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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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1 **Strawberry consumption improves aging-associated impairments, mitochondrial biogenesis**  
2 **and functionality through the AMP-Activated Protein Kinase signaling cascade**  
3

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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  $\gamma$  co-activator 1 $\alpha$  (PGC-1 $\alpha$ ) 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 homeostasis, 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% CO<sub>2</sub> 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 O<sub>2</sub><sup>•-</sup> accumulation in plasma, by the hypoxanthine/xanthine oxidase O<sub>2</sub><sup>•-</sup> 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 10<sup>6</sup>, 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 (nMI/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  $\mu$ l of oligomycin  
208 (2.5  $\mu$ g/mL) at injection in port A, 61  $\mu$ l of FCCP (2  $\mu$ M) at injection in port B, and 68  $\mu$ l of  
209 antimycin/rotenone (10  $\mu$ M/1 $\mu$ M) at injection in port C. For isolated rat liver mitochondria, the  
210 following compounds were used: 50  $\mu$ l of NADH (final concentration 300  $\mu$ M final) at injection A,  
211 55  $\mu$ l of rotenone (final concentration 2  $\mu$ M) at injection B, 60  $\mu$ l of succinate (final concentration  
212 10 mM) at injection C and 65  $\mu$ l antimycin A (final concentration 4  $\mu$ M) at injection D. Five wells  
213 were utilized per condition in any given experiment and data are expressed as  $\mu$ mol of O<sub>2</sub> consumed  
214 per minute normalized to 1000 cells ( $\mu$ mol O<sub>2</sub>/1000 cells/min) or as as  $\mu$ mol of O<sub>2</sub> 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  $\mu$ 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  $\mu$ 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- $\mu$ 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 $\alpha$ , 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,

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

265

### 266 *3.1.3 Mitochondrial biogenesis and functionality*

267 The S group showed a significant reduction of mitochondrial ROS (39.1%,  $P < 0.05$ ), when  
268 compared to C group (Table 1). In addition, a significant increase (42.3%,  $P < 0.05$ ) in citrate  
269 synthase activity, a marker of mitochondrial mass, was found after two months of strawberry  
270 consumption (Table 1). These results were confirmed by the measurement of ATP levels, which  
271 were lower in the control group and significantly increased after strawberry supplementation (Table  
272 1).

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

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

322

### 323 3.2.3 Up-regulation of AMPK pathway *in vitro*

324 The capacity of strawberry polyphenols to activate AMPK was confirmed in our *in vitro*  
325 experimental model: the expression levels of p-AMPK, SIRT-1 and consequently of PGC-1 $\alpha$  were  
326 significantly increased ( $P < 0.05$ ) in strawberry pre-treated cells, together with the expression levels  
327 of Nfr2 (Fig. 3). As expected, treatment with strawberry extract alone caused an increase in the  
328 expression of p-AMPK, SIRT1 and PGC1- $\alpha$  as previously demonstrated, while pretreatment of cells  
329 with compound C clearly prevented the strawberry-induced AMPK activation (Fig. 4) and impaired  
330 SIRT-1 and PGC1- $\alpha$  stimulation, confirming the capacity of the extract to induce AMPK activation  
331 and the functional linkage between AMPK, SIRT1 and PGC1- $\alpha$ .

332

## 333 4. Discussion

334 To the best of our knowledge, this is the first study that analyzes the involvement of AMPK  
335 pathway *in vivo* after strawberry consumption and its effect on the aging condition. Previous studies  
336 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<sup>+</sup> 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- $\alpha$ . 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- $\alpha$  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-  $\alpha$  and  
395 Nfr2) 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- $\alpha$ , SIRT1, PGC1- $\alpha$ , 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.

597  
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.

604  
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- $\alpha$ ,  
607 SIRT1, PGC1- $\alpha$ , 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 ( $P < 0.05$ ).

610  
611 Fig. 4. Blockage of AMPK signalling abolished strawberry effects in HepG2. Cells were pre-treated  
612 with or without compound C (10  $\mu$ M) and with strawberry (10  $\mu$ g/ml). Whole cell lysates were  
613 subjected to Western Blot analysis with antibodies against p-AMPK, SIRT1 and PGC1- $\alpha$ .

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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 <sup>a</sup>	143.00 $\pm$ 1.37 <sup>b</sup>
Protein carbonyl content (nmol/mg)	0.78 $\pm$ 0.01 <sup>a</sup>	0.41 $\pm$ 0.01 <sup>b</sup>
TBARS (nmol/mg)	0.35 $\pm$ 0.01 <sup>a</sup>	0.23 $\pm$ 0.01 <sup>b</sup>
<i>Liver:</i>		
Protein carbonyl content (nmol/mg)	9.01 $\pm$ 0.02 <sup>a</sup>	3.35 $\pm$ 0.26 <sup>b</sup>
TBARS (nmol/mg)	0.26 $\pm$ 0.06 <sup>a</sup>	0.11 $\pm$ 0.04 <sup>b</sup>
GPx (nmol/min/mg)	230.31 $\pm$ 7.79 <sup>b</sup>	295.90 $\pm$ 7.57 <sup>a</sup>
GR (nmol/min/mg)	119.94 $\pm$ 2.11 <sup>b</sup>	167.25 $\pm$ 5.72 <sup>a</sup>
GST (nmol/min/mg)	420.39 $\pm$ 3.36 <sup>b</sup>	502.61 $\pm$ 4.25 <sup>a</sup>
SOD (IU/mg)	104.80 $\pm$ 1.73 <sup>b</sup>	163.45 $\pm$ 1.41 <sup>a</sup>
Catalase (IU/min/mg)	21.79 $\pm$ 0.11 <sup>b</sup>	25.48 $\pm$ 0.52 <sup>a</sup>
<i>Liver mitochondria:</i>		
Total ROS (nM nitroxide/min/mg prot)	1.38 $\pm$ 0.06 <sup>a</sup>	0.84 $\pm$ 0.04 <sup>b</sup>
ATP (nM/mg prot)	36.05 $\pm$ 14.90 <sup>b</sup>	82.75 $\pm$ 17.20 <sup>a</sup>
Citrate synthase (specific activity)	179.77 $\pm$ 20.72 <sup>b</sup>	255.90 $\pm$ 29.41 <sup>a</sup>
OCR (pmol/min)		
Complex I	93.00 $\pm$ 2.34 <sup>b</sup>	153.80 $\pm$ 2.82 <sup>a</sup>
Complex II	98.10 $\pm$ 0.58 <sup>b</sup>	126.30 $\pm$ 3.25 <sup>a</sup>

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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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<b>Parameters</b>	<b>Control group</b>	<b>Strawberry group</b>
<i>Cellular biomarkers</i>		
Total ROS (nM nitroxide/min/mg prot)	82%	46%
Protein carbonyl content (nmol/mg)	29.41 $\pm$ 0.10 <sup>a</sup>	13.63 $\pm$ 0.2 <sup>b</sup>
TBARS (nmol/mg)	1.05 $\pm$ 0.02 <sup>a</sup>	0.68 $\pm$ 0.01 <sup>b</sup>
GPx ( $\mu$ mol/min/mg)	1.86 $\pm$ 0.03 <sup>b</sup>	3.52 $\pm$ 0.02 <sup>a</sup>
GR ( $\mu$ mol/min/mg)	1.37 $\pm$ 0.01 <sup>b</sup>	2.34 $\pm$ 0.02 <sup>a</sup>
GST ( $\mu$ mol/min/mg)	3.55 $\pm$ 0.09 <sup>b</sup>	7.08 $\pm$ 0.07 <sup>a</sup>
SOD (IU/mg)	14.38 $\pm$ 0.03 <sup>b</sup>	25.57 $\pm$ 0.01 <sup>a</sup>
Catalase (IU/min/mg)	56.94 $\pm$ 0.11 <sup>b</sup>	92.15 $\pm$ 0.52 <sup>a</sup>
<i>Mitochondrial biomarkers</i>		
ATP (nM/mg prot)	19.40 $\pm$ 4.90 <sup>b</sup>	33.51 $\pm$ 2.10 <sup>a</sup>
Citrate synthase (specific activity)	176.27 $\pm$ 12.07 <sup>b</sup>	256.57 $\pm$ 12.08 <sup>a</sup>

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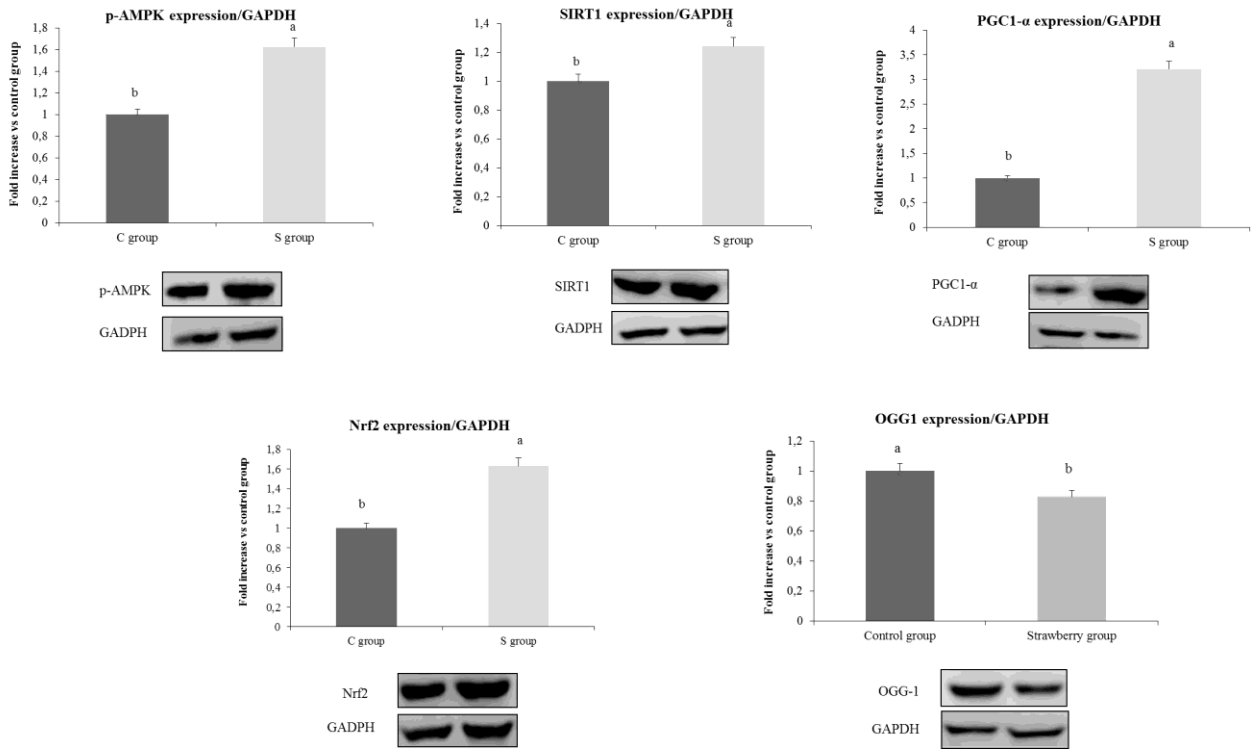
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650 Figure 1



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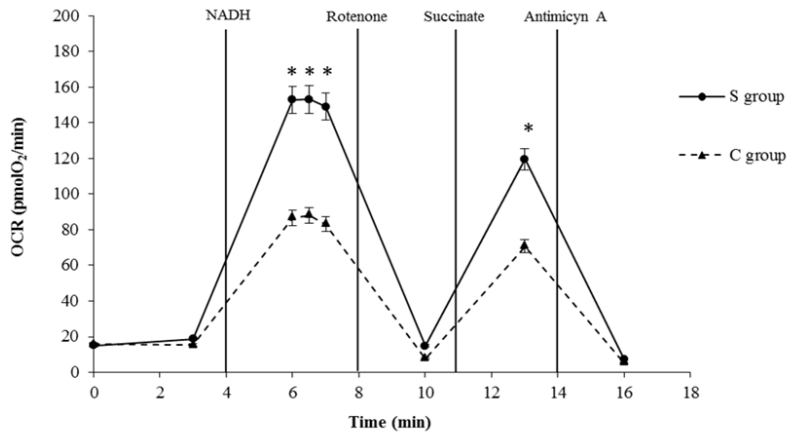
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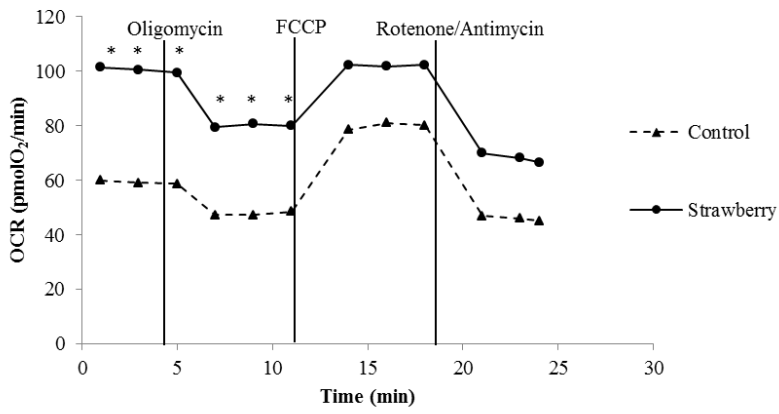
679 Figure 2

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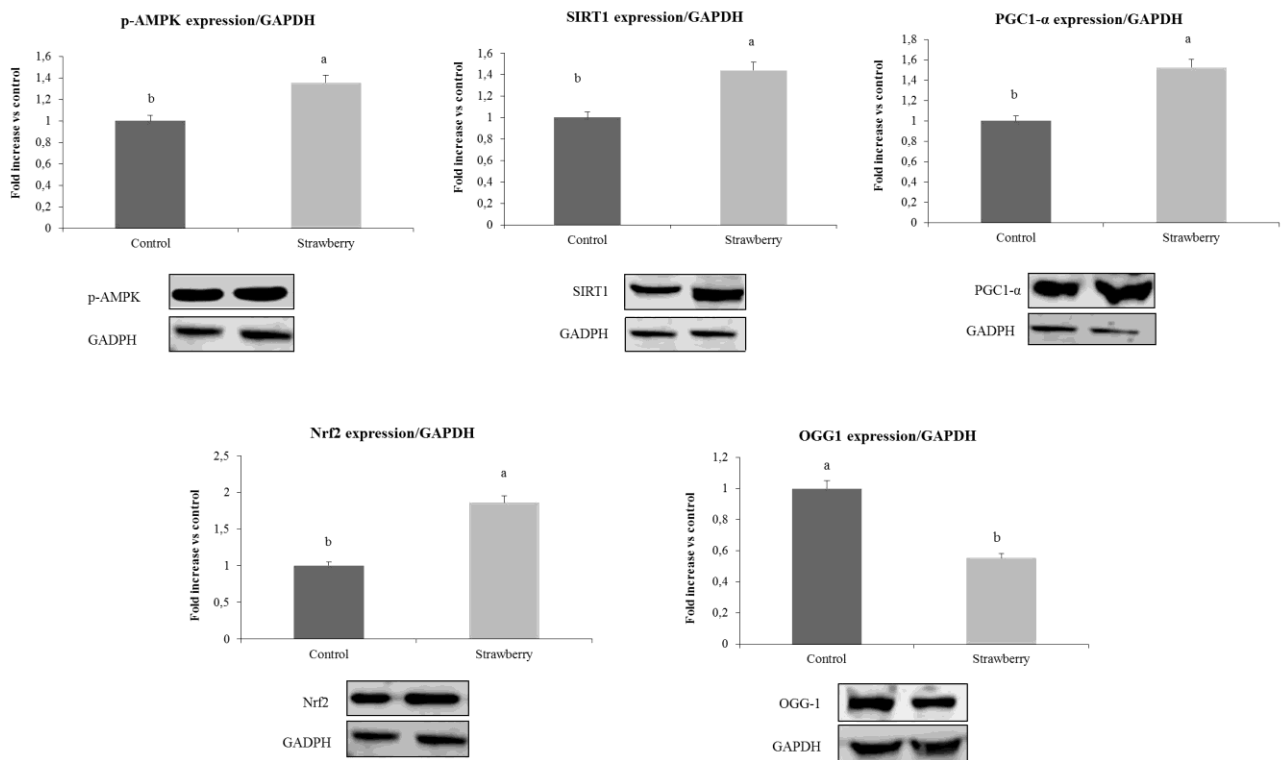
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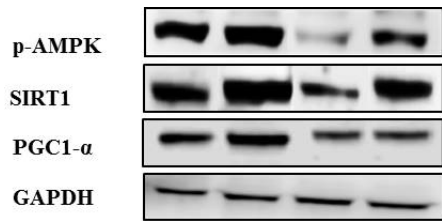
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