

1 **NUTRACEUTICAL PROPERTIES OF CHESTNUT FLOURS: BENEFICIAL EFFECTS**
2 **ON SKELETAL MUSCLE ATROPHY.**

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24 Atrophy protection by chestnut tocopherols

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31 **Keywords: chestnut flour, tocopherols, skeletal muscle atrophy, sphingolipids**

32

33 **Abstract**

34

35 Plants contain a wide range of non-nutritive phytochemicals, many of which have protective
36 or preventive properties for human diseases. The aim of the present work has been to investigate the
37 nutraceutical properties of sweet chestnut flour extracts obtained from fruits collected from 7
38 geographic areas of Tuscany (Italy), and their ability in modulating skeletal muscle atrophy. We
39 found that the cultivars from different geographic areas are characterized by composition and
40 quantity of various nutrients and specific bioactive components, such as tocopherols, polyphenols
41 and sphingolipids. The nutraceutical properties of chestnut sweet flours have been evaluated in
42 C2C12 myotubes induced to atrophy by serum deprivation or dexamethasone. We found that the
43 pretreatment with both total extract of tocopherols and sphingolipids is able to counterbalance cell
44 atrophy, reducing the decrease in myotube size and myonuclei number, and attenuating protein
45 degradation and the increase in expression of MAFbx/atrogen-1 (a muscle-specific atrophy marker).
46 By contrast, polyphenol extracts were not able to prevent atrophy. Since we also found that γ -
47 tocopherol is the major form of tocopherol in sweet flour and its content differs depending on the
48 procedure of sweet flour preparation, the mechanisms by which γ -tocopherol as well as
49 sphingolipids affect skeletal muscle cell atrophy have been also investigated. This is the first
50 evidence that chestnut sweet flour is a natural source of specific bioactive components with a
51 relevant role in the prevention of cell degeneration and maintaining of skeletal muscle mass,
52 opening important implications in designing appropriate nutritional therapeutic approaches to
53 skeletal muscle atrophy.

54

55 Introduction

56 Fruits and vegetables contain vitamins, minerals, fiber and potentially bioactive compounds
57 known as phytochemicals. These compounds are not considered as nutrients but have protective or
58 preventive properties for a variety of human diseases including cancer¹ cardiovascular diseases² and
59 Alzheimer's disease³, mostly due to antioxidant and/or anti-inflammatory activities. Recent studies
60 performed on Portuguese cultivars⁴⁻⁶ and Croatian cultivars of *Castanea Sativa Mill* extracts⁷ and
61 on Mugello's marrons (Tuscany, Italy)^{8,9} have evidenced chestnuts and marrons as an important
62 natural source of antioxidants, particularly of polyphenols and vitamin E.

63 Polyphenols are the biggest group of phytochemicals that contribute to important
64 characteristics of food such as taste, odor, colour and oxidative stability¹⁰.

65 Many studies including epidemiological data suggest that long term consumption of food
66 rich in plant polyphenols offers some protection against the principal chronic diseases, such as
67 cancer, cardiovascular diseases, and neurodegenerative diseases¹⁰, mainly related to oxidative stress
68 response¹¹. Vitamin E is a generic term for all tocopherol and tocotrienol derivatives. The most
69 abundant form of vitamin E isoforms in mammals is α -tocopherol and it has long been recognized
70 as a classic free radical scavenging antioxidant whose deficiency impairs mammalian fertility¹².
71 New biological activities including anti-inflammatory, antineoplastic, and natriuretic functions have
72 been reported for other tocopherols, such as γ -tocopherol¹². In particular, the tocotrienol subfamily
73 of natural vitamin E possesses powerful neuroprotective, anticancer, and cholesterol-lowering
74 properties; for example, nanomolar concentration of α -tocotrienol, but not of α -tocopherol, prevents
75 neurodegeneration¹³. Epidemiological data suggest that γ -tocopherol is a better negative risk factor
76 for certain types of cancer and myocardial infarction than is a α -tocopherol^{1, 12, 14}.

77 Oxidative stress induces unloading-mediated muscle atrophy that is prevented by
78 polyphenols¹⁵. In addition, Vitamin E is a nutrient widely used to attenuate muscle injury associated
79 with eccentric exercise¹⁶.

80 Sphingolipids (SLs), components of the membranes of all eukariotic cells, participate in the
81 control of a variety of important cell functions, such as cell growth, differentiation, inflammation,
82 and apoptosis^{17,18}. The first evidence of SL importance as functional nutrients was obtained by
83 Merrill's group (reviewed in¹⁷) that reported that dietary sphingomyelin (SM) inhibits colon
84 carcinogenesis in animal models. In addition, other studies have shown that consumption of SLs
85 reduces serum low-density lipoprotein cholesterol and elevates high-density lipoproteins¹⁷. SLs are
86 found in all foodstuff; however, only a few of the published studies reported the analysis of their
87 content in food, found to be some hundreds of mg/kg, a relatively low concentration¹⁷. Degradation
88 of the most abundant SLs, SM, by the action of several sphingomyelinases generates ceramide that

89 can also be formed from serine by *de novo* synthesis. Deacylation of ceramide leads to the
90 formation of sphingosine that can be further phosphorylated by sphingosine kinases (SK) to yield
91 sphingosine 1-phosphate (S1P)¹⁸. Among bioactive SLs, S1P acts as survival and trophic factor,
92 increases cell proliferation and differentiation and inhibits apoptosis¹⁸. Recently, it has been
93 demonstrated that SK1/S1P axis promotes myoblast differentiation¹⁹, exerts a trophic action on
94 denervated or damaged skeletal muscle fibres¹⁹⁻²⁰ and it is crucial for satellite cell activation and
95 tissue regeneration²⁰⁻²².

96 Skeletal muscle exhibits great plasticity in response to altered activity levels, ultimately
97 resulting in substantial changes in tissue mass: an increase have been associated with resistance
98 exercise or after anabolic agent administration, whereas a loss in skeletal muscle mass (atrophy) has
99 been observed in several conditions, such as starvation, ageing and physical inactivity, as well as in
100 disease states including cancer cachexia, diabetes and AIDS^{23,24}. At present effective treatments that
101 can prevent, attenuate or reverse muscle atrophy are not clearly defined. Emerging evidence
102 implicates oxidative stress as a key regulator of cell signaling pathways, leading to increased
103 proteolysis and muscle atrophy during periods of prolonged disuse²³. The discovery of two muscle-
104 specific E3 ubiquitin ligases, Muscle atrophy F-Box (MAFbx/ atrogin-1) and Muscle RING (Really
105 Interesting New Gene) Finger-1 (MuRF1), prompted renewed expectation in identifying muscle-
106 specific targets for therapeutic manipulation²⁴, as the two enzymes belong to the ubiquitin
107 proteasome pathway, the predominant pathway involved in protein degradation in skeletal muscle.

108 Autophagy is also involved in skeletal muscle homeostasis: it can be detrimental and
109 contribute to tissue degeneration, but can also be a compensatory mechanism for cell survival²⁵.
110 Autophagy is characterized by sequestration of cytoplasmic components in double-membraned
111 autophagosomes and their fusion with lysosomes. Autophagosomal membrane nucleation is
112 promoted by phosphatidylinositol-3-phosphate generated by the Beclin-1-interacting complex²⁵.

113 The aim of the present work has been to investigate the nutraceutical properties of sweet
114 chestnut flour extracts obtained from fruits of seven different areas of Tuscany and to examine
115 whether these extracts could modulate skeletal muscle cell atrophy in C2C12 myotubes, reported to
116 be a good model for the study of skeletal muscle cell biology^{19, 26-28}.

117

118 **Results**

119

120 *Composition of tuscan chestnut flour. Content of bioactive molecules*

121

122 Since chestnut flour from Mugello area is obtained essentially from a single cultivar
123 (Marrone Fiorentino) (Table 1), we used this sample for the chemical composition of this tuscan
124 chestnut flour. Sweet chestnut flour was analysed and characterized for the content of proteins, fat,
125 carbohydrates and ions. Data reported in Table 2 indicate that the four has an high content of
126 carbohydrates, starch being the most representative and similar in content to Spanish chestnut
127 cultivars³⁹. Previous studies on Spanish and Portuguese chestnut cultivars showed a protein content
128 of 4.9-7.4 g/100 g dry weight, a higher value respect to that reported in Table 2⁴⁰. Moreover, in the
129 same study the content of fat (1.7-3.1 g/100g dry weight) is lower of that found in Marrone
130 Fiorentino. The high content of complex carbohydrates, associated with the low content of fat,
131 makes the chestnut flour a healthy food representative of the Meditteranean diet. The chemical
132 composition in metals was not significantly different from what previously reported⁶. From a
133 nutritional point of view, chestnut flour can be an important source of minerals: potassium is
134 present in a significant concentration as well as magnesium and calcium.

135 In order to investigate the nutraceutical properties of chestnut flour, the relative values of
136 polyphenols, tocopherols and “functional” SLs were quantified (Table 3). Polyphenols vary from
137 1688 to 2799 mg/Kg as (gallic acid), whereas the content in SLs, similar in all the samples, ranges
138 from 48 to 56 mg/Kg dry weight. Chromatographic analysis revealed the presence of several
139 vitamin E isoforms. γ -Tocopherol was the major tocopherol; the profile was similar along all the
140 assayed tuscan sweet chestnut flours (γ -tocopherol >>> δ -tocopherol > α -tocopherol). Tocopherol
141 content, ranging from 18 to 114 mg/Kg, appears to be dependent from several parameters: the
142 drying method (traditional vs other not traditional), cultivar tipology and geographic locations in the
143 same area.

144

145 *Effect of total polyphenol and tocopherol extracts on skeletal muscle cell growth*

146

147 The effects of total polyphenol and tocopherol extracts on cell growth and viability have been
148 quantified both by cell counting and MTS assay, a method that measures the ability of
149 dehydrogenases in living cells to reduce MTS to formazan (Fig. 1). Both tocopherol and polyphenol
150 extracts decrease cell growth in a time-dependent manner compared to control cells, but
151 polyphenols have a more-pronounced effect than tocopherols. Of note, the cell number at day 4 is

152 not significantly different from day 1 in cells treated with tocopherol extract and only slightly with
153 polyphenol extract (Fig.1A). The difference is more evident after four days of culture, when MTS
154 reduction capability is reduced by polyphenol or tocopherol extract to 12% and 50% of the control
155 cells, respectively (Fig.1B). Therefore, the reduction of formazan formation might be due either to
156 a low number of cells metabolizing the MTS and to a decrease of mitochondrial activity in each
157 cell.

158 Since polyphenol and tocopherol extract interfered with cell growth, we evaluated the ability of
159 both compounds to induce cell death by lactate dehydrogenase (LDH) assay. The slight effect of
160 tocopherol and polyphenol extracts in releasing LDH activity into cell medium at 4 days of
161 incubation ($7\pm 3\%$ and $10\pm 3\%$, respectively) compared with the effect of 0.1% Triton X100 (set to
162 100%) and the absence of apoptotic markers expression (cytosolic cytochrome C and mitochondria-
163 associated Bax) at the concentration used (data not shown), indicate the lack of cytotoxicity,
164 suggesting that C2C12 myoblasts likely undergo cell cycle arrest and, presumably, start the
165 myogenic program.

166

167 *Effect of total polyphenol and tocopherol extracts on starvation- and Dexa-induced muscle atrophy*

168

169 In order to investigate the involvement of functional molecules identified in chestnut flour on
170 skeletal muscle cell atrophy, we used C2C12 myoblasts, a well known cell model for studying
171 myogenesis induction¹⁹, the biology of terminal myogenic differentiated cells, and cell
172 atrophy/hypertrophy. The contrast-phase images of C2C12 muscle myoblasts induced to
173 differentiate as described in Methods is reported in Supplemental data (Fig 2). Myogenic
174 differentiation is characterized by consistent and progressive modifications of cellular morphology
175 such as tendency to single myoblast to elongate and fuse leading to the formation of syncytia with
176 more nuclei (myotube) and modifications of the protein expression pattern with the appearance of
177 typical protein markers of myogenic differentiation, such as sarcomeric actin, caveolin-3¹⁹,
178 phospho-p38 MAPK and the nuclear translocation of the transcription factor, myogenin²⁰
179 (Supplemental data). Following atrophy induction by serum starvation or treatment with Dexa, as
180 reported in supplemental data, C2C12 myotubes show a clear modification of cell morphology with
181 a significant decrease in myotube size. It is worthy to note a significant decrease to a third and to a
182 half of the control value after treatment with 24 nM total polyphenol and tocopherol extracts,
183 respectively. While 4 nM tocopherol does not have any significant effect, 4 nM polyphenol extract
184 is still able to significantly reduce cell size.

185 Interestingly, the incubation with total polyphenol extract did not prevent the reduction of
186 the cellular diameter elicited by Dexa or serum starvation (Table 4), whereas the pre-treatment with
187 tocopherols is able to revert the atrophic phenotype measured as cellular diameter as well as
188 myonuclei number. Table 4 also shows, as a positive control, the effect of protein synthesis
189 inhibitor cycloheximide (3 μ M), that promotes cell size and micronuclei number decrease, similarly
190 to what observed with serum starvation and Dexa treatment.

191 In another set of experiments we have investigated the modifications on cell atrophy by
192 measuring the expression of the protein marker MAFbx/atrogen-1 (Fig.2). When compared to
193 control cells, the basal level of expression of MAFbx/atrogen-1 decreases after treatment with
194 polyphenol and tocopherol extracts while, as expected, increases after Dexa addition (Fig.2A). In
195 contrast, the treatment with polyphenol extract induces, whereas with tocopherol extracts reduces
196 the MAFbx/atrogen-1 levels in atrophy-induced cells (Fig.2A). A significant reduction of ubiquitin
197 ligase content is also found in serum-starved cells treated with total tocopherol (Fig.2B and
198 polyphenol extract (Fig.2C). Successively, we have investigated the effect of pure γ -tocopherol, the
199 main tocopherol found in chestnuts. Treatment with γ -tocopherol (24 nM) has a different effect
200 compared to that promoted by total tocopherol extract on atrophic myotubes (Fig.2D). Indeed, the
201 addition of the pure compound increases 3.1-fold the expression of MAFbx/atrogen-1 in control
202 cells, whereas, added to Dexa-treated cells, it slightly reduces the expression of the atrophy marker
203 (Fig.2D, see also 5A).

204

205 *Effect of total polyphenol and tocopherol extracts on protein synthesis in C2C12 myotubes*

206

207 In order to quantify the effect of polyphenol and tocopherol extracts on cell muscle atrophy we have
208 also measured the incorporation of [3 H]-leucine into proteins (Table 5) in both starved- and Dexa -
209 treated cells in the presence and absence of total extracts. The incubation with Dexa for 24 hours
210 does not modify the incorporation of radioactivity into proteins in respect to control cells, whereas
211 both polyphenol and tocopherol extracts increase it by approximately 1.7-fold and 2.0-fold,
212 respectively, in agreement with their role in the promotion of myogenic program. The incubation
213 with both Dexa and polyphenol extract does not significantly changes incorporation of [3 H]-leucine
214 into proteins compared to polyphenol extract alone, but radioactivity content remains significantly
215 different from Dexa-treated myotubes. In contrast, treatment with both Dexa and tocopherol extract
216 changes protein radioactivity to 1.40-fold and 0.66-fold compared to Dexa and tocopherol-treated
217 cells, respectively. Serum starvation induces a 48% reduction in the incorporation of radioactivity
218 into proteins. In this experimental condition, the presence of tocopherol extract increases [3 H]-

219 leucine incorporation (1.37-fold), whereas polyphenol extracts do not significantly change protein
220 synthesis rate compared to control cells. However, both extracts are able to prevent the effects of
221 serum starvation.

222 223 *Effect of polyphenols and tocopherols on skeletal muscle cell autophagy*

224
225 Since autophagy is reported to be involved in protein breakdown in skeletal muscle atrophy, we
226 have also analyzed the morphology of C2C12 myotubes following treatment with Dexa alone and in
227 combination with total polyphenol or tocopherol extracts, and quantified the relative expression of
228 Beclin-1, a known autophagic marker (Fig. 3). Notably, the treatment of atrophic myotubes with
229 Dexa and polyphenol extract, but not tocopherol extract, leads to the appearance of
230 autophagosomes, a hallmark of autophagy and to an additive effect on Beclin-1 expression
231 compared to Dexa alone. Total tocopherol extract does not prevent Dexa-induced autophagy.

232 233 *Involvement of ERK1/2 on γ -tocopherol action*

234
235 In order to have an insight into the mechanism of action of tocopherol, we have incubated C2C12
236 myotubes with γ -tocopherol and we have studied the activation of ERK1/2, recently reported to be
237 an upstream regulator of Atrogin-1²⁵. As reported in Fig. 4, in the presence of Dexa, γ -tocopherol is
238 able to increase the level of phospho-ERK1/2, the active form of this MAPK, supporting a role of
239 pure compound in the activation of ERK1/2 signaling.

240 241 *Effect of sphingolipids on Dexa-induced skeletal muscle cell atrophy*

242
243 Finally, we have investigated whether SL extract is able to modify the expression of the atrophy
244 protein marker MAFbx/atrogin-1. As shown in Fig. 5A, although SL extract does not have any
245 significant effect on the basal level of the atrophy protein, it is able to prevent the effect of Dexa,
246 decreasing MAFbx/atrogin-1 levels of approximately 40% , similarly to what observed with total
247 tocopherol extract (Fig. 3A), but not with γ -tocopherol (Fig. 3C and 5A).

248 Among the SLs, it is reported that the bioactive lipid S1P, derived from sphingosine kinase
249 (SK) activation, plays a crucial role as trophic survival factor in skeletal muscle¹⁹⁻²⁰. In order to
250 investigate whether skeletal muscle cell atrophy is correlated to S1P content, we have treated
251 C2C12 myotubes with the SK inhibitor compound II (iSK)⁴¹ (Fig. 5B). We have observed that the
252 inhibition of S1P generation promotes MAFbx/atrogin-1 expression, and slightly reduces Dexa

253 effect. In contrast, serum deprivation does not have any effect on the up-regulation of
254 MAFbx/atrogen-1 expression elicited by iSK.

255

256 **Experimental**

257

258 *Materials and chemicals*

259

260 Chemicals, standards, cell culture reagents, Dulbecco's Modified Eagle's Medium
261 (DMEM), fetal calf serum (FCS), protease inhibitor cocktail, bovine serum albumin, horse serum
262 (HS) and dexamethasone (Dexa) were purchased from Sigma (St. Louis, MO, USA); C2C12 cells
263 were obtained from American Type Culture Collection (ATCC CRL1772, Manassa, VA, USA);
264 Coomassie Blue reagent was from Bio-Rad (Hercules, CA, USA); D-erythro-Sphingosine 1-
265 phosphate (S1P) was from Calbiochem (San Diego, CA, USA); tocopherol set was obtained from
266 Sigma (St. Louis, MO, USA). Sphingosine kinase inhibitor (SKI-II) was from Tocris (Bristol, UK);
267 CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was from Promega (Promega
268 Madison, WI, USA); penicillin/streptomycin were from Invitrogen (Carlsbad, CA, USA); anti-
269 MAFbx/atrogen-1 antibody, anti-beclin, anti-phospho-ERK1/2 antibodies from Cell Signalling
270 (Danvers, MA, USA); (C20); anti-myogenin, anti-caveolin-3, anti- β -actin antibodies and secondary
271 antibodies conjugated to horse radish peroxidase were from Santa Cruz (Santa Cruz Biotechnology,
272 CA, USA); L-[3,4,5-³H]-Leucine ([³H]-leucine), (3.7-5.56 TBq/mmol) was from Perkin Elmer
273 (Monza, Italy). Enhance chemiluminescence kit (Luminata Western HRP) were from Millipore
274 (Merck Millipore Headquarters Billerica, MA) and high-performance chemiluminescence film was
275 from GE Healthcare Life Sciences (Chalfont St. Giles, UK).

276

277 *Sample preparation*

278

279 Flours directly furnished by 12 producers from 7 different area of Tuscany (Supplemental
280 data) immediately after milling have been kept at $16 \pm 2^\circ\text{C}$ till the time of analysis. Table 1
281 describes the areas of origin, the varieties of the fruits and the characteristics of the chain of
282 production such as the mode of essication (wood essicator "*metato*" or air oven) the temperature
283 and time of essication, and the type of mill (stone, or hammer, water mill or electrical mill).

284

285 *Traditional drying*

286

287 The drying of chestnuts and/or marrons has represented for centuries the most common method of
288 transformation of these fruits since it permitted to use the product all year round. The drying

289 process is still carried out in a traditional structure called “*metato*”, located directly in the orchards
290 or close to the producer’s house (Supplemental Material).

291 The “*metato*” is a rectangular structure built in stone without using cement (the so called “a
292 secco” technique: dry-stone technique). The only room is divided, at half height, by a layer of
293 chestnut wooden boards. Through a window, located on the higher part of the structure, fruits are
294 introduced and placed on the boards, ready for drying. Normally a 30 cm thick layer of chestnuts is
295 left on the wooden board to be dried. In the lower part of the structure a fire exclusively made of
296 chestnut wood and chaff is lighted. The fire must be rigorously kept without flame in order to
297 maintain a constant heating of 35°C-40°C. The drying process lasts about 40 days. Considering that
298 the loading into the “*metato*” is carried out progressively during the harvest, it is important to turn
299 over the chestnuts each time (or on a weekly basis) a new load is added so that the fresh fruits are
300 always placed as close as possible to the heat. After drying, the fruit is mechanically separated from
301 the skin and the pellicle (threshing). The threshing is followed by a fruit selection for a further
302 quality control.

303

304 *Flour preparation*

305 In some samples, grinding is carried out with traditional millstones and other samples have been
306 obtained with modern techniques using heat pumps, thresher drums and hammer mills that improve
307 the productivity. After the collection, the chestnut flour were analyzed as described in²⁹⁻³⁰.

308

309 *Determination of chestnut and chestnut flour chemical composition*

310

311 The moisture content was determined by drying in an oven at 103°C for 6 h as described in Bellini
312 et al.²⁹ The total nitrogen was quantified using the Kjeldahl method and the percentage of nitrogen
313 was transformed into proteins by using the conversion factor of Mc Carthy (Nx5.3)²⁹.

314 For the fat determination, the sample was hydrolyzed by heating in the presence of 25%
315 hydrochloric acid. The acidic solution, mixed with ethyl alcohol, was then extracted with ethyl ether
316 and petroleum ether. The solvent was removed and the fat residue weighed²⁹. For the evaluation of
317 the percentage of saturated, monounsaturated and polyunsaturated fatty acids, fat extract was cooled
318 and transesterified in the presence of potassium hydroxide methanolic solution. The methyl esters
319 were separated by GC with FID detector and expressed as % m/m.

320 The determination of sucrose, fructose and glucose was carried out on the pulp of the fruit of
321 flour by extraction in water, sonication of the solution (power 50-60%, room temperature) for 30

322 minutes and HPLC analysis (mobile phase acetonitrile: water 80:20, quaternary amine column
323 Spheri Amino-5, 5µm 4.6 x 220 mm, refractive index detector)²⁹.

324 Determination of starch content was performed using the enzymatic kit from Boeringer
325 Mannheim (Roche .Daemstadt, Germany). Ash analysis was conducted after incineration of
326 samples at 525°C for approximately 8 h. For the determinations of metals, ash residues were
327 dissolved in 1% nitric acid solution. The solutions were then analyzed by atomic absorption
328 spectrophotometer Spectr AA220 (Varian, Polo Alto, USA).²⁹

329

330 *Preparation of phenolic extracts*

331

332 A 20 g sample was weighed in a centrifugal tube and then added to 70 ml of 80 % methanol
333 solution. The suspension was extracted in a ultrasonic bath for 15 min (90 % intensity, room
334 temperature) in order to obtain better efficiency in the destruction of cell walls that contained
335 phenolic compounds. The extract was separated by vacuum filtration and then transferred into a 100
336 ml flask, adding the necessary volume of deionized water to bring it to 100 ml. That solution was
337 transferred into 250 ml round-bottom conical flask, added with 11 ml of hydrochloric acid at 37%,
338 then hydrolyzed for 2 h at 90 °C to obtain aglycone form of the phenols. When the extract was cool,
339 it was adjusted to pH 7.0 with sodium hydroxide 6 M and the volume was brought to 200 ml with
340 80% methanol³¹.

341

342 *Estimation of total phenolic content*

343

344 The total phenolic content of the extracts was determined with Folin-Ciocalteu colorimetric
345 reaction³². External calibration curve were performed using gallic acid as a standard. Five hundred
346 µl of the phenolic extract and 2.5 ml of Folin-Ciocalteu reagent (diluted 1:10) were placed into a 5
347 ml flask and then, after 2 minutes, taken up to 5 ml with 75 g/l Na₂CO₃. The solution was mixed
348 and incubated at 50 °C for 15 minutes. After cooling, absorbance was measured at 760 nm using a
349 UV-VIS Cary 1-E spectrophotometer (Varian, Palo Alto, USA).

350

351 *Estimation of total flavonoid content*

352

353 Strata-X 6 ml/500 mg SPE cartridge (Phenomenex, Macclesfield, United Kingdom) was activated
354 in order with 10 ml of ethyl acetate, 10 ml of methanol and 10 ml of 0.01 M hydrochloric acid. 10
355 ml of the phenolic extract were filtered through 0.45 µm RC syringe filter. 5 ml of the filtrate were

356 loaded into activated cartridge. 6 ml of 0.01 M hydrochloric acid were flushed through cartridge
357 and then discarded. 40 ml of ethyl acetate were used to elute the flavonoid fraction. Flavonoid
358 fraction obtained from SPE treatment was evaporated to dryness on a rotary evaporator, then
359 dissolved in 10 ml of 80% methanol. Aluminum chloride colorimetric method was used for
360 determination of flavonoids³³. Seventy five μ l of 50 g/l sodium nitrite were added to 2 ml of extract
361 (appropriately diluted). Aluminum chloride (0.15 ml of 100 g/l) was added after 5 min; 0.5 ml of 1
362 M sodium hydroxide and 0.25 ml of water were added 6 min later. Absorbance was measured at
363 510 nm, using UV-VIS Cary 1-E spectrophotometer (Varian, Palo Alto, USA). External calibration
364 curve was performed using quercetin as standard.

365

366 *Preparation of tocopherol and sphingolipid extracts*

367

368 The tocopherol extract was prepared performing the saponification procedure³⁴ that consists of
369 mixing in a 250 ml round-bottom conical flask 20 g flour with a solution containing 30 ml absolute
370 ethanol, 60 ml of 500 g/l potassium hydroxide, 15 ml of 100 g/l ascorbic acid and 30 ml sodium
371 chloride saturated solution. The round flask were equipped with a reflux condenser and then heated
372 at 70°C for 45 min. After cooling, the saponified mixture was extracted three times with 100 ml of
373 10% ethyl acetate in hexane. Extracts were collected and filtered through anhydrous Na₂SO₄. The
374 extract was evaporated to dryness and reconstituted in 8 ml of heptane.

375 SL extracts were prepared from chestnut flours obtained from the specific areas. The sample
376 was weighed in a screw tube and then added to 5 ml of CHCl₃: MeOH (1:2). The suspension was
377 extracted in a ultrasonic bath for 10 min. The mixture was then incubated at 37.5 °C for 1 h in a
378 closed screw tube, cooled down to room temperature and filtered. Saponification, used to remove
379 phospholipids and neutral lipids from SLs, was performed using 1 M KOH for 6 h at 40°C with
380 stirring, and successively the samples were neutralized and washed using the method of Folch et al.
381 ³⁵. After solvent partition of SLs, HP-TLC analysis was performed accordingly to standardized
382 procedures with some modifications³⁶. Briefly, the plates (silica gel 60) were activated for 30 min at
383 120°C before use. Standards and reconstituted samples were applied onto the plate with a
384 microdispenser and plate developed with mobile phase (chloroform:methanol:water, 65:25:4). To
385 quantify each lipid species (SM, cerebrosides, galactosylceramide), calibration curves were
386 obtained by running in parallel known amounts of purified lipid standards. The plates were
387 visualized with sulfuric acid-CuSO₄-ammonium molybdate spray reagent followed by heating at
388 110°C for 15 min (SM) or with 3% cupric acetate in 8% phosphoric acid solution for 10 min at
389 180°C (ceramide) or with 0.5% α -naphthol in methanol-water, 1:1, dried at room temperature,

390 sprayed with sulfuric acid- water, 95:5, and heated at 100°C. Densitometry of standard and sample
391 zones was performed and the SLs content determined by interpolation from the graph.

392

393 *Estimation of total tocopherols content*

394

395 Tocopherols were quantified according to the official NF ISO method 9936³⁷ by high-performance
396 liquid chromatography (HPLC-1100 series, Hewlett-Packard) equipped with fluorescence detector
397 (excitation λ 295 nm, emission λ 330 nm). Analysis of tocopherols was performed using a
398 thermostated Phenomenex Luna silica column (25 cm \times 4.6 mm i.d. \times 5 μ m), at 25 °C, with the
399 corresponding analysis guard column. The HPLC mobile phase consisted of heptane/t-butyl methyl
400 ether (94:6 v/v), eluted at a flow rate of 1.5 ml/min.

401

402 *Cell culture and treatments*

403

404 Murine C2C12 myoblasts were routinely grown in DMEM supplemented with 10% FCS. For
405 myogenic differentiation experiments, cells were grown in until 95% of confluency and induced to
406 differentiate by switching to differentiation medium (DM), DMEM containing 1-2% horse serum
407 (HS) for the following 3-5 days. To obtain the phenotype of atrophic cells, C2C12 myotubes (MT)
408 were maintained in a culture medium free of nutrients and growth factors (-FCS, starvation) for 24-
409 48 hours, or treated with Dexa, a compound which enhances the degradation of proteins²⁵.

410 The analysis of the atrophic phenotype was performed a) by evaluating cell morphology
411 under a phase contrast microscope and quantifying the number of myonuclei and the cell diameter
412 (fiber size), b) by a molecular approach consisting in the evaluation by Western Blotting analysis of
413 the expression of MAFbx/Atrogin-1, the ubiquitin ligase typically expressed in atrophic cells²⁴.

414

415 *LDH activity determination*

416 Cell toxicity was determined by lactic dehydrogenase (LDH) activity release in cell medium.
417 Positive control of cell lysis was performed adding 0.1% Triton X 100 for 30 min to cell monolayer.
418 Values were expressed as mean % \pm SD of two independent experiments, each at least in duplicate.

419

420 *Viability MTS assay*

421

422 Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-
423 sulfophenyl)-2H-tetrazolium (MTS) assay²⁰. Briefly, C2C12 cells in 96-well plates were plated at

424 50% confluence and synchronized in serum-free medium for 24 h. Then, cells were incubated in
425 medium containing 10% FCS, and treated with bioactive molecules or specific inhibitors for the
426 indicated times and incubated for further 24 h. MTS assay kit was utilized to measure production of
427 formazan, which is directly proportional to the cell viability. Optical density at 490 nm wavelength
428 was determined using an ELISA microplate reader (Bio-Rad, Hercules, CA, USA).

429

430 *Protein synthesis measurement by [³H]-leucine incorporation*

431

432 To measure the rate of protein synthesis, C1C12 differentiated cells (24-well plates), treated or not
433 with Dexa or starved as indicated for each specific experimental condition, were incubated in Leu-
434 labeling medium RPMI (without Leu), and labeled with 0.037 MBq /ml of [³H]-leucine for 2 hours.
435 At the indicated time points, the monolayer was washed with phosphate buffered saline and
436 incubated in ice cold 10% trichloroacetic acid (TCA) for 30 min at 4°C. The TCA-precipitable
437 material was washed with cold 5% TCA, and then solubilized in 0.1 M NaOH. [³H]-leucine
438 incorporation was measured by liquid scintigraphy in a beta counter (Beckman). Decays per minute
439 (DPM) were normalized by total protein for each well.

440

441 *Western blotting analysis*

442

443 Immunoblotting was performed using enhanced chemiluminescence as previously reported^{27, 38}.
444 Cells were lysed for 30 min at 4 °C in a buffer containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl,
445 1 mM EDTA, 6 mM EGTA, 15 mM Na₄P₂O₇, 20 mM NaF, 1% Nonidet and protease inhibitor
446 cocktail (1.04 mM AEBSF, 0.08 μM aprotinin, 0.02 mM leupeptin, 0.04 mM bestatin, 15 μM
447 pepstatin A, 14 μM E-64) essentially as described in³⁸. Cell extracts were centrifuged for 15 min at
448 10,000xg at 4 °C. Proteins (10-25 μg) from lysates were resuspended in Laemmli's sodium
449 dodecylsulfate (SDS) sample buffer. Samples were subjected to SDS-polyacrylamide gel
450 electrophoresis (SDS-PAGE) and Western Blotting analysis as previously described³⁸. The blots were
451 first blocked in 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature
452 for 1 h, and then incubated in the same blocking solution containing primary antibodies overnight at
453 4°C. After sufficient washes with TBST, blots were incubated with appropriate horseradish
454 peroxidase-conjugated secondary antibodies for 1 h. Blots were again washed in TBST and,
455 thereafter, signals were detected by using Luminata Western HRP reagent. Finally, blots were
456 exposed to high-performance chemiluminescence film. Densitometric analysis of the bands was
457 performed using NIH IMAGE (ImageJ software, Bethesda, MD, USA) and Quantity-One (Imaging

458 and Analysis Software by Bio-Rad Laboratories, Hercules, CA) and band intensity was reported as
459 relative percentage (means \pm SEM), obtained by calculating the ratio of specific protein on β -actin
460 or β -tubulin intensity and normalizing to control, set as 100.

461

462 *Statistical analysis*

463

464 For all the experiments at least three samples were analysed and all the assays were carried out in
465 triplicates. The results are expressed as range of mean values and standard error (S.E.M.) or
466 standard deviation (S.D.). The differences between the chestnut extracts were analysed using one-
467 way analysis of variance (ANOVA), considering statistical significant $P < 0.05$.

468 **Discussion**

469

470 The present study points out that tuscan chestnut sweet flour is a natural source of specific
471 bioactive components (tocopherols and SLs) able to counteract the degenerative processes (atrophy
472 and autophagy) responsible for the loss of skeletal muscle mass. The basic composition and
473 nutraceutical properties of sweet chestnut flours obtained from fruits collected from 7 different
474 areas of Tuscany (Italy) were found to be very similar; the differences seemed to be dependent on
475 several parameters from the methodologies used to obtain the flours to the tipology of cultivar or
476 regions of origin. The chemical composition was also similar to that published for fruits of other
477 territorial origins reviewed in⁶. In the present manuscript, we also show the vitamin E profile that
478 appears to be similar along all the assayed tuscan sweet chestnut flours (γ -tocopherol > δ -tocopherol
479 > α -tocopherol > β -tocopherol), and to what has been reported from other chestnut fruits⁴.

480 It is worth noting that the content in tocopherols measured in sweet chestnut flours (18-114
481 mg/kg) is higher than that found in fruits by Barreira et al.,⁴ and de Vasconcelos et al.⁶, that have
482 reported a value of 4 and 4-27 mg/kg for Portuguese chestnuts, respectively, likely due to the loss of
483 water during flour preparation, or other factors such as harvesting year, sampling or methodological
484 analytical procedures.

485 Although almonds, hazelnuts, walnuts, pistachios and pecans are not generally used to
486 produce flour, the comparison between the composition of chestnut flours and these fruits indicates
487 a quite dissimilar qualitative vitamin E profile from almond or hazelnut, but very similar to that
488 obtained from walnut, pistachios or pecans⁴².

489 The high γ -tocopherol dominance in chestnut flour may represent an important feature for
490 the nutraceutical properties of this product, since γ -tocopherol is known to be the most effective
491 isoform in scavenging free radicals and nitrogen oxygen species and to have anticancer and anti-
492 inflammatory action^{1, 43}.

493 Table 3 indicates that γ -tocopherol content was in the same range for all geographic areas
494 considered (89-96%). We have found some statistical differences in γ -tocopherol content among the
495 analysed samples, also of the same area, suggesting an influence of the drying parameters in
496 preserving the content of bioactive compounds.

497 Numerous studies performed using animal models have confirmed that prolonged skeletal
498 muscle inactivity/or atrophic conditions promote oxidative stress, leading to an increase of reactive
499 oxygen species (ROS) produced in both inactive and contracting skeletal muscles. When ROS
500 production in cells exceeds the antioxidant capacity to maintain the normal redox balance, a pro-
501 oxidant state occurs altering redox signaling.

502 The here reported findings demonstrate that long-term incubation with chestnut flour
503 tocopherol and polyphenol extracts decreases myoblast proliferation. Since no apoptotic hallmarks
504 are found, the decrease in cell growth appears due to cell cycle arrest and, likely, in the induction of
505 myogenic differentiation (E.M., personal communication).

506 A growing number of *in vitro* studies reveal that selective antioxidants can delay disuse
507 muscle atrophy. Notably, it is worth noting that total tocopherol extracts from tuscan chestnut sweet
508 flour protect from skeletal muscle cell atrophy and this protection appears not to be due to a general
509 antioxidant action, since the total polyphenol extract, that are also considered antioxidants, fails to
510 protect C2C12 cells either from changes in morphology than from reduction in protein synthesis. In
511 addition, chestnut flour tocopherol extract does not affect Dexamethasone-induced autophagy, whereas,
512 polyphenol extract potentiates the effect of Dexamethasone on Beclin-1 expression and on the acquisition of
513 an autophagic phenotype, indicating a more severe cytotoxic effect after treatment with this latter
514 bioactive molecule. Notably, although both total tocopherol extract and γ -tocopherol can prevent
515 Dexamethasone-induced atrophy, each of the compounds differently affects Atrogin-1 expression. In
516 particular, pure γ -tocopherol alone increases expression of the atrophy marker MAFbx/atrogin-1
517 and alters cell morphology in accordance with recent studies that have cast doubt on the benign
518 effects of long-term antioxidant supplementation⁴⁴. In contrast, although γ -tocopherol accounts for
519 more than 90% of the tocopherol extracts, the latter has a positive effect both in control and in
520 atrophic myotubes. On the other hand, the total extract may contain other marginal compound(s)
521 responsible for the beneficial effects.

522 Therefore, tocopherol mixtures, obtained from natural products, might offer beneficial advantages
523 in some patients and in aging populations because they possess enhanced anti-inflammatory activity
524 compared to pure compounds, as also reported in various studies^{1,43}. Moreover, the properties of
525 tocopherol chestnut extracts likely able to counteract ROS-mediated processes and/or directly exert
526 antioxidant effects, might be useful for ameliorating skeletal muscle performance. This *in vitro*
527 study needs to be confirmed in animal models since the bioavailability of the active compounds
528 could preclude the putative beneficial effects. Moreover, the translation from animal models to
529 humans, already occurred for other molecules such as curcumin, will allow to address the
530 pharmacokinetics, safety, and efficacy of this nutraceutical in human health. Since chestnut flour is
531 a gluten-free flour, the nutraceutical properties reported in this study and others⁵⁻⁹ allows to
532 consider this functional food as a part of a diet for people affected by coeliac disease.

533 A novel and relevant finding of the present study is the presence of SLs in chestnut flour and
534 the protective role played by SLs extracts on skeletal muscle cell atrophy. Although, SLs represent
535 an important source of bioactive molecules, only a few of the published studies reported the

536 analysis of their content in food, found to be some hundreds of mg/kg¹⁷. The content of SLs
537 approximately 50 mg/kg dry weight is a relatively low concentration, but appears to be sufficient to
538 prevent MAFbx/atrogen-1 expression.

539 SLs are first and/or second messengers involved in the regulation of cell fate, inflammation
540 and migration¹⁸ and are known to play an important role in maintaining cellular redox homeostasis
541 through regulation of NADPH oxidase, mitochondrial integrity, and antioxidant enzymes⁴⁵. It is
542 worth noting that diseases which result in muscle weakness, such as heart failure, are characterized
543 by alteration in SLs metabolism⁴⁶: elevated sphingomyelinase (SMase) activity that, in intact
544 muscle, increases mitochondrial ROS that contribute to diminished muscle force⁴⁷.

545 Bioactive SLs (ceramide, S1P and ceramide-1-phosphate) play a crucial role in skeletal
546 muscle cell biology, and, since they are interconvertible molecules^{18,46}, they often exhibit opposite
547 actions also in skeletal muscle cells^{26,36}: S1P is a prosurvival and promyogenic factor¹⁹⁻²¹ whereas
548 ceramide inhibits myogenesis⁴⁸.

549 The characterization of the specific bioactive lipids of chestnut extracts able to affect
550 myotube atrophy, was not an aim of the present work. However, on the basis of the relevance of
551 these compounds for the physiopathology of many tissues, it will be worthy to be investigated.

552 The here reported protective effect might be mediated by S1P since in our experimental
553 conditions SK inhibitor increases the expression of the atrophy marker Atrogen-1/MAFbx, and
554 decreases the nuclei number and cell diameter (E.M., personal communication), supporting a
555 positive role of SK/S1P axis on the maintenance of a normal differentiated skeletal muscle
556 phenotype. Whether the protective effect of chestnut sweet flour SL extracts is due to the specific
557 bioactive lipid S1P and whether it is related to maintenance of redox homeostasis or to a different
558 mechanism is worth to be further investigated. Indeed, it might help to clarify the nutraceutical
559 properties relevant for skeletal muscle mass regulation and to address an important question on
560 which conditions, in the chestnut flour chain of production (methodological, industrial, etc.) must
561 be kept under control to assure the preservation of these nutraceutical properties.

562 Moreover, since many studies have suggested that a low vitamin E nutritional status and the
563 level of SLs in plasma are associated with pathological condition⁴⁹⁻⁵¹, a diet enriched in natural
564 products containing tocopherol and SL extracts might be advantageous for maintaining a healthy
565 status and wellness.

566

567 **Conclusion**

568

569 The present work provide the first evidence that chestnut sweet flour is a source of specific
570 bioactive components, such as tocopherols and SLs, with a relevant role in the prevention of cell
571 degeneration and maintaining of skeletal muscle mass. Therefore, these observations might be
572 useful in designing appropriate nutritional therapeutical approaches in skeletal muscle cell atrophy,
573 consisting in a diet enriched of food naturally rich in SLs or developing products fortified with SLs
574 that may be of help for an aged population that undergo to a physiological decrease of skeletal
575 muscle mass and tone.
576

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578

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582

583 **Non conflict of interest statement.**

584

585 The authors declare that there are no conflicts of interest with any financial organization regarding
586 the results presented in this research manuscript.

587

588

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732

733 LEGENDS

734

735 **Fig. 1.** Effect of total polyphenol and tocopherol extracts on skeletal muscle cell growth.

736

737 A) Cell counting. C2C12 cells were serum-starved for 24 h and, successively, incubated in growth
738 medium in the absence or in the presence of vehicle (control), polyphenols (100 nM) or tocopherols
739 (100 nM) for the indicated time (days). Cells were collected by trypsinization and counted using
740 Burker camera. Values are means \pm S.E.M. of at least three independent experiments performed in
741 quadruplicate (*P < 0.05 vs. vehicle).

742 B) MTS-dye reduction assay. C2C12 cells were treated as above and formazan product was
743 quantified by reading optical density at 490 nm. Values are means \pm S.E.M. of three independent
744 experiments performed in quadruplicate (*P < 0.05 vs. vehicle t0, §P<0.05 vs. specific control).

745

746 **Fig. 2.** Effect of polyphenol and tocopherol extracts on skeletal muscle cell atrophy.

747

748 A-D) Western blot analysis of MAFbx/atrogin-1 (Atrogin-1) expression. C2C12 myotubes were
749 incubated with vehicle or with polyphenol or tocopherol extracts (100 nM) for 1 hour before the
750 addition of Dexa (A) or serum-starved as indicated (B,C) or treated with γ -tocopherol (24 nM) as
751 indicated in (D). Thirty six hours later, cells were harvested and lysed as described in Methods.
752 Total lysates (20 μ g) were used to immunodetect the level of MAFbx/atrogin-1 with specific
753 antibodies and revealed by enhanced chemoluminescence. Equally loaded protein was checked by
754 expression of the non-muscle specific β isoform of actin. The band intensity is reported in the
755 graphics as percentage relative to the intensity of the band corresponding to the respective
756 unchallenged control set as 100. Values are means \pm S.E.M. of three independent experiments (*P <
757 0.05 vs control (vehicle) in 3A and 3D or serum starvation (-FCS) in 3B and 3C, §P<0.05 vs. Dexa).

758

759 **Fig. 3.** Effect of polyphenols and tocopherols on skeletal muscle cell autophagy.

760

761 A) Images of a representative field were obtained by phase contrast microscopy. C2C12 myotubes
762 (MT) were treated in the presence or absence of total polyphenol or tocopherol extracts before the
763 addition of Dexa. Note in the magnification the typical autophagic vacuoles.

764 B) Western Blotting analysis. C2C12 differentiated cells were treated as in A). Twenty-four hours
765 later, myotubes were harvested and lysated as described in Methods. Specific antibodies were used

766 to immunodetect Beclin-1 (Beclin), revealed by enhanced chemoluminescence. Equally loaded
767 protein was checked by evaluating the expression level of β -actin. Band intensity is reported in the
768 graphic as percentage relative to the intensity of the band corresponding to the respective
769 unchallenged control set as 100. Values are means \pm S.E.M. of at least three independent
770 experiments (Student's *t* test **P* < 0.05 vs. Dexa).

771

772 **Fig. 4.** Involvement of ERK1/2 on γ -tocopherol action.

773

774 Western blot analysis of phospho-ERK1/2 expression was performed using C2C12 myotubes
775 incubated with vehicle or with γ -tocopherol (γ -Toco) for 1 hour before the addition of Dexa as
776 indicated. Thirty six hour later, cells were harvested, lysed and total lysates (20 μ g) immunoblotted
777 to detect the level of the active form of ERK1/2 (phospho-ERK1/2, p-ERK1/2). Band intensity
778 corresponding to p-ERK was normalized on the value of β -actin and it is reported in the graphic as
779 percentage relative control set as 100. Values are means \pm S.E.M. of at least three independent
780 experiments (Student's *t* test, **p* < 0.05 vs. control (vehicle)).

781

782 **Fig. 5** Effect of sphingolipid extract on skeletal muscle cell atrophy.

783

784 A) Western blot analysis of MAFbx/atrogen-1 (Atrogen-1) expression. C2C12 myotubes were
785 incubated with vehicle or with total SLs extract for 1 hour before the addition of Dexa or treated
786 with γ -tocopherol (24 nM) as indicated. Thirty six hours later, cells were harvested and lysed as
787 described in Methods. Total lysates (20 μ g) were used to immunodetect the level of
788 MAFbx/atrogen-1 (Atrogen-1).

789 B) Western blot analysis of MAFbx/atrogen-1 (Atrogen-1) expression. C2C12 myotubes were
790 incubated with vehicle or with sphingosine kinase inhibitor (iSK) for 1 hour before the addition of
791 Dexa or serum-starved as indicated. Thirty six hour later cells were lysed and MAFbx/atrogen-1
792 immunodetected. Equally loaded protein was checked by expression of the non-muscle specific
793 protein (β -actin or β -tubulin). Band intensity is reported in the graphics as percentage relative to
794 the intensity of the band corresponding to the respective unchallenged control set as 100. Student's *t*
795 test, **P* < 0.05 vs. control; #*P* < 0.05 vs. SLs alone (in A) or serum-starved control cells in (B).