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

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KEYWORDS

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.

Highlights

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

ABSTRACT

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

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

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

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

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

1. INTRODUCTION

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

Oxidative stress results in damage of different cellular components by excessive generation of pro-oxidant species, as well as deficiency of antioxidant defense mechanisms [7]. Lipids are susceptible targets of oxidation and plasma oxidized LDLs (ox-LDLs) play a major role in atherosclerosis development [2]. Ox-LDLs can be estimated with a reliable and sensitive technique based on monoclonal antibodies [8]. Several studies in adults demonstrated significant correlations between high circulating levels of ox-LDL and prevalence of CVDs, diabetes and metabolic syndrome [3]. Moreover, children with familiar hypercholesterolemia (FH) showed higher ox-LDL levels as compared to controls [9], although data in children are scanty and, at times, contradictory [10,11].

Severe accumulation of endogenous pro-oxidants in cells could also generate DNA damage, including modified DNA bases, that may contribute to cardiovascular dysfunction. In this context, there is a growing amount of evidence documenting that oxidative stress-induced DNA damage may contribute to atherosclerotic plaque formation [12]. Oxidized DNA damage can be evaluated through different methodologies. The “comet assay”, also called “single cell gel electrophoresis”, is a sensitive and rapid technique for quantifying and analyzing DNA damage, evaluated for example as single- and double- DNA strand breaks in individual cells. Direct measurement of oxidative DNA damage may be obtained through modifications of the comet assay, which allow the detection of oxidised DNA bases through the use of specific enzymes, i.e. formamidopyrimidine DNA glycosylase (FPG) or endonuclease III, able to detect oxidised purines and pyrimidines, respectively [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.
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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

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

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

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

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

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

2.2 Blood sample collection, separation and storage

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

2.3 Evaluation of oxidatively induced DNA damage in PBMCs

The evaluation of cell resistance to oxidatively induced DNA damage was performed by comet assay, as previously reported [23]. Oxidative stress in fresh PBMCs was induced using 500 uM H_2O_2. Two slides were prepared for each subject: one was treated with H_2O_2 (500 µmol/l in PBS)

for 5 min at room temperature in the dark; the other was treated for 5 min with a solution of PBS only (control slide). Following the oxidative treatment, slides were immersed in a lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, 1% N-lauroylsarcosine sarcosine sodium salt, pH 10 with NaOH; 1% Triton X-100 and 1% DMSO) for 1 h at 4°C in the dark. Slides were then transferred in a horizontal electrophoresis tank containing an alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA) and kept for 40 min at 4°C. Then, electrophoresis was carried out (25 V, 300 mA, 20 min) in the same alkaline solution at 4 °C in the dark. The samples were subsequently washed in a neutralizing buffer (0.4 M Tris–HCl, pH 7.5) for 15 min at 4°C in the dark, stained with ethidium bromide (2 μg/ml) for 20 min, washed in PBS, drained, and coverslipped. DNA damage produced was compared to that obtained in control cells. The lower the DNA damage detected, the higher the ability of the cells to protect themselves from an induced oxidative stress.

2.4 Evaluation of FPG-sensitive sites in PBMCs

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

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

2.5 Quantification of DNA damage

One hundred images of nucleoids, or comets, per slide were electronically captured at 20x magnification, using an epifluorescence microscope (Olympus CX 41; Olympus Italia) attached to a high sensitivity CCD video camera (CFW 1808M; Scion Corporation, Germany), and to a computer equipped with an image analysis system (Cometa 1.5; Immagini e Computer, Bareggio, Milan, Italy). The level of DNA damage was calculated as the percentage of DNA in the tail. For each subject, the percentage of DNA in the tail of control cells (slides not treated with H_2O_2 or FPG) was subtracted from the percentage of DNA in the tail of cells incubated with H_2O_2 or FPG.

2.6 Analysis of ox-LDL

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

2.7 Statistical analysis

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

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

3. RESULTS

3.1 Baseline characteristics

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

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

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

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

3.3 Effect of intervention on DNA strand breaks and H_2O_2-induced -DNA damage in PBMCs

The effect of intervention on the levels of DNA strand breaks and H_2O_2-induced -DNA damage in PBMCs is reported in **Table 1**. The consumption of hazelnuts and control treatment did not affect the levels of DNA strand breaks. With regard to cell protection against an ex-vivo induced oxidative stress, HZN+S treatment significantly decreased (time effect, p=0.002) the level of oxidatively-induced DNA strand breaks by 16.0% (95% CI: −32.9%, +0.89%), while no significant effect was observed after HZN-S and control treatment (**Table 1**).

3.4 Effect of intervention on oxidized LDL levels

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

3.5 Correlation between serum lipid profile and markers of oxidative stress

Correlations between DNA and lipid damage and serum lipid profile previously published [20] have been performed to ascertain the contribution of dyslipidemia on oxidative stress in the population under study. At baseline, a positive correlation between LDL-C concentrations and H_2O_2-induced DNA damaged (r= 0.34, p= 0.04) was detected. Furthermore, serum TC, LDL-C and non-HDL-C 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, respectively) in the subgroup of subjects analyzed (n=40).

Interestingly, a direct association between ox-LDL concentrations and the levels of TC, LDL-C 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)

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

4. DISCUSSION

Our study shows the effect of hazelnuts intake on oxidative stress markers in children and adolescents with primary hyperlipidemia. In particular, hazelnut consumption was associated with reduced levels of DNA strand breaks, FPG-sensitive site and H_2O_2-induced DNA strand breaks. On the other hand, we could not demonstrate an effect of HZN+S on ox-LDLs, whose levels remained unchanged in treated and untreated patients.

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

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

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

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

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

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

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

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

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

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

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

6. CONCLUSIONS

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

7. ACKNOWLEGMENTS

Federica Guaraldi and Valeria Deon contributed equally to the work and manuscript preparation as first authors. Federica Guaraldi enrolled the subjects, verified compliance with dietary protocol and reviewed the manuscript. Valeria Deon and Cristian Del Bo' wrote the draft manuscript, performed the analysis of DNA damage and oxidized-LDL, the statistical analysis and contributed to data interpretation. Stefano Vendrame contributed to sample analysis and reviewed the manuscript. Marisa Porrini supervised the analysis and critically revised the manuscript. Patrizia Riso and Ornella Guardamagna designed the study, obtained funding and critically revised the manuscript.

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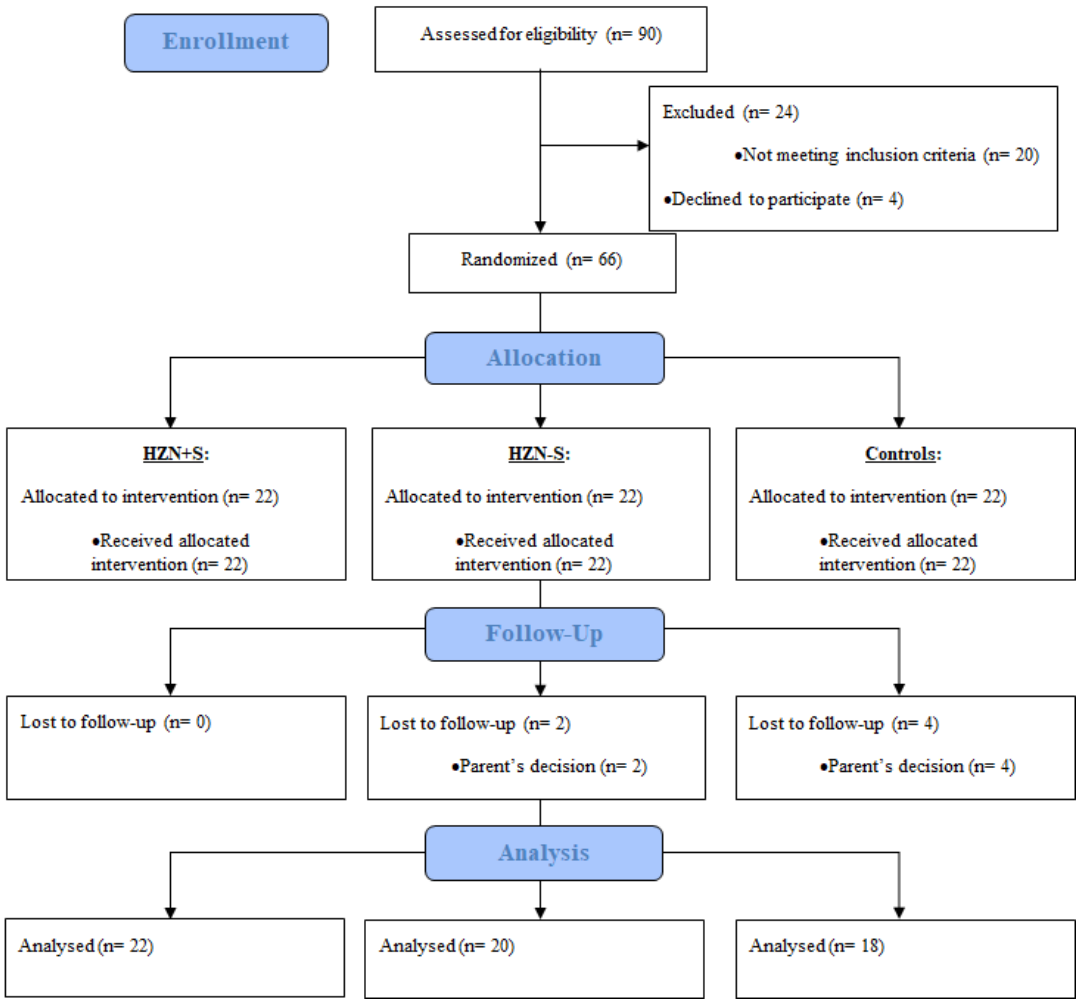
FIGURE CAPTION 1

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

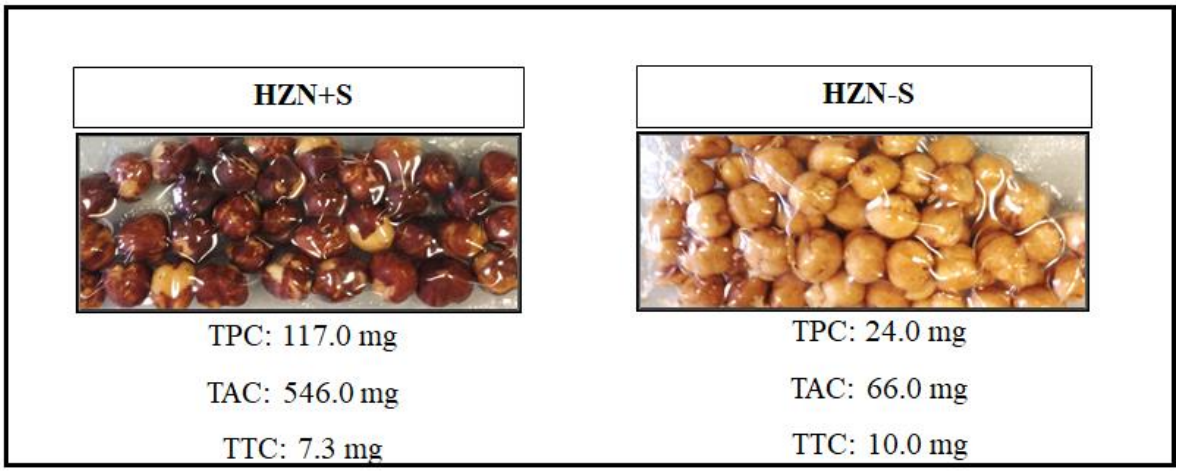
Figure 1



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

544
545 **FIGURE CAPTION 2**
546
547 **Figure 2** – Contribution in antioxidants provided by a portion (30 g) of HZN+S and HZN-S
548

Figure 2



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551 **Legend:** TPC, Total Phenolic Content; TAC, Total Antioxidant Capacity; TTC, Total Tocopherol
552 Content; GAE, Gallic Acid Equivalents; TE, Trolox Equivalent
553

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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* Effect	t Effect	*T* *Inter*
	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8	*p*	*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_2O_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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556

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* Effect	t Effect	*T x t Interaction*
	Baseline	Week 8	Baseline	Week 8	*p*	*p*	*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 densisty 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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