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Strawberry consumption improves aging-associated impairments, mitochondrial biogenesis and functionality through the AMP-Activated Protein Kinase signaling cascade

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39 **Abstract**

40 Dietary polyphenols have been recently proposed as activators of the AMP-activated protein kinase
41 (AMPK) signaling pathway and this fact might explain the relationship between the consumption of
42 polyphenol-rich foods and the slowdown of the progression of aging. In the present work, the
43 effects of strawberry consumption were evaluated on biomarkers of oxidative damage and on aging-
44 associated reductions in mitochondrial function and biogenesis for 8 weeks in old rats. Strawberry
45 supplementation increased antioxidant enzyme activities, mitochondrial biomass and functionality,
46 and decreased intracellular ROS levels and biomarkers of protein, lipid and DNA damage ($P <$
47 0.05). Furthermore, a significant ($P < 0.05$) increase in the expression of the AMPK cascade genes,
48 involved in mitochondrial biogenesis and antioxidant defenses, was also detected after strawberry
49 intake. These in vivo results were then verified in vitro on HepG2 cells, confirming the involvement
50 of AMPK in the beneficial effects exerted by strawberry against aging progression.

51

Keywords: AMPK activation, aging, oxidative stress, mitochondrial functionality, strawberry polyphenols

52 **1. Introduction**

53 It is known that oxidative stress, mitochondrial dysfunction and bioenergetic alterations are the
54 main factors involved in the aging process and in the development of age-related diseases, such as
55 metabolic syndrome, type 2 diabetes and cardiovascular diseases (the “free radical theory of aging”
56 and the “mitochondrial theory of aging” respectively described by Harman in 1956 and Ochoa et al.
57 in 2011). The production of reactive oxygen species (ROS), the major source of cellular damage,
58 mostly occurs in mitochondria and accumulates during aging. Oxidative stress induced by increased
59 ROS production leads in turn to accumulated damage in the mitochondrial DNA (mtDNA) genome,
60 thus perpetuating the increased production of ROS and aberrant electron transfer chain components,
61 and reduced ATP synthesis and impaired mitochondrial function, in a self-perpetuating cycle
62 (Ochoa et al., 2011). Mitochondrial biogenesis is a crucial process for cell viability and survival,
63 since its dysfunction impairs maintenance of energy production and metabolism regulation as well
64 as oxidative stress resistance (Gesing et al., 2011). In eukaryotic cells, mitochondrial biogenesis is
65 prompted through the modulation of the ATP/ADP ratio, activation of AMP-activated protein
66 kinase (AMPK) pathway, and the subsequent expression of peroxisomal proliferator activator
67 receptor γ co-activator 1 α (PGC-1 α) and nuclear respiratory factor-1 (Nrf1) transcription factors
68 (Hardie, Ross & Hawley, 2012). The AMPK cascade, one of the main systems to ensure the
69 maintenance of energy homoeostasis, is also involved in the cellular response against ROS-induced
70 oxidative stress damage, through increased expression levels of nuclear factor (erythroid-derived 2)-
71 like 2 (Nrf2), Mn superoxide dismutase (MnSOD) and catalase, (Colombo & Moncada, 2009;
72 Cordero et al., 2013; Steinberg & Kemp, 2009). Indeed, activation of the AMPK cascade has been
73 associated with the improvement of glucose and lipid metabolism, with the inhibition of platelet
74 aggregation and thrombi reduction, as well as with neuroprotective and anticancer effects
75 (Takikawa Inoue, Horio, & Tsuda, 2010; Park, Inoue, Horio, & Tsuda, 2014; Lee, Lee, Kim, &
76 Park, 2010; Zhang, Wang, Wang, Liu, & Xia, 2013). Furthermore, the contribution of aging-

77 associated reductions of AMPK activity in mitochondrial dysfunction and increased oxidative
78 damage associated with aging has been already advanced (Reznick et al ., 2007).
79 Dietary polyphenols have been recently proposed as activators of the AMPK signaling pathway,
80 and this fact might explain the relationship between consumption of polyphenol-rich foods, disease
81 prevention, and the slowdown of aging progression (Gasparrini et al., 2015). In spite of the high
82 polyphenolic content of berries, literature data evaluating the *in vivo* anti-aging effects of berry
83 bioactive compounds through the activation of the AMPK cascade are still scarce.
84 The aim of the present study is to evaluate the protective effect of strawberry consumption against
85 oxidative damage, antioxidant defence and mitochondrial impairment in old rats, paying particular
86 attention to the implication of the AMPK pathway. To do this, a 2-month animal feeding trial with
87 19-21 old Wistar rats was carried out. Biomarkers of DNA, protein and lipid oxidation damage,
88 antioxidant enzyme activities and other mitochondrial bioenergetic parameters were analyzed. The
89 obtained results were also corroborated in human hepatoma HepG2, by assessing the capacity of
90 the strawberry extract to activate the AMPK signalling pathway and so counteract oxidative stress
91 and improve mitochondrial functionality.

92

93 **2. Materials & methods**

94 *2.1 Reagents*

95 Media and reagents for cell culturing were purchased from Carlo Erba Reagents (Milan, Italy),
96 while Tali™ CellROX® Orange Reagents and ATP kit were obtained from Invitrogen™, Life
97 Technologies (Milan, Italy). All chemicals and solvents were acquired from Sigma-Aldrich
98 Chemical (Milan, Italy), while EPR probes were purchased from Noxygen, Elzach, Germany.
99 Primary and secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas,
100 USA).

101

102

103 2.2 *Strawberry fruit analysis*

104 Strawberry fruits (*Fragaria* × *ananassa*, Alba cultivar) were hand harvested, detached from the
105 sepals, frozen and lyophilized. The strawberry powder was kept under vacuum, in the dark, at a
106 temperature of -80 °C until compositional analysis and meal preparation. Fruit powder analysis
107 included measuring total antioxidant capacity, total phenol and flavonoid content, vitamin C, and
108 HPLC–DAD/ESI–MS-driven anthocyanin characterisation, as described in our publications
109 (Giampieri et al., 2016, 2017).

110

111 2.3 *Animal study design*

112 Wistar rats (*Rattus norvegicus*) were chosen for the aged animal model, as previously described (
113 Shi, Liu, Zhang, Xue, Liu, & Chen, 2014). Sixteen old male rats (19-21 months, initial
114 weight of 500-550 g) were provided by the “Istituto Nazionale di Ricovero e Cura per gli Anziani”
115 (INRCA, Ancona, Italy), were housed individually and maintained on a 12 h light/12 h darkness
116 cycle with free access to drinking water. Prior to the feeding trial, the rats were randomly assigned
117 to receive either a standard diet (C group, n =8) or a strawberry-enriched diet (S group, n = 8) for 8
118 weeks. Both diets were supplied in the form of powder and daily prepared by mixing each
119 individual ingredient using a rotating mixer and kept in the dark at a temperature of 4°C. Compared
120 to the standard diet (AIN93M), the strawberry enriched diet was prepared by substituting 15% of
121 the total calories with freeze-dried strawberry powder, and the amount of macro- and micronutrient
122 adjusted to be identical between the two diets (Giampieri et al 2017).

123 The animals received their respective food and drink at libitum. The amount of food consumed by
124 each animal was monitored by weighing each day the amount of food present in the feeder before
125 giving the following daily food ration and this was taken into account when calculating the total
126 food consumption per animal. Rats were weighed once a week for the whole experimental period.

127 At the end of the two months, the rats were anesthetized with 4% isoflurane inhalation at the
128 same time of day to avoid any circadian fluctuation and samples were collected. Blood was

129 collected by intra-cardiac puncture and immediately transferred into heparin-containing tubes.
130 Heparinized plasma was isolated by centrifugation at 1130 g for 20 min at 15 °C and stored at -80
131 °C until analyses. After exsanguination, the whole livers were carefully removed, washed with ice-
132 cold 0.9 % NaCl solution, weighed and divided into two portions: one was used for the fresh
133 isolation of mitochondrial fractions as previously described (Pedersen et al., 1978), while the other
134 portion was frozen under liquid nitrogen and stored at -80 °C for biochemical and Western blotting
135 analyses.

136 For biochemical analyses, the livers were homogenized on ice in 5 mM Tris-HCl buffer (pH 7.4)
137 containing 1 mM EDTA (Sigma-Aldrich, Milan, Italy), in a ratio (10 %) of 1 g of wet tissue to 10
138 ml of buffer, using a IKA-Werk (Janke Kunkel, UE) homogenizer. After centrifugation at 12.040 g
139 for 20 min at 4 °C, the supernatant was kept and then stored at -80 °C until analysis. Proteins were
140 measured by the Bradford procedure (Bradford, 1976) using BSA as standard.

141 The animals were handled in compliance with all applicable laws and regulations and according
142 to the statements of the European Union (86/609/EEC), concerning the protection of animals used
143 for experimental and other scientific purposes. Experimental protocols were approved by the
144 Institutional Animal Care Committee of the Ministry of Health (Italy) and by the Animal Research
145 Ethics Committee of INRCA.

146

147 *2.4 Cell culture and treatment*

148 Human HepG2 cells were obtained from the American Type Culture Collection (Manassas, Va,
149 USA). Cells were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100
150 µg/ml streptomycin, and 5.5 mmol/l D-glucose and were incubated in a humidified atmosphere of
151 5% CO₂ at 37°C. Strawberry extract was prepared as previously described (Amatori et al., 2016),
152 concentrated under vacuum and resuspended in DMEM to achieve a final concentration of 10
153 µg/ml. This concentration represents the lowest effective dose of strawberry extract, which gave the
154 best results in terms of cell viability and reproducibility according to the MTT assay for cytotoxicity

155 studies (data not shown). Cells were incubated for 48 h with strawberry extract, while control cells
156 were incubated only with DMEM.

157 For biochemical analysis, cells were treated with RIPA buffer, incubated on ice for 5 minutes and
158 stored at -80 °C until analyses.

159

160 *2.5 ROS production*

161 In HepG2, the determination of intracellular ROS levels was performed using the probe CellROX®
162 Orange reagent, as previously described (Gasparrini et al., 2017). Results were expressed as the
163 percentage of cells with increased ROS levels compared with the control.

164 In animals, Electron Paramagnetic Resonance (EPR) spectroscopy was used to measure the kinetic
165 rate of O₂[•] accumulation in plasma, by the hypoxanthine/xanthine oxidase O₂[•] generating system in
166 the presence of the hydroxylamine spin probe PPH (1-hydroxy-4-phosphono-oxy-2,2,6,6-
167 tetramethyl-piperidine) following the procedure described by Watanabe et al. (2007). EPR spectra
168 were recorded on a Bruker EMX EPR spectrometer (Bruker, Karlsruhe, Germany) operating at X-
169 Band equipped with an XL microwave frequency counter and a temperature controller, with the
170 following settings: frequency 9.78 GHz, field width 100 G (Gauss), power 20 mW, modulation
171 amplitude 2 G, gain 2 x 10⁶, time constant 40.96 ms, scan time 42 s, number of scans 7. As a
172 control, the EPR spectrum obtained from a reaction mixture containing PPH, xanthine and xanthine
173 oxidase (positive control) was used and the peak height of the low field component of the spectrum
174 was compared to those recorded in the presence of plasma. Kinetics were determined by plotting the
175 nitroxide concentration, as determined from a calibration curve for intensity of the signal of 4-
176 hydroxy-2,2,6,6-tetramethyl-piperidin-1-oxyl at various known concentrations *vs* time (7 min). The
177 results are expressed as concentration of nitroxide radical per minute per mg protein (nM/min/mg
178 prot).

179

180

181 2.6 Biomarkers of oxidative stress and antioxidant enzymes

182 In cellular lysates from HepG2 and in rat plasma and liver homogenates, protein carbonyl
183 content and lipid peroxidation levels were determined as reported in our previous publications
184 (Giampieri et al., 2016). Antioxidant enzyme activities (catalase, superoxide dismutase, glutathione
185 peroxidase, glutathione reductase, glutathione transferase) were evaluated spectrophotometrically as
186 previously reported (Giampieri et al., 2016)

187

188 2.7 Mitochondrial bioenergetic functions

189 2.7.1 Total mitochondrial ROS production

190 Total mitochondrial ROS production was measured by EPR spectroscopy in rat liver, as previously
191 described by Panov, et al. (2005). The results are expressed as concentration of nitroxide radical per
192 minute per mg protein (nMl/min/mg prot).

193

194 2.7.2 Measurement of citrate synthase and ATP levels

195 The specific activity of citrate synthase was measured in cellular lysates and liver homogenate at
196 412 nm minus 360 nm (13.6 mmol/L/cm) by using 5,5-dithio-bis(2-nitrobenzoic acid) to detect free
197 sulfhydryl groups in coenzyme A, as previously described (Bullon, Cordero, Quiles, Morillo, del
198 Carmen Ramirez-Tortosa, & Battino, 2011). Citrate synthase data were expressed as enzyme
199 activity. ATP levels were determined by a bioluminescence assay using an ATP determination kit
200 according to the instructions of the manufacturer.

201

202 2.7.3 Determination of mitochondrial respiration rate

203 Oxygen consumption rate (OCR) in HepG2 cells and rat liver mitochondria was measured in real-
204 time using a XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica MA, USA) as
205 previously reported by Richardson et al. (2012) et Giampieri et al. (2016), respectively. For cells,
206 after an OCR baseline measurement, a profiling of mitochondrial function was performed by

207 sequential injection of four compounds that affect bioenergetics as follows: 55 μ l of oligomycin
208 (2.5 μ g/mL) at injection in port A, 61 μ l of FCCP (2 μ M) at injection in port B, and 68 μ l of
209 antimycin/rotenone (10 μ M/1 μ M) at injection in port C. For isolated rat liver mitochondria, the
210 following compounds were used: 50 μ l of NADH (final concentration 300 μ M final) at injection A,
211 55 μ l of rotenone (final concentration 2 μ M) at injection B, 60 μ l of succinate (final concentration
212 10 mM) at injection C and 65 μ l antimycin A (final concentration 4 μ M) at injection D. Five wells
213 were utilized per condition in any given experiment and data are expressed as *pmol* of O₂ consumed
214 per minute normalized to 1000 cells (*pmol* O₂/1000 cells/min) or as as *pmol* of O₂ consumed per
215 minute for animal analysis.

216

217 2.8 Immunoblotting analysis

218 After treatment, HepG2 were collected, washed with PBS, lysed in 100 μ l lysis buffer (120 mmol/L
219 NaCl, 40 mmol/L Tris [pH 8], 0.1% NP40) containing protease and phosphatase inhibitor cocktails
220 and centrifuged at 13000 *g* for 15 minutes.

221 Proteins (100 μ g/ml) from cell supernatants and from liver homogenate were separated on a 10-15%
222 acrylamide SDS/PAGE (Bio-Rad, Hercules, CA, USA). Proteins were transferred onto a
223 nitrocellulose 0.2- μ m membrane, using the trans-blot SD semidry electrophoretic transfer cell (Bio-
224 Rad, Hercules, CA, USA) and then membranes were blocked with TBS-T containing 5% non-fat
225 milk for 1 h at room temperature. Phosphorylated AMPK (p-AMPK), SIRT1, PGC-1 α , Nrf-2 and
226 OGG-1 antibodies were used to detect proteins by Western blotting. Membranes were incubated at
227 4°C overnight with the respective primary antibody solution, diluted at 1:500 and then membranes
228 were probed 1 hour at room temperature with their specific alkaline phosphatase conjugated
229 secondary antibodies (1:80000). Immunolabeled proteins were detected by using a
230 chemiluminescence method (C-DiGit Blot Scanner, LI-COR, Bad Homburg, Germany). The protein
231 was determined by the Bradford method (1976).

232

233 2.9 Statistical Analysis

234 Statistical analyses were performed using STATISTICA software package (Statsoft Inc., Tulsa,
235 OK, USA). Plasma, liver and mitochondria data were subjected to the Wilcoxon paired samples
236 test. The mean of three analyses was used and the results reported as mean \pm standard error (SE)
237 and as % changes from the control group. Differences at $P < 0.05$ were considered statistically
238 significant.

239

240 3. Results

241 3.1 Effects of strawberry intake on old rats

242 3.1.1 Body weight

243 Data on body weight and liver ratios showed no significant variations between groups, indicating
244 that the strawberry supplementation did not interfere with normal animal maintenance (Giampieri et
245 al., 2017). These results were confirmed by plasma biochemical parameters and liver histological
246 analysis, which showed no difference between control group (C-group) and strawberry group (S-
247 group) (Giampieri et al., 2017); no significant changes were observed even for daily food intake.

248

249 3.1.2 Biomarkers of oxidative stress and antioxidant defences in plasma and liver

250 As shown in Table 1, strawberry supplementation resulted in a significant decrease of $O_2^{\bullet-}$
251 accumulation in plasma (17.8% reduction, $P < 0.05$) compared to the rats fed the standard diet. The
252 consumption of strawberries also led to a significant decrease of circulating biomarkers of protein
253 (47.4% reduction of carbonyls levels, $P < 0.05$) and lipid oxidation (34.2% reduction of TBARS
254 levels, $P < 0.05$) (Table 1). Compared to the control group, an improvement in biomarkers of
255 oxidative stress was also observed in the liver of old rats fed the strawberry enriched diet, showing a
256 reduction of about 62.8% in protein carbonyls ($P < 0.05$) and 57.7% TBARS ($P < 0.05$) levels
257 (Table 1). In agreement with these results, the expression of OGG-1 (8-Oxoguanine glycosylase),
258 an enzyme responsible for the excision of a mutagenic base that occurs as a result of ROS exposure,

showed a significant decrease (56.8%, $P < 0.05$) in S group compared to C group (Fig. 1), highlighting a marked protective effect of strawberry consumption on all the biological macromolecules against oxidative stress. Furthermore, a positive effect of strawberry enriched-diet was observed on liver antioxidant enzymes (Table 1): GPx, GR, GST activities significantly increased in the S group (28.5%, 39.4% and 19.5% respectively, $P < 0.05$) and the same trends were also found for catalase and SOD activities (16.9% and 55.9% respectively, $P < 0.05$) (Table 1).

265

3.1.3 Mitochondrial biogenesis and functionality

The S group showed a significant reduction of mitochondrial ROS (39.1%, $P < 0.05$), when compared to C group (Table 1). In addition, a significant increase (42.3%, $P < 0.05$) in citrate synthase activity, a marker of mitochondrial mass, was found after two months of strawberry consumption (Table 1). These results were confirmed by the measurement of ATP levels, which were lower in the control group and significantly increased after strawberry supplementation (Table 1).

To confirm the potential improvement of strawberries on mitochondrial function, the OCR was measured in isolated liver mitochondria, exposed sequentially to each of four well-defined modulators of oxidative phosphorylation: NADH, rotenone, succinate and antimycin A (Fig. 2A). Addition of NADH, which is a substrate that transfers electrons to the mitochondrial NADH-ubiquinone oxidoreductase (complex I), caused a significant OCR increase (65.4%, $P < 0.05$) in S-group (Table 1). The subsequent addition of rotenone, an inhibitor of mitochondrial NADH-ubiquinone oxidoreductase, arrested electron flow through the mitochondrial respiratory complexes and caused a dramatic decrease in OCR, in both groups examined, with values close to those of the basal respiratory rate (Fig. 2A). The subsequent addition of succinate, a substrate that transfers electrons to the succinate-ubiquinone reductase (complex II), caused a marked increase of OCR in both experimental groups, with a significant increase (28.7%, $P < 0.05$) in S-group compared to C-group (Table 1). Finally, addition of antimycin A, an inhibitor of ubiquinol-cytochrome c

285 oxidoreductase (complex III), completely stopped OCR in both experimental groups confirming
286 total blocking of mitochondrial activity (Fig. 2A).

287

288 *3.1.4 Up-regulation of AMPK pathway in vivo*

289 Rats fed with the strawberry enriched diet showed significantly higher levels of p-AMPK, SIRT-1,
290 PGC1- α and Nrf2 ($P < 0.05$) compared to rats fed with the standard diet (Fig. 1), suggesting that
291 strawberry intake is associated with enhanced mitochondrial biogenesis and antioxidant defences
292 through AMPK expression.

293

294 *3.2 Effects of strawberry treatment on cells*

295 *3.2.1 Biomarkers of oxidative stress and antioxidant defences*

296 The protective effect of strawberries in reducing oxidative damage and improving mitochondrial
297 functionality was then confirmed *in vitro*, on HepG2 cells. Treatment with the strawberry extract
298 decreased intracellular ROS concentration (43.9%, $P < 0.05$) and consequently attenuated the levels
299 of carbonyl groups (53.6%, $P < 0.05$), TBARS (35.2%, $P < 0.05$) (Table 2) and OGG-1 (46.3%, P
300 < 0.05) (Fig. 3). The strawberry extract also exerted a positive effect on the activities of the
301 principal antioxidant enzymes: in fact, a significant increase in GPx, GR, GST activities (89.2%,
302 70.8%, 99.4%, respectively, $P < 0.05$) was observed (Table 2). The same favorable results were
303 detected for SOD and catalase activities (77.8% and 61.8%, respectively, $P < 0.05$) (Table 2) as
304 well.

305

306 *3.2.2 Mitochondrial biogenesis and functionality*

307 Besides the antioxidant capacity, strawberry treatment also stimulated mitochondrial biogenesis
308 and functionality. Indeed, a significant increase in citrate synthase activity (45.5%, $P < 0.05$) and
309 ATP levels (42.1%, $P < 0.05$) was found in cells treated with strawberries (Table 2), highlighting
310 an increase in mitochondrial mass. In addition, the protective capacity of strawberry extract on

mitochondrial functionality was confirmed by measuring OCR. Cells were sequentially exposed to different well-defined modulators of oxidative phosphorylation: oligomycin (an inhibitor of F_1F_0 -ATPase or complex V), FCCP (an uncoupler of oxidative phosphorylation from the electron transport chain) and antimycin + rotenone (inhibitors of complex I and III, respectively) (Fig. 2B). Basal OCR was markedly increased in cells treated with strawberry extract (41.0%, $P < 0.05$) compared to control, mainly due to the increase in mitochondrial biomass. Addition of oligomycin caused an inhibition of electron flow resulting in marked decreases of OCR in both groups, while the FCCP increased OCR to the maximal mitochondrial respiration the cells could reach; the addition of rotenone + antimycin arrested the electron flow through the mitochondrial respiratory complexes causing a considerable decrease in oxygen consumption, as shown by the drop in OCR levels in all cells.

3.2.3 Up-regulation of AMPK pathway *in vitro*

The capacity of strawberry polyphenols to activate AMPK was confirmed in our *in vitro* experimental model: the expression levels of p-AMPK, SIRT-1 and consequently of PGC-1 α were significantly increased ($P < 0.05$) in strawberry pre-treated cells, together with the expression levels of Nrf2 (Fig. 3). As expected, treatment with strawberry extract alone caused an increase in the expression of p-AMPK, SIRT1 and PGC1- α as previously demonstrated, while pretreatment of cells with compound C clearly prevented the strawberry-induced AMPK activation (Fig. 4) and impaired SIRT-1 and PGC1- α stimulation, confirming the capacity of the extract to induce AMPK activation and the functional linkage between AMPK, SIRT1 and PGC1- α .

4. Discussion

To the best of our knowledge, this is the first study that analyzes the involvement of AMPK pathway *in vivo* after strawberry consumption and its effect on the aging condition. Previous studies have shown that two months of berry supplementation exerts beneficial effects on cognition, motor

337 behavior and neuronal function as well as on markers of inflammation and oxidative stress in rats
338 aged 19 to 21 months (Poulose, Bielinski, Carey, Schauss & Shukitt-Hale, 2016; Shukitt-Hale,
339 Bielinski, Lau, Willis, Carey, & Joseph, 2015; Malin, et al., 2011). In this study, we aimed to
340 evaluate the effects of two months of strawberry consumption on aging-associated reductions in
341 mitochondrial function and biogenesis and on biomarkers of oxidative damage in old rats,
342 evaluating, in particular, the involvement of the AMPK pathway. The choice of strawberry fruits as
343 feeding material was justified by their commercial relevance in the Mediterranean area, being the
344 most consumed berries in fresh or processed form (such as jams, juices and jellies). Moreover,
345 strawberries provide noteworthy health benefits because of their high nutritional value and content
346 of phenolic compounds, which exert anti-microbial, anti-inflammatory, anti-atherosclerotic and
347 anticarcinogenic effects both *in vitro* and *in vivo* models (Amatori et al., 2016; Giampieri et al.,
348 2012; Park et al., 2016; Basu, Betts, Nguyen, Newman, Fu, & Lyons, 2014). These biological and
349 functional activities are related not only to the antioxidant capacity but also to the modulation of
350 many cellular pathways involved in metabolism, survival, proliferation and antioxidant defenses
351 (Forbes-Hernandez et al., 2016; Giampieri et al., 2014). In addition, among berries, strawberries
352 have received increasing attention in recent years and a growing amount of scientific evidence has
353 demonstrated how short- or long-term intake of strawberries could be beneficial for consumers
354 (Park et al., 2016; Basu et al., 2014; Tulipani et al., 2014; Alvarez-Suarez et al., 2014). The dose of
355 strawberry fruit, used in this study, corresponded to a substitution of 300 calories with strawberry
356 intake (approximately 90 g of dried fruits) in a human 2000 Kcal daily diet.

357 Traditionally, the best known role of AMPK has been the regulation of energy production from
358 glucose and fatty acids during stress and the inhibition of energy consumption for cholesterol and
359 glycogen synthesis as well as for protein (Hardie, Roos & Hawley, 2012; Steinberg & Kemp, 2009).
360 However, emerging findings show that the role of AMPK is not constrained to energy metabolism
361 maintenance during increased energy consumption, but this kinase can also regulate several
362 biological mechanisms, i.e. oxidative stress, endoplasmatic reticulum stress, autophagocytosis, and

363 inflammation, thus increasing stress resistance in many body tissues (Salminen & Kaarniranta,
364 2012). Beside this, AMPK seems to play a critical role also within the complex signaling network
365 that regulates mitochondrial biogenesis. Mitochondrial biogenesis and functionality decrease with
366 aging, with devastating consequences: indeed, in aged subjects mitochondrial turnover is slower,
367 leading to the further accumulation of modified proteins, lipids and DNA, and exasperating the
368 situation resulting from the insufficient mitochondrial activity (López-Lluch, Irusta, Navas, & de
369 Cabo, 2008). Interestingly, the responsiveness of AMPK activation seems to decline during the
370 aging process and indeed all the above-mentioned processes and conditions are affected during
371 aging: oxidative stress and endoplasmic stress are increased, autophagic capacity and mitochondrial
372 biogenesis are reduced, while low-grade inflammation appears in old subjects (Salminen &
373 Kaarniranta, 2012). Consequently, AMPK seems to be an important key factor in modulating
374 several age-associated processes. The signalling cascade predicts that AMPK activates SIRT1,
375 which, responding to the increase in cellular NAD^+ concentration, is the principal regulator of
376 energy metabolism and survival process, such as proliferation and apoptosis (Gasparrini et al.,
377 2015; Salminen & Kaarniranta, 2012). Once activated, SIRT1 regulates both some stress resistance
378 pathways, including FoxO and NF-KB signalling and downstream targets involved in different
379 biological processes, i.e. PGC1- α . This protein is the crucial factor for the activation of the full
380 program of mitochondriogenesis and acts as a common intracellular mediator during
381 mitochondriogenesis induced by hormones (Hsieh, Yang, Choudhry, Yu, Rue, Bland, & Chaudry,
382 2005). Once activated, PGC1- α coordinates the activities of several transcription factors involved in
383 mitochondrial biogenesis, including nuclear respiratory factor 1, peroxisome proliferator-activated
384 receptor and estrogen-related receptor.

385 Finally, recent studies have demonstrated that AMPK can activate the Nrf2 signaling pathway, a
386 potent inducer of cellular defenses against oxidative stress (Salminen & Kaarniranta, 2012). On the
387 one hand, this pathway seems to be deregulated or inactive during aging and in age-related
388 degenerative pathologies, thus worsening oxidative stress in these conditions. On the other hand, the

389 activation of Nrf2 signaling pathway has been shown to extend the lifespan of different model
390 organisms (Salminen & Kaarniranta, 2012).

391 In the present work, we have reported for the first time that strawberry consumption is associated
392 with an *in vivo* up-regulation of AMPK during aging, explaining, in part, its beneficial effects on
393 health. We found that rats fed with a strawberry enriched diet for two months presented higher
394 levels ($P < 0.05$) of p-AMPK and of the proteins related to this pathway (SIRT-1, PGC1- α and
395 Nrf2) compared to rats fed with standard diet. In addition, our results link, for the first time,
396 strawberry AMPK activation with an improvement of oxidative stress and bioenergetic status *in*
397 *vivo*. Indeed, alterations induced by oxidative stress generally affect all biological macromolecules
398 and are the basis of the free radical and mitochondrial theory of aging as well as the development of
399 several degenerative diseases. We have shown that strawberry consumption exerted favourable
400 effects against oxidative stress, increasing total antioxidant capacity and decreasing radical levels in
401 plasma, liver and mitochondria of old rats. Even if many ROS, such as hydroxyl radical, are highly
402 reactive and can significantly contribute to cellular oxidative stress, in the present work our
403 attention mainly addressed $O_2^{\bullet-}$, since, in most cases, it is the first radical that is produced by
404 cellular oxidase and during mitochondrial respiration (Shang-U & Frederick, 2012; Turrens, 2003).

405 As a consequence all biomarkers of oxidative stress, such as protein carbonyls, TBARS and OGG-
406 1, decreased with a concomitant stimulation of antioxidant enzymes, like GPx, GR, GST, SOD and
407 catalase. At the same time, we found a relevant increase in mitochondrial mass and bioenergetic
408 status, indicated by the increase in citrate synthase activity and ATP levels, respectively, and a
409 marked improvement in mitochondrial functionality, shown by the improvement in OCR, after
410 strawberry consumption. Interestingly, these results are closely similar to those we obtained on
411 young rats fed with the same strawberry cultivar, in the same amount, for the same period; these
412 findings show that strawberry consumption may lead, in old rats, to a reversion of oxidative stress
413 and mitochondrial functionality comparable to those found for young animals (Giampieri et al.,
414 2016).

415 **5. Conclusion**

416 In conclusion, according to these data, strawberry consumption improves the aging condition
417 through AMPK activation, reinforcing once again the importance of a correct diet in health
418 maintenance, even in the elderly. The reduction of oxidative damage and improvement of
419 mitochondrial functionality after strawberry consumption could, in fact, be an important protective
420 approach to ameliorate the “aging phenotype” and delay the onset of aging-related metabolic
421 diseases. For these reasons, we propose AMPK as a novel way to understand and treat aging and
422 age-associated conditions. Further analyses involving different animal models or a longer period of
423 supplementation are strongly required to confirm these observations. In addition, these effects need
424 to be evaluated also in adult animals, in order to verify if strawberry consumption could delay aging
425 and prevent the occurrence of age-related diseases.

426 Information from these studies is necessary to promote the use of dietary compounds that directly
427 affect the AMPK pathway for the next generation of functional foods and nutraceuticals, such as
428 strategic tools to expand longevity and improve aging.

429

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435

436 **Conflicts of interest**

437 The authors declare no conflicts of interest.

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590 **Figure captions**

591 Fig. 1. Strawberry supplementation increased expression levels of proteins related to mitochondrial
592 biogenesis and cellular antioxidant defence in liver of old rats. The levels of phosphorylated
593 AMPK- α , SIRT1, PGC1- α , Nrf2 and OGG1 in rat livers were determined by using Western blotting
594 (representative subset is shown). Mean values belonging to the same set of data with different
595 superscript letters are significantly different ($P < 0.05$). C group: standard diet; S group: strawberry
596 diet.

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598 Fig. 2. Strawberries improved oxygen consumption rate. (A) In isolated mitochondria of rats fed
599 with standard or strawberry enriched diet and (B) in HepG2, treated with DMEM or strawberry
600 extract, mitochondria oxygen consumption was monitored after sequential injection of different
601 compounds that affect bioenergetics at the indicated time points into each well, after baseline rate
602 measurement. Values are means \pm SE. Mean values with different superscript letters are
603 significantly different ($P < 0.05$). C group: standard diet; S group: strawberry diet.

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605 Fig. 3. Strawberry treatment increased expression levels of proteins related to mitochondrial
606 biogenesis and cellular antioxidant defence on HepG2 cells. The levels of phosphorylated AMPK- α ,
607 SIRT1, PGC1- α , Nrf2 and OGG1 in cells were determined by using Western blotting
608 (representative subset is shown). Mean values belonging to the same set of data with different
609 superscript letters are significantly different ($P < 0.05$).

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611 Fig. 4. Blockage of AMPK signalling abolished strawberry effects in HepG2. Cells were pre-treated
612 with or without compound C (10 μ M) and with strawberry (10 μ g/ml). Whole cell lysates were
613 subjected to Western Blot analysis with antibodies against p-AMPK, SIRT1 and PGC1- α .

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615

616 Table 1. Biomarkers of oxidative stress and antioxidant status in plasma, liver and liver
617 mitochondria of old rats. Data are presented as means \pm SE; mean values belonging to the same set
618 of data with different superscript letters are significantly different ($P < 0.05$).
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	Control group	Strawberry group
<i>Plasma:</i>		
Total ROS (nM nitroxide/min/mg prot)	174.10 \pm 0.33 ^a	143.00 \pm 1.37 ^b
Protein carbonyl content (nmol/mg)	0.78 \pm 0.01 ^a	0.41 \pm 0.01 ^b
TBARS (nmol/mg)	0.35 \pm 0.01 ^a	0.23 \pm 0.01 ^b
<i>Liver:</i>		
Protein carbonyl content (nmol/mg)	9.01 \pm 0.02 ^a	3.35 \pm 0.26 ^b
TBARS (nmol/mg)	0.26 \pm 0.06 ^a	0.11 \pm 0.04 ^b
GPx (nmol/min/mg)	230.31 \pm 7.79 ^b	295.90 \pm 7.57 ^a
GR (nmol/min/mg)	119.94 \pm 2.11 ^b	167.25 \pm 5.72 ^a
GST (nmol/min/mg)	420.39 \pm 3.36 ^b	502.61 \pm 4.25 ^a
SOD (IU/mg)	104.80 \pm 1.73 ^b	163.45 \pm 1.41 ^a
Catalase (IU/min/mg)	21.79 \pm 0.11 ^b	25.48 \pm 0.52 ^a
<i>Liver mitochondria:</i>		
Total ROS (nM nitroxide/min/mg prot)	1.38 \pm 0.06 ^a	0.84 \pm 0.04 ^b
ATP (nM/mg prot)	36.05 \pm 14.90 ^b	82.75 \pm 17.20 ^a
Citrate synthase (specific activity)	179.77 \pm 20.72 ^b	255.90 \pm 29.41 ^a
OCR (pmol/min)		
Complex I	93.00 \pm 2.34 ^b	153.80 \pm 2.82 ^a
Complex II	98.10 \pm 0.58 ^b	126.30 \pm 3.25 ^a

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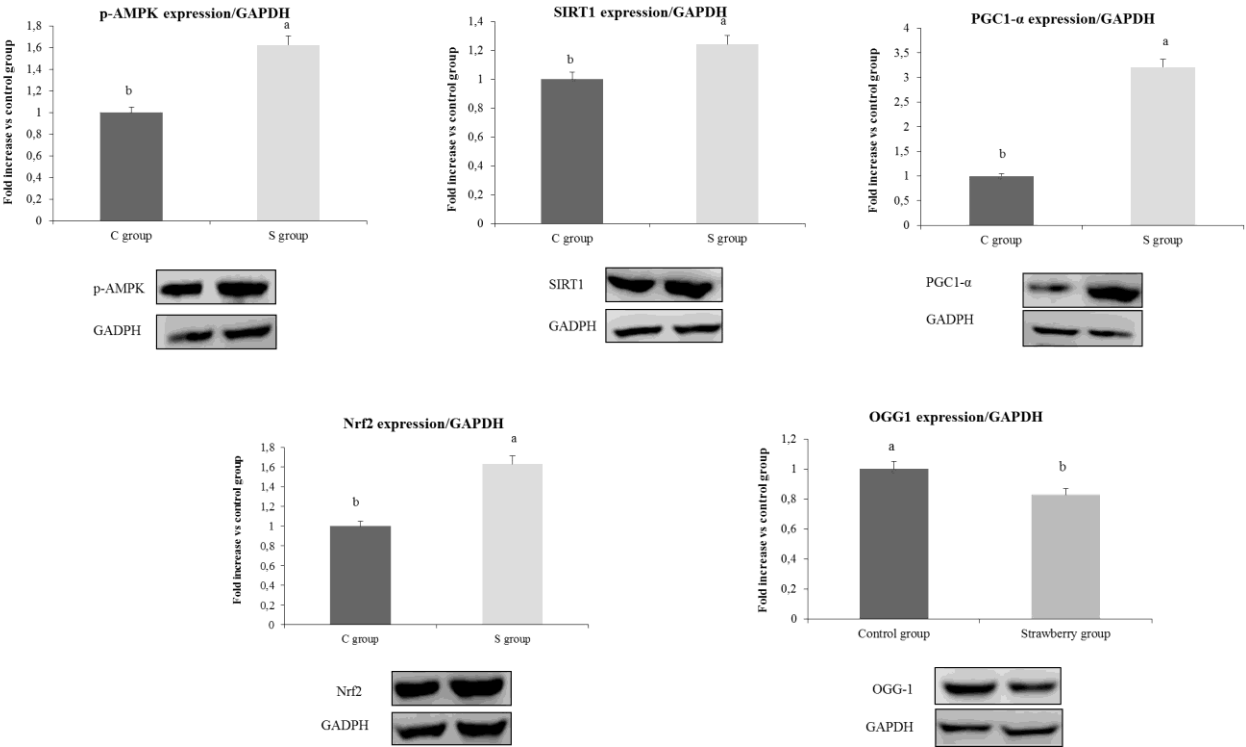
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630 Table 2. Biomarkers of oxidative stress, antioxidant status and mitochondrial functionality in
631 HepG2. Data are presented as means \pm SE; mean values belonging to the same set of data with
632 different superscript letters are significantly different ($P < 0.05$).
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Parameters	Control group	Strawberry group
<i>Cellular biomarkers</i>		
Total ROS (nM nitroxide/min/mg prot)	82%	46%
Protein carbonyl content (nmol/mg)	29.41 \pm 0.10 ^a	13.63 \pm 0.2 ^b
TBARS (nmol/mg)	1.05 \pm 0.02 ^a	0.68 \pm 0.01 ^b
GPx (μ mol/min/mg)	1.86 \pm 0.03 ^b	3.52 \pm 0.02 ^a
GR (μ mol/min/mg)	1.37 \pm 0.01 ^b	2.34 \pm 0.02 ^a
GST (μ mol/min/mg)	3.55 \pm 0.09 ^b	7.08 \pm 0.07 ^a
SOD (IU/mg)	14.38 \pm 0.03 ^b	25.57 \pm 0.01 ^a
Catalase (IU/min/mg)	56.94 \pm 0.11 ^b	92.15 \pm 0.52 ^a
<i>Mitochondrial biomarkers</i>		
ATP (nM/mg prot)	19.40 \pm 4.90 ^b	33.51 \pm 2.10 ^a
Citrate synthase (specific activity)	176.27 \pm 12.07 ^b	256.57 \pm 12.08 ^a

650 Figure 1



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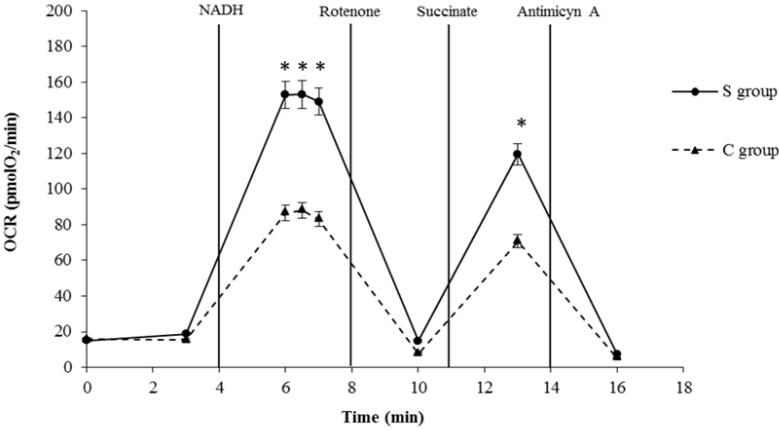
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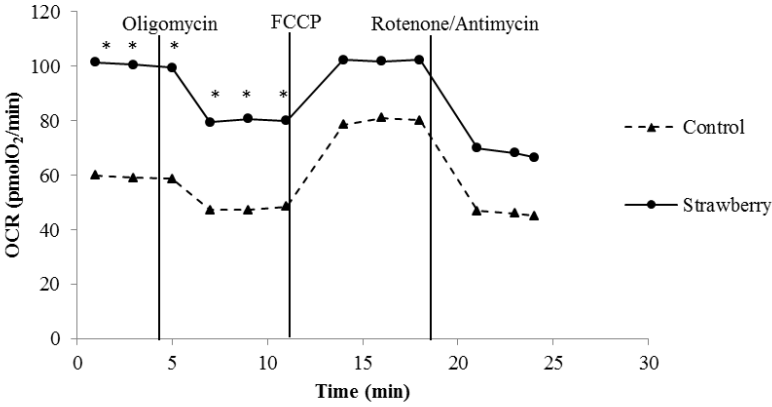
679 Figure 2

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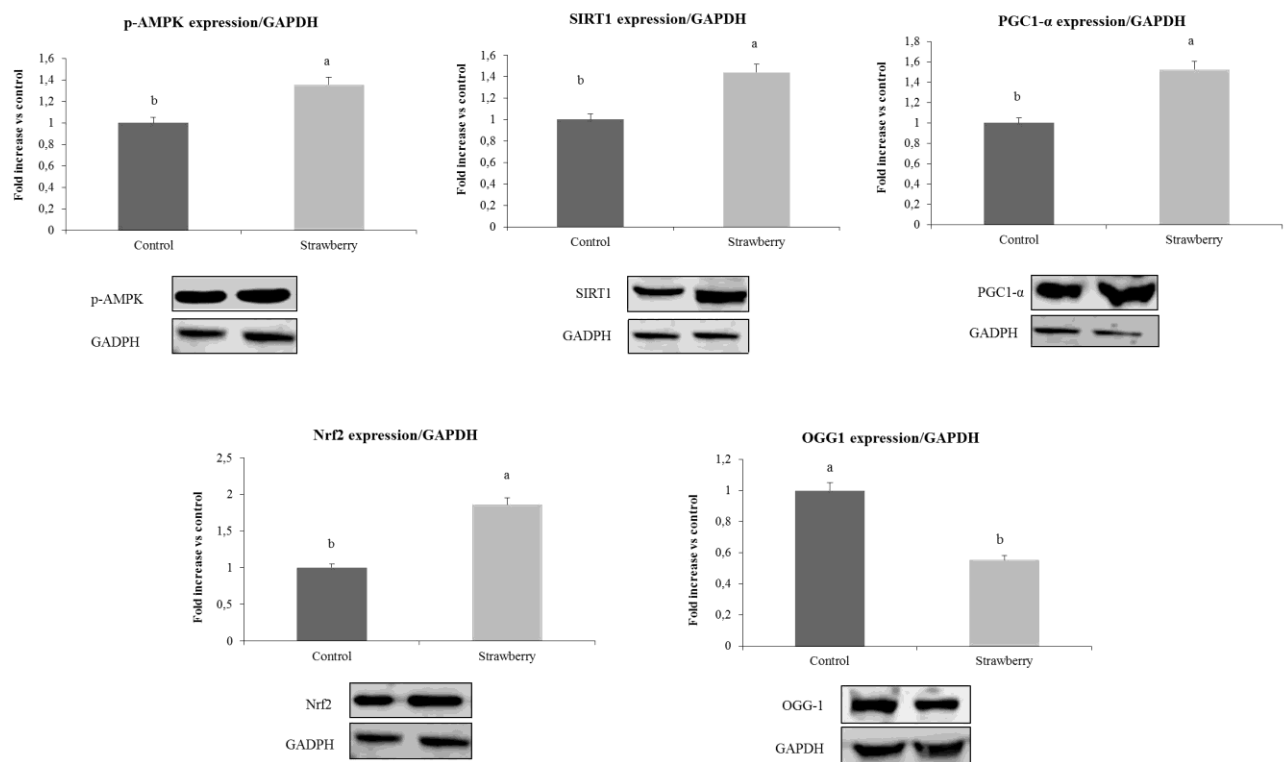
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704 Figure 3

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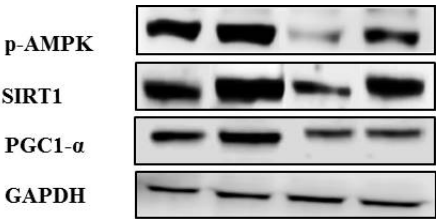


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734 Figure 4

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Strawberry - + - +

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