis³³. Interestingly, TMAO was also able to significantly induce IL-6 release alone from renal fibroblast, strengthening the link to renal fibrosis. Moreover, TMAO was also found to enhance TNF- α mediated secretion of several chemokines CXCL-6, MCP-1, MCP-2, MCP-3 and CCL20 compared to TNF- α alone. All these chemokines participate in the pathogenesis of kidney disease according to previous publications^{39–43}. Inhibition of MCP-1 in renal disease has been shown to lead to patient improvements⁴⁰. CCL20 has also been found to be increased in kidney disease. CCL20 promotes T-cell recruitment, renal tissue injury and reduced renal function⁴³. We also found, using the Olink proteomic platform, that TNF- α and TMAO enhanced the secretion of additional inflammatory-and growth mediators associated with kidney disease; VEGFA, GDNF, CDCP1, OPG, uPA, AXIN1, MMP-1, MMP-10, PD-L1, HGF, Flt3L, 4E-BP1, CD40, CASP-8, ADA, TNFRSF9, TWEAK^{44–61}. Increased systemic levels of TWEAK has been shown to trigger kidney injury, inflammation, and renal fibrosis. The Ras/ERK/ NF κ B pathways play a crucial role in TWEAK-induced fibroblast proliferation and inflammation⁶¹. Circulating levels of sCD40 and sCD40L have also been shown to be associated with renal injury⁵⁶. Taken together, our results suggest that the TMAO can enhance TNF- α mediated kidney inflammation by inducing the release of several cytokines, chemokines, inflammatory-and growth mediators from renal fibroblasts.

Our study is the first aiming to elucidate the combined effect of TMAO and TNF- α on renal fibroblasts through assessing aspects of renal fibrosis and inflammation. The current existing research has focused mainly on how TMAO or TNF- α alone affects the progress of kidney disease. However, the co-existence of TMAO and TNF- α (and/or other pro-inflammatory factors) in the renal interstitium is closer to the pathophysiological background of kidney disease.

In conclusion, our findings showed that TMAO enhances TNF- α mediated renal fibroblast proliferation and collagen production via Akt/mTOR/ERK signaling pathway. We also observed that the combination of TMAO and TNF- α increased the release of several inflammatory mediators associated with kidney disease. To the best of our knowledge, this is the first study elucidating the synergistic effects of TMAO and TNF- α on renal inflammation and fibrosis. Our results can promote further research evaluating the combined effect of TMAO and inflammatory mediators on the development of kidney disease.

Data availability

All data generated or analysed during this study are included in this published article.

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S.K., I.D., K.P. A.K.K. design the study. S.K., I.D., G.V.P., A.K.K. conducted the experiments. S.K., I.D., G.V.P., K.P., A.K.K. analyzed the data. S.K., I.D. drafted the article. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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