Article

Effects of Trimethylamine and Trimethylamine Oxide on Human Serum Albumin Observed by Tryptophan Fluorescence and Absorbance Spectroscopies

Laura Bordoni [1], Roberta Galeazzi [2,†], Giorgia Giorgini [2] and Rosita Gabbianelli [1,*,†]

1   Unit of Molecular Biology and Nutrigenomics, School of Pharmacy, University of Camerino, 62032 Camerino, Italy
2   Departement of Life and Environmental Science (DISVA), Università Politecnica delle Marche, Via Brecce Bianche, 60131 Ancona, Italy
*   Correspondence: rosita.gabbianelli@unicam.it
†   These authors contributed equally to this work.

Abstract: Trimethylamine (TMA) is an aliphatic tertiary amine produced by gut microbiota, starting from dietary precursors such as L-choline, L-carnitine and betaine. TMA and its metabolite trimethylamine-N-oxide (TMAO) are elevated in the plasma of cardiovascular disease (CVD) patients. Despite extensive literature on this topic, the scientific community is still divided on which of the two molecules is responsible for the harmful effects on human health. To assess whether the plasma levels of these molecules are also modulated by interactions with macromolecules present in the plasma, the weak bonds between TMA or/and TMAO with human serum albumin (HSA) were studied via molecular docking and spectrofluorimetric assay. The impact of TMA and TMAO on HSA and low-density lipoproteins (LDL) oxidation was also evaluated. Docking analysis shows three main binding sites for TMA and two for TMAO. Spectrofluorimetric results show interactions of HSA with TMA and TMAO; a significant ($p = 0.010$) decrease in Trp-214 intrinsic fluorescence of HSA was measured starting from the lowest concentrations of both TMA and TMAO (3.26 nM and 29.2 nM, respectively). Furthermore, at all concentrations tested, no significant effect on the formation of carbonyls in HSA was measured ($p > 0.05$) in the presence of TMA or TMAO. However, 28.6 mM TMAO significantly increased ($p < 0.05$) the degree of oxidation of LDL, suggesting that TMAO has a pro-oxidant role on LDL.

Keywords: HSA; molecular docking; fluorescence; oxidative stress; TMA; TMAO

1. Introduction

Human serum albumin (HSA), is a multidomain monomeric protein made up of 585 amino acid residues, structurally organized into three domains, establishing two subunits connected with 17 disulphide bonds [1]. It represents about 50% of the total mass of plasma proteins, making it the most abundant protein. Due to its negative charge, it is responsible for about 70% of the oncotic pressure of plasma, thus playing a key role in the distribution of fluids between the various compartments of the organism. HSA has an important reversible binding capacity toward several compounds, thus allowing their distribution to different tissues during plasma circulation. Furthermore, HSA plays a key antioxidant role in plasma [2].

HSA's primary structure includes a single tryptophan residue (Trp) in position 214 [3]; the importance of this residue lies in the fact that, in proteins, tyrosine (Tyr) and Trp residues are endowed with intrinsic fluorescence; moreover, using a wavelength equal to 295 nm, only the Trp residues are excited [4]. It is therefore clear that, since there is only one Trp residue in HSA, this can be used for spectrofluorimetric studies aimed at evaluating the conformational changes of HSA when placed in certain environmental conditions, or to

determine if and which conformational changes are induced by a particular molecule or chemical species in cases where it binds to HSA. In fact, the intensity of Trp fluorescence depends on the surrounding environment; in particular, if Trp is in an apolar environment and is exposed to a solvent or forms hydrogen bonds, its emission spectrum shifts towards higher wavelengths and its quantum fluorescence yield (ΦF) decreases; moreover, the fluorescence intensity can increase or decrease according to the specific microenvironment and the presence of quenching groups.

HSA also contains 35 Cys residues, 34 of which form intramolecular disulphide bridges that significantly determine the stability and the relatively long half-life of the protein (around 20 days) [1]. The remaining residue of Cys in position 34 represents the most abundant thiol group in plasma; for about 75%, it is in a reduced form, consequently playing a crucial role as an antioxidant in the blood. The reactivity of this residue is limited by the steric hindrance provided by the surrounding environment; this prevents the formation of intermolecular disulphide bridges between two albumin molecules, a phenomenon that could cause negative effects due to the consequent lowering of oncotic pressure.

Trimethylamine (TMA) is an aliphatic tertiary amine produced by gut microbiota, starting from precursors present in the diet such as L-choline, L-carnitine and betaine [5]. Choline can be present in free or esterified form, thus having different ways of absorption in the intestine; the form most converted to TMA is mainly the free one; carnitine and its metabolite γ-butyrobetaine also derive from the consumption of animal products, such as meat, fish and milk, while betaine is contained mainly in vegetables such as spinach, beets and cereals [6]. Starting from these precursors, intestinal bacteria produce TMA via TMA lyases; trimethylamine oxide (TMAO) is generated in the liver by flavin-monooxygenases (FMOs) to serve as the final form and this is eliminated through the kidney [7]. A positive correlation between elevated plasma TMAO and an increased risk for major adverse cardio and cerebrovascular events suggests that TMAO can contribute to CVD aetiology [8,9]. However, the mechanisms contributing to the increase in plasma TMAO levels in CVD patients is still to be elucidated; conflicting findings are reported in the literature, and not only TMAO but also TMA metabolism has been considered as a risk factor for CVD [10]. For these reasons, TMA-producing bacteria might be suitable predictive biomarkers of risk. Even though several studies aimed at evaluating the association between TMA/TMAO metabolism and human health have been conducted, the complete picture is far from being elucidated [7–15].

The aim of the present study was therefore to first investigate whether TMA and/or TMAO can interact with HSA to understand if the plasma concentration of TMA and TMAO can be modified by their interaction with HSA. In addition, differences in the binding of TMA and/or TMAO with HSA were tested. Secondly, this study aimed to determine whether the incubation of HSA with TMA and/or TMAO could promote protein oxidation or lipid peroxidation by the measurement of the impact of TMA/TMAO on low density lipoproteins (LDL).

2. Materials and Methods

2.1. Materials

All reagents were of pure and analytical grade; human albumin, TMA, LDL and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma Chemical Co., (St. Louis, MO, USA), while the TMAO was from Alfa Aesar, (Kandel, Germany).

2.2. Molecular Docking-In Silico Methods

HSA structure was retrieved from Brookhaven Protein Data Bank (Uniprot P02768, pdb code 1ao6) (http://www.wwpdb.org (accessed on 27 October 2020), processed within the CHIMERA software [16] and minimized using the AMBERff14 force field within the AMBER 2018 suite [17]; a progressive minimization was carried out until the average root mean square deviation (RMSD) of the non-hydrogen atoms reached 0.3 Å and the resulting structure was then used in the molecular docking calculations. TMA structure

and TMAO were built in and minimized using Gaussian16 at the DFT/6-311G* level of theory [18]. Autodock 4.2/MGLTools5.4 was used to perform the molecular docking calculations [19,20], using the previously calculated charges at the QM/DFT level for TMA. Initially, a blind docking approach was used in order to identify every putative site on the albumin protein surface. Subsequently, on the lowest energy and most populated poses, a focused docking protocol has been applied to better refine both pose and its energy. For the blind docking, we used three different grid maps calculations ($126 \times 126 \times 126$ Å^3), to include all the protein surface; in the focused docking protocol, the grid map was centred on the ligand in the considered pose and extended around the cleft ($40 \times 40 \times 40$ Å^3) with points spaced equally at 0.375 Å. The number of GA (genetic algorithm) runs was set to 150, the energy evaluations (25,000,000), the maximum number of top individuals that automatically survive (0.1), and the step size for translation (0.2 Å). All the docking calculations were carried out in triplicate using three different CPUs of random seed. The final docked albumin-TMA and albumin-TMAO complexes were ranked according to the predicted binding energy and arranged into clusters with a 2.0 Å cut-off. The final structures were minimized using the AMBERff14 force field within the AMBER18 software package [18].

2.3. Spectrofluorimetric Assay

Human serum albumin, at a concentration of 0.19 mg/mL [3], was incubated with or without different concentrations of TMA (3.26 nM, 0.82, 2.46, 5.74, 24,6 mM) and/or TMAO (29.2 nM, 2.86, 6.68, 9.54, 28.6 mM) in 20 mM Tris/HCl, 0.1 mM EDTA and 15 mM NaCl, pH 7.4. These concentrations were selected considering the plasma mean value of TMA and TMAO in CVD patients, as measured and reported in Bordoni et al. [15]. A ratio of mean value plasma HSA with TMA or TMAO was calculated; the ratio was converted in order to achieve the same in vitro ratio that would mimic physiological and pathological conditions (i.e., physiological condition was obtained at 3.26 nM TMA and 29.2 nM TMAO). Four samples were prepared for each experiment: the first with only albumin (control), the second with albumin and TMA, the third with albumin and TMAO, the fourth with albumin, TMA and TMAO. The samples were incubated at room temperature (25 °C); the measurements were taken at an excitation wavelength 295 nm at time 0 and at 60 min. The emission spectra were measured in the range from 310 nm to 450 nm in a spectrofluorometer Hitachi F-4500. All experiments were repeated at least three times under the same conditions. Data were presented as the % of variation of Trp emission spectra peak of samples containing TMA, TMAO or both compounds versus the control.

2.4. Evaluation of HSA Oxidation

Samples previously used for the spectrofluorimetric assay were used to measure carbonyl group formation in HSA incubated with TMA or TMAO, or TMA+TMAO. Each sample was incubated for 60 min with 0.5 mL of 10 mM 2,4-dinitrophenylhydrazine in water solution, then 0.5 mL of trichloroacetic acid in water solution was added and centrifuged. The precipitate obtained was washed three times with 1 mL of the EtOH/EtOAc mixture and dissolved in 200 μL of 6 M guanidine in water solution. The absorbance readings were carried out on a microplate at a wavelength of 370 nm.

2.5. Measurement of LDL Oxidation

To measure the effect of TMA and TMAO on LDL oxidation, conjugated dienes (a measure of lipid oxidation) were monitored in the LDL samples incubated in the presence or absence of TMA, TMAO or TMA+TMAO in a 96-well microplate reader. LDL, at a concentration of 0.01 mg/mL, were incubated with or without different concentrations of TMA (0.6 μM, 0.82, 2.46, 5.74, 24.6 mM) and/or TMAO (5.37 μM, 2.86, 9.54, 28.6 mM) starting from the plasma mean value reported by Bordoni et al., [15]. As a positive control to induce LDL oxidation, Cu^{++} (5 μM) was used as reported by Venditti et al. [16]. Five samples were prepared for each experiment: the first with LDL (control), the second with

LDL and 5 μM of Cu^{++} (positive control), the third with LDL and TMA, the fourth with LDL and TMAO, the fifth with LDL, TMA and TMAO. The samples were incubated at 25 °C; the absorbance measurements at 234 nm were carried out at different incubation times (0, 30, 60, 80, 90, 100, 120, 140, 150, 160 and 180 min). All experiments were repeated at least twice under the same conditions.

2.6. Statistical Data Analysis

Data were analysed using the Statistical Package for Social Science (SSPS, IBM, Armonk, NY, USA). The normality of the data distribution was tested via the Kolmogorov–Smirnov test. Concerning spectrofluorometric data and LDL oxidation, statistically significant differences between groups were assessed by Kruskal–Wallis or ANOVA tests followed by post-hoc analysis (LSD), respectively, for non-parametric or parametric analyses based on data distribution. The level of statistical significance was defined by a two-tailed *p* value < 0.05 throughout the study.

3. Results

3.1. Molecular Docking of TMA, TMAO and Human Serum Albumin

The 3D human serum albumin (HSA) structure can be divided into three homologous domains, each one is known to be made up of two separate helical subdomains (named A and B), connected by random coil. This modular structural organization of HSA provides a variety of ligand binding sites [20,21]. From the docking of TMA targeting HSA, we detected the existence of three main cluster poses (Figure 1, Energy reported in Table S1, Supplementary Material), whilst from the TMAO and HSA docking, we found two cluster poses both located quite externally at the proximity of the HSA surface (Figure 1, Energy reported in Table S1, Supplementary Material). Furthermore, for TMA, the lowest energy pose lies in the opposite side of HSA with respect to the TMAO's one (Figure 1). In addition, the external localization of the TMAO binding poses, despite being easily reached, could result in less stable binding interactions in time; the opposite is instead observed for TMA, whose two lowest energy poses are buried inside the HSA helices and with the third positioned close to the S–S bond between Cys514–Cys559. No binding sites were found at the dimeric interface, suggesting the accessibility of the found clefts.
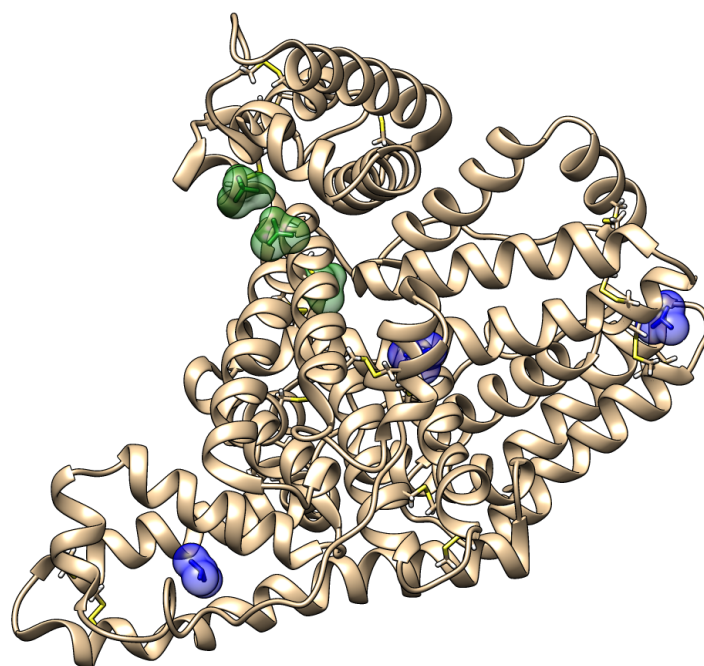

Figure 1. HAS in complex with TMA (blue coloured) and TMAO (green coloured) in their respective lowest energy binding poses; the ligands are reported in a stick model with a VdW transparent surface.

Analysing, in detail, TMA interactions with HAS, we observed that the two most stable TMA poses are located in the proximity of the disulphide bridges (Figure 2): in the most stable binding pose, Cys316–Cys361 and Cys360–Cys369 lie close to TMA (Figure 2A,B), whilst in the second pose, even if the cysteine residues are not directly involved in binding interactions with the ligand, TMA's binding site is buried within a cleft whose conformation is directly affected by the presence of S–S bonds involving Cys289–Cys278 and Cys279–Cys265 (Figure 2C,D). In order to evaluate if the oxidation state of HSA can affect TMA binding, we reduced the state of Cys316, Cys 361, Cys360 and Cys369, converting the S–S bond in the corresponding thiol moieties S–H, and redocked the ligand. Prior to proceeding with the docking experiments, a full relaxation and minimization of the reduced protein was carried out to solve any local strains. As a result, we obtained a significantly higher binding energy (-10.8 vs. -14.1 kJ/mol), thus associated with a corresponding much lower binding affinity.
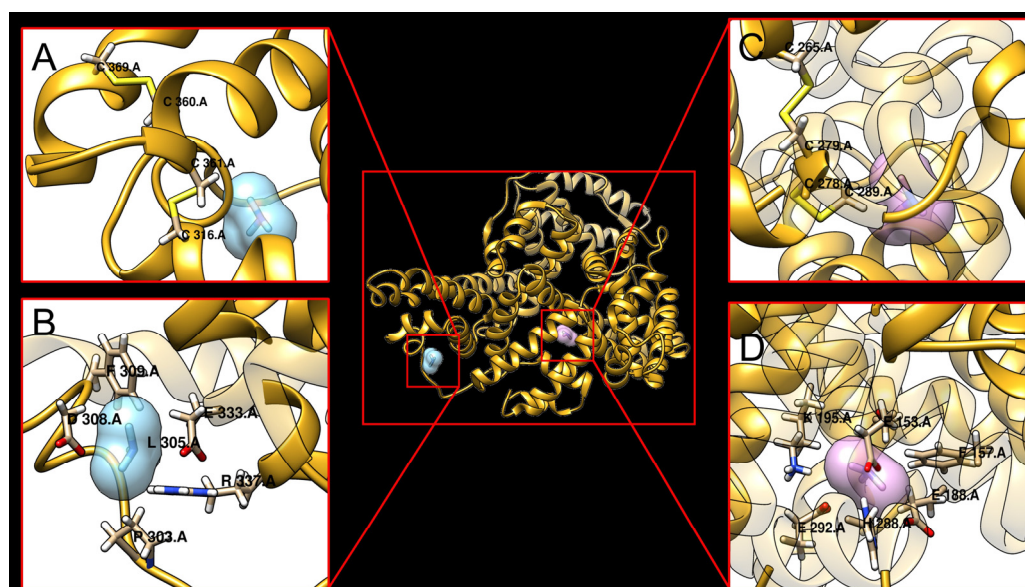

Figure 2. Left: HAS in complex with TMA's lowest energy cluster (blue transparent VdW surface) (**A**) focusing on S–S bonds involved in the interaction site; (**B**) a focus on the specific TMA interaction at its corresponding binding cleft. **Right**: HAS in complex with the second TMA energy cluster (violet transparent VdW surface); (**C**) S–S bonds involved are highlighted; (**D**) a focus on TMA's second cluster interaction at its binding cleft.

3.2. Fluorescence Study

Trp emission spectra in the samples containing HSA, TMA or TMAO, or both compounds at physiological concentrations (3.26 nM and 29.2 nM, respectively), showed a significant decrease in Trp peak in the sample containing TMA + TMAO compared to the other ones, both at time 0′ ($p = 0.002$) and at 60 min ($p = 0.001$) (Figure 3A). Similar outcomes were observed at all the other concentrations of TMA and TMAO used (Figure 3B–F). When experiments were performed with 0.82 mM or 4.1 mM of TMA and 0.954 mM or 4.77 mM TMAO, no significant changes were measured (data not shown).
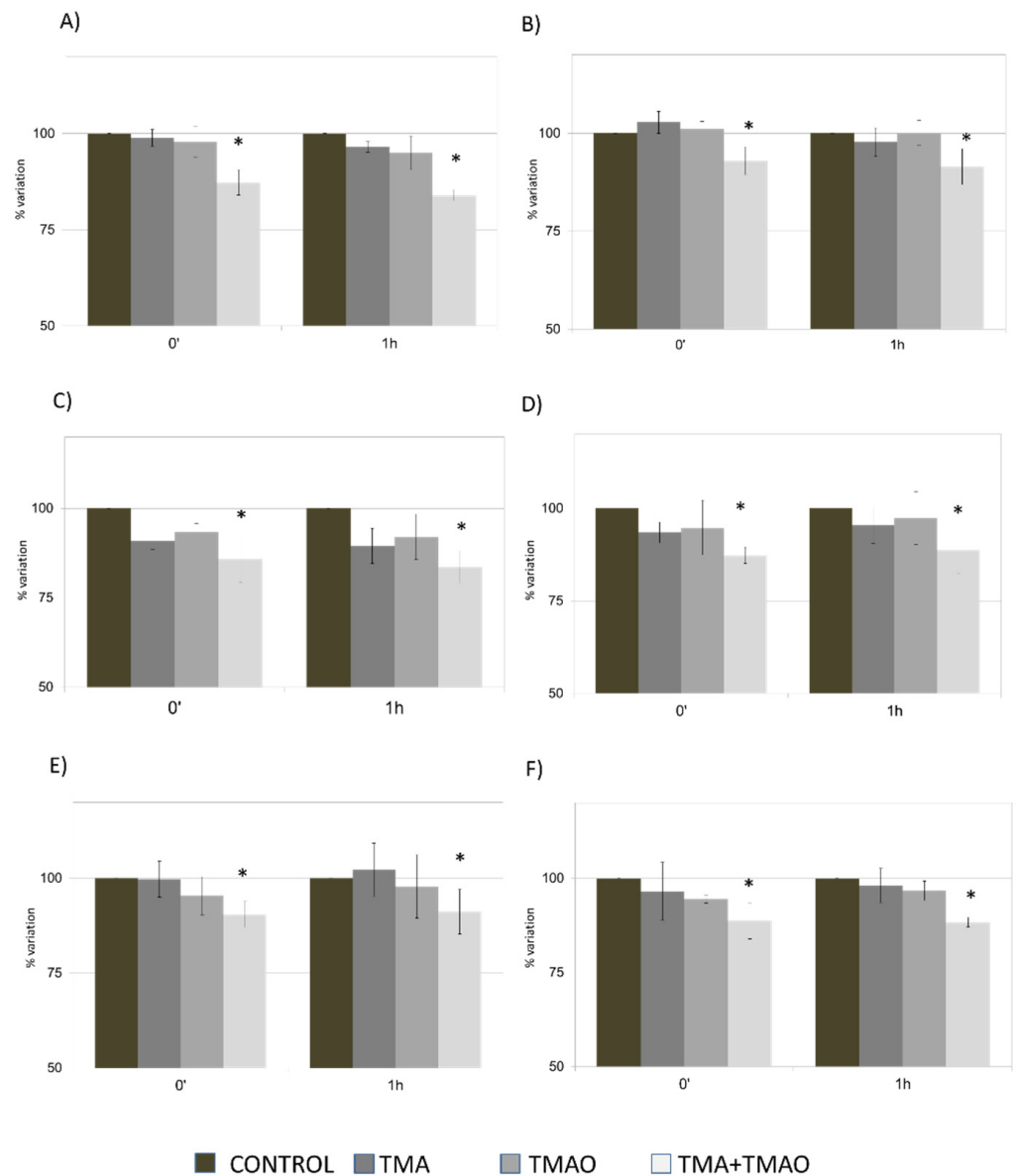
Figure 3. Percentage variation in Trp emission spectra (mean value $\pm$ SD) of 190 μg/mL HSA incubated at 25 °C at 0$'$ and after 1 h with: (**A**) 3.26 nM TMA, 29.2 nM TMAO; (**B**) 0.82 mM TMA, 9.54 mM TMAO; (**C**) 2.46 mM TMA, 2.86 mM TMAO; (**D**) 2.46 mM TMA, 28.6 mM TMAO; (**E**) 5.74 mM TMA, 6.68 mM TMAO; and (**F**) 24.6 mM TMA, 2.86 mM TMAO and in 20 mM Tris, 0.1 mM EDTA, 15 mM NaCl, pH 7.4. * $p = 0.010$.

3.3. Measurement of LDL and HSA Oxidation

Studies on the impact of TMA or TMAO or both on LDL oxidation show that in the presence of 2.46 mM TMA + 28.6 mM TMAO or only TMAO, the degree of oxidation of LDL increases significantly ($p < 0.05$) starting from 60 min (Figure 4C). When TMA is present at a higher concentration than TMAO (24.6 mM TMA, 2.86 mM TMAO), no significant differences in LDL oxidation were observed (Figure 4D). To evaluate the impact of TMAO at lower concentrations (5.37 μM–9.54 mM), additional experiments were conducted; no changes were observed at these concentrations (Figure 4A,B).
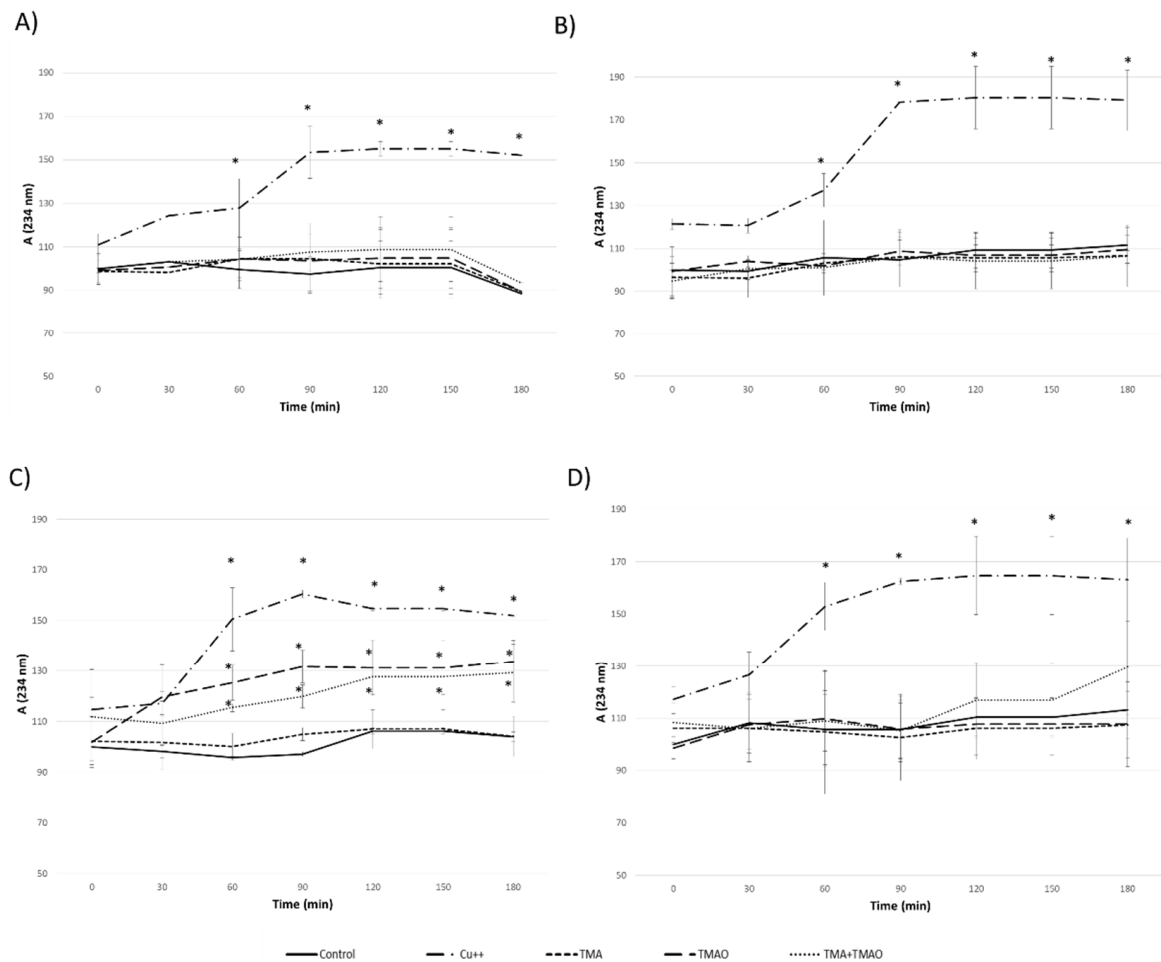
Figure 4. LDL oxidation was measured by a change in absorbance (mean value $\pm$ SD) at 234 nm of LDL (0.01 mg/mL) incubated with 5 μM Cu^{++} with (**A**) 0.6 μM TMA, 5.37 μM TMAO; (**B**) 0.82 mM TMA, 9.54 mM TMAO; (**C**) 2.46 mM TMA, 28.6 mM TMAO; (**D**) 24.6 mM TMA, 2.86 mM TMAO; * $p < 0.05$.

HSA oxidation was evaluated by carbonyl groups formation; no change in the carbonyl group formation was observed (data not shown).

4. Discussion

Variations in plasma levels of TMA and its metabolite TMAO are associated with CVD, thus representing a potential biomarker for the prevention of these diseases; however, it is still unknown which of the two compounds (if any) is decisive as a risk factor for CVD.

Previous studies have reported that the plasma level of TMA is slightly higher in men than in women, while that of TMAO is similar in the two genders [22]. By comparing two groups of people, the first made up of vegetarians and vegans, the second made up of omnivores, it was found that the plasma levels of TMAO were significantly lower in the first group [23]. Moreover, a reduced ability to produce TMAO starting from carnitine was noted in the first group, probably because of the reduced ability of the microbiota of the vegetarian and vegan subjects to convert carnitine into TMA; this finding suggests that dietary habits have a significant impact on plasma levels of TMA and TMAO [23]. Moreover, due to its low molecular weight (75.1 Da), TMAO is easily excreted in the kidney. Studying the renal excretion of radioactive isotope-labelled TMA and TMAO, it has been shown that both compounds are eliminated by approximately 95% within the first 24 h. However, while TMA is mostly eliminated within the first 3 h following exponential

dynamics, the elimination of TMAO presents as constant kinetics during the first 9 h after administration [23].

Physiologically, TMAO is an osmolyte that acts as a stabilizer of proteins. It helps to maintain correct protein folding, counteracts the effect of denaturants such as changes in pH, urea and high pressure, and as such, is important for maintaining the enzymatic activity of the enzymes to which it binds [24].

Recently, numerous studies have examined TMAO and its precursor TMA for their potential role in the onset of CVD [11–15,25–28]. However, considering the literature and the present research, the results remain contradictory; although some studies suggest that TMAO produces harmful effects on the cardiovascular system, others attribute this role to TMA, even hypothesizing that TMAO has a protective effect from damage induced by TMA [27].

From the molecular docking analysis, it emerges that, even if TMAO and TMA have a calculated comparable free binding energy with HSA (Table S1), they do not have the same binding affinity. In fact, TMAO binds only to externally located binding sites, i.e., in proximity of the HSA molecular surface limit. This corresponds to labile and not durable binding interactions, that are instead observed for TMA, whose poses are buried inside the HSA helices and fixed via direct interaction with a huge number of residues, such as disulphide linkages. These in silico findings are coherent with previous observations suggesting that incubation with TMA but not TMAO resulted in the change of conformation of the protein structure of albumin [28]. However, in the present study, the tryptophan emission peak was significantly ($p = 0.010$) lower when albumin was incubated with both TMA+TMAO starting from low concentrations, which mimics physiological conditions [15]; this suggests a synergistic cooperative interaction of TMA and TMAO with HSA. The decrease in Trp fluorescence emission intensity may be associated with the changes in the microenvironment around Trp 214 following the interaction with TMA and TMAO. It can be hypothesized that the weak interactions with TMA and TMAO do not promote any HSA oxidation since no significant differences were observed in carbonyl groups formation. According to this outcome, under our experimental conditions, a stabilizing effect of TMA+TMAO on HSA could be hypothesized. Nevertheless, when this outcome is compared with the in silico study, especially under conditions of increased oxidative stress, a more stable HSA–TMA complex can be expected. On the other hand, in the presence of 2.46 mM TMA and 28.6 mM TMAO, or only 28.6 mM TMAO, a significant increase in LDL oxidation was observed, suggesting that other factors can be involved in the unfavourable association between TMA or TMAO and CVD.

A limit of this study is that we did not use the TMA and TMAO induced changes in Trp emission of HSA for the evaluation of their binding constants. Future studies investigating the spectrofluorimetric titration of the protein by TMA and TMAO, and an analysis of the binding curves, are warranted.

5. Conclusions

This study shows that both TMA and TMAO interact with has, favouring an alteration in the environment of Trp 214 and HSA conformational changes. Molecular docking analysis identified different sites and energy of interaction between TMA and TMAO with HSA, and the synergistic cooperative link observed in vitro might contribute to explain the differences in plasma TMA or TMAO levels. Furthermore, it seems that these two compounds, at the concentrations used in the present study, are not able to influence protein oxidation in HSA; despite this, a high concentration of TMAO significantly increases the degree of oxidation of LDL.

Based on the data obtained, further studies are needed to accurately elucidate the controversial role of TMA and TMAO in CVD.

References

1. Fanali, G.; di Masi, A.; Trezza, V.; Marino, M.; Fasano, M.; Ascenzi, P. Human serum albumin: From bench to bedside. *Mol. Asp. Med.* **2012**, *33*, 209–290. [CrossRef] [PubMed]

2. Ascenzi, P.; Fasano, M. Allostery in a monomeric protein: The case of human serum albumin. *Biophys. Chem.* **2010**, *148*, 16–22. [CrossRef] [PubMed]

3. Zolese, G.; Falcioni, G.; Bertoli, E.; Galeazzi, R.; Wozniak, M.; Wypych, Z.; Gratton, E.; Ambrosini, A. Steady-state and time resolved fluorescence of albumins interacting with N-oleylethanolamine, a component of the endogenous N-acylethanolamines. *Proteins Struct. Funct. Genet.* **2000**, *40*, 39–48. [CrossRef]

4. Sugio, S.; Kashima, A.; Mochizuki, S.; Noda, M.; Kobayashi, K. Crystal structure of human serum albumin at 2.5 Å resolution. *Protein Eng. Des. Sel.* **1999**, *12*, 439–446. [CrossRef] [PubMed]

5. Onyszkiewicz, M.; Jaworska, K.; Ufnal, M. Short chain fatty acids and methylamines produced by gut microbiota as mediators and markers in the circulatory system. *Exp. Biol. Med.* **2020**, *245*, 166–175. [CrossRef] [PubMed]

6. Zeisel, S.H.; Warrier, M. Trimethylamine N-Oxide, the Microbiome, and Heart and Kidney Disease. *Annu. Rev. Nutr.* **2017**, *21*, 157–181. [CrossRef] [PubMed]

7. Bennett, B.J.; de Aguiar Vallim, T.Q.; Wang, Z.; Shih, D.M.; Meng, Y.; Gregory, J.; Allayee, H.; Lee, R.; Graham, M.; Crooke, R.; et al. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab.* **2013**, *17*, 49–60. [CrossRef]

8. Heianza, Y.; Ma, W.; Manson, J.E.; Rexrode, K.M.; Qi, L. Gut Microbiota Metabolites and Risk of Major Adverse Cardiovascular Disease Events and Death: A Systematic Review and Meta-Analysis of Prospective Studies. *J. Am. Heart Assoc.* **2017**, *6*, e004947. [CrossRef]

9. Schiattarella, G.G.; Sannino, A.; Toscano, E.; Giugliano, G.; Gargiulo, G.; Franzone, A.; Trimarco, B.; Esposito, G.; Perrino, C. Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: A systematic review and dose-response meta-analysis. *Eur. Heart J.* **2017**, *38*, 2948–2956. [CrossRef]

10. Jaworska, K.; Bielinska, K.; Gawrys-Kopczynska, M.; Ufnal, M. TMA (trimethylamine), but not its oxide TMAO (trimethylamine-oxide), exerts haemodynamic effects: Implications for interpretation of cardiovascular actions of gut microbiome. *Cardiovasc. Res.* **2019**, *115*, 1948–1949. [CrossRef]

11. Olek, R.A.; Samulak, J.J.; Sawicka, A.K.; Hartmane, D.; Grinberga, S.; Pugovics, O.; Lysiak-Szydlowska, W. Increased trimethylamine N-oxide is not associated with oxidative stress markers in healthy aged women. *Oxid. Med. Cell. Longev.* **2019**, *2019*, 6247169. [CrossRef]

12. Samulak, J.J.; Sawicka, A.K.; Hartmane, D.; Grinberga, S.; Pugovics, O.; Lysiak-Szydlowska, W.; Olek, R.A. L-carnitine supplementation increases trimethylamine-N-oxide but not markers of atherosclerosis in healthy aged women. *Ann. Nutr. Metab.* **2019**, *74*, 11–17. [CrossRef]

13. Yin, J.; Liao, S.X.; He, Y.; Wang, S.; Xia, G.H.; Liu, F.T.; Zhu, J.J.; You, C.; Chen, Q.; Zhou, L.; et al. Dysbiosis of gut microbiota with reduced trimethylamine-N-oxide level in patients with large-artery atherosclerotic stroke or transient ischemic attack. *J. Am. Heart Assoc.* **2020**, *4*, e002699. [CrossRef]

14. Landfald, B.; Valeur, J.; Berstad, A.; Raa, J. Microbial trimethylamine-N-oxide as a disease marker: Something fishy? *Microb. Ecol. Health Dis.* **2017**, *28*, 327309. [CrossRef]

15. Bordoni, L.; Petracci, I.; Pelikant-Malecka, I.; Radulska, A.; Piangerelli, M.; Samulak, J.J.; Lewicki, L.; Kalinowski, L.; Gabbianelli, R.; Olek, R.A. Mitochondrial DNA copy number and trimethylamine levels in the blood: New insights on cardiovascular disease biomarkers. *FASEB J.* **2021**, *35*, e21694. [CrossRef]

16. Venditti, E.; Bacchetti, T.; Tiano, L.; Carloni, P.; Greci, L.; Damiani, E. Hot vs. cold water steeping of different teas: Do they affect antioxidant activity? *Food Chem.* **2010**, *119*, 1597–1604. [CrossRef]

17. Pettersen, E.F.; Goddard, T.D.; Huang, C.C.; Couch, G.S.; Greenblatt, D.M.; Meng, E.C.; Ferrin, T.E. UCSF Chimera—A visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *25*, 1605–1612. [CrossRef]

18. Case, D.; Ben-Shalom, I.; Brozell, S.; Cerutti, D.; Cheatham, T., III; Cruzeiro, V.; Darden, T.; Duke, R.; Ghoreishi, D.; Gilson, M.; et al. *AMBER 2018*; University of California: San Francisco, CA, USA, 2018.

19. Frisch, M.J.; Trucks, G.W.; Schlegel, H.B.; Scuseria, G.E.; Robb, M.A.; Cheeseman, J.R.; Scalmani, G.; Barone, V.; Petersson, G.A.; Nakatsuji, H.; et al. *Gaussian 16, Revision C.01*; Gaussian, Inc.: Wallingford, CT, USA, 2016.

20. Morris, G.M.; Huey, R.; Lindstrom, W.; Sanner, M.F.; Belew, R.K.; Goodsell, D.S.; Olson, A.J. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J. Comput. Chem.* **2009**, *30*, 2785–2791. [CrossRef]

21. Fasano, M.; Curry, S.; Terreno, E.; Galliano, M.; Fanali, G.; Narciso, P.; Notari, S.; Ascenzi, P. The extraordinary ligand binding properties of human serum albumin. *IUBMB Life* **2005**, *57*, 787–796. [CrossRef]

22. Bordoni, L.; Fedeli, D.; Piangerelli, M.; Pelikant-Malecka, I.; Radulska, A.; Samulak, J.J.; Sawicka, A.K.; Lewicki, L.; Kalinowski, L.; Olek, R.A.; et al. Gender-Related Differences in Trimethylamine and Oxidative Blood Biomarkers in Cardiovascular Disease Patients. *Biomedicines* **2020**, *23*, 238. [CrossRef]

23. Koeth, R.A.; Wang, Z.; Levison, B.S.; Buffa, J.A.; Org, E.; Sheehy, B.T.; Britt, E.B.; Fu, X.; Wu, Y.; Li, L.; et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat. Med.* **2013**, *19*, 576–585. [CrossRef]

24. Al-Waiz, M.; Mitchell, S.C.; Idle, J.R.; Smith, R.L. The metabolism of 14C-labelled trimethylamine and its N-oxide in man. *Xenobiotica* **1987**, *17*, 551–558. [CrossRef]

25. Liao, Y.T.; Manson, A.C.; DeLyser, M.R.; Noid, W.G.; Cremer, P.S. Trimethylamine N-oxide stabilizes proteins via a distinct mechanism compared with betaine and glycine. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 2479–2484. [CrossRef]

26. Wang, Z.; Tang, W.H.W.; O'Connell, T.; Garcia, E.; Jeyarajah, E.J.; Li, X.S.; Jia, X.; Weeks, T.L.; Hazen, S.L. Circulating trimethylamine N-oxide levels following fish or seafood consumption. *Eur. J. Nutr.* **2022**, *61*, 2357–2364. [CrossRef]

27. Hamaya, R.; Ivey, K.L.; Lee, D.H.; Wang, M.; Li, J.; Franke, A.; Sun, Q.; Rimm, E.B. Association of diet with circulating trimethylamine-N-oxide concentration. *Am. J. Clin. Nutr.* **2020**, *112*, 1448–1455. [CrossRef]

28. Jaworska, K.; Hering, D.; Mosieniak, G.; Bielak-Zmijewska, A.; Pilz, M.; Konwerski, M.; Gasecka, A.; Kapłon-Cieślicka, A.; Filipiak, K.; Sikora, E.; et al. TMA, A Forgotten Uremic Toxin, but Not TMAO, Is Involved in Cardiovascular Pathology. *Toxins* **2019**, *11*, 490. [CrossRef]