

# 1 PUFA and Oxidative Stress. Differential Modulation of the Cell 2 Response by DHA

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## 12 **Abstract:**

13 Although an increased dietary intake of long-chain n-3 PUFA is considered an effective  
14 preventive strategy, a theoretical concern related to the possible increase of lipid  
15 peroxidation induced by a PUFA-rich diet still remains. In this study, the effects of  
16 different PUFA (**linoleic,  $\alpha$ -linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic**  
17 **acid**) on cytotoxicity, lipid oxidation, and modulation of antioxidant defenses were  
18 evaluated in HepG2 cells submitted to an oxidative stress (H<sub>2</sub>O<sub>2</sub>). Results clearly evidenced  
19 that all supplemented PUFA but DHA enhanced cell susceptibility to H<sub>2</sub>O<sub>2</sub>. Overall, our  
20 results underline that PUFA cannot be considered as a single category but as individual  
21 compounds, and research on mechanisms of action and preventive effects should deal with  
22 the individual fatty acids, particularly in the case of DHA.

23 **Keywords:** HepG2 cells; hydrogen peroxide; cytotoxicity; antioxidant enzymes  
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## 26 **1. Introduction**

27 There are converging opinions among experts, organizations and health professionals that a  
28 recommendation for a daily individual consumption of 500 mg of eicosapentaenoic (EPA)/  
29 docosahexaenoic (DHA) acids would provide health benefits (Salem & Eggersdorfer, 2015). An  
30 increased dietary intake of n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), EPA and DHA,  
31 is considered an effective strategy in the prevention and modulation of cardiovascular (Ohnishi &  
32 Saito, 2013; Cao et al., 2015) and neurological diseases (Patrick & Ames, 2015), for the optimization  
33 of cognitive performance (Muldoon et al., 2014) and brain development (Brenna & Carlson, 2014).

34 Since DHA and other PUFA are highly oxidizable molecules (Miyashita, 2014), a theoretical  
35 concern remains on their use at high concentration in the prevention of chronic diseases having the  
36 oxidative stress as one of the underlying mechanisms.

37 Besides being prone to oxidation, PUFA have been reported to activate the stress-signaling pathway  
38 controlling the genetic program used by cells to induce the synthesis of proteins responsible for  
39 detoxification and counteraction of the oxidative stress (Ma, 2013). In this way, PUFA can modulate  
40 the activity of antioxidant enzymes (Wang et al., 2004) to ensure survival and reduced status.

41 Supplementing cultured cells with individual PUFA we previously observed diverse effects on  
42 oxidation and modulation of antioxidant defenses. The different effects were not simply related to the  
43 length of the carbon chain, or to the number and position of double bonds in the fatty acid (Di Nunzio  
44 et al., 2011). In particular, DHA supplementation did not cause any adverse effect, suggesting that its  
45 use as preventive dietary strategy does not induce a higher sensitivity to oxidation. It is conceivable  
46 that the overall effects of the different PUFA are related to their individual ability to increase the  
47 intracellular concentration of pro- or antioxidants.

48 In this light, the concomitant oxidative status of the cell could have a role in its response to PUFA  
49 supplementation, and the differential effect of DHA than other PUFA needs to be confirmed in cells  
50 submitted to an oxidative insult. To evaluate it, in the present work, we supplemented HepG2 cells  
51 with **linoleic acid (18:2n-6, LA)**,  **$\alpha$ -linolenic acid (18:3n-3, ALA)**, **arachidonic acid (20:4n-6, ARA)**,  
52 **eicosapentaenoic acid (20:5n-3, EPA)**, and **docosahexaenoic acid (22:6n-3, DHA)**, then a mild  
53 oxidative stress **reflecting physiological condition** was induced (Halliwell et al., 2000; González-  
54 Flecha et al., 1993; Khassaf et al., 2001). HepG2 cells were used as model system since liver is a major  
55 organ attacked by reactive oxygen species (ROS) (Sánchez-Valle et al., 2012), and parenchymal cells  
56 are primary cells subjected to oxidative stress induced injury in the liver. Cells were supplemented  
57 with physiological concentration (60  $\mu$ M) of n-6 and n-3 PUFA, and cell survival, lipid oxidation, and  
58 antioxidant defenses were evaluated after exposure to hydrogen peroxide.

## 59 **2. Experimental Section**

### 60 *2.1. Chemical*

61 Dulbecco's modified Eagle's medium (DMEM), and Dulbecco's phosphate-buffered saline (DPBS)  
62 were purchased from Lonza (Milan, Italy). All chemicals and solvents were of the highest analytical  
63 grade and were purchased from Sigma-Aldrich (Milan, Italy).

### 64 *2.2. HepG2 cells tissue culture*

65 HepG2 human hepatoma cells were maintained at 37°C, 95% air, 5% CO<sub>2</sub> in DMEM supplemented  
66 with 10% (v/v) fetal calf serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Di Nunzio et al.,  
67 2010). Once a week cells were split 1:20 into a new 75cm<sup>2</sup> flask, and medium was refreshed after 72 h.

### 68 *2.3. Fatty acid supplementation and oxidative stress*

69 Cells were seeded in 6-well plates, and after 24 hours, at 75-80% confluence, they were incubated  
70 for 21 h with the different fatty acids at 60  $\mu$ M concentration. Free fatty acids were dissolved in 100%

71 isopropanol, and complexed to bovine serum albumin (BSA). Fatty acid-BSA complexes were  
72 prepared fresh each time, and BSA final concentration was 0.5% in serum free DMEM.  
73 Unsupplemented (US) cells received corresponding amounts of BSA and isopropanol, which final  
74 concentration in the media was kept below 1% (v/v). LA, ALA, ARA, EPA, and DHA were used for  
75 cell supplementation. After 21 h incubation cells were washed twice with warm DPBS, and then  
76 exposed for 20 or 60 min to 0.2 mM H<sub>2</sub>O<sub>2</sub> in Earle's Balanced Salt Solution (EBSS) (116 mM NaCl,  
77 5.4 mM KCl, 0.8 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, 2.38 mM CaCl<sub>2</sub>, 0.39 mM MgSO<sub>4</sub>). Some  
78 unsupplemented cells received EBSS only (control cells). Then EBSS medium was collected,  
79 centrifuged at 400 g for 3 min, and used to evaluate LDH release and TBARS concentration. Cells  
80 were washed twice with warm DPBS, and processed for further analysis.

#### 81 *2.4. Lactate dehydrogenase (LDH) release*

82 LDH activity in the EBSS was assessed following spectrophotometrically for 1 min the rate of  
83 NADH oxidation at 340 nm (Di Nunzio et al., 2013), and enzyme activity was expressed as  
84 mUnits/ml.

#### 85 *2.5. Thiobarbituric acid reactive substances (TBARS) concentration*

86 TBARS, the end products of lipid peroxidation, were assayed in EBSS as previously reported by  
87 Valli *et al.* (Valli et al., 2012), and their concentration was expressed as relative fluorescence units  
88 (RFU) normalized for mg protein in the corresponding well.

#### 89 *2.6. Cell viability*

90 Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
91 (MTT) assay, based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystal by  
92 metabolic active cells (Danesi et al., 2011). Results were expressed as percent of control cell viability,  
93 assigned as 100%.

#### 94 *2.7. Conjugated diene (CD) level*

95 Cellular level of CD containing lipids was assayed as reported by Di Nunzio *et al.* (Di Nunzio et al.,  
96 2011), and expressed as percent of the value detected in control cells, assigned to 100%.

#### 97 *2.8. Cytoplasmic total antioxidant activity (TAA)*

98 Cytoplasmic TAA was measured as reported by Di Nunzio *et al.* (Di Nunzio et al., 2013), based on  
99 the ability of the antioxidant molecules in the sample to reduce the radical cation of ABTS. Values  
100 were normalized for protein content in the sample, and expressed as micromoles of trolox equivalent  
101 (TE)/mg protein.

#### 102 *2.9. Reduced glutathione (GSH) content*

103 Cytoplasmic GSH content was determined as previously described (Valli et al., 2012), normalized  
104 for protein content in the sample, and expressed as nmoles of GSH/mg protein.

### 105 2.10. Antioxidant enzymes activity

106 Cytoplasmic superoxide dismutase (SOD) activity was assayed using a commercial kit (Cayman  
107 Chemical, Michigan, USA) following the manufacture instructions. SOD activity (Units) was then  
108 normalized for mg of protein in the sample. Cytoplasmic catalase (CAT) activity was assayed  
109 spectrophotometrically at 240 nm following the extinction of H<sub>2</sub>O<sub>2</sub> (Danesi et al., 2006), normalized  
110 for protein content in the sample, and expressed as Units/mg protein. Cytoplasmic glutathione-S-  
111 transferase (GST) activity was assayed as previously reported by Di Nunzio *et al.* (Di Nunzio et al.,  
112 2011). GST activity (nmol/min) was then normalized for protein content in the sample. Cytoplasmic  
113 glutathione peroxidase (GPx) activity was assayed using a commercial kit (Cayman Chemical,  
114 Michigan, USA) following the manufacture instructions. GPx activity (nmol/min) was then normalized  
115 for protein content in the sample.

### 116 2.11. Protein content

117 Protein content was determined according to Bradford (Bradford, 1976), using BSA as standard.

### 118 2.12. Statistical analysis

119 Data are reported as mean  $\pm$  SD of at least six samples derived from three independent cell cultures.  
120 Statistical analysis was by the one way ANOVA with Dunnett as post-test.

## 121 3. Results

122 To evidence the detrimental effect of H<sub>2</sub>O<sub>2</sub> addition, control cells were compared to stressed cells,  
123 both US and fatty acid supplemented. Then a statistical comparison was performed among stressed  
124 cells, to verify the differential effect of the single fatty acids, and to evidence whether the diverse  
125 supplementation could protect or exacerbate the outcomes of the oxidative stress.

### 126 3.1. H<sub>2</sub>O<sub>2</sub>-induced cell oxidative damage

127 The H<sub>2</sub>O<sub>2</sub>-induced cell damage was evaluated by measuring cell viability, LDH release, TBARS  
128 level and CD content (figure 1). As shown in figure 1A, in all cells H<sub>2</sub>O<sub>2</sub> exposure caused a significant  
129 reduction of viability with respect to controls. Among stressed cells, viability decreased in all  
130 supplemented cells, but DHA.

131 The oxidative stress caused a significant increase in LDH activity and TBARS concentration in the  
132 medium of all cells except the US and DHA supplemented ones (figure 1B and 1C, respectively).  
133 Notwithstanding the increase of TBARS in almost all stressed cells, the level of CD, the first products  
134 of lipid peroxidation, increased in LA and ARA supplemented cells only (figure 1D).

135

### 136 3.2. Modulation of antioxidant defenses

137 To evaluate the effect of PUFA supplementation on the cell response to an oxidative stress,  
138 cytosolic TAA and GSH content (figure 2), and the activity of SOD, CAT, GST and GPx enzymes  
139 (figure 3) were determined.

140 Compared to controls, H<sub>2</sub>O<sub>2</sub> exposure caused a significant decrease of cytosolic TAA in US and LA  
141 supplemented cells only (figure 2A), and no modification in GSH content apart from a significant  
142 increase in DHA supplemented cells (figure 2B). Comparing stressed cells, TAA appeared higher in  
143 ARA and DHA supplemented hepatocytes, the latter also showing a higher GSH concentration.

144 Compared to controls, upon H<sub>2</sub>O<sub>2</sub> exposure a significant increase of SOD activity was detected in  
145 LA and n-3 PUFA supplemented cells (figure 3A), and CAT activity increased in all cells (figure 3B).  
146 GST activity was not modified in US cells, while it significantly increased in all supplemented cells  
147 but DHA ones (figure 3C). Furthermore, a significant increase in GPx activity was detected in LA  
148 supplemented cells only (figure 3D). Comparing stressed cells, a differential influence of the  
149 supplemented fatty acids on enzyme activities was evidenced. SOD activity being higher in EPA and  
150 DHA supplemented, CAT in ARA and EPA supplemented, GST in ARA supplemented, and GPx in  
151 LA supplemented cells than in US ones.

152 Since cellular oxidative status may be influenced by nonlethal oxidative stress exposure in a time  
153 depending manner (Brunk et al., 1995), SOD, CAT, GST and GPx activities were also evaluated after  
154 20 min H<sub>2</sub>O<sub>2</sub> exposure (figure 4A, 4B, 4C and 4D respectively).

155 Compared to controls, upon the shorter H<sub>2</sub>O<sub>2</sub> exposure time SOD activity increased in ARA and  
156 EPA supplemented cells only, and no significant modification in CAT and GPx activity was detected.  
157 GST activity was reduced in all supplemented cells except DHA and US ones. Comparing stressed  
158 cells a differential effect of the supplementation on SOD, CAT and GST activities was still detected,  
159 while no differences were evidenced in GPx activity.

#### 160 **4. Discussion**

161 n-6 and n-3 PUFA are well known for their physiological functions and preventive actions (Liu et  
162 al., 2015; Pelliccia et al., 2013) that are ascribable to different mechanisms including regulation of  
163 membrane functionality, cell signaling, and gene expression (Robichaud & Surette, 2015; Righi et al.,  
164 2011; Bordoni et al., 2007). In contrast to their health effects, PUFAs are known to be prone to rapid  
165 peroxidation, which has been shown to impair membrane functions and inactivate proteins and  
166 enzymes, eventually leading to various disorders and diseases (Csala et al., 2015). To explain this  
167 apparent oxymoron, it has been speculated that the lipid peroxidation occurring in PUFA-rich cells  
168 causes a low level, sub-toxic stress that could induce a protective adaptations over time (i.e. hormesis),  
169 contributing to PUFA health effects. Actually, n-3 PUFA have been shown to up-regulate the  
170 antioxidant systems in various cells (Giordano & Visioli, 2014), so counteracting the oxidative stress  
171 (Zanatta et al., 2014; Tourtas et al., 2012). Although different PUFA share common chemical  
172 characteristics, in a previous work we evidenced that they differently modulate the activity of  
173 antioxidant enzymes, and have a different impact on cell oxidative status (Di Nunzio et al., 2011). A  
174 concomitant oxidative stress could have an impact on the effects of PUFA, this representing an  
175 important issue since a higher intake of PUFA, particularly n-3 PUFA, is suggested for the  
176 prevention/modulation of a wide range of diseases associated with an increased production of ROS

177 (Fischer & Maier, 2015; Reuter et al., 2010; Pashkow, 2011; Salomone et al., 2016). To further  
178 elucidate it, in the present work we have supplemented different PUFA to HepG2 cells, which were  
179 then submitted to a mild oxidative stress.

180 Results herein reported clearly evidence that the cell response to a subsequent oxidative stress can  
181 be modulated by a preceding PUFA supplementation.

182 All supplemented fatty acids except DHA enhance cell susceptibility to H<sub>2</sub>O<sub>2</sub>. This could be due to  
183 an increased lipid peroxidation inducing disturbance of fine structures, alteration of integrity, fluidity,  
184 and permeability, and functional loss of biomembranes, and generates potentially toxic products  
185 (Ayala et al., 2014), as indicated by the increase in TBARS concentration in the medium of all  
186 supplemented cells but DHA supplemented ones. Since CD containing lipids, the first products of lipid  
187 peroxidation, are usually quickly cleaved to their corresponding breakdown products (Yin et al., 2011),  
188 the higher CD concentration in n-6 PUFA than n-3 PUFA supplemented hepatocytes could indicate a  
189 longer time lasting lipoperoxidation.

190 Although we already observed a PUFA sensitizing effect in basal condition (Di Nunzio et al.,  
191 2011), results herein reported cannot be simply considered as its consequence but as an additive effect  
192 due to the concomitant presence of PUFA and H<sub>2</sub>O<sub>2</sub>. It can be clearly evidenced comparing present  
193 and previous data; as example, in basal condition LDH release was significantly higher in ARA  
194 supplemented cells only (Di Nunzio et al., 2011), while it is significantly increased in all supplemented  
195 cells except DHA ones after H<sub>2</sub>O<sub>2</sub> addition, the increase being higher in ARA supplemented cells. It is  
196 worth noting that DHA supplementation has no adverse effect either in basal condition (Di Nunzio et  
197 al., 2011) or after the oxidative insult.

198 This differential modulation of the cell response to the oxidative stress is in part ascribable to the  
199 modulation of the antioxidant enzymes activities. The effect of PUFA on the antioxidant enzymes  
200 status have been extensively studied leading to controversial results. To the Authors knowledge,  
201 previous studies have been performed using fish oil (Avramovic et al., 2012; Romieu et al., 2008) or  
202 other oils (Umesha & Naidu, 2015; Taranu et al., 2014; Haggag et al., 2014) containing different  
203 combinations of PUFA, this not allowing to extrapolate the effect of the single fatty acid. Results  
204 herein reported clearly evidence that each fatty acid has specific modulatory effects on antioxidant  
205 enzymes, and promotes a specific profile of antioxidant enzyme activities that cannot be predicted  
206 using oils containing different proportion of PUFA. The importance of the proportion of PUFA in a  
207 mixture has been also shown by Molinar-Toribio *et al.* (Molinar-Toribio et al., 2015) who evidenced in  
208 rat supplemented with EPA plus DHA mixtures that the activity of antioxidant enzymes increases with  
209 magnitudes depending on the EPA:DHA ratio. Notably, the highest DHA concentration reduced the  
210 oxidative stress.

211 Our study confirms that exposure time to H<sub>2</sub>O<sub>2</sub> modulates anti-oxidant enzymes activity, as  
212 previously reported by Shull *et al.* (Shull et al., 1991) and Franco *et al.* (Franco et al., 1999). In  
213 addition, our results evidence that PUFA supplementation exacerbates this time-depending  
214 modulation. Assuming that the observed increased activity of antioxidant enzymes is based on an  
215 increased transcription of the corresponding encoding genes and an increased synthesis of the  
216 enzymatic proteins, a lag time between the insult and the cell response is conceivable. This can explain  
217 the less evident changes in antioxidant enzymes activities after 20 than 60 min H<sub>2</sub>O<sub>2</sub> exposure. In  
218 addition, the weaker cell response after shorter time of exposure could be due to the lower intracellular

219 concentration of H<sub>2</sub>O<sub>2</sub>. In fact, H<sub>2</sub>O<sub>2</sub> diffusion across the plasma membrane is low during the first 10  
220 minutes of exposure (Alexandre et al., 2006).

221 Oxidative stress underlies a complex array of metabolic alterations. Under this condition,  
222 derangement of cell biochemistry and function is known to involve misregulation of key components  
223 responsible for cell signaling, such as membrane receptors, kinases, phosphatases, and transcription  
224 factors (Leonarduzzi et al., 2011; Brigelius-Flohé et al., 2011). In this view, the overall differential  
225 effect of supplemented DHA could rely on different mechanisms. Among them, a different extent of  
226 incorporation/metabolism of the supplemented fatty acid inside the cell. Although all supplemented  
227 PUFA are readily incorporated by HepG2 cells at the expense of monounsaturated fatty acids, in a  
228 previous work we observed different conversion rate (Di Nunzio et al., 2010). LA and ALA are further  
229 metabolized upon cellular uptake, and EPA is partially desaturated/elongated to DHA. On the contrary,  
230 no DHA retro-conversion is detected. Therefore the effects of all PUFA except DHA could be  
231 ascribable to both the supplemented PUFA and its derivatives. The differential metabolism of  
232 supplemented PUFA could also impact on the modification of membrane composition, and  
233 consequently functionality, since the majority of PUFA is incorporated in the cell membrane  
234 phospholipids (Abbott et al., 2012). The oxidative stress may selectively impair cell PUFA  
235 concentration, which in turn may modulate the effect of the oxidative stress (Zaloga et al., 2006).  
236 Oxidative condition modifies fatty acid composition in neonatal rat cardiomyocytes (Bordoni et al.,  
237 2005), and a slight increase in the saturated and a decrease in monounsaturated and PUFA content has  
238 been detected in the liver of pro-oxidant pesticide-treated rats (Nakbi et al., 2010). At membrane level,  
239 exposure to an oxidative stress leads not only to fatty acid oxidation but also to phospholipid  
240 breakdown due to activation of phospholipase A<sub>2</sub> (Nalbone et al., 1990). DHA released from  
241 phospholipids is the substrate for the formation of resolvins and protectins, that act through distinct  
242 receptors to unfold biological effects (Calder, 2015) including oxidation preventing effects (Liu et al.,  
243 2014; Tian et al., 2015).

244 A higher DHA cellular content may play an important role in the protection from oxidative damage  
245 because 4-hydroxyhexenal, an aldehyde derived from DHA peroxidation, up-regulates Nrf2-HO-1  
246 antioxidant signaling pathway, which induces antioxidant effects (Gao et al., 2007).

247 Many studies indicate that PUFA, particularly EPA and DHA, control gene expression also by  
248 directly governing the transcription/activity of peroxisome proliferator-activated receptor-alpha  
249 (PPAR $\alpha$ ) (Di Nunzio et al., 2009; Georgiadi et al., 2012), whose activation may result in enhanced  
250 expression/activity of antioxidant enzymes such as CAT and SOD (Toyama et al., 2004; Inoue et al.,  
251 2001). PPARs exert their effect on gene transcription by dimerization with the 9-cis retinoic acid  
252 receptors (RXRs) (Feige et al., 2005). RXR is also able to bind PUFA, mainly DHA (Crawford et al.,  
253 2003). Other PUFAs such as EPA or ARA can activate RXR but with lower efficiency (Egea et al.,  
254 2002). Thus, DHA can be considered a more efficient activator of PPAR $\alpha$  (Bordoni et al., 2006).

255 In conclusion, based on reported results each PUFA seems to possess specific pro- and antioxidative  
256 characteristics, not predictable on chemical basis such as the length of the carbon chain or the  
257 unsaturation degree. According to many *in vivo* (Mas et al., 2010; Patten et al., 2013) and *in vitro*  
258 (González-Pérez et al., 2006; Mukherjee et al., 2004) studies, DHA appears the only fatty acid not  
259 increasing cell susceptibility to H<sub>2</sub>O<sub>2</sub>, although its effect is not ascribable to a stronger induction of  
260 antioxidant enzymes activity.

261 Overall, results herein reported clearly evidence that fatty acid supplementation can modify the cell  
262 response to an oxidative stress, and the response is dependent on the supplemented fatty acid. We are  
263 beginning to understand the physiology and molecular basis of the effects of n-3 and n-6 fatty acids,  
264 and thereby gradually encompass the full biological potential of these compounds. Our results  
265 underline that PUFA cannot be considered as a single category, but as individual compounds. It  
266 appears particularly evident for DHA, and although observed results must be confirmed *in vivo*, this  
267 apparent “DHA paradox” represents an important step ahead in the use of DHA supplementation as  
268 preventive strategy in the clinical practice.

## 269 **Disclosure statement**

270 The authors declare no conflict of interest.

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## 276 **References**

- 277 1. Abbott SK, Else PL, Atkins TA, Hulbert AJ. 2012. Fatty acid composition of membrane  
278 bilayers: importance of diet polyunsaturated fat balance. *Biochim Biophys Acta.* 1818:1309-  
279 1317.
- 280 2. Alexandre J, Batteux F, Nicco C, Chéreau C, Laurent A, Guillevin L, Weill B, Goldwasser F.  
281 2006. Accumulation of hydrogen peroxide is an early and crucial step for paclitaxel-induced  
282 cancer cell death both in vitro and in vivo. *Int J Cancer.* 119:41-48.
- 283 3. Avramovic N, Dragutinovic V, Krstic D, Colovic M, Trbovic A, de Luka S, Milovanovic I,  
284 Popovic T. 2012. The effects of omega 3 fatty acid supplementation on brain tissue oxidative  
285 status in aged wistar rats. *Hippokratia.* 16:241-245.
- 286 4. Ayala A, Muñoz MF, Argüelles S. 2014. Lipid peroxidation: production, metabolism, and  
287 signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid Med Cell Longev.*  
288 2014:360438.
- 289 5. Bordoni A, Angeloni C, Leoncini E, Danesi F, Maranesi M, Biagi PL, Hrelia S. 2005.  
290 Hypoxia/reoxygenation alters essential fatty acids metabolism in cultured rat cardiomyocytes:  
291 protection by antioxidants. *Nutr Metab Cardiovasc Dis.* 15:166-73.
- 292 6. Bordoni A, Astolfi A, Morandi L, Pession A, Danesi F, Di Nunzio M, Franzoni M, Biagi P,  
293 Pession A. 2007. N-3 PUFAs modulate global gene expression profile in cultured rat  
294 cardiomyocytes. Implications in cardiac hypertrophy and heart failure. *FEBS Lett.* 581:923-929.
- 295 7. Bordoni A, Di Nunzio M, Danesi F, Biagi PL. 2006. Polyunsaturated fatty acids: From diet to  
296 binding to ppars and other nuclear receptors. *Genes Nutr.* 1:95-106.



- 297 8. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities  
298 of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72:248-254.
- 299 9. Brenna JT, Carlson SE. 2014. Docosahexaenoic acid and human brain development: evidence  
300 that a dietary supply is needed for optimal development. *J Hum Evol.* 77:99-106.
- 301 10. Brigelius-Flohé R, Flohé L. 2011. Basic principles and emerging concepts in the redox control of  
302 transcription factors. *Antioxid Redox Signal.* 15:2335-2381.
- 303 11. Brunk UT, Zhang H, Dalen H, Ollinger K. 1995. Exposure of cells to nonlethal concentrations of  
304 hydrogen peroxide induces degeneration-repair mechanisms involving lysosomal destabilization.  
305 *Free Radic Biol Med.* 19:813-822.
- 306 12. Calder PC. 2015. Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms  
307 and clinical relevance. *Biochim Biophys Acta.* 1851:469-484.
- 308 13. Cao Y, Lu L, Liang J, Liu M, Li X, Sun R, Zheng Y, Zhang P. 2015. Omega-3 Fatty Acids and  
309 Primary and Secondary Prevention of Cardiovascular Disease. *Cell Biochem Biophys.* 72:77-81.
- 310 14. Crawford MA, Golfetto I, Ghebremeskel K, Min Y, Moodley T, Poston L, Phylactos A, Cunnane  
311 S, Schmidt W. 2003. The potential role for arachidonic and docosahexaenoic acids in protection  
312 against some central nervous system injuries in preterm infants. *Lipids.* 38:303-315.
- 313 15. Csala M, Kardon T, Legeza B, Lizák B, Mandl J, Margittai É, Puskás F, Száraz P, Szelényi P,  
314 Bánhegyi G. 2015. On the role of 4-hydroxynonenal in health and disease. *Biochim Biophys*  
315 *Acta.* 1852:826-838.
- 316 16. Danesi F, Ferioli F, Caboni MF, Boschetti E, Di Nunzio M, Verardo V, Valli V, Astolfi A,  
317 Pession A, Bordoni A. 2011. Phytosterol supplementation reduces metabolic activity and slows  
318 cell growth in cultured rat cardiomyocytes. *Br J Nutr.* 106:540-548.
- 319 17. Danesi F, Malaguti M, Di Nunzio M, Maranesi M, Biagi PL, Bordoni A. 2006. Counteraction of  
320 adriamycin-induced oxidative damage in rat heart by selenium dietary supplementation. *J Agric*  
321 *Food Chem.* 54:1203-1208.
- 322 18. Di Nunzio M, Danesi F, Bordoni A. 2009. n-3 PUFA as regulators of cardiac gene transcription:  
323 a new link between PPAR activation and fatty acid composition. *Lipids.* 44:1073-1079.
- 324 19. Di Nunzio M, Toselli M, Verardo V, Caboni MF, Bordoni A. 2013. Counteraction of oxidative  
325 damage by pomegranate juice: influence of the cultivar. *J Sci Food Agric.* 93:3565-3573.
- 326 20. Di Nunzio M, Valli V, Bordoni A. 2011. Pro- and anti-oxidant effects of polyunsaturated fatty  
327 acid supplementation in HepG2 cells. *Prostaglandins Leukot Essent Fatty Acids.* 85:121-127.
- 328 21. Di Nunzio M, van Deursen D, Verhoeven AJ, Bordoni A. 2010. n-3 and n-6 Polyunsaturated  
329 fatty acids suppress sterol regulatory element binding protein activity and increase flow of non-  
330 esterified cholesterol in HepG2 cells. *Br J Nutr.* 103:161-167.
- 331 22. Egea PF, Mitschler A, Moras D. 2002. Molecular recognition of agonist ligands by RXRs. *Mol*  
332 *Endocrinol.* 16:987-997.
- 333 23. Feige JN, Gelman L, Tudor C, Engelborghs Y, Wahli W, Desvergne B. 2005. Fluorescence  
334 imaging reveals the nuclear behavior of peroxisome proliferator-activated receptor/retinoid X  
335 receptor heterodimers in the absence and presence of ligand. *J Biol Chem.* 280:17880-17890.
- 336 24. Fischer R, Maier O. 2015. Interrelation of oxidative stress and inflammation in  
337 neurodegenerative disease: role of TNF. *Oxid Med Cell Longev.* 2015:610813.

- 338 25. Franco AA, Odom RS, Rando TA. 1999. Regulation of antioxidant enzyme gene expression in  
339 response to oxidative stress and during differentiation of mouse skeletal muscle. *Free Radic Biol*  
340 *Med.* 27:1122-1132.
- 341 26. Gao L, Wang J, Sekhar KR, Yin H, Yared NF, Schneider SN, Sasi S, Dalton TP, Anderson ME,  
342 Chan JY, Morrow JD, Freeman ML. 2007. Novel n-3 fatty acid oxidation products activate Nrf2  
343 by destabilizing the association between Keap1 and Cullin3. *J Biol Chem.* 282:2529-2537.
- 344 27. Georgiadi A, Boekschoten MV, Müller M, Kersten S. 2012. Detailed transcriptomics analysis of  
345 the effect of dietary fatty acids on gene expression in the heart. *Physiol Genomics.* 44:352-361.
- 346 28. Giordano E, Visioli F. 2014. Long-chain omega 3 fatty acids: molecular bases of potential  
347 antioxidant actions. *Prostaglandins Leukot Essent Fatty Acids.* 90:1-4.
- 348 29. González-Flecha B, Cutrin JC, Boveris A. 1993. Time course and mechanism of oxidative stress  
349 and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. *J Clin Invest.* 91:456-  
350 464.
- 351 30. González-Pérez A, Planagumà A, Gronert K, Miquel R, López-Parra M, Titos E, Horrillo R,  
352 Ferré N, Deulofeu R, Arroyo V, Rodés J, Clària J. 2006. Docosahexaenoic acid DHA blunts liver  
353 injury by conversion to protective lipid mediators: protectin D1 and 17S-hydroxy-DHA. *FASEB*  
354 *J.* 20:2537-2539.
- 355 31. Haggag Mel-S, Elsanhoty RM, Ramadan MF. 2014. Impact of dietary oils and fats on lipid  
356 peroxidation in liver and blood of albino rats. *Asian Pac J Trop Biomed.* 4:52-58.
- 357 32. Halliwell B, Clement MV, Long LH. 2000. Hydrogen peroxide in the human body. *FEBS*  
358 *Letters.* 486:10-13.
- 359 33. Inoue I, Goto S, Matsunaga T, Nakajima T, Awata T, Hokari S, Komoda T, Katayama S. 2001.  
360 The ligands/activators for peroxisome proliferator-activated receptor alpha (PPARalpha) and  
361 PPARgamma increase Cu<sup>2+</sup>, Zn<sup>2+</sup>-superoxide dismutase and decrease p22phox message  
362 expressions in primary endothelial cells. *Metabolism.* 50:3-11.
- 363 34. Khassaf M, Child RB, McArdle A, Brodie DA, Esanu C, Jackson MJ. 2001. Time course of  
364 responses of human skeletal muscle to oxidative stress induced by nondamaging exercise. *J Appl*  
365 *Physiol.* 90:1031-1035.
- 366 35. Leonarduzzi G, Sottero B, Testa G, Biasi F, Poli G. 2011. New insights into redox-modulated  
367 cell signaling. *Curr Pharm Des.* 17:3994-4006.
- 368 36. Liu JJ, Green P, John Mann J, Rapoport SI, Sublette ME. 2015. Pathways of polyunsaturated  
369 fatty acid utilization: implications for brain function in neuropsychiatric health and disease.  
370 *Brain Res.* 1597:220-246.
- 371 37. Liu M, Boussetta T, Makni-Maalej K, Fay M, Driss F, El-Benna J, Lagarde M, Guichardant M.  
372 2014. Protectin DX, a double lipoxygenase product of DHA, inhibits both ROS production in  
373 human neutrophils and cyclooxygenase activities. *Lipids.* 49:49-57.
- 374 38. Ma Q. 2013. Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol.* 53: 401-  
375 426.
- 376 39. Mas E, Woodman RJ, Burke V, Puddey IB, Beilin LJ, Durand T, Mori TA. 2010. The omega-3  
377 fatty acids EPA and DHA decrease plasma F2-isoprostanes: Results from two placebo-controlled  
378 interventions. *Free Radic Res.* 44, 983-990.

- 379 40. Miyashita K. Paradox of omega-3 PUFA oxidation. 2014. *Eur J Lipid Sci Technol.* 116:1268-  
380 1279.
- 381 41. Molinar-Toribio E, Pérez-Jiménez J, Ramos-Romero S, Romeu M, Giralt M, Taltavull N,  
382 Muñoz-Cortés M, Jáuregui O, Méndez L, Medina I, Torres JL. 2015. Effect of n-3 PUFA  
383 supplementation at different EPA:DHA ratios on the spontaneously hypertensive obese rat model  
384 of the metabolic syndrome. *Br J Nutr.* 113:878-887.
- 385 42. Mukherjee PK, Marcheselli VL, Serhan CN, Bazan NG. 2004. Neuroprotectin D1: a  
386 docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from  
387 oxidative stress. *Proc Natl Acad Sci USA.* 101:8491-8496.
- 388 43. Muldoon MF, Ryan CM, Yao JK, Conklin SM, Manuck SB. 2014. Long-chain omega-3 fatty  
389 acids and optimization of cognitive performance. *Mil Med.* 179:95-105.
- 390 44. Nakbi A, Tayeb W, Grissa A, Issaoui M, Dabbou S, Chargui I, Ellouz M, Miled A, Hammami  
391 M. 2010. Effects of olive oil and its fractions on oxidative stress and the liver's fatty acid  
392 composition in 2,4-Dichlorophenoxyacetic acid-treated rats. *Nutr Metab (Lond).* 7:80.
- 393 45. Nalbone G, Grynberg A, Chevalier A, Leonardi J, Termine E, Lafont H. 1990. Phospholipase A  
394 activity of cultured rat ventricular myocyte is affected by the nature of cellular polyunsaturated  
395 fatty acids. *Lipids.* 25:301-306.
- 396 46. Ohnishi H, Saito Y. 2013. Eicosapentaenoic acid EPA reduces cardiovascular events:  
397 relationship with the EPA/arachidonic acid ratio. *J Atheroscler Thromb.* 20:861-877.
- 398 47. Pashkow FJ. 2011. Oxidative Stress and Inflammation in Heart Disease: Do Antioxidants Have a  
399 Role in Treatment and/or Prevention? *Int J Inflam.* 2011:514623.
- 400 48. Patrick RP, Ames BN. 2015. Vitamin D and the omega-3 fatty acids control serotonin synthesis  
401 and action, part 2: relevance for ADHD, bipolar disorder, schizophrenia, and impulsive behavior.  
402 *FASEB J.* 29:2207-2222.
- 403 49. Patten AR, Brocardo PS, Christie BR. 2013. Omega-3 supplementation can restore glutathione  
404 levels and prevent oxidative damage caused by prenatal ethanol exposure. *J Nutr Biochem.*  
405 24:760-769.
- 406 50. Pelliccia F, Marazzi G, Greco C, Franzoni F, Speziale G, Gaudio C. 2013. Current evidence and  
407 future perspectives on n-3 PUFAs. *Int J Cardiol.* 170:S3-7.
- 408 51. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. 2010. Oxidative stress, inflammation, and  
409 cancer: how are they linked? *Free Radic Biol Med.* 49:1603-1616.
- 410 52. Righi V, Di Nunzio M, Danesi F, Schenetti L, Mucci A, Boschetti E, Biagi P, Bonora S, Tugnoli  
411 V, Bordoni A. 2011. EPA or DHA supplementation increases triacylglycerol, but not  
412 phospholipid, levels in isolated rat cardiomyocytes. *Lipids.* 46:627-636.
- 413 53. Robichaud PP, Surette ME. 2015. Polyunsaturated fatty acid-phospholipid remodeling and  
414 inflammation. *Curr Opin Endocrinol Diabetes Obes.* 22:112-118.
- 415 54. Romieu I, Garcia-Esteban R, Sunyer J, Rios C, Alcaraz-Zubeldia M, Velasco SR, Holguin F.  
416 2008. The effect of supplementation with omega-3 polyunsaturated fatty acids on markers of  
417 oxidative stress in elderly exposed to PM2.5. *Environ Health Perspect.* 116:1237-1242.
- 418 55. Salem N Jr, Eggersdorfer M. 2015. Is the world supply of omega-3 fatty acids adequate for  
419 optimal human nutrition? *Curr Opin Clin Nutr Metab Care.* 18:147-154.

- 420 56. Salomone F, Godos J, Zelber-Sagi S. 2016. Natural antioxidants for non-alcoholic fatty liver  
421 disease: molecular targets and clinical perspectives. *Liver Int.* 36:5-20.
- 422 57. Sánchez-Valle V, Chávez-Tapia NC, Uribe M, Méndez-Sánchez N. 2012. Role of oxidative  
423 stress and molecular changes in liver fibrosis: a review. *Curr Med Chem.* 19:4850-4860.
- 424 58. Shull S, Heintz NH, Periasamy M, Manohar M, Janssen YM, Marsh JP, Mossman BT. 1991.  
425 Differential regulation of antioxidant enzymes in response to oxidants. *J Biol Chem.* 266:24398-  
426 24403.
- 427 59. Taranu I, Gras M, Pistol GC, Motiu M, Marin DE, Lefter N, Ropota M, Habeanu M. 2014.  $\omega$ -3  
428 PUFA rich camelina oil by-products improve the systemic metabolism and spleen cell functions  
429 in fattening pigs. *PLoS One.* 9:e110186.
- 430 60. Tian Y, Zhang Y, Zhang R, Qiao S, Fan J. 2015. Resolvin D2 recovers neural injury by  
431 suppressing inflammatory mediators expression in lipopolysaccharide-induced Parkinson's  
432 disease rat model. *Biochem Biophys Res Commun.* 460:799-805.
- 433 61. Tourtas T, Birke MT, Kruse FE, Welge-Lüssen UC, Birke K. 2012. Preventive effects of omega-  
434 3 and omega-6 Fatty acids on peroxide mediated oxidative stress responses in primary human  
435 trabecular meshwork cells. *PLoS One.* 7:e31340.
- 436 62. Toyama T, Nakamura H, Harano Y, Yamauchi N, Morita A, Kirishima T, Minami M, Itoh Y,  
437 Okanoue T. 2004. PPAR $\alpha$  ligands activate antioxidant enzymes and suppress hepatic fibrosis  
438 in rats. *Biochem Biophys Res Commun.* 324:697-704.
- 439 63. Umesha SS, Naidu KA. 2015. Antioxidants and antioxidant enzymes status of rats fed on n-3  
440 PUFA rich Garden cress *Lepidium Sativum* L seed oil and its blended oils. *J Food Sci Technol.*  
441 52:1993-2002.
- 442 64. Valli V, Gómez-Caravaca AM, Di Nunzio M, Danesi F, Caboni MF, Bordoni A. 2012. Sugar  
443 cane and sugar beet molasses, antioxidant-rich alternatives to refined sugar. *J Agric Food Chem.*  
444 60:12508-12515.
- 445 65. Wang HH, Hung TM, Wei J, Chiang AN. 2004. Fish oil increases antioxidant enzyme activities  
446 in macrophages and reduces atherosclerotic lesions in apoE-knockout mice. *Cardiovasc Res.*  
447 61:169-176.
- 448 66. Yin H, Xu L, Porter NA. 2011. Free radical lipid peroxidation: mechanisms and analysis. *Chem*  
449 *Rev.* 111:5944-5972.
- 450 67. Zaloga GP, Ruzmetov N, Harvey KA, Terry C, Patel N, Stillwell W, Siddiqui R. 2006. (N-3)  
451 long-chain polyunsaturated fatty acids prolong survival following myocardial infarction in rats. *J*  
452 *Nutr.* 136:1874-1878.
- 453 68. Zanatta AL, Miranda DT, Dias BC, Campos RM, Massaro MC, Michelotto PV Jr, West AL,  
454 Miles EA, Calder PC, Nishiyama A. 2014. Fish oil supplementation decreases oxidative stress  
455 but does not affect platelet-activating factor bioactivity in lungs of asthmatic rats. *Lipids.* 49:665-  
456 675.

457 **Figure 1. Cell viability A), LDH activity B), TBARS concentration C) and CD level D) in basal**  
458 **condition and after 1h H<sub>2</sub>O<sub>2</sub> exposure.**

459 *Data are means ± SD of at least six samples in each group, coming from three independent cell*  
460 *cultures. Statistical analysis was by the one-way ANOVA ( $p < 0.001$ ) using Dunnett's post-test to*  
461 *compare: 1) stressed cells both US and supplemented) to controls (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ );*  
462 *and 2) supplemented stressed cells to US stressed cells ( $^{\circ}p < 0.05$ ;  $^{\circ\circ}p < 0.001$ ).*

463

464 **Figure 2. TAA A) and GSH level B) in basal condition and after 1h H<sub>2</sub>O<sub>2</sub> exposure.**

465 *Data are means ± SD of at least six samples in each group, coming from three independent cell*  
466 *cultures. Statistical analysis was performed by the one-way ANOVA ( $p < 0.01$ ) using Dunnett's as post-*  
467 *test to compare: 1) stressed cells both US and supplemented) to controls (\* $p < 0.05$ ); and 2)*  
468 *supplemented stressed cells to US stressed cells ( $^{\circ}p < 0.05$ ;  $^{\circ\circ}p < 0.01$ ).*

469

470 **Figure 3. SOD A), CAT B), GST C) and GPx D) activity in basal condition and after 1h H<sub>2</sub>O<sub>2</sub>**  
471 **exposure.**

472 *Data are means ± SD of at least six samples in each group, coming from three independent cell*  
473 *cultures. Statistical analysis was performed by the one-way ANOVA A, B and C ( $p < 0.001$ ); D ( $p < 0.01$ )*  
474 *using Dunnett's as post-test to compare: 1) stressed cells both US and supplemented) to controls*  
475 *\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ); and 2) supplemented stressed cells to US stressed cells ( $^{\circ}p < 0.05$ ;*  
476  *$^{\circ\circ}p < 0.01$ ;  $^{\circ\circ\circ}p < 0.001$ ).*

477

478 **Figure 4 SOD A), CAT B), GST C) and GPx D) activity in basal condition and after 20 min H<sub>2</sub>O<sub>2</sub>**  
479 **exposure.**

480 *Data are means ± SD of at least six samples in each group, coming from three independent cell*  
481 *cultures. Statistical analysis was performed by the one-way ANOVA A and B ( $p < 0.01$ ); C ( $p < 0.001$ )*  
482 *using Dunnett's as post-test to compare: 1) stressed cells both US and supplemented) to controls*

483 \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ); and 2) supplemented stressed cells to US stressed cells ° $p < 0.05$ ;  
484 °° $p < 0.01$ ; °°° $p < 0.001$ ).