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TMA, beyond TMAO, might contribute to vascular inflammation by disturbing mitochondrial functions in macrophages

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ABSTRACT

Emerging evidence highlights conflicting data regarding the roles of trimethylamine (TMA) and trimethylamine-N-oxide (TMAO) plasma levels in cardiovascular diseases. In this study, we investigate in THP-1 monocytes the pro-inflammatory effects of TMA and TMAO at both physiological and pathological concentrations previously measured in a human cohort, focusing on their impact on ATP production, mitochondrial gene expression, mitochondrial membrane potential (ΔΨm), and mitochondrial DNA copy number (mtDNAcn). Results show that 0.6 μM and 1.2 μM TMA as well as 40 μM TMAO increase the expression levels of the pro-inflammatory IL-8, while the anti-inflammatory cytokine IL-10 was upregulated by 1.2 μM TMA and 40 μM TMAO. An increase in the expression levels of mitochondrial genes *MT-ATP6*, *MT-CO1*, *MT-CYB* and *MT-ND6* was measured on all conditions tested, while no significant changes in mtDNAcn were observed. Remarkably, TMA (0.6 μM and 1.2 μM), but not TMAO, decreases ATP content and increases the mitochondrial membrane potential in THP-1 cells after 24 h of incubation. In conclusion, our study suggests that not only circulating TMAO but also TMA may contribute to vascular inflammation by disturbing mitochondrial functions in monocytes. This evidence underscores the need for further investigations to better understand the effects of these metabolites on cardiovascular health.

1. Introduction

Cardiovascular disease (CVD) is a leading global cause of death, with risk factors including high blood pressure, obesity, smoking, and unbalanced diet. In particular, the Western diet, high in fats, red meat, and refined carbohydrates but low in fruits and vegetables, is strongly linked to CVD. Recent research has highlighted the potential of trimethylamine-N-oxide (TMAO), a metabolite derived from diets rich in animal products [1], to serve as a biomarker for CVD [2]. Evidence indicates that higher circulating TMAO levels are associated with an increased risk of conditions such as atherosclerosis, hypertension, heart failure, arrhythmias, coronary artery disease (CAD), diabetes, and chronic kidney disease [3]. TMAO originates from the oxidation of trimethylamine (TMA), which is produced by gut bacteria from dietary precursors like choline, betaine, and carnitine, predominantly found in animal products [4]. After TMA is absorbed into the bloodstream, it is transported to the liver, where it is converted into TMAO. Despite its rapid turnover (most of it is excreted through urine, with small amounts eliminated via respiration or feces), TMAO is also reduced back to TMA and re-enters circulation [3]. Various mechanisms have been proposed to explain TMAO's role in atherosclerosis and CVD, including its ability to induce inflammation, promote cholesterol accumulation, and impair endothelial functions [5]. However, emerging evidence presents a more nuanced picture [6], with some studies suggesting that TMAO may also have protective effects [7]. For instance, fish, a major source of dietary TMAO, is widely recommended for CVD prevention.

Plasma TMA has also garnered interest in this context [8,9]. Studies on rats have shown that TMA increases blood pressure and adversely affects vascular smooth muscle cells [10]. Nevertheless, despite its measurement in plasma, there is limited research on TMA's cellular and molecular effects [8,11,12]. One proposed mechanism underlying TMA and TMAO's influence on inflammation is the disruption of mitochondrial homeostasis [11]. Mitochondria, which are affected by both internal and external factors such as diet, play a central role in inflammation and may also serve as targets for inflammatory processes. Mitochondrial DNA copy number (mtDNAcn), in particular, has emerged as a potential biomarker for predicting CVD [13,14]. In a previous study [15], we measured lower mtDNAcn in CAD patients than

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healthy controls, which correlated with TMA, but not with TMAO levels. Additionally, we have recently shown that TMA excess reduced intracellular mtDNAcn and ATP in Caco-2 cells [16].

Considering that low-grade inflammation is a proposed mechanism by which diet-derived metabolites contribute to vascular damage and CVD, this study explores the effects of TMA and TMAO on circulating macrophages using THP-1 monocytes as an *in vitro* model. Specifically, it examines whether TMA and TMAO trigger the pro-inflammatory cascade and affect mitochondrial dynamics, focusing on ATP production, cellular membrane potential, and markers of mitochondrial functions.

2. Materials and methods

2.1. Cell culture

THP-1 cells (ATCC, Rockville, MD) were cultured in RPMI 1640 medium 1X with L-glutamine (Corning, Turin, Italy) supplemented with 10 % heat-inactivated Fetal Bovine Serum (FBS; Euroclone, Milano, Italy), 1 % penicillin/streptomycin (Euroclone, Milano, Italy) and 50 μM β-Mercaptoethanol (Euroclone, Milano, Italy). The cells were kept at 37 °C in a humidified atmosphere containing 5 % CO_2. Medium was changed every 2 days and cells were passaged at 80 % confluence.

2.2. Viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to evaluate the cytotoxic effect of TMA and TMAO on THP-1 cells. Briefly, THP-1 cells were seeded in 96-well plates (1×10^4 cell/well) in RPMI 1640 1X with L-glutamine, cultured for 24 h and treated with several concentrations of TMA (50 μM, 25 μM, 10 μM, 1 μM, 0.5 μM, 0.1 μM) and TMAO (400 μM, 100 μM, 50 μM, 25 μM, 5 μM, 1 μM). At the end of the incubation period, 5 mg/ml MTT solution was added to the cells and incubated for 4 h. The insoluble formazan salt product was solubilized by adding dimethyl sulfoxide (DMSO) and its amount was determined by measuring the optical density at 540 nm using a microplate reader. Cell viability was calculated according to equation (T/C) × 100 %, where T and C represent respectively the mean optical density of the treated group and the control group.

2.3. Cell treatments

THP-1 cells were differentiated for 24 h with phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) [17]to induce differentiation of monocytes into the M0 phenotype macrophages. The TMA and TMAO concentration that were tested in this study were selected from TMA and TMAO levels previously measured in plasma of CAD patients [15]. Specifically, the highest concentration and the mean concentration of either TMA or TMAO measured in CAD patients were chosen as the highest and lowest dose tested on THP-1 cells, respectively (i.e., 0.6 μM TMA, 1.2 μM TMA, 5 μM TMAO and 40 μM TMAO). Negative controls (differentiated macrophages with vehicle only) were also added. Experiments were run in duplicate. After 24 h of incubation, cells were collected. Cells were mechanically detached and centrifuged at 300×*g* for 10 min; the supernatant was removed, and cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C until further use.

2.4. mtDNA quantification and gene expression analysis

Total DNA was extracted using the Genomic DNA Isolation Kit (Cat. 24700, Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's instructions. Relative quantification by quantitative PCR (Biorad CFX96) was chosen to quantify mtDNAcn, considering nuclear DNA as a normalizer, as previously described [18]. Primers used (listed in supplementary materials table 1) have been previously validated for their specificity (unique amplification of mtDNA) and the absence of coamplified nuclear insertions of mitochondrial origin (NUMTs). Relative mtDNAcn was determined using the $2^{-\Delta Ct}$ method. Each analysis was run in duplicate. An inter-run calibrator sample was applied to adjust the results obtained from different amplification plates.

Total RNA was extracted from treated THP-1 cells (Total RNA Purification Plus Kit, Cat. 48300, Norgen Biotek, Thorold, ON, Canada), and retrotranscribed to cDNA using the PrimeScript RT-PCR Kit (Cat. RR037A, Takara Bio, Göteborg, Sweden) according to the manufacturer's instructions. Gene expression analyses were carried out by quantitative real-time PCR (Biorad CFX96), using the TB Green® Premix Ex Taq™ (Cat. RR420A, Takara Bio, Göteborg, Sweden). The amplification conditions were: 30 s at 95 °C followed by 5 s at 95 °C and 30 s at 60 °C, these latter repeated for 40 cycles. To check the specificity of each amplification, a melting curve was also performed. The expression levels of the target genes were normalized relative to β-actin, using the $2^{-\Delta\Delta Ct}$ method. The target genes analysed were the inflammation-related *IL-6, IL-8,* and *IL-10* and the mitochondrial *ND6*, *CYTB*, *CO1*, *ATP6*. The sequences of the primers used in the study are listed in Supplementary Table 1.

2.5. ATP quantification

The ATP content from treated THP-1 cells was quantified using the ATP Colorimetric Assay Kit (Cat. MAK190, Sigma-Aldrich, Germany) according to the manufacturer's instructions. Briefly, pellets from treated THP-1 cells (0.6 μM and 1.2 μM TMA, 5 μM and 40 μM TMAO) were lysed, and the ATP content was determined by phosphorylating glycerol. Negative controls were also included. The absorbance was read at 570 nm and data an ATP calibration curve was used for accurate ATP quantification. All analyses were run in triplicates.

2.6. Mitochondrial membrane potential

For the assessment of the mitochondrial membrane potential (ΔΨm) in treated THP-1 cells, the Mitochondrial Membrane Potential Kit (Cat. MAK159, Sigma-Aldrich, Germany) was used according to manufacturer's instructions. This assay uses cationic, lipophilic dye JC-10 that can discriminate between living cells and apoptotic cells through differences in their ΔΨm. Briefly, 8×10^4 cells were seeded into a 96-well plate. Different concentrations of TMA (0.1 μM, 0.6 μM, 1.2 μM, 10 μM, 25 μM, 50 μM) and TMAO (5 μM, 25 μM, 40 μM, 100 μM, 200 μM, 400 μM) were added to the cells. Treated cells were incubated 24 h at 37 °C. Afterword, treated cells were incubated with the JC-10 dye at 37 °C for 60 min and the fluorescence was red at $\lambda_{ex} = 490/\lambda_{em} = 525$ nm and at $\lambda_{ex} = 540/\lambda_{em} = 590$ nm) through a fluorometer. Each experimental condition was set up in duplicate.

2.7. Statistics analysis

Statistical analysis was performed by using SPSS (IBM SPSS Statistics for Windows, Version 24.0, USA) and R version 3.5.3 (R Core Team, Vienna, Austria). The ANOVA test was used to compare the difference between group means, with Dunnett's post hoc test to adjust for multiple comparisons of each treatment vs the control. A p-value <0.05 was considered significant throughout the study.

3. Results

3.1. Effect of TMA and TMAO on macrophages viability

Results from MTT assay showed no cytotoxic effect of neither TMA not TMAO at the concentrations tested on THP-1 cell (Supplementary Fig. 1). In particular, no significant difference in cell viability was measured in cells exposed to different TMA (50uM, 25uM, 10uM, 1uM, 0.5uM, 0.1uM; $p > 0.05$) or TMAO (400uM, 100uM, 50uM, 25uM, 5uM, 1uM; $p > 0.05$) concentrations compared to the control (Supplementary

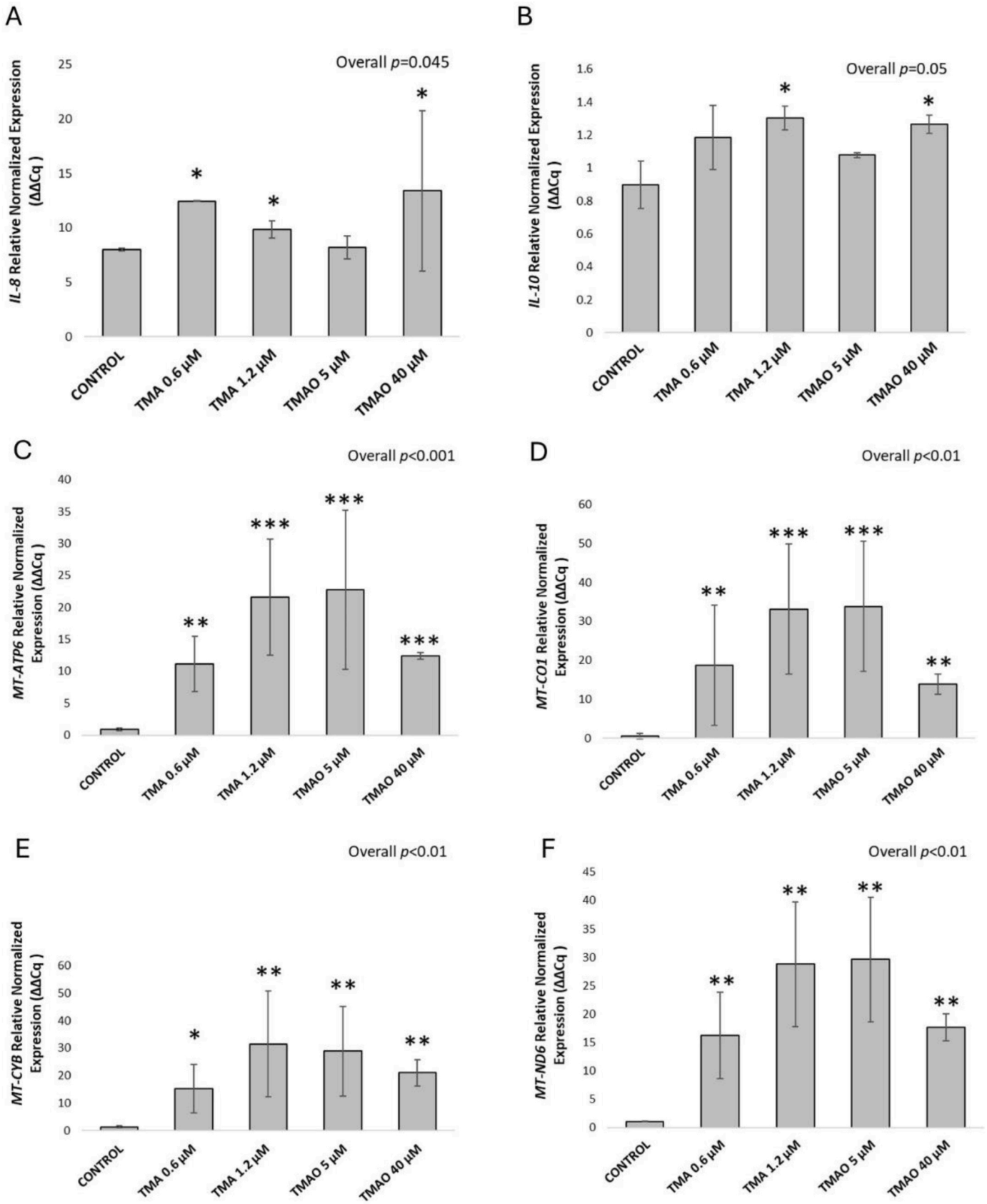

Fig. 1. A) Expression levels of IL-8 (A), IL-10 (B) mt-ATP6 (C), mt-COI (D), mt-CYB (E) and mt-ND6 (F) in THP-1 cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs control.

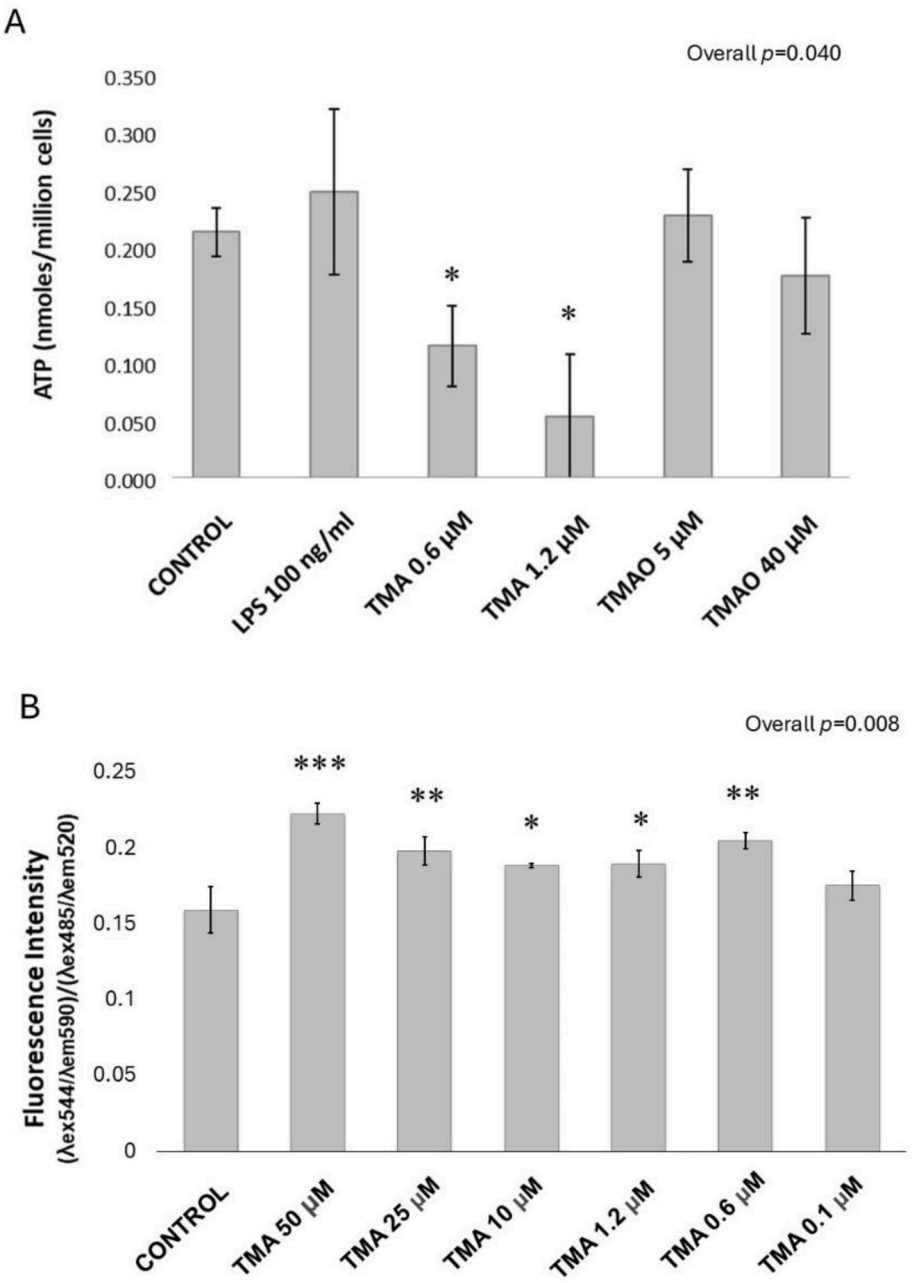

Fig. 2. A) Intracellular ATP levels in THP-1 cells. ATP contents is expressed as nmoles ATP per 1 million cells; B) Mitochondrial membrane potential (ΔΨm) of THP-1 cells after 24 h TMA exposure.

Fig. 1A and B).

3.2. Effect of TMA and TMAO on inflammation-related or mitochondrial gene expression and mtDNAcn

A significant increase of the expression levels of the pro-inflammatory IL-8 cytokine was observed at all TMA concentrations tested (0.6 μM TMA; 1.2 μM TMA) and at 40 μM TMAO (Fig. 1A) relative to controls. We found that the expression of the anti-inflammatory IL-10 was upregulated by TMA and TMAO, at the highest doses respectively (1.2 μM TMA; 40 μM TMAO) (Fig. 1B). No significant changes were measured for IL-6 gene expression (overall $p > 0.05$). Both TMA and TMAO, significantly increased the expression levels of the analysed mitochondrial genes at all concentrations tested (Fig. 1C, D, E, F). When investigating the mtDNAcn in treated cells, no significant differences were measured compared to control (overall $p > 0.05$) (Supplementary Fig. 2).

3.3. Effect of TMA and TMAO on ATP production and mitochondrial membrane potential

After 24 h exposure of TMA, a significant decrease in ATP content relative to control was observed in cells exposed to all TMA concentration tested (0.6 μM TMA, $p < 0.05$; 1.2 μM TMA, $p < 0.05$) (Fig. 2A). In contrast, no significant differences were found in cells exposed to TMAO compared to the control (Fig. 2A).

In accordance with this result, a significant increase of the ΔΨm was induced by 24 h TMA treatments up to 0.6 μM concentration compared to the control (overall $p < 0.01$) (Fig. 2B). In contrast, no significant effect on ΔΨm was measured in cells exposed to different TMAO treatments in respect to the control (overall $p > 0.05$) (Supplementary Fig. 3).

4. Discussion

The present study investigated the effects that plasma TMA and TMAO may potentially exert on circulating macrophages, using human THP-1 cells as an *in vitro* model, and focusing on inflammatory processes and mitochondrial dynamics. Cells were exposed to two different concentrations of TMA and TMAO for 24 h. The concentrations were selected based on levels previously measured by Bordoni et al. [15] in the plasma of CAD patients and control subjects. These concentrations were designed to mimic physiological conditions (lower doses of TMA and TMAO) and pathological conditions (higher doses of TMA and TMAO).

Our results indicate that TMA (at all tested concentrations) and TMAO (only at the highest dose) can activate macrophages and trigger an inflammatory response, as demonstrated by the increased expression of the pro-inflammatory cytokine IL-8 (with previous studies showing its role as an independent risk factor for CVD [19,20]). These findings suggest that only high plasma TMAO levels may pose a risk, while TMA appears to be harmful at any concentration, potentially contributing to CVD onset by promoting inflammation. We observed that the anti-inflammatory cytokine IL-10 was upregulated at the highest TMA and TMAO concentrations, likely as a compensatory response to counteract IL-8-induced inflammation. IL-10 is known to inhibit IL-8 production in LPS-activated mononuclear cells and suppress immune responses in both humans and mice [21,22]. Based on our findings, TMA and TMAO may create a complex cytokine environment where pro-inflammatory signals, like IL-8, are balanced by inhibitory cytokines, such as IL-10.

Since the perturbation of mitochondrial homeostasis has been suggested as one possible cause of TMA and TMAO-induced inflammation, in the present study mitochondrial functions have been investigated. Under the conditions tested, neither TMA nor TMAO induced significant changes in the mtDNA content of THP-1 cells, even though previous studies in a human cohort detected a direct correlation between mtDNAcn and TMA levels measured in plasma from CAD patients [15]. However, we found that a 24-h exposure to TMA, but not TMAO, was linked to decreased ATP levels and altered mitochondrial membrane potential in our model. In contrast, both TMA and TMAO treatments resulted in the upregulation of selected mitochondrial genes, each encoding a protein involved in a distinct complex of the respiratory chain (complexes I, III, IV, and V, respectively). Our findings are consistent with previous evidence reported by Jalandra et al. [11] showing a time and dose-dependent decreased intracellular ATP content in adenocarcinoma cell lines after 24 h, 48 h, and 72 h treatment with TMA. In contrast, in our study TMAO did not induce changes in ATP production, even though a previous work on cardiomyocytes reported that TMAO inhibited the activity of pyruvate dehydrogenase in mitochondria, impairing the production of ATP [23]. Given that the production of ATP is strictly dependent on the electron transfer, we investigated the effect of TMA and TMAO on the $\Delta\Psi m$. Our results revealed that only TMA, and not TMAO, affected the $\Delta\Psi m$. Specifically, TMA treatment led to an increase in $\Delta\Psi m$. Given that elevated $\Delta\Psi m$ typically correlates with enhanced ATP production, we speculate that the increased $\Delta\Psi m$ observed following TMA treatment likely reflects an upregulation of the respiratory process (supported by the increased expression of mitochondrial genes), as the mitochondria attempt to compensate for the ATP depletion induced by TMA.

In conclusion, our study reveals that both TMA and TMAO, can trigger inflammatory pathways in macrophages. However, TMA, more than TMAO, may disturb cellular homeostasis at concentrations previously measured in human plasma by altering mitochondrial dynamics. Altogether, our findings contribute to reinforcing the hypothesis that TMA, and not only TMAO, may have a contributing role in CVD, even though the exact mechanisms remain to be fully elucidated. These preliminary findings highlight the need for further research to elucidate the mechanistic pathways underlying TMA-induced vascular inflammation as well as, new studies on circulating TMA levels in human cohorts to enhance CVD prevention, improve risk stratification, and advance personalized therapeutic approaches.

CRediT authorship contribution statement

Laura Bordoni: Conceptualization, Data curation, Project administration, Writing – original draft, Writing – review & editing. **Irene Petracci:** Data curation, Writing – original draft, Writing – review & editing. **Rosita Gabbianelli:** Project administration, Resources, Supervision, Writing – review & editing.

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Declaration of competing interest

The authors declare they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2025.151529.

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