

1 **Effect of short term hazelnut consumption on DNA damage and oxidized-LDL in children**
2 **and adolescents with primary hyperlipidemia: a randomised controlled trial**

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16 **RUNNING TITLE:** Hazelnuts consumption reduces DNA damage

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20

21 **KEYWORDS**

22 Primary hyperlipidemia; children; hazelnuts; oxidative stress; DNA damage; oxidized-LDL

Abbreviations: BMI, body mass index; CI, confidence interval; CVD, cardiovascular disease; FCHL, familial combined hyperlipidemia; FH, familial hypercholesterolemia; FPG, formamidopyrimidine DNA glycosylase; HDL-C, high-density lipoprotein cholesterol; HZN+S, hazelnuts with skin; HZN-S, hazelnuts without skin; LDL-C, low-density lipoprotein cholesterol; MUFA, monounsaturated fatty acids; non-HDL-C, non-high density lipoprotein cholesterol; ox-LDL, oxidized LDL; PBMC, peripheral blood mononuclear cell; PHC, polygenic hypercholesterolemia; SD, standard deviation; TC, total cholesterol; TG, triglycerides.

23 **Highlights**

- 24 - Hazelnuts contain unsaturated fatty acids, vitamin E and bioactives as polyphenols
- 25 - Regular intake may contribute to improve lipid profile and reduce oxidative stress
- 26 - An 8-week hazelnut intervention was performed in hyperlipidemic children
- 27 - Markers of DNA damage, but not oxidised LDL, decreased after intervention
- 28 - Hyperlipidemic children may benefit from the inclusion of hazelnuts in their diet
- 29

30 **ABSTRACT**

31 Children with primary hyperlipidemia are prone to develop premature atherosclerosis, possibly
32 associated with increased oxidative stress. Nutritional therapy is the primary strategy in the
33 treatment of hyperlipidemia and associated conditions. Dietary interventions with bioactive-rich
34 foods, such as nuts, may contribute to the modulation of both lipid profile and the
35 oxidative/antioxidant status. Our study aimed to assess the impact of a dietary intervention with
36 hazelnuts on selected oxidative stress markers in children and adolescents with primary
37 hyperlipidemia.

38 A single blind, 8-week, randomized, controlled, three-arm, parallel-group study was performed.
39 Children and adolescents diagnosed with primary hyperlipidemia (n=60) received dietary guidelines
40 and were randomized into three groups: group 1 received hazelnuts with skin (HZN+S), and group
41 2 hazelnuts without skin (HZN-S), at equivalent doses (15-30 g/day, based on body weight); group
42 3 (controls) received only dietary recommendations (no nuts). At baseline and after 8 weeks, plasma
43 oxidized LDL (ox-LDL) concentrations, oxidative levels of DNA damage in lymphocytes, and
44 potential correlation with changes in serum lipids were examined.

45 A reduction of endogenous DNA damage by $18.9\pm 51.3\%$ ($p=0.002$) and $23.1\pm 47.9\%$ ($p=0.007$) was
46 observed after HZN+S and HZN-S, respectively. Oxidatively-induced DNA strand breaks
47 decreased by $16.0\pm 38.2\%$ ($p=0.02$) following HZN+S treatment. Ox-LDL levels did not change
48 after HZN+S intervention, but positively correlated with total cholesterol (TC) and low-density
49 lipoprotein cholesterol (LDL-C).

50 A short-term hazelnut intervention improves cell DNA protection and resistance against oxidative
51 stress but not ox-LDL in hyperlipidemic pediatric patients.

52 The trial was registered at ISRCTN.com, ID no. ISRCTN12261900.

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56 **1. INTRODUCTION**

57 Hyperlipidemia plays a key role in the pathogenesis of atherosclerosis through several steps.
58 Increased lipid peroxidation and associated oxidative stress trigger endothelial dysfunction [1],
59 activate plaque progression [2], and induce cardiovascular diseases (CVDs) [3,4]. This course starts
60 early in life, as demonstrated in hypercholesterolemic children, who reveal premature markers of
61 subclinical atherosclerosis, including endothelial dysfunction[5] in relation to prolonged exposure
62 to oxidative stress [6].

63 Oxidative stress results in damage of different cellular components by excessive generation of pro-
64 oxidant species, as well as deficiency of antioxidant defense mechanisms [7]. Lipids are susceptible
65 targets of oxidation and plasma oxidized LDLs (ox-LDLs) play a major role in atherosclerosis
66 development [2]. Ox-LDLs can be estimated with a reliable and sensitive technique based on
67 monoclonal antibodies [8]. Several studies in adults demonstrated significant correlations between
68 high circulating levels of ox-LDL and prevalence of CVDs, diabetes and metabolic syndrome [3].
69 Moreover, children with familiar hypercholesterolemia (FH) showed higher ox-LDL levels as
70 compared to controls [9], although data in children are scanty and, at times, contradictory [10,11].
71 Severe accumulation of endogenous pro-oxidants in cells could also generate DNA damage,
72 including modified DNA bases, that may contribute to cardiovascular dysfunction. In this context,
73 there is a growing amount of evidence documenting that oxidative stress-induced DNA damage
74 may contribute to atherosclerotic plaque formation [12]. Oxidized DNA damage can be evaluated
75 through different methodologies. The “comet assay”, also called “single cell gel electrophoresis”, is
76 a sensitive and rapid technique for quantifying and analyzing DNA damage, evaluated for example
77 as single- and double- DNA strand breaks in individual cells. Direct measurement of oxidative
78 DNA damage may be obtained through modifications of the comet assay, which allow the detection
79 of oxidised DNA bases through the use of specific enzymes, i.e. formamidopyrimidine DNA
80 glycosylase (FPG) or endonuclease III, able to detect oxidised purines and pyrimidines, respectively
81 [13]. Furthermore, DNA damage is often used to estimate cell resistance to *ex vivo* oxidative

82 treatments (i.e. hydrogen peroxide) [13,14]. The principle behind this approach is that, antioxidants
83 deriving from dietary supplementation reach the cells and enhance their ability to resist against an
84 oxidative attack. Limited studies have evaluated the levels of DNA damage in hyperlipidemic
85 subjects to assess the effect of oxidative/antioxidant status on DNA stability in lymphocytes
86 [15,16].

87 The Mediterranean diet is thought to increase protection against atherosclerosis and CVDs, and this
88 effect has been attributed to a variety of dietary components. Cardioprotective effects are also
89 associated with regular consumption of nuts due to their optimal composition in bioactive
90 compounds, such as unsaturated fatty acids, fiber, L-arginine, tocopherols, polyphenols and
91 phytosterols [17,18]. Furthermore, the ingestion of whole nuts, including their skin where a
92 significant part of their antioxidant polyphenols reside, could also contribute to the cholesterol-
93 lowering effect [19].

94 The aim of this study was to compare the effect of hazelnut consumption, with skin (HZN+S) or
95 without (HZN-S), on oxidative stress markers, evaluated by ox-LDL, endogenous and oxidatively-
96 induced DNA damage, in children and adolescents with primary hyperlipidemia.

97

98 **2. MATERIALS AND METHODS**

99 **2.1 Experimental design**

100 The study was approved by the Ethics Committee of the City of Health and Science University
101 Hospital of Turin (Italy) (EC:CS377) in accordance with the principles outlined of the Declaration
102 of Helsinki and was registered under ISRCTN.com (identifier no. ISRCTN12261900).

103 The full experimental design was previously reported [20]. Briefly, sixty-six hyperlipidemic
104 children and adolescents (mean age 11.6 ± 2.6 years) were enrolled within pediatric patients cared
105 at the Department of Public Health and Pediatric Sciences of the University of Turin to participate
106 in a trial evaluating the effect of hazelnuts *Corylus avellana* L. (cultivar 'Tonda Gentile delle
107 Langhe' from Piedmont, Italy) on serum lipid profile and fatty acid composition of erythrocyte

108 phospholipids [20]. To be eligible, screened children and adolescents were required to be normal-
109 weight [body mass index (BMI) <90th percentile for age and sex] with diagnosis of primary
110 hyperlipemia - including familial hypercholesterolemia (FH), familial combined hyperlipidemia
111 (FCHL) or polygenic hypercholesterolemia (PHC) - with total serum cholesterol (TC) and/or
112 triglycerides (TG) levels higher than age- and sex-specific 90th percentile. Diagnostic criteria of
113 primary hyperlipidemia were based on accepted international standards as previously reported [21].
114 Subjects with secondary hyperlipidemia or other disorders, obesity, lipid-lowering treatments
115 (including functional foods), allergy or aversion to nuts were excluded. All participants and their
116 legal guardians agreed to participate in the second step of the trial and to collect blood in order to
117 perform further analysis.

118 The intervention study was an 8-week, randomized, single-blind, controlled trial, with three parallel
119 treatment arms and was performed between January 2015 and October 2015. Detailed information
120 on study intervention and its impact on lipid profile have been previously reported [20].

121 A pediatrician who was not involved in the study and in sample analysis was appointed to allocate
122 patients to the different treatments according to a randomization list obtained through the center
123 database. The numbers of participants who were randomly assigned to different study groups, the
124 rate of patients completing the study and analyzed for the primary outcome are depicted in **Figure**
125 **1**. Subjects were deprived of nuts for 3 weeks before the beginning of the study, and were randomly
126 assigned to one of three groups with a 1:1:1 ratio (22 subjects each): 1) HZN+S group received
127 unpeeled hazelnuts; 2) HZN-S group received peeled hazelnuts; 3) control group received only
128 dietary recommendations. Participants in the two hazelnut groups were instructed to consume one
129 daily portion of roasted HZN-S or HZN+S for 8 weeks, while those in the control group were asked
130 to avoid nut consumption for the entire intervention period. The amount of hazelnuts was calculated
131 on the basis of children's body weight and corresponded to servings of 15-30 g. Hazelnuts provided
132 about 50% of fat, mainly monounsaturated fatty acids (MUFAs) and in particular oleic acid (>80%
133 of total fat). Moreover, hazelnuts are source of phytosterols, tocopherols and minerals. Moreover,

134 HZN+S provided a concentration of polyphenols - mainly gallic acid and procyanidin B2 - three
135 fold higher than HZN-S, and exhibited a higher antioxidant capacity (**Figure 2**) [20].

136 At recruitment, children and their families received nutritional recommendations based on the
137 cardiovascular health integrated lifestyle diet (CHILD-1) as reported [22]. All participants were
138 encouraged to maintain the same dietary habits throughout the study period. To check the
139 compliance to the instructions patients were asked to fill weekly food diaries during each
140 intervention phase and to return any residual package of hazelnuts. At the beginning and at the end
141 of the study, each participant underwent medical examination for the analysis under study.

142

143 **2.2 Blood sample collection, separation and storage**

144 Venous blood samples were collected early in the morning after an overnight fast into vacutainers
145 containing lithium heparin. PBMCs were separated by density gradient, using Histopaque 1077. A
146 total of 100µl of whole blood was gently mixed with 900 µl of cold RPMI 1640 medium in
147 microfuge tubes. Then, 100 µl Histopaque 1077 was carefully added to the bottom of the tube and
148 centrifuged at 200 ×g for 4 min at room temperature. The PBMCs were removed, washed with
149 PBS, and centrifuged for 10 s at 5000×g at room temperature to pellet the cells. The supernatant
150 was poured off and the pellet resuspended in 50 µl of PBS and used immediately for the
151 determination of *ex-vivo* resistance to oxydatively-induced DNA strand breaks. A different batch of
152 isolated PBMC was diluted into an appropriate freezing medium made of 50% fetal bovine serum,
153 40% RPMI 1640 and 10% DMSO as cryoprotectant, and stored at -80°C for the subsequent
154 determination of endogenous DNA damage.

155

156 **2.3 Evaluation of oxidatively induced DNA damage in PBMCs**

157 The evaluation of cell resistance to oxidatively induced DNA damage was performed by comet
158 assay, as previously reported [23]. Oxidative stress in fresh PBMCs was induced using 500 uM
159 H₂O₂. Two slides were prepared for each subject: one was treated with H₂O₂ (500 µmol/l in PBS)

160 for 5 min at room temperature in the dark; the other was treated for 5 min with a solution of PBS
161 only (control slide). Following the oxidative treatment, slides were immersed in a lysis buffer (2.5
162 M NaCl, 0.1 M Na₂EDTA, 10 mM Tris, 1% N-lauroylsarcosine sarcosine sodium salt, pH 10 with
163 NaOH; 1% Triton X-100 and 1% DMSO) for 1 h at 4°C in the dark. Slides were then transferred in
164 a horizontal electrophoresis tank containing an alkaline electrophoresis buffer (0.3 M NaOH, 1 mM
165 Na₂EDTA) and kept for 40 min at 4°C. Then, electrophoresis was carried out (25 V, 300 mA, 20
166 min) in the same alkaline solution at 4 °C in the dark. The samples were subsequently washed in a
167 neutralizing buffer (0.4 M Tris–HCl, pH 7.5) for 15 min at 4°C in the dark, stained with ethidium
168 bromide (2 µg/ml) for 20 min, washed in PBS, drained, and coverslipped. DNA damage produced
169 was compared to that obtained in control cells. The lower the DNA damage detected, the higher the
170 ability of the cells to protect themselves from an induced oxidative stress.

171

172 **2.4 Evaluation of FPG-sensitive sites in PBMCs**

173 Endogenous DNA damage (FPG-sensitive sites) was analysed on cryopreserved PBMCs by means
174 of the enzyme formamidopyrimidine DNA glycosylase, able to detect the oxidized purines (mainly
175 8-oxo-7,8-dihydroguanine), as previously reported [24]. In brief, cryopreserved PBMCs were
176 rapidly thawed at 37°C and washed with fresh RPMI medium and cold PBS. Cell suspension was
177 embedded in LMP agarose (1.5% wt/vol in Tris–acetate–EDTA buffer at pH 7.4, 37°C) and
178 pipetted on fully frosted slides previously precoated with NMP agarose (1% wt/vol in Tris–acetate–
179 EDTA buffer). After the lysis phase (2.5 M NaCl, 0.1 M Na₂EDTA, 10 mM Tris, 1% N-
180 lauroylsarcosine sarcosine sodium salt, pH 10 with NaOH; 1% Triton X-100 and 1% DMSO, for 1
181 h at 4°C in the dark), slides were washed three times (5 min each) in 40 mM HEPES, 0.1 M KCl
182 and 0.5 mM EDTA pH 8.0, with KOH buffer. Then, one slide was treated with a solution of FPG
183 enzyme (100 ng/ml in enzyme buffer: 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/ml
184 bovine serum albumin, pH 8.0 with KOH), while the other slide (control) with buffer without FPG.

185 Incubation was performed at 37°C for 45 min. The slides were then transferred to electrophoresis
186 buffer and processed, as previously described (see oxidatively induced DNA damage).

187

188 **2.5 Quantification of DNA damage**

189 One hundred images of nucleoids, or comets, per slide were electronically captured at 20x
190 magnification, using an epifluorescence microscope (Olympus CX 41; Olympus Italia) attached to a
191 high sensitivity CCD video camera (CFW 1808M; Scion Corporation, Germany), and to a computer
192 equipped with an image analysis system (Cometa 1.5; Immagini e Computer, Bareggio, Milan,
193 Italy). The level of DNA damage was calculated as the percentage of DNA in the tail. For each
194 subject, the percentage of DNA in the tail of control cells (slides not treated with H₂O₂ or FPG) was
195 subtracted from the percentage of DNA in the tail of cells incubated with H₂O₂ or FPG.

196

197 **2.6 Analysis of ox-LDL**

198 The serum ox-LDL concentrations were measured by an ELISA kit (MercoDIA, Uppsala, Sweden),
199 according to the manufacturer instructions. The absorbance was evaluated at 450 nm using a plate
200 reading spectrophotometer (mod. F200 Infinite, TECAN Milan, Italy). Each sample was determined
201 in duplicate. The analysis was performed in a subsample of 40 subjects, belonging to HZN+S and
202 control groups. Ratio of ox-LDL/LDL ratio and ox-LDL/HDL were also calculated.

203

204 **2.7 Statistical analysis**

205 Sample size was calculated taking into account the expected variation in the primary endpoints
206 considered based on results previously obtained from our group. In particular, 16 subjects per group
207 were estimated to be sufficient to detect significant differences in DNA damage after HZN
208 interventions, with a power of 80% and $p=0.05$. All data are presented as mean \pm standard deviation
209 (SD). Two-way ANOVA was used to compare the effect of dietary treatment (HZN+S, HZN-S or
210 control group) and time (baseline vs. 8 weeks) on the levels of DNA damage and ox-LDL.

211 Differences were considered significant at $p \leq 0.05$; post hoc analysis of the differences between
212 treatments was assessed by the Least Significant Difference test considering $p \leq 0.05$ for statistical
213 significance. Regression analysis was used to verify correlations between the variables under study
214 (DNA and lipid damage vs. serum lipids) at baseline and percent changes observed between pre-to-
215 post intervention in the three groups. Statistical analysis was performed using STATISTICA
216 software (Statsoft Inc, Tulsa, OK, USA).

217

218 **3. RESULTS**

219 **3.1 Baseline characteristics**

220 Baseline levels of DNA strand breaks, FPG-sensitive sites and oxidatively-induced DNA damage in
221 the three treatment groups of hyperlipidemic subjects are reported in Table 1. No significant
222 differences were found by one-way ANOVA ($p \geq 0.05$) at baseline among groups.

223

224 **3.2 Effect of intervention on DNA strand breaks and FPG-sensitive sites in PBMCs**

225 The effect of intervention on the levels of DNA strand breaks and FPG-sensitive sites in PBMCs is
226 reported in **Table 1**. The regular intake of hazelnuts was associated with the reduction (*time effect*)
227 of the levels of DNA strand breaks by 18.8% (95% CI: -36.8%, -2.71%, $p=0.003$) and by 13.9%
228 (95% CI: -28.6%, 0.84%; $p=0.001$), respectively after HZN+S and HZN-S intake. A significant
229 decrease (*time x treatment interaction*, $p=0.0006$) was also observed for the levels of FPG-sensitive
230 sites (endogenous DNA damage). In particular, DNA damage was reduced by 18.9% (95% CI: -
231 41.7%, +3.9%; $p=0.002$) and by 23.1% (95% CI: -45.5%, -0.66%; $p=0.007$), respectively after the
232 consumption of HZN+S and HZN-S.

233 On the contrary, an increase of 26.8% (96% CI: -0.84%, +54.5%; $p=0.04$) in FPG-sensitive sites
234 and a reduction (*time effect*) in the levels of DNA strand breaks of 21.2% (95% CI: -39.1%, -3.38%,
235 $p=0.015$) was registered in the control group at the end of the study period (**Table 1**).

236

237 **3.3 Effect of intervention on DNA strand breaks and H₂O₂-induced -DNA damage in PBMCs**

238 The effect of intervention on the levels of DNA strand breaks and H₂O₂-induced -DNA damage in
239 PBMCs is reported in **Table 1**. The consumption of hazelnuts and control treatment did not affect
240 the levels of DNA strand breaks. With regard to cell protection against an ex-vivo induced oxidative
241 stress, HZN+S treatment significantly decreased (time effect, $p=0.002$) the level of oxidatively-
242 induced DNA strand breaks by 16.0% (95% CI: -32.9%, +0.89%), while no significant effect was
243 observed after HZN-S and control treatment (**Table 1**).

244

245 **3.4 Effect of intervention on oxidized LDL levels**

246 In **Table 2** are reported the levels of ox-LDL and its ratio with LDL and HDL concentrations.
247 Data regarding the serum lipid profile was previously published [20]. Mean baseline ox-LDL levels
248 in hyperlipidemic children were 54.4 ± 15.4 U/L, and did not significantly change in the control or
249 HZN+S groups after intervention (**Table 2**). No effect was also observed in the ratio of ox-
250 LDL/HDL. A time effect was observed in the levels of ox-LDL/LDL; post hoc analysis revealed a
251 difference between baseline values of HZN+S group and control group after 8 weeks

252

253 **3.5 Correlation between serum lipid profile and markers of oxidative stress**

254 Correlations between DNA and lipid damage and serum lipid profile previously published [20] have
255 been performed to ascertain the contribution of dyslipidemia on oxidative stress in the population
256 under study. At baseline, a positive correlation between LDL-C concentrations and H₂O₂-induced
257 DNA damaged ($r= 0.34$, $p= 0.04$) was detected. Furthermore, serum TC, LDL-C and non-HDL-C
258 levels were directly related to ox-LDL ($r= 0.84$, $p< 0.001$; $r= 0.85$, $p< 0.001$; $r= 0.87$, $p< 0.001$,
259 respectively) in the subgroup of subjects analyzed (n=40).

260 Interestingly, a direct association between ox-LDL concentrations and the levels of TC, LDL-C
261 and non-HDL in both control ($r= 0.57$, $p= 0.014$; $r= 0.73$, $p= 0.001$; $r= 0.72$, $p= 0.001$, respectively)

262 and HZN+S group ($r= 0.60, p= 0.004$; $r= 0.56, p= 0.008$; $r= 0.69, p< 0.001$, respectively) was
263 evidenced.

264

265 **4. DISCUSSION**

266 Our study shows the effect of hazelnuts intake on oxidative stress markers in children and
267 adolescents with primary hyperlipidemia. In particular, hazelnut consumption was associated with
268 reduced levels of DNA strand breaks, FPG-sensitive site and H₂O₂-induced DNA strand breaks. On
269 the other hand, we could not demonstrate an effect of HZN+S on ox-LDLs, whose levels remained
270 unchanged in treated and untreated patients.

271 According to previous literature, hyperlipidemia is associated with increased oxidative stress
272 [12,15,16,25–27] from the pediatric age [5,28,29]. A proper nutritional intervention is considered
273 the primary strategy to prevent the onset of chronic degenerative diseases, especially in children
274 with primary hyperlipidemia, presenting with higher risk of developing CVD in adulthood [30].

275 In particular, clinical and epidemiological studies in hyperlipidemic and healthy adults have
276 consistently demonstrated the favorable effects of regular nut intake on health [31–33], which
277 appear attributable to their high content in bioactive compounds with antioxidant properties (i.e.
278 vitamin E, polyphenols, phytosterols), able to reduce both cholesterol levels and oxidative
279 stress[34]. Clinical trials have shown a correlation between regular nut consumption and the
280 reduction of some markers of oxidative stress, including the levels of DNA damage (calculated as
281 the percentage of DNA strand breaks in lymphocytes, or levels of urinary 8-hydroxy-2'-
282 deoxyguanosine concentrations, [8-OH-dG]); *in vivo* antioxidant capacity; ox-LDL;
283 malondialdehyde concentrations; conjugated diene formation; plasma or urine isoprostane
284 concentrations; and antioxidant non-enzymatic and enzymatic activity [34–45]. However, most of
285 the studies investigated the effects of almonds, walnuts or pistachios, while data on hazelnuts are
286 scanty.

287 Hazelnuts are among the most popular tree nuts consumed worldwide, and the second richest source
288 of MUFAs - mainly oleic acid- and phytochemicals with remarkable antioxidant properties, i.e. α -
289 tocopherol, proanthocyanidins and carotenoids [18,34]. In our study, Italian hazelnuts *Corylus*
290 *avellana* L. ‘Tonda Gentile delle Langhe’ were administered peeled or unpeeled (HZN-S and HZN+S,
291 respectively) to evaluate potential differences in the antioxidant properties associated with hazelnut
292 skin. Indeed, a portion of HZN+S or HZN-S provides similar amounts of MUFAs, phytosterols, α -
293 tocopherol and minerals, but different amount of polyphenols (mostly gallic acid), which are mainly
294 present in the skin. Although generally considered a byproduct of peeled HZNs, skins are an
295 interesting edible source of polyphenol compounds with antioxidant properties [46].

296 Several *in vivo* studies have documented the capacity of polyphenol-rich foods to decrease
297 oxidative stress and/or to increase antioxidant protection by reducing DNA oxidized bases and
298 strand breaks [47,48]. The comet assay is a valid, widely used method for the evaluation of DNA
299 damage and the protective effects of dietary bioactives in human cells [49]. To our knowledge, no
300 previous study has investigated the impact of hazelnut intake on DNA damage in patients at risk of
301 oxidative stress, like children with primary hyperlipidemia, nor the potential difference associated
302 with the consumption of peeled vs. unpeeled hazelnuts. Based on our data, both types of hazelnuts
303 significantly reduced the levels of DNA strand breaks and FPG-sensitive sites (as marker of
304 endogenous DNA damage), but only HZN+S significantly decreased the *ex-vivo* oxidatively-
305 induced DNA damage in PBMCs, a suggested marker of antioxidant protection or cell ability to
306 protect from an oxidative insult. Indeed, it is noteworthy that high amounts of vitamin E, with well-
307 recognized antioxidant properties, are regularly introduced through hazelnut intake. Moreover, it
308 may be hypothesized that the higher amount of polyphenols, and antioxidant capacity in HZN+S
309 previously showed [20] increased treatment efficacy (**Figure 2**).

310 Regarding control treatment, we found a significant increase in the levels of the FPG-sensitive sites
311 possibly attributed to the significant reduction in the levels of DNA strand breaks (background SBs
312 expressed as % DNA in tail, EB) following the 8 weeks of intervention. The reduction observed is

313 difficult to explain and it may be due to an improvement of endogenous antioxidant status following
314 the dietary advices.

315 The ability of nuts to reduce markers of oxidative DNA damage was also observed in other
316 intervention studies conducted in adults supplemented with almonds [36,37], brazil nuts [39], or
317 mixed nuts (i.e. walnuts, almonds and hazelnuts) [38]. Two studies carried out in healthy smokers
318 demonstrated that the oxidative DNA damage levels significantly decreased after daily almond
319 supplementations for 4 weeks, suggesting that almonds could counteract the oxidative stress
320 mediated by tobacco [36,37]. After an 8-week intake of selenium-rich Brazil nuts, Cominetti et al.
321 [39] found a significant decrease of DNA strand breaks in a group of obese women with wild-type
322 genotype of glutathione peroxidase1 Pro198Leu polymorphism, but not in the groups with other
323 genotype variants. However, studies investigating the relationship between hazelnut consumption
324 and oxidative stress in humans are limited. López-Uriarte et al. [38] evaluated the role of mixed
325 nuts, including hazelnuts, in adult patients with metabolic syndrome, and found a significant
326 reduction of DNA damage, evaluated as 8-OH-dG, after 8-week treatment.

327 The unchanged ox-LDL levels found after hazelnut consumption in our trial were also observed in
328 other studies following the intake of different nuts [35,38]. Despite the high content of MUFAs,
329 which have been associated with reduced susceptibility of LDL to oxidation [50], after HZN+S
330 treatment we did not observe significant changes in the levels of ox-LDL, and its ratio with LDL-C
331 and HDL-C concentrations. A possible explanation is that our study was carried out in children with
332 primary hyperlipidemia showing mild LDL-C elevations and instructed to healthy lifestyle
333 recommendations. As expected, cholesterol values were directly correlated to ox-LDL. Moreover,
334 the LDL-C concentrations were positively associated with the levels of oxidatively-induced DNA
335 damage, suggesting that both markers may be considered of special interest from the clinical point
336 of view, providing an overall indication of the oxidative status secondary to hyperlipidemia.

337 The cardiometabolic health benefits associated with nut consumption were mainly described in
338 studies conducted in adults reporting a favorable effect on plasma lipid profile as we have also

339 demonstrated in children. Indeed, only two intervention trials were performed in children and
340 adolescents to evaluate the efficacy of nuts in reducing CV risk [51,52], but none of them included
341 hyperlipidemic patients. Maranhão et al. [51] demonstrated that regular intake of Brazil nuts for 16
342 weeks positively influenced the lipid profile, microvascular function and ox-LDL levels in obese
343 children, attributed to nuts high content of unsaturated fatty acids and bioactives.

344 A possible limitation of the present study is the absence of a control group of healthy children and
345 adolescents, which makes data applicable only to children with primary hyperlipidemia.

346

347 **6. CONCLUSIONS**

348 In conclusion, hazelnuts supplementation can be recommended to children with primary
349 hyperlipidemia in association with an appropriate, balanced diet, to improve the lipid profile and
350 reduce the oxidative stress.

351

352 **7. ACKNOWLEDGMENTS**

353 Federica Guaraldi and Valeria Deon contributed equally to the work and manuscript preparation as
354 first authors. Federica Guaraldi enrolled the subjects, verified compliance with dietary protocol and
355 reviewed the manuscript. Valeria Deon and Cristian Del Bo' wrote the draft manuscript, performed
356 the analysis of DNA damage and oxidized-LDL, the statistical analysis and contributed to data
357 interpretation. Stefano Vendrame contributed to sample analysis and reviewed the manuscript.
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364

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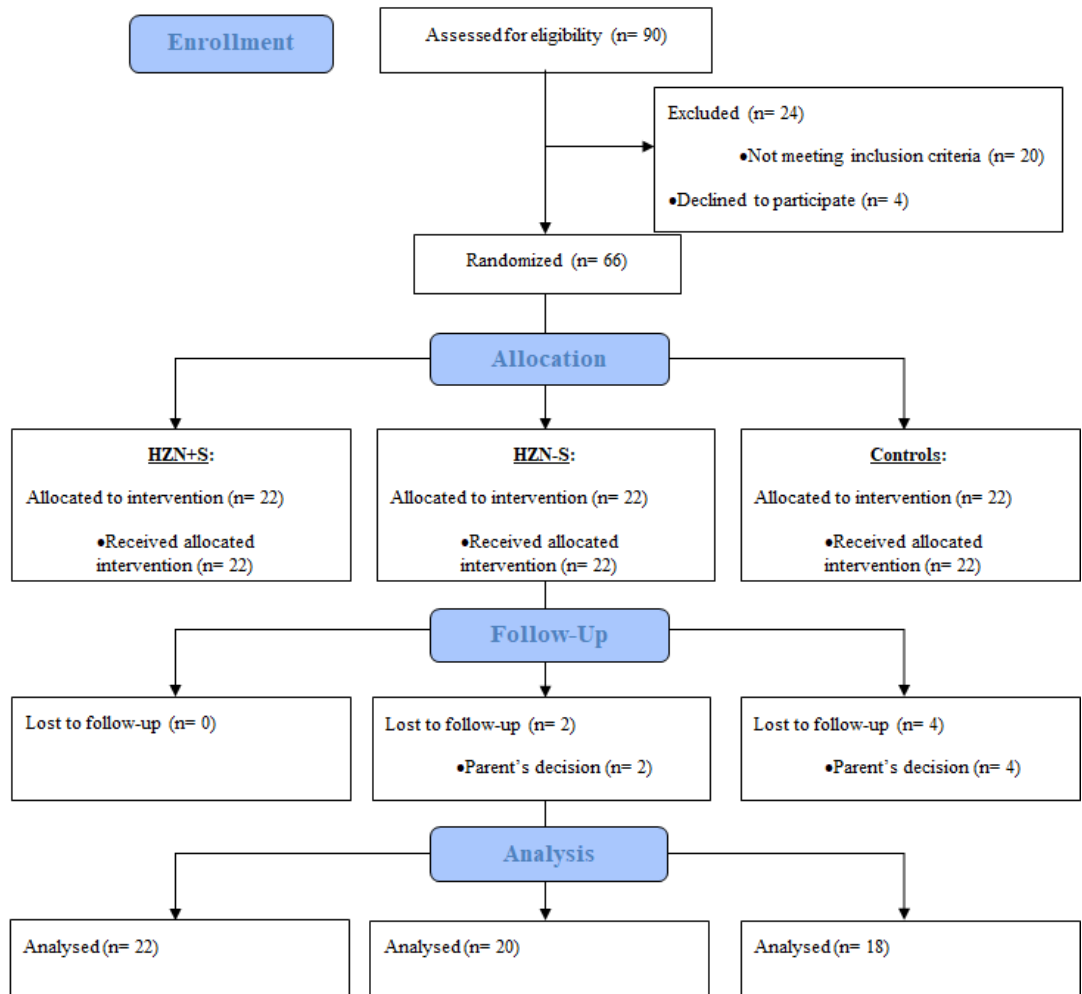
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FIGURE CAPTION 1

Figure 1 – Flow-chart of participant selection, allocation to the study arms and follow-up

Figure 1



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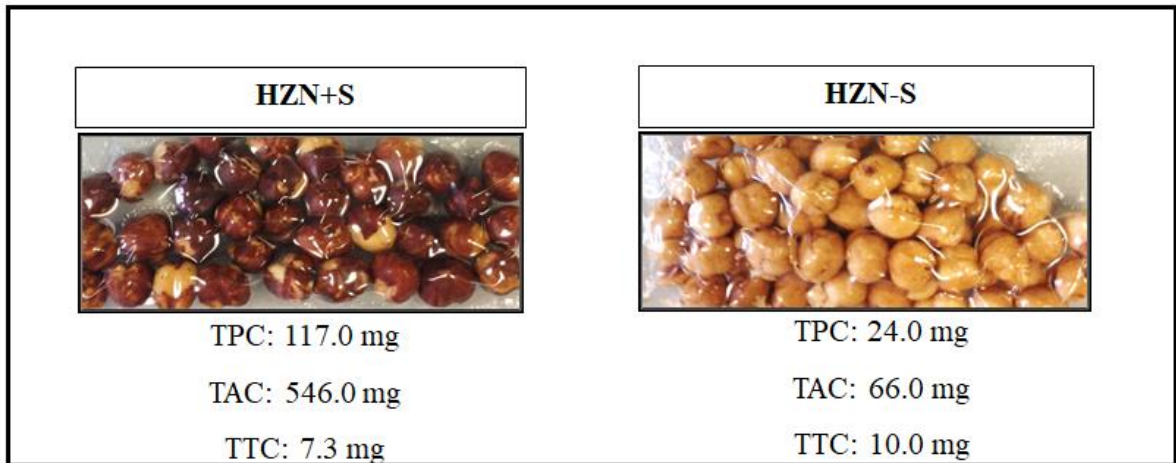
Legend: Control, no treatment group; HZN+S, group treated with hazelnuts with skin; HZN-S, group treated with hazelnuts without skin.

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FIGURE CAPTION 2

Figure 2 – Contribution in antioxidants provided by a portion (30 g) of HZN+S and HZN-S

Figure 2



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Legend: TPC, Total Phenolic Content; TAC, Total Antioxidant Capacity; TTC, Total Tocopherol Content; GAE, Gallic Acid Equivalents; TE, Trolox Equivalent

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Table 1. Levels of DNA strand breaks, FPG-sensitive sites and oxidatively-induced DNA damage at baseline and after 8-weeks of each treatment in the three groups of hyperlipidemic children and adolescents.

DNA damage (% DNA in tail)	Control (n=18)		HZN+S (n=22)		HZN-S (n=20)		T	t	T x t
	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8	Effect p	Effect p	Interaction p
Background SBs (% DNA in tail, EB)	17.44 ± 5.10 ^a	13.65 ± 7.38 ^b	18.66 ± 6.27 ^a	13.41 ± 4.72 ^b	19.70 ± 5.45 ^a	16.00 ± 5.53 ^b	0.293	0.0001	0.
Net FPG-sensitive sites (% DNA in tail)	15.9 ± 7.9 ^a	18.9 ± 9.7 ^b	14.7 ± 5.1 ^a	10.5 ± 4.1 ^c	13.9 ± 3.3 ^a	10.1 ± 4.8 ^c	0.005	0.035	0.
Background SBs (% DNA in tail, PBS)	6.85 ± 1.70	6.25 ± 1.57	6.53 ± 1.54	6.83 ± 1.73	6.15 ± 1.59	6.64 ± 1.82	0.787	0.796	0.
Net H ₂ O ₂ -induced DNA damage (% DNA in tail)	35.3 ± 8.0 ^{ab}	29.6 ± 12.8 ^b	36.6 ± 12.4 ^a	28.7 ± 12.2 ^{bc}	37.4 ± 14.2 ^a	32.0 ± 8.3 ^{ac}	0.702	0.002	0.

HZN+S, hazelnuts with skin; HZN-S, hazelnuts without skin; SB, strand breaks; EB, endonuclease buffer; PBS, phosphate buffer saline; T, treatment effect; t, time effect; T x t, treatment x time interaction. Values are expressed as mean ± SD. ^{a,b,c} Data with different letters within the same row differ significantly ($p < 0.05$).

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Table 2. Levels of ox-LDL, ox-LDL/LDL ratio and ox-LDL/HDL ratio evaluated at baseline and after 8-weeks of treatment in controls and HZN+S groups.

	Control (n=18)		HZN+S (n=22)		T	t	T x t
	Baseline	Week 8	Baseline	Week 8	Effect p	Effect p	Interaction p
Ox-LDL, U/L	54.1 ± 16.9	55.1 ± 15.3	54.5 ± 14.4	53.3 ± 13.6	0.874	0.926	0.462
Ox-LDL/LDL ratio	0.40 ± 0.09 ^{ab}	0.43 ± 0.08 ^b	0.39 ± 0.06 ^a	0.41 ± 0.05 ^{ab}	0.477	0.022	0.720
Ox-LDL/HDL ratio	1.05 ± 0.43	1.05 ± 0.40	0.95 ± 0.44	0.91 ± 0.43	0.357	0.636	0.607

HZN+S, hazelnuts with skin; HZN-S, hazelnuts without skin; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; ox-LDL, oxidized LDL; T, treatment effect; t, time effect; T x t, treatment x time interaction. Values are expressed as mean ± SD. ^{a,b} Data with different letters within the same row differ significantly ($p < 0.05$).

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