Trimethylamine N-oxide does not impact viability, ROS production and mitochondrial membrane potential of adult rat cardiomyocytes

(Article begins on next page)

21 November 2019

1   *Research Article*

2 Trimethylamine N-oxide does not impact viability,
3 ROS production and mitochondrial membrane
4 potential of adult rat cardiomyocytes

5   **Giulia Querio [1], Susanna Antoniotti [1], Renzo Levi [1] and Maria Pia Gallo [1,*]**

6   [1]   Department of Life Sciences and Systems Biology, University of Turin, Via Accademia Albertina 13, 10123,
7   Turin, Italy
8   *   Correspondence: mariapia.gallo@unito.it; Tel.: +390116704671

9

10 **Abstract:** Trimethylamine N-oxide (TMAO) is an organic compound derived from dietary choline
11 and L-carnitine. It behaves as an osmolyte, a protein stabilizer and an electron acceptor, showing
12 different biological functions in different animals. Recent works point out that in humans high
13 circulating levels of TMAO are related with the progression of atherosclerosis and other
14 cardiovascular diseases. Nevertheless, studies on a direct role of TMAO on cardiomyocytes
15 parameters are still limited. This work focuses on the effects of TMAO on isolated adult rat
16 cardiomyocytes. TMAO both 100μM and 10mM, from 1 to 24 h of treatment, does not affect cell
17 viability, sarcomere length, intracellular ROS and mitochondrial membrane potential. Furthermore,
18 the simultaneous treatment with TMAO and known cardiac insults, H_2O_2 or Doxorubicin, does not
19 affect the effect of the treatment. In conclusion, TMAO cannot be considered a direct cause or an
20 exacerbating risk factor of cardiac damage at the cellular level in acute conditions.

21 **Keywords:** Trimethylamine N-oxide; cardiomyocytes; cardiotoxicity; ROS; mitochondrial
22 membrane potential
23

24 1. Introduction

25 Trimethylamine N-oxide (TMAO) is an amine oxide directly introduced through the diet or
26 synthetized from its precursors, primarily L-carnitine and choline, that are transformed into
27 trimethylamine (TMA) by the gut microbiota. Once absorbed, TMA in most mammals is oxidized by
28 hepatic FMOs to form TMAO which enters the systemic circulation. Several studies illustrates
29 different biological functions of TMAO in other animals. In elasmobranchs and deep sea fishes it acts
30 as an osmolyte able to counteract either osmotic or hydrostatic pressure. It is a protein stabilizer
31 preserving protein folding and it also acts as an electron acceptor balancing oxidative stress [1,2].
32 TMAO is also reduced to TMA in the anaerobic metabolism of a number of bacteria. Although TMAO
33 is involved in several reactions within cells, recent studies highlight its detrimental role when present
34 in high plasmatic concentrations in some mammals. In fact, TMAO seems to be involved in
35 accelerating endothelial cell senescence, enhancing vascular inflammation and oxidative stress [3,4];
36 it also could be involved in the stimulation of platelets hyperreactivity and in the onset of thrombosis,
37 exacerbating atherosclerotic lesions [5]. Several studies also underline the role of TMAO in the
38 pathogenesis of type 2 diabetes mellitus [6]. There are limited data on its function in mediating direct
39 cardiac injuries, and they are mainly focused on its role in the impairment of mitochondrial
40 metabolism [7] and calcium handling [8]. Instead, recent papers revalue the role of TMAO,

41 underlining the emerging debate on its direct effect in causing or exacerbating cardiovascular
42 diseases (CVD) [9,10]. First criticisms point out that populations that have diets with high
43 concentrations of TMAO, like those rich in fish products, when compared to Western diets rich in its
44 precursors, have reduced risks of CVD or diseases assumed to be related to high TMAO plasma levels
45 [11]. Another study demonstrate that TMAO does not affect macrophage foam cells formation and
46 lesion progression in ApoE$^{-/-}$ mice expressing human cholesteryl ester transfer protein, suggesting
47 that the molecule does not worsen atherosclerosis [12]. Furthermore, administration of TMAO seems
48 to ameliorate symptoms related to streptozotocin induced diabetes in rats and mice, highlighting
49 no direct contribution of the molecule in exacerbating this condition [13]. Finally, data about TMAO
50 plasma concentrations in health and pathological subjects are not clear: lack of plasma concentration
51 ranges of the molecule highlights the difficulties in referring to TMAO as a protective or a damaging
52 factor in CVD. Starting from these conflicting considerations, aim of this work is to evaluate for the
53 first time the effect of TMAO in an *in vitro* model of adult rat cardiomyocytes exposed to different
54 concentrations of the compound from 1 h to 24 h of treatment. To show whether TMAO exacerbates
55 or reduces induced cell stress, cardiomyocytes are simultaneously treated with TMAO and H_2O_2 or
56 Doxorubicin (DOX). Investigations have been focused to cell viability after TMAO or TMAO and
57 stressors co-treatment, assessing cell morphology and functionality with α-actinin staining and
58 specific probes that measure oxidative stress status and mitochondrial membrane potential.

59 **2. Results**

60 *2.1 TMAO and cell viability*

61 In order to investigate the effect of TMAO on cell viability, cardiomyocytes are treated with
62 TMAO 100μM, TMAO 10mM, H_2O_2 50μM and H_2O_2 50μM+TMAO 100μM. After 1 h or 24 h of
63 treatment, cardiomyocytes are labeled with PI and marked nuclei of suffering cells are detected by
64 confocal microscopy at 568nm. Concentrations used have been taken from literature: TMAO 100μM
65 is recognized as a marker of cardiovascular risk, TMAO 10mM is over the physiological range and
66 here tested to detect any effect induced by high concentrations of the compound [14]. As shown in
67 Figure 1a, there is no effect of TMAO 100μM nor TMAO 10mM at either time of treatment, while
68 H_2O_2, here used as positive control, has effects only after 24 h. Simultaneous treatment with H_2O_2 and
69 TMAO does not ameliorate nor worsen the effect of the stressor on cell viability (1 h: CTRL:
70 83.95±6.59, n=3, 52 cells; TMAO 100μM: 85.52±7.01, n=5, 81 cells; TMAO 10mM: 84.08±5.84, n=5, 92
71 cells; H_2O_2: 52.92±16.46, n=3, 56 cells; H_2O_2+TMAO: 50.93±18.50, n=3, 58 cells. 24 h: CTRL: 83.42±2.29,
72 n=3, 101 cells; TMAO 100μM: 85.66±6.48, n=3, 91 cells; TMAO 10mM: 62.22±10.47 n=3, 119 cells; H_2O_2:
73 2.38±2.38, n=3, 82 cells(***P<0.001); H_2O_2+TMAO: 1.33±1.33, n=3, 41 cells(***P<0.001)). Figure 1b displays
74 confocal images of cardiomyocytes from a representative experiment of PI staining after 24 h of
75 treatment. White arrows point out PI-stained, damaged cardiomyocytes.

a)

b)
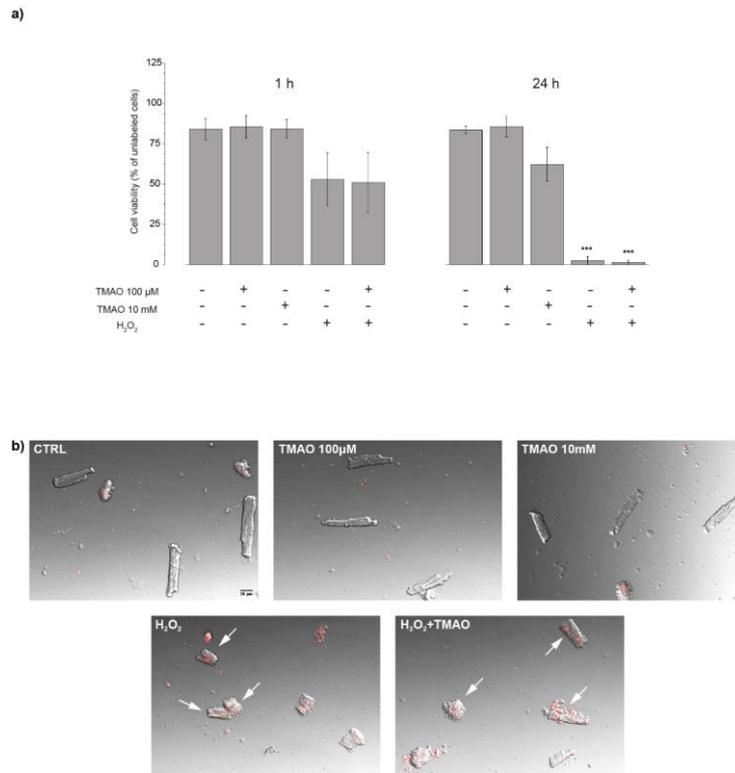
Figure 1. Cell viability after TMAO exposure. a) Bar graph of cell viability after 1 h and 24 h of treatment. Cells viability results reduced only after H_2O_2 treatment for 24 h, condition not ameliorated nor worsened by the simultaneous treatment with TMAO (refer to the main text for numerical values). b) Merged images in bright field and fluorescence of cells treated for 24 h with TMAO 100μM and TMAO 10mM, H_2O_2 and H_2O_2 + TMAO and labeled with propidium iodide (PI) (20X magnification). White arrows point out PI-stained, damaged cardiomyocytes.

2.2 TMAO and sarcomere length

To evaluate if TMAO is able to alter sarcomere structures after 24 h of treatment, sarcomere length is measured in α-actinin stained cardiomyocytes. As shown in Figure 2, no changes in sarcomere length are observed in cells treated with TMAO, while H_2O_2 50μM used as a positive control, cause cardiomyocytes shrinkage, condition that is not ameliorated nor worsened by the simultaneous treatment with TMAO. In cardiomyocytes treated with DOX 1μM for 24 h we do not observe sarcomere length variations, as in our model DOX treatment is designed to induce a mild damage preceding cell shortening. Even so, 100μM TMAO does not modify DOX-treated cardiomyocytes (sarcomere length in μm is: CTRL: 1.69±0.01, n=7, 42 cells; TMAO 100μM: 1.69±0.01, n=6, 34 cells; TMAO 10mM: 1.67±0.02, n=3, 19 cells; H_2O_2: 1.22±0.04, n=5, 33 cells (***P<0.001); H_2O_2+TMAO: 1.28±0.03, n=3, 24 cells (***P<0.001); DOX: 1.62±0.02, n=3, 16 cells; DOX+TMAO: 1.65±0.01, n=3, 15 cells).

96
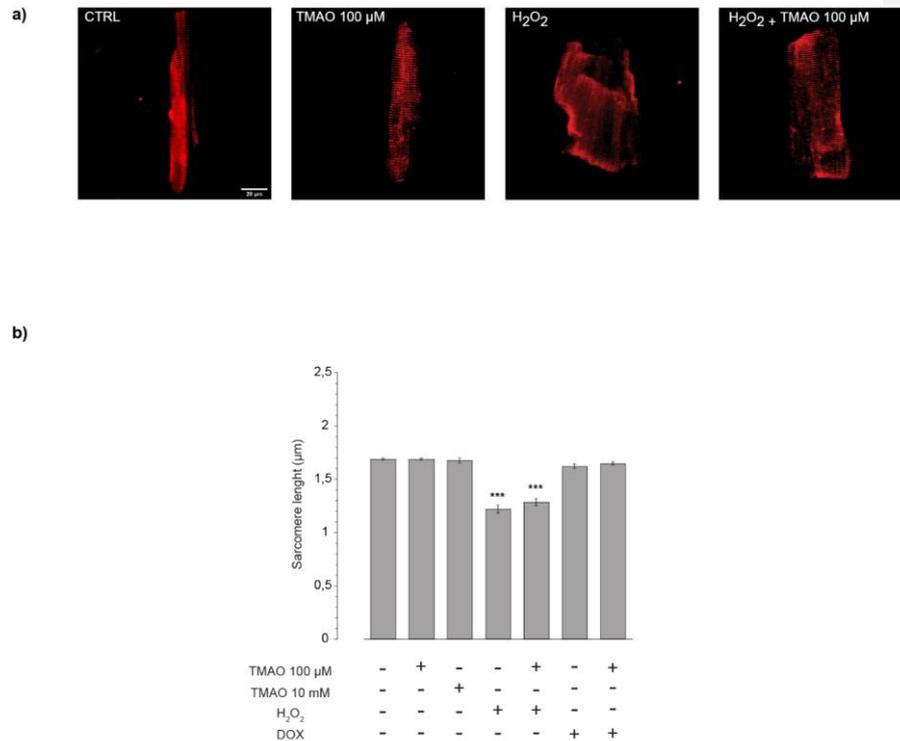
Figure 2. Sarcomere length after TMAO treatment. a) Confocal microscopy images of fixed cells labeled for α-actinin protein (40X magnification). b) Bar graph showing sarcomere length after 24 h of treatment with TMAO and other stressors: no cell shrinkage is measured when cells are exposed to different TMAO concentrations (refer to the main text for numerical values).

2.3 TMAO and intracellular Reactive Oxygen Species (ROS)

In order to determine a variation in total ROS produced after treatment with TMAO for 1 h, 3 h or 24 h, cells are labeled with DCF-DA probe and its fluorescence is quantified and related to control. As shown in Figures 3 (1 h, 3 h) and 4 (24 h) no fluorescence variations after TMAO treatment is detected at any concentration and time used. As a positive control we employ DOX 1μM for 24 h [15]; this drug caused a significant variation in ROS production referred to control condition. TMAO 100μM does not modify ROS production in DOX treated cardiomyocytes (Fig. 4). (1h: TMAO 100μM: 1.30±0.21, n=3, 34 cells; TMAO 10mM: 1.32±0.23, n=3, 40 cells, vs CTRL; 3h: TMAO 100μM: 0.96±0.05, n=3, 45 cells; TMAO 10mM: 1.15±0.09, n=3, 52 cells, vs CTRL; 24h: TMAO 100μM: 1.18±0.04, n=5, 40 cells; TMAO 10mM: 1.24±0.16, n=3, 52 cells; DOX: 1.33±0.11, n=4, 21 cells (**P<0.01); DOX+TMAO: 1.27±0.01, n=6, 31 cells (***P<0.001) vs CTRL).

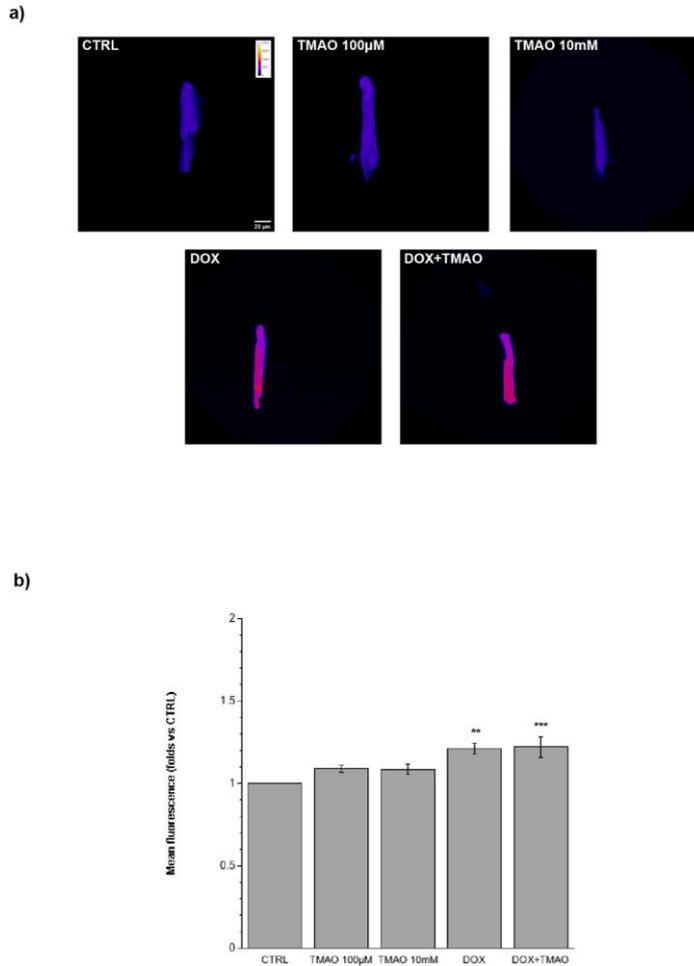112

113
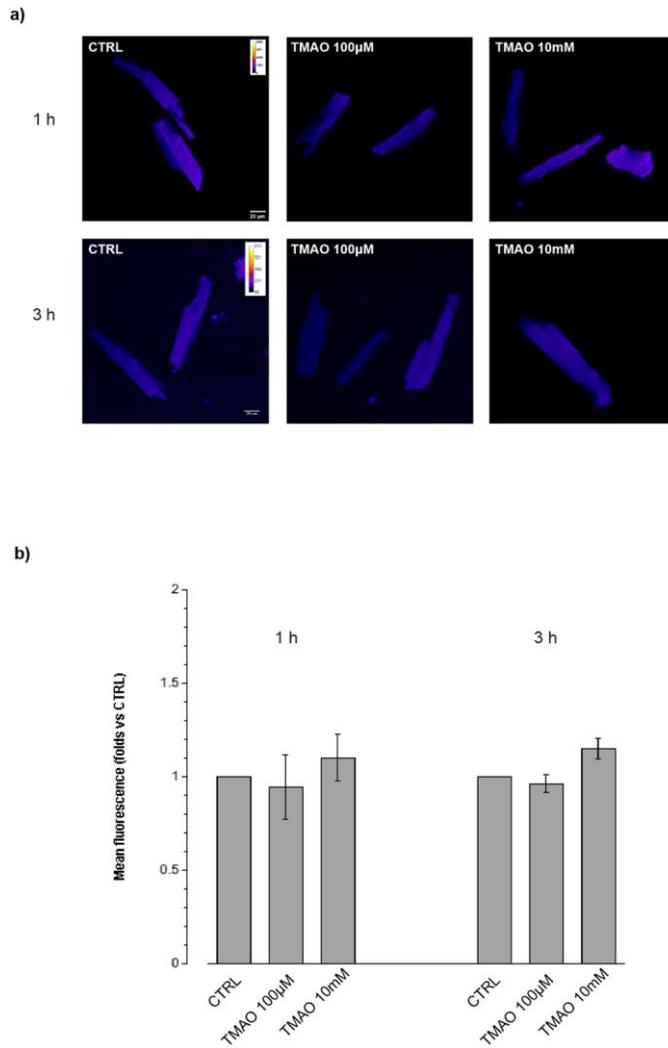
114 **Figure 3.** ROS production after 1 h and 3 h of treatment. a) Confocal microscopy images of cells treated
115 with TMAO 100µM and TMAO 10mM for 1 h and 3 h and labeled with DCF-DA probe (60X
116 magnification). b) Bar graph showing mean fluorescence after 1 h and 3 h of treatment, no variations
117 or ROS produced are detectable (refer to the main text for numerical values).

118

a)

b)

119

120 **Figure 4.** ROS production after 24 h of treatment. a) Confocal microscopy images of cells treated with
121 TMAO 100µM and TMAO 10mM for 24 h and labeled with DCF-DA probe. In these experiments
122 Doxorubicin (DOX) is used as positive control (60X magnification). b) Bar graph showing mean
123 fluorescence after 24 h of treatment. No variations of ROS produced are detectable after TMAO
124 treatment, and a small but significant increase is visible after DOX treatment (here used as positive
125 control), this increase is not changed by a simultaneous treatment with TMAO (refer to the main text
126 for numerical values).

127 *2.4 TMAO and mitochondrial membrane potential*

128 To investigate the potential metabolic damage induced by TMAO, cardiomyocytes treated with
129 TMAO 100µM and 1mM for 1 h, 3 h or 24 h are labeled with the JC-1 probe. Figures 5 (1 h, 3 h) and 6
130 (24 h) show variations in mitochondrial membrane potential (red/green fluorescence ratio) detected
131 by confocal microscopy in living cells. TMAO treatment from 1 to 24 h does not cause any difference
132 towards control, indicating no mitochondrial effect of the molecule, while, as expected, DOX cause a
133 depolarization of mitochondrial membrane potential after 24 h of treatment. TMAO 100µM does not

modify mitochondrial membrane potential in DOX treated cardiomyocytes (Fig. 6) (1 h: TMAO 100μM: 1.09±0.08, n=5, 65 cells; TMAO 10mM: 1.11±0.11, n=3, 49 cells, vs CTRL. 3 h: TMAO 100μM: 1.02±0.06, n=3, 26 cells; TMAO 10mM: 0.88±0.05, n=4, 39 cells, vs CTRL; 24 h: TMAO 100μM: 1.08±0.11, n=3, 21 cells; TMAO 10mM: 0.95±0.15, n=3, 54 cells; DOX: 0.73±0.02, n=3, 24 cells (**P<0.01); DOX+TMAO: 0.69±0.05, n=3, 22 cells (**P<0.01), vs CTRL).
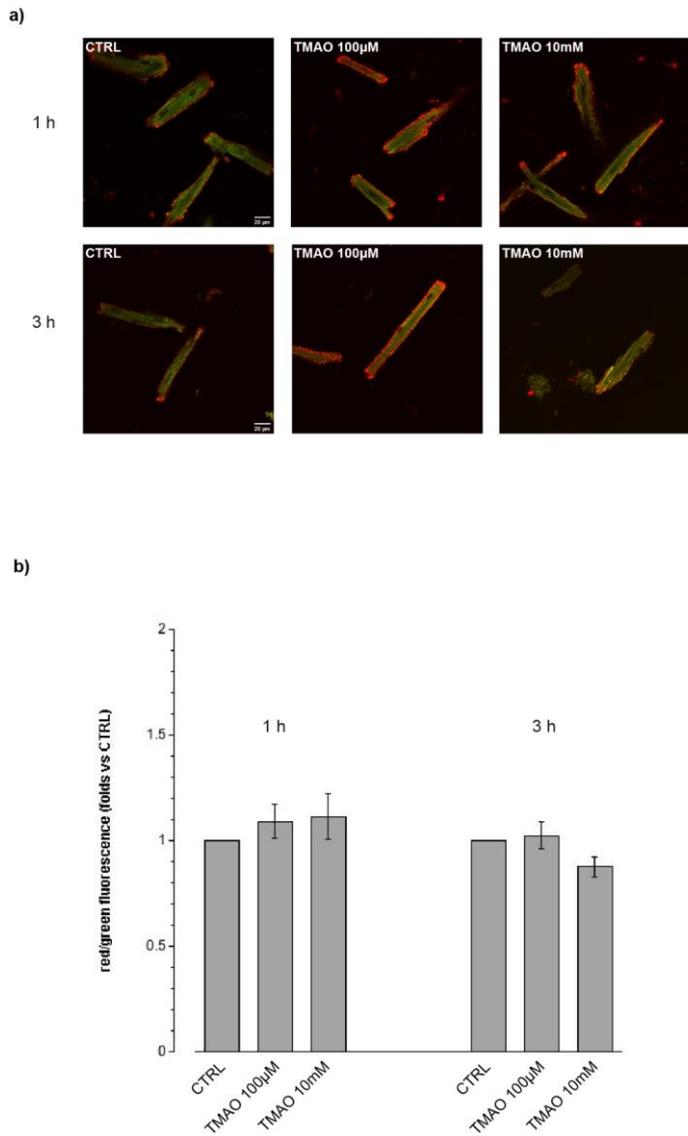
Figure 5. Mitochondrial membrane potential variation following 1 h and 3 h of treatment. a) Confocal microscopy images of cells treated with TMAO 100μM and TMAO 10mM for 1 h and 3 h and labeled with JC-1 probe (60X magnification). b) Bar graph showing red/green fluorescence after 1 h and 3 h of treatment, no variations of mitochondrial membrane potential is detected (refer to the main text for numerical values).

a)

b)
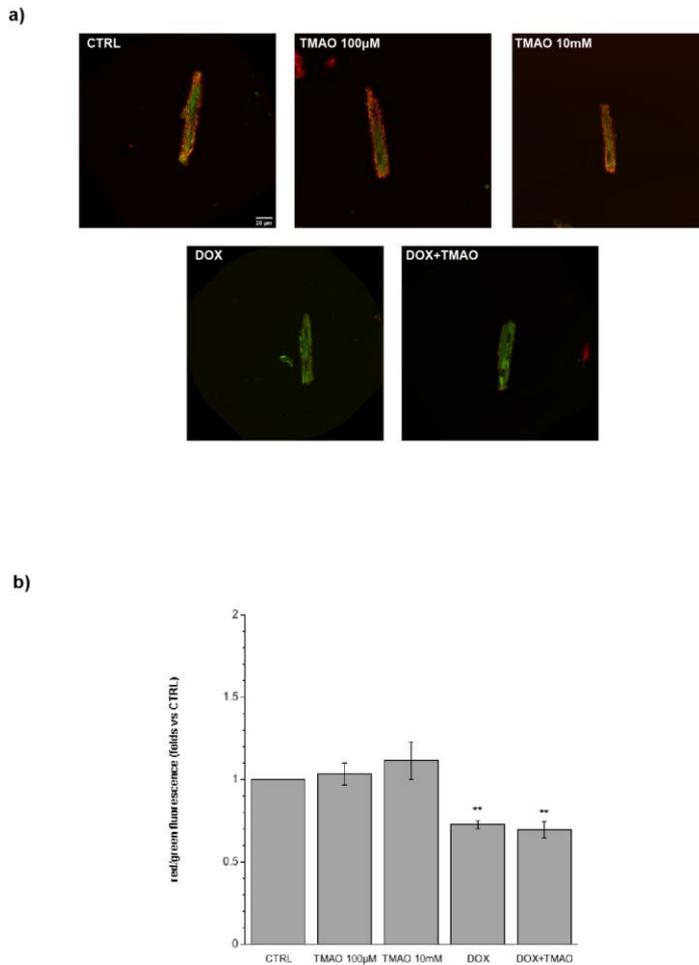
Figure 6. Mitochondrial membrane potential variation following 24 h treatment. a) Confocal microscopy images of cells treated with TMAO 100μM and TMAO 10mM for 24 h and labeled with JC-1 probe. In these experiments Doxorubicin (DOX) is used as positive control (60X magnification). b) Bar graph showing red/green fluorescence after 24 h of treatment, no variations of mitochondrial membrane potential is detected, a little but significant reduction of the ratio is visible after DOX treatment here used as positive control, condition not changed in a simultaneous treatment with TMAO (refer to the main text for numerical values).

3. Discussion

This study provides novel insights into the physiological role of TMAO on isolated adult rat cardiomyocytes. Our findings do not show effects of TMAO on cell viability, sarcomere length, ROS

159 production and mitochondrial membrane potential within the range of concentration used.
160 Moreover, we demonstrate that TMAO does not exacerbate or counteract the effect of known insults
161 such as H_2O_2 or Doxorubicin, tested for up to 24 h of treatment. Taken together these results suggest
162 that TMAO should not be considered a primary cause of acute cardiac damage and that the molecule
163 could not revert or worsen existing risk factors of cardiac damage.
164 In the last few years, many studies suggest a strong relationship among diet, gut microbiota and
165 cardiovascular diseases [16]. In particular, some attention has been pointed to either TMAO directly
166 coming from diet (fish), or produced from L-carnitine and choline conversion by gut microbiota into
167 TMA and oxidized in the liver by FMO3 enzymes [17, 14].
168 Experiments have mainly now focused on endothelial cells damaging role of TMAO. It has been
169 described to upregulate cellular senescence reducing cell proliferation, increasing the expression of
170 senescence markers, as p53 and p21, and impairing cell migration [3]. TMAO also increases
171 endothelial cells oxidative stress through a down-regulation of SIRT-1 and impairs NO production
172 that causes endothelial dysfunction [4]. ~~Prolonged hypertensive~~Hypertensive effects of TMAO
173 ~~angiotensin II~~ have been evaluated by Ufnal and colleagues who demonstrate that TMAO has a role
174 in stabilizing the action of Ang II and in prolonging its hypertensive effect, underlining the role of
175 TMAO in stabilizing protein conformation and no direct role of the molecule in mediating
176 hypertension ~~promoting this condition~~ [18]. Koeth and colleagues underline the strong relationship
177 among high consumption of TMAO precursors, high TMAO plasma concentrations and the
178 development of atherosclerosis [19], while another study underlines the effect of the metabolite in
179 enhancing platelet hyperreactivity and thrombosis risk in subjects with high TMAO plasma
180 concentrations [5]. In relation with cardiovascular effects of TMAO, Dambrova and collaborators
181 evidence that high plasma concentrations of the molecule are linked with increased body weight,
182 insulin resistance and it directly correlates with an augmented risk of diabetes [20].
183 Only a few studies are centered on the direct effect of the molecule on cardiac cells; in particular
184 they focus on the impairment of mitochondrial metabolism in the heart and underline TMAO as an
185 agent that increases the severity of cardiovascular events or that enhances the progression of
186 cardiovascular diseases [7]. Savi et colleagues show a damaging effect of TMAO in cardiomyocytes
187 because it worsens intracellular calcium handling with a reduced efficiency in the intracellular
188 calcium removal and consequent loss in functionality of cardiac cells, furthermore TMAO seems to
189 alter energetic metabolism and to facilitate protein oxidative damage [8].
190 This scenario presents TMAO as either a marker or a direct agent involved in vascular and
191 cardiac outcomes, but recent papers seem to oppose this point of view, highlighting uncertainty about
192 the causative relation between TMAO and CVD [9]. It is still debated the function of TMAO that is,
193 for example, controversial in fish-rich diets, because of the higher bioavailability of the compound in
194 seafood products and their well-known role in lowering risk of CVD. Also, TMAO does not enhance
195 atherosclerosis development because it seems not to be involved in foam cell formation even at higher
196 concentrations than physiological ones [12], and, there is no direct correlation between high plasma
197 TMAO concentrations and coronary heart diseases [21, 22]. Last findings by Huc et al., underline also
198 a protective role of TMAO in reducing diastolic dysfunction and fibrosis in pressure-overloaded heart
199 [23].
200 The present study fits into this debate and the results presented agree with other works
201 supporting TMAO as a non-damaging factor. In fact, it is well known that loss of vital cardiac cells is
202 a damaging condition that hampers primarily the functionality of the heart and has several
203 aggravating responses also in peripheral tissues. Our first investigations underline no toxic effect in
204 cardiomyocytes exposed to high concentrations of TMAO highlighting that the molecule is not
205 involved in inducing cardiac tissue cells loss ~~(Fig. 1)~~, and, no alterations of cardiac structure emerge
206 from the evaluation of sarcomere length and cytoskeletal organization ~~(Fig. 2)~~. Oxidative stress could
207 be considered one of the causative factors of senescence in cells, and one of the promoters of
208 cardiometabolic reorganization in response to injury. Considering TMAO as a possible inducer of
209 ROS rising, both in cytoplasmic and mitochondrial environment, we show no variation in ROS
210 production even after 24 h of treatment ~~(Fig. 3 and 4)~~ and we detect no depolarization of

mitochondrial membrane potential underling no direct influence of the molecule in inducing cardiac cell senescence (Fig. 5 and 6).

Formatted: Strikethrough

4. Materials and Methods

4.1 Animal care and sacrifice

Experiments are performed on female adult rats which are allowed *ad libitum* access to tap water and standard rodent diet. The animals receive human care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and in accordance with Italian law (DL-116, Jan. 27, 1992). The scientific project is supervised and approved by the Italian Ministry of Health, Rome, and by the ethical committee of the University of Torino. Rats are anaesthetized by i.p. injection of Pentobarbital (Nembutal, 100 mg/Kg) and killed by stunning and cervical dislocation.

4.2 Solutions and drugs

Tyrode standard solution containcontaining (in mM): 154 NaCl, 4 KCl, 1 $MgCl_2$, 5.5 D-glucose, 5 HEPES, 2 $CaCl_2$, pH adjusted to 7.34 with NaOH. Ca^{2+} free Tyrode solution contain (in mM): 154 NaCl, 4 KCl, 1 $MgCl_2$, 5.5 D-glucose, 5 HEPES, 10 2,3-Butanedione monoxime, 5 taurine, pH 7.34. All drug-containing solutions are prepared fresh before the experiments and Tyrode solutions are oxygenated (O_2 100%) before each experiment. Unless otherwise specified, all reagents for cells isolation and experiments are purchased from Sigma-Aldrich.

4.3 Adult rat ventricular cells isolation

Isolated cardiomyocytes are obtained from the hearts of adult rats (200–300 g body wt) according to the previously described method [2324]. Briefly, after sacrifice, the rat hearts are explanted, washed in Ca^{2+} free Tyrode solution and cannulated via the aorta. All the following operations are carried on under a laminar flow hood. The heart is perfused at a constant flow rate of 10 ml/min with Ca^{2+} free Tyrode solution (37°C) with a peristaltic pump for approximately 5 min to wash away the blood and then with 10 ml of Ca^{2+} free Tyrode supplemented with collagenase (0.3mg/ml) and protease (0.02mg/ml). Hearts are then perfused and enzymatically dissociated with 20ml of Ca^{2+} free Tyrode containing 50μM $CaCl_2$ and the same enzymatic concentration as before. Atria and ventricles are then separated and the ventricles are cut in small pieces and shaken for 10 minutes in 20ml of Ca^{2+} free Tyrode solution in presence of 50μM $CaCl_2$, collagenase and protease. Calcium ions concentration is slowly increased to 0.8mM. Cardiomyocytes are then plated on glass cover slips or glass bottom dishes (Ibidi, Germany), both treated with laminin to allow cell adhesion.

4.4 Cell viability

Cell viability is evaluated by propidium iodide (PI) staining on glass bottom dishes adherent cells. At the end of the treatments cells are incubated with PI (10μg/ml, Invitrogen) for 5 min in the dark. Nuclei of suffering cells are detected with confocal microscopy using Olympus Fluoview 200 microscope at 568nm (magnification 20X). Merged images are created with ImageJ (Rasband, W. S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2017) and cells viability is calculated as percentage of (total cells-labeled cells)/total cells.

4.5 Evaluation of sarcomere length

Cardiomyocytes on glass coverslips are stimulated with TMAO and H_2O_2, as a positive control. Cells are treated for 24 h with TMAO, at 100μM and 10mM, then the sarcomere protein α-actinin, localized in the Z lines, has been detected using confocal microscopy. Then cells are fixed in 4% PFA for 40 min. After two washes with PBS, cells are incubated for 20 min with 0.3% Triton and 1% bovine serum albumin (BSA) in PBS and stained for 24 h at +4°C with a mouse monoclonal anti-α-actinin primary antibody (Sigma, 1:800). Cover slides are washed twice with PBS and incubated 1 h at room

256 temperature with the secondary antibody (1:2000, anti-mouse Alexa Fluor 568, Thermo Fisher). After
257 two washes in PBS, coverslips are mounted on standard slides with DABCO and observed after 24 h
258 under confocal microscope. Confocal fluorimetric measurements are acquired using a Leica SP2 laser
259 scanning confocal system, equipped with a 40X water-immersion objective. Image processing and
260 analysis are performed with ImageJ software. Sarcomere length is evaluated measuring the distance
261 between Z lanes in n=10 sarcomeres/cell.

262   *4.6 Intracellular Reactive Oxygen Species (ROS) measurement*

263   Production of ROS is evaluated by fluorescence microscopy using the 2'-7'-Dichlorofluorescein
264 diacetate probe (DCF-DA). After adhesion on glass bottom dishes, DCF-DA solution (5µg/ml) is
265 added to each dish 30min prior to the end of the treatment, then cells are washed with standard
266 Tyrode solution. Fluorescence images at 488nm have been acquired with Olympus Fluoview 200
267 microscope (magnification 60X). Fluorescence variations are calculated with the definition and
268 measurement of Regions Of Interest (ROIs) using the software ImageJ and expressed as relative
269 Medium Fluorescence Index (MFI) compared to control, fixed at 1.

270   *4.7 Mitochondrial membrane potential measurement*

271   Mitochondrial membrane potential is evaluated by staining cardiomyocytes with the dye
272 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1)**.** JC-1 solution (10µM) is
273 added to each dish 30 min prior to the end of the treatment, then cells are washed with Tyrode
274 standard. Fluorescence images at 488nm and 568nm have been acquired with Olympus Fluoview 200
275 microscope (magnification 60X). Amounts of the monomeric form of the dye are quantified using the
276 red/green fluorescence ratio in the ROIs using the software ImageJ and expressed as folds towards
277 control, fixed at 1.

278   *4.8 ~~Statitical~~Statistical analysis*

279   All data are expressed as mean±Standard Errors of the mean. For differences between mean
280 values Bonferroni's multiple comparisons test has been performed. Differences with P<0.05 are
281 regarded as statistically significant.

5. Conclusions

283   In summary, this study demonstrates that TMAO is not directly involved in causing or
284 exacerbating cardiac damage in an acute stress model (Fig. 7). However, there are some limitation of
285 this study: a very wide range of plasmatic TMAO concentrations is presented in literature, ~~and even~~
286 within different mammals, and also between different sexes of the same species, several orders of
287 magnitude can be considered physiological [24, 25, 26], so, to test direct effect of the molecule, it has
288 been used high concentrations, even over human physiological ones. Another weakness of the study
289 could be linked to the time of treatment, because evaluations are no longer than 24 h and they could
290 only represent an acute exposure to TMAO. ~~In~~Moreover, in order to evaluate more deeply the
291 mechanism involved in TMAO-mediated responses it could be necessary to treat cells for longer time
292 to assess a chronic stress compatible with the development of CVD. Furthermore we only study
293 TMAO effect on female ventricular cardiomyocytes and it could be interesting to extend the analysis
294 also to male cardiomyocytes as gender differences have been observed in cardioprotective
295 mechanisms [27] and TMAO induced intracellular calcium imbalance has been described in male
296 cardiomyocytes [8]. Finally, our findings provide new insights into cardiac effect of TMAO, exploring
297 the direct treatment of isolated cardiomyocytes.

298
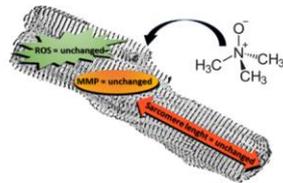
Figure 7. No effects of TMAO is detectable on isolated adult rat cardiomyocytes viability, sarcomere length, ROS production and mitochondrial membrane potential (MMP).

Abbreviations

TMAO	Trimethylamine N-oxide
PI	Propidium iodide
ROS	Reactive Oxygen Species
DCF-DA	2′-7′-Dichlorofluorescein diacetate
JC-1	5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide

References

1. Ufnal, M.; Zadlo, A.; Ostaszewski, R. TMAO: A small molecule of great expectations. *Nutrition* **2015**, *31*(11), 1317–1323. https://doi.org/10.1016/j.nut.2015.05.006

2. Yancey, P. H. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology* **2005**, *208*(Pt 15), 2819–2830. https://doi.org/10.1242/jeb.0173

3. Ke, Y.; Li, D.; Zhao, M.; Liu, C.; Liu, J.; Zeng, A.; Shi, X.; Cheng, S.; Pan B.; Zheng , L.; Hong, H. Gut flora-dependent metabolite Trimethylamine-N-oxide accelerates endothelial cell senescence and vascular aging through oxidative stress. *Free Radical Biology & Medicine* **2018**, *116*, 88–100. https://doi.org/10.1016/j.freeradbiomed.2018.01.007

4. Li, T.; Chen, Y.; Gua, C.; Li, X. Elevated Circulating Trimethylamine N-Oxide Levels Contribute to Endothelial Dysfunction in Aged Rats through Vascular Inflammation and Oxidative Stress. *Frontiers in Physiology* **2017**, *8*. https://doi.org/10.3389/fphys.2017.00350

5. Zhu, W.; Gregory, J. C.; Org, E.; Buffa, J. A.; Gupta, N.; Wang, Z.; Li, L.; Fu, X.; Wu, Y.; Mehrabian, M.; Balfour Sartor, R.; McIntyre, T. M.; Silverstein, R. L.; Tang, W. H. W.; DiDonato, J. A.; Brown, J. M.; Lusis,

324   A. J.; Hazen, S. L. Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk.
325   *Cell* **2016**, *165*(1), 111–124. https://doi.org/10.1016/j.cell.2016.02.011

326   6.   Shan, Z.; Sun, T.; Huang, H.; Chen, S.; Chen, L.; Luo, C.; Yang, W.; Yang, X.; Yoa, P.; Cheng, J.; Hu, F. B.;
327   Liu, L. Association between microbiota-dependent metabolite trimethylamine-N-oxide and type 2 diabetes.
328   *The American Journal of Clinical Nutrition* **2017**, ajcn157107. https://doi.org/10.3945/ajcn.117.157107

329   7.   Makrecka-Kuka, M.; Volska, K.; Antone, U.; Vilskersts, R.; Grinberga, S.; Bandere, D.; Liepinsh, E.;
330   Dambrova, M. Trimethylamine N-oxide impairs pyruvate and fatty acid oxidation in cardiac mitochondria.
331   *Toxicology Letters* **2017**, *267*, 32–38. https://doi.org/10.1016/j.toxlet.2016.12.017

332   8.   Savi, M.; Bocchi, L.; Bresciani, L.; Falco, A.; Quaini, F.; Mena, P.; Brighenti, F.; Crozier, A.; Stilli, D.; Del Rio,
333   D. Trimethylamine-N-Oxide (TMAO)-Induced Impairment of Cardiomyocyte Function and the Protective
334   Role of Urolithin B-Glucuronide. *Molecules* **2018**, *23*(3), 549. https://doi.org/10.3390/molecules23030549

335   9.   Arduini, A.; Zammit, V. A.; Bonomini, M. Identification of trimethylamine N-oxide (TMAO)-producer
336   phenotype is interesting, but is it helpful? *Gut* **2019**, *0*, 1. https://doi.org/10.1136/gutjnl-2018-318000

337   10. Nowiński, A.; Ufnal, M. Trimethylamine N-oxide: A harmful, protective or diagnostic marker in lifestyle
338   diseases? *Nutrition* **2018**, *46*, 7–12. https://doi.org/10.1016/j.nut.2017.08.001

339   11. Dumas, M.-E.; Maibaum, E. C.; Teague, C.; Ueshima, H.; Zhou, B.; Lindon, J. C.; Nicholson, J. K.; Stamler,
340   J.; Elliott, P.; Chan, Q.; Holmes, E. Assessment of analytical reproducibility of 1H NMR spectroscopy based
341   metabonomics for large-scale epidemiological research: the INTERMAP Study. *Analytical Chemistry* **2006**,
342   *78*(7), 2199–2208. https://doi.org/10.1021/ac0517085

343   12. Collins, H. L.; Drazul-Schrader, D.; Sulpizio, A. C.; Koster, P. D.; Williamson, Y.; Adelman, S. J.; Owen, K.;
344   Sanli, T.; Bellamine, A. L-Carnitine intake and high trimethylamine N-oxide plasma levels correlate with
345   low aortic lesions in ApoE−/− transgenic mice expressing CETP. *Atherosclerosis* **2016**, *244*(C), 9–37.
346   https://doi.org/10.1016/j.atherosclerosis.2015.10.108

347   13. Lupachyk, S.; Watcho, P.; Stavniichuk, R.; Shevalye, H.; Obrosova, I. G. Endoplasmic Reticulum Stress
348   Plays a Key Role in the Pathogenesis of Diabetic Peripheral Neuropathy. *Diabetes* **2013**, DB_120716.
349   https://doi.org/10.2337/db12-0716

350   14. Zeisel, S. H.; Warrier, M. Trimethylamine N-Oxide, the Microbiome, and Heart and Kidney Disease. *Annual
351   Review of Nutrition* **2017**, *37*, 157–181. https://doi.org/10.1146/annurev-nutr-071816-064732

352   15. Sarvazyan, N. Visualization of doxorubicin-induced oxidative stress in isolated cardiac myocytes. *The
353   American Journal of Physiology* **1996**, *271*(5 Pt 2), H2079-2085.
354   https://doi.org/10.1152/ajpheart.1996.271.5.H2079

355   16. Zununi Vahed, S.; Barzegari, A.; Zuluaga, M.; Letoumeur, D.; Pavon-Djavid, G. Myocardial infarction and
356   gut microbiota: an incidental connection. *Pharmacological Research* **2018**, 129, 308-317.
357   http://doi.org/10.1016/j.phrs.2017.11.008

358 17. Cho, C. E.; Taesuwan, S.; Malysheva, O. V.; Bender, E.; Tulchinsky, N. F.; Yan, J.; Sutter, J. L.; Caudill, M.
359 A. Trimethylamine-N-oxide (TMAO) response to animal source foods varies among healthy young men
360 and is influenced by their gut microbiota composition: A randomized controlled trial. *Molecular Nutrition*
361 *& Food Research* **2017**, *61*(1). https://doi.org/10.1002/mnfr.201600324

362 18. Ufnal, M.; Jazwiec, R.; Dadlez, M.; Drapala, A.; Sikora, M.; Skrzypecki, J. Trimethylamine-N-oxide: a
363 carnitine-derived metabolite that prolongs the hypertensive effect of angiotensin II in rats. *The Canadian*
364 *Journal of Cardiology* **2014**, *30*(12), 1700–1705. https://doi.org/10.1016/j.cjca.2014.09.010

365 19. 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.;
366 Smith, J. D.; DiDonato, J. A.; Chen, J.; Li, H.; Wu, G. D.; Lewis, J. D.; Warrier, M.; Brown, J. M.; Krauss, R.
367 M.; Tang, W. H. W.; Bushman, F. D.; Lusis, A. J.; Hazen, S. L. Intestinal microbiota metabolism of L-
368 carnitine, a nutrient in red meat, promotes atherosclerosis. *Nature Medicine* **2013**, *19*(5), 576.
369 https://doi.org/10.1038/nm.3145

370 20. Dambrova, M.; Latkovskis, G.; Kuka, J.; Strele, I.; Konrade, I.; Grinberga, S.; Hartmane, D.; Pugovics, O.;
371 Erglis, A.; Liepinsh, E. Diabetes is Associated with Higher Trimethylamine N-oxide Plasma Levels.
372 *Experimental and Clinical Endocrinology & Diabetes: Official Journal, German Society of Endocrinology [and]*
373 *German Diabetes Association* **2016**, *124*(4), 251–256. https://doi.org/10.1055/s-0035-156933

374 21. Mueller, D. M.; Allenspach, M.; Othman, A.; Saely, C. H.; Muendlein, A.; Vonbank, A.; Drexel, H.; von
375 Eckardstein, A. Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and
376 poor metabolic control. *Atherosclerosis* **2015**, *243*(2), 638–644.
377 https://doi.org/10.1016/j.atherosclerosis.2015.10.091

378 22. Yin, J.; Liao, S.-X.; He, Y.; Wang, S.; Xia, G.-H.; Liu, F.-T.; Zhu, J.-J.; You, C.; Chen, Q.; Zhou, L.; Pan, S.-Y.;
379 Zhou, H.-W. Dysbiosis of Gut Microbiota With Reduced Trimethylamine-N-Oxide Level in Patients With
380 Large-Artery Atherosclerotic Stroke or Transient Ischemic Attack. *Journal of the American Heart Association*
381 **2015**, *4*(11). https://doi.org/10.1161/JAHA.115.002699

382 23. Huc, T.; Drapala, A.; Gawrys, M.; Konop, M.; Bielinska, K.; Zaorska, E.; Samborowska, E.; Wyczalkowska-
383 Tomasik, A.; Paczek, L.; Dadlez, M.; Ufnal, M. Chronic, low-dose TMAO treatment reduces diastolic
384 dysfunction and heart fibrosis in hypertensive rats. American Journal of Physiology Heart and circulatory
385 Physiology **2018**, 315, H1805-H1820. https://doi.org/10.1152/ajpheart.00536.2018

386 23.24. Gallo, M. P.; Femminò, S.; Antoniotti, S.; Querio, G.; Alloatti, G.; Levi, R. Catestatin induces glucose
387 uptake and GLUT4 trafficking in adult rat cardiomyocytes. BioMed Research International **2018**,
388 https://doi.org/10.1155/2018/2086109

389 24.25. Lenky, C. C.; McEntyre, C. J.; Lever, M. Measurement of marine osmolytes in mammalian serum by
390 liquid chromatography-tandem mass spectrometry. Analytical Biochemistry **2012**, 420, 7-12.
391 https://doi.org/10.1016/j.ab.2011.09.013

392 25.26. Somero, G. N.; From dogfish to dogs: trimethylamines protect proteins from urea. America Journal
393 of Physiology **1986**, 1, 9-12. https://doi.org/10.1152/physiologyonline.1986.1.1.9

27. Lagranha, C. L.; Deschamps, A.; Aponte, A.; Steenbergen, C.; Murphy, E. Sex differences in the phosphorylation of mitochondrial proteins result in reduced production of reactive oxygen species and cardioprotection on females. Circulation Research **2010**, 106(11), 1981-91. http://doi.org/10.1161/CIRCRESAHA.109.213645