Effect of hazelnut on serum lipid profile and fatty acid composition of erythrocyte phospholipids in children and adolescents with primary hyperlipidemia: a randomized controlled trial

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SUMMARY

Background and Aim: Regular intake of nuts improves lipid profile and thus reduces the cardiovascular (CV) risk associated with hyperlipidemia. The aim of the study was to investigate the effect of a dietary intervention with hazelnuts (HZNs, 15-30 g/day, depending on patient weight) on serum lipid profile, anthropometric parameters and fatty acids (FAs) composition of erythrocyte phospholipids in children and adolescents with primary hyperlipidemia.

Methods: Eight-week randomized, single blind, controlled, three-arm, parallel-group study. Sixty-six subjects were enrolled and randomized in 3 groups receiving: 1) hazelnuts with skin (HZN+S); 2) hazelnuts without skin (HZN-S); 3) dietary advices for hyperlipidemia only (controls). Before and after intervention, clinical parameters were measured and blood samples were collected for the evaluation of serum lipid levels and phospholipid FA composition of erythrocytes.

Results: Two-way ANOVA showed a significant effect of *time* on serum low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C)/LDL-C ratio and non-HDL-C ($p \leq 0.001$), but not of *treatment* and *time x treatment* interaction. In particular, HZN+S and HZN-S significantly reduced the concentrations of LDL-C and increased HDL-C/LDL-C ratio. HZNs also had a favorable impact on FAs composition of erythrocyte phospholipids, as demonstrated by *time x treatment* interaction, with a significant increase of monounsaturated fatty acids (MUFAs) ($p=0.008$) and MUFAs/saturated fatty acids (SFAs) ratio ($p=0.002$) with respect to the control group.

Conclusions: For the first time, we documented a positive effect of HZN consumption on lipid profile and FA composition of erythrocyte phospholipids in children with primary hyperlipidemia. Further studies are encouraged to better define HZN impact on the markers of CV risk in this population.

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

47 **Keywords:** Children, primary hyperlipidemia, hazelnuts, serum lipids, omega-3 index, erythrocyte
48 fatty acids.

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; CHILD, cardiovascular health integrated lifestyle diet; FA, fatty acid; FCHL, familial combined hyperlipidemia; FH, familial hypercholesterolemia; GAE, gallic acid equivalents; HDL-C, high density lipoprotein cholesterol; HZN, hazelnuts; HZN+S, hazelnuts with skin; HZN-S, hazelnuts without skin; LDL-C, low-density lipoprotein cholesterol; MUFAs, monounsaturated fatty acids; non–HDL-C, non-high density lipoprotein cholesterol; PHC, polygenic hypercholesterolemia; PUFAs, polyunsaturated fatty acids; RBCs, red blood cells; SFAs, saturated fatty acids; TC, total cholesterol; TE, trolox equivalent; TG, triglycerides.

1. Introduction

Hyperlipidemia is a well established risk factor of atherosclerosis with detrimental effects on blood vessels since childhood. Therefore, children with primary hyperlipidemia are at increased risk to develop cardiovascular disease (CVD) later in life [1].

Dietary treatments, designed to replace dietary saturated fatty acids (SFAs) and cholesterol with unsaturated fatty acids, are considered the primary approach in the management of dyslipidemias and CVD prevention [2]. The American Academy of Pediatrics has suggested specific dietary programs for children (CHILD1 and CHILD2) to promote growth, avoid adiposity and ameliorate the lipoprotein profile. These guidelines reinforce the need to limit the intake of total fat and SFAs to 30% and 7-10% of total daily energy, respectively [3].

The benefits associated with replacing SFAs with monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) on lipid profile and other CV risk factors have been increasingly demonstrated [4], together with the advantages on CV risk and metabolic profile deriving from healthy dietary patterns, e.g. the Mediterranean diet, rich in fruit, vegetables, legumes, nuts, whole grains, dairy products, fish and olive oil [4].

Moreover, several studies have shown a remarkable cardioprotective effect associated with regular nut consumption, attributed to the bioactive components contained in the nut kernel and skin, in particular MUFAs and PUFAs, phytosterols, antioxidant vitamins (e.g. tocopherols), fibers, and polyphenols [5]. In particular, hazelnuts (HZNs) are characterized by a relatively high content of polyphenols, playing an important role in improving serum lipid profile through several mechanisms, including the regulation of cholesterol absorption, the inhibition of triglycerides synthesis and secretion, and the reduction of low density lipoprotein oxidation [6].

A number of intervention studies have assessed health benefits associated with the consumption of various types of nuts in adults, while only few have been performed in children [7], and none of them evaluated CVD risk in adult age of children affected by familial hyperlipidemias.

Hyperlipidemia and diet composition are two important determinants of cell fatty acid (FA) composition, which is critical for cell behavior and function [8]. Thus, changes in membrane properties induced by specific dietary interventions may have important consequences on targeted cellular functions and, consequently, on CV risk. In this regard, the FA composition of erythrocyte, which mirrors the tissue FA status [8-10], has been postulated as a useful marker of serum lipoprotein changes [11].

Based on these premises, our study aimed at investigating the effect of a dietary intervention with roasted Italian HZNs *Corylus avellana* L., consumed daily for 8 weeks, on anthropometrical and clinical parameters, serum lipid profile, and FA composition of RBC phospholipids in children and adolescents with primary hyperlipidemia. Since HZNs can be consumed with skin or peeled, two intervention arms were considered: the first was represented by patients treated with hazelnut with skin (HZN+S), the second by patients treated with hazelnuts without skin (HZN-S). A third group of patients receiving only dietary advices was used as control.

2. Subjects and methods

2.1. Subjects recruitment

A total of 66 children and adolescents with primary hyperlipidemia (31 females; mean age: 11.6 ± 2.6 years) were selected among 90 followed at the Department of Health Sciences and Pediatrics of the University of Turin, Turin, Italy. To be enrolled in the study, patients had to be diagnosed with primary hyperlipidemia, which included familial hypercholesterolemia (FH), familial combined hyperlipidemia (FCHL) and polygenic hypercholesterolemia (PHC), with serum levels of total cholesterol (TC) and/or triglycerides (TG) above the age- and sex-specific 90th percentile. Diagnosis of the various forms of hyperlipidemia was made according to international criteria [12]. In particular, FH was diagnosed in presence of low-density lipoprotein cholesterol (LDL-C) ≥ 95th percentile, parental LDL-C ≥ 190 mg/dL, tendon xanthomas and/ or cardiovascular disease (phenotype IIA). FCHL was diagnosed in children showing TC and/or TG >90th age- and sex-specific percentile, with

100 at least one parent affected by hypercholesterolemia, hypertriglyceridemia, or both (IIA, IV, or IIB
101 phenotype, respectively), with concomitant individual and familial lipid phenotype variability.
102 Children with LDL-C levels >90th percentile and a family history of dominant inherited
103 hypercholesterolemia, but not fulfilling the biochemical international diagnostic criteria of FH or
104 FCHL were diagnosed with PHC.
105 Exclusion criteria were food allergies or specific aversion for nut consumption; secondary forms of
106 hyperlipidemia; obesity, defined as a body mass index (BMI) ≥ 97th percentile, for age and sex (to
107 exclude a confounding variable); chronic diseases requiring medical treatment; smoking habit;
108 treatment with lipid-lowering treatment or functional foods in the previous 3 months. Finally, patients
109 should demonstrate a good dietary compliance, at least in the previous 3 months, assessed by a
110 questionnaire on food intake and preference. The process of patient selection and allocation to the
111 different study groups is depicted in **Figure 1**.
112 The study protocol complied with the principles of the Declaration of Helsinki, and was
113 approved by the Ethics Committee of the Città della Salute e della Scienza, University Hospital of
114 Turin, Turin, Italy (EC:CS377). The study purpose and protocol were exhaustively explained to all
115 participants and their parents, who signed an informed consent before study enrollment. The trial was
116 registered under ISRCTN.com (identifier no. ISRCTN12261900).
117
118 *2.2. Hazelnut preparation for the intervention study*
119 Italian HZNs *Corylus avellana* L., (cultivar 'Tonda Gentile delle Langhe', from Piedmont, Italy),
120 were provided with (HZN+S) or without (HZN-S) skin, in pre-weighed vacuum packed portions.
121 Roasted nuts were chosen, since they are commonly consumed by the Italian population. All
122 hazelnuts were roasted for 31 minutes at 135°C. To minimize skin falling, hazelnuts were roasted in
123 a tunnel oven and collected before the entrance in the cooling tower. Then, only hazelnuts with >80%
124 of skin were selected and used as "HZN+S" for the intervention trial. Other roasted hazelnuts were
125 peeled mechanically in the peeling tower and used as "HZN-S".

126 The amount of HZNs per portion was calculated based on the doses advised to adults, adjusted on
127 children body weight (0.43 g/kg of body weight on average, corresponding to 15-30 g portions).
128
129 *2.3. Fat and bioactive composition of hazelnuts*
130 HZNs composition in terms of total fat, sterols and tocopherols content was determined using
131 standardized methods. The HZN oil was extracted using a cold-pressing method. The recovered oil
132 was clarified and stored at -18 °C until analyses. Fatty acids composition was obtained through the
133 analysis of derived methyl esters by gas-chromatography, following the method described by Ficarra
134 et al. [13]. The fatty acid concentration (expressed as mg of fatty acid/g of oil) was calculated
135 according to AOAC 963.22 method. The content of minerals was measured by inductively coupled
136 plasma mass spectroscopy (ICP-MS; Varian 820 ICP-MS) [14]. Polyphenol compounds were
137 extracted following the method suggested by Ghirardello et al. [15]. The total phenolic content was
138 assessed by spectrophotometry by means of the modified Folin–Ciocalteu method, and was expressed
139 as mg of gallic acid equivalents (GAE) per g of sample. Polyphenol composition was determined
140 using a Thermo-Finnigan Spectra-System HPLC equipped with a Finnigan Surveyor PDA Plus
141 detector. The separation was achieved at 22 °C on a C18 RP Lichrospher 250 × 4.6 mm, 5-µm column
142 equipped with a C18 RP Lichrospher 5-µm guard column. The mobile phase was trifluoroacetic
143 acid/ultrapure water (solvent A) and methanol (solvent B); the flow rate was 0.8 mL min^{-1}, and the
144 injection volume was 20 µL. The PDA spectra were recorded over a wavelength (λ) range of 200 to
145 600 nm, and quantification was performed recording the peak area at a maximum λ of each
146 compound. Identification was achieved by comparing the retention times and spectra of our samples
147 with those of authentic standards. In addition, the antioxidant capacity (radical scavenging activity)
148 of the HZN extracts was evaluated according to method of Ghirardello et al. [15]. The results of total
149 antioxidant activity were expressed as µmoles of Trolox equivalent (TE) per g of sample.
150

151 *2.4. Experimental design*

152 The intervention study was an 8-week randomized, single blind, controlled, three-arm, parallel-group
153 (**Figure 1**). Patients were allocated to different treatment groups (with a 1:1:1 ratio, 22 subjects per
154 group) by a pediatrician who was not involved in the study and did not participate to sample analysis,
155 according to a randomization list obtained through the investigating center database. Group 1 was
156 treated with one daily portion of HZN+S; group 2, with one daily portion of HZN-S; group 3
157 (controls) received only dietary advices. At the beginning of the study, patients assigned to treatment
158 groups received a HZNs supply sufficient for the complete duration of treatment, portioned into pre-
159 weighed packages, and were asked to consume one portion per day for 8 consecutive weeks. The
160 control group was advised to follow a nut-free diet for the subsequent 8 weeks. At enrollment, all
161 children and their parents received nutritional recommendations based on the cardiovascular health
162 integrated lifestyle diet (CHILD1) guidelines for children with identified hyperlipidemia as supported
163 by the American Academy of Pediatrics [3]. The essential diet features were: 55% of daily energy
164 from carbohydrates, 15% from proteins, and 30% from fats (saturated fat 7-10%); dietary cholesterol
165 <100 mg/1000 kcal and no more than 300 mg/day and 10-25 g/day of soluble fiber.

166 All participants were encouraged to maintain the same dietary and lifestyle habits throughout
167 the study period. To check the compliance to the dietary instructions, the patients and their families
168 were asked to fill weekly food diaries, before and after enrollment in the study, and were periodically
169 interviewed for the duration of the study. A nutritionist provided detailed instructions to patients and
170 their parents on how to record food intake. At the end of the study, dietary records were analyzed to
171 estimate the average daily energy and nutrient intake. The nutritional evaluation was performed with
172 Software MètaDieta® (Me.Te.Da S.r.l., San Benedetto del Tronto, Italy) using Italian Food
173 Composition databases.

174 At baseline and at the end of the study, each patient underwent a medical examination after
175 an overnight fast, during which blood samples and the physical parameters (i.e. height, weight and
176 blood pressure) were obtained. Participants in the HZN groups were asked to give back any uneaten

177 HZN package at the last visit. Compliance was assessed by weighing the eventual packages returned,
178 and by analysing weekly food diaries filled at baseline and during the study.
179
180 *2.5. Physical evaluation*
181 Height and weight were measured to the nearest 0.1 cm and 0.1 kg respectively (Wunder C-202 and
182 HR1; Wunder Sa.Bi. srl, Trezzo sull'Adda, Italy). Body mass index (BMI) was calculated as body
183 weight in kilograms, divided by height squared in meters (kg/m^2). The absolute and Z-score values
184 for weight, height and BMI were reported at baseline. Z-scores were assessed using reference tables
185 for the Italian pediatric population defined by Cacciari et al. [16]. Blood pressure was measured in
186 triplicate with a five-minute interval between each measurement using a validated manual mercury
187 sphygmomanometer (Tycos Classic Hand Aneroid model 5098-02, Welch Allyn, USA) and the mean
188 of these values was calculated.
189
190 *2.6. Biochemical analysis*
191 Fasting venous blood samples were collected into vacutainer tubes containing silicon. Serum levels
192 of TC, HDL-C and TG were directly determined by an automatic biochemical analyzer (Olympus
193 AU2700, Japan). The coefficient of variation (CV) was 1.3%, 1.8% and 2% for CT, HDL-C and TG
194 measurements, respectively. LDL-C concentration was estimated using the Friedewald formula:
195 LDL-C = TC (mg/dL) − HDL-C (mg/dL) − TG (mg/dL)/5 [17]. Non-high density lipoprotein
196 cholesterol (non–HDL-C) was calculated subtracting HDL-C from TC.
197
198 *2.7. Analysis of FA composition of erythrocyte phospholipids*
199 Fasting venous blood samples of 2.5 ml were drawn into vacutainer tubes containing lithium heparin.
200 Plasma was separated by tube centrifugation (1400 g x 15 min, 4°C) and stored at -80°C for further
201 analysis. The buffy layer of white blood cells was removed using a pipette, and erythrocytes were
202 washed twice in an equal volume of a physiologic solution (0.9% NaCl, w/v). An aliquot (0.5 g) of

erythrocytes was stored at –80°C for further analysis. FAs extraction from erythrocyte phospholipids and gas chromatographic analysis were performed in accordance to the method described by Simonetti et al. [18].

2.8. Statistical analysis

Sample size was calculated from previous studies in order to detect significant differences in the serum lipid concentrations and FA composition of erythrocyte phospholipids with a p value of 0.05 and a power of 80% [14, 19]. Based on data from previous studies, eighteen subjects per group were estimated sufficient to demonstrate a 5% variation of LDL-C concentration with a p value of 0.05 and a power of 80%. A total of 66 subjects were included considering possible drop-outs. STATISTICA software (Statsoft Inc., Tulsa, OK, USA) was used for statistical analysis of data. Two-way ANOVA was applied to compare the effect of *treatment* and *time* (before and after treatment) on serum lipid levels and erythrocyte phospholipids composition. Differences were considered significant for $p \leq 0.05$; post hoc analysis of differences between treatments was assessed by the least significant difference test with $p \leq 0.05$ as level of statistical significance. Finally, regression analysis was used to define correlations between erythrocyte MUFA levels and serum lipid profile.

3. Results

3.1. Characterization of hazelnuts

The fat and bioactives composition of 100 g of HZN+S and HZN-S are reported in **Table 1**.

The major components of HZNs was fat, with a high prevalence of MUFAs, in particular oleic acid. Furthermore, HZNs contained phytosterols, tocopherols (mainly α-tocopherol), minerals (mainly potassium, phosphorus, magnesium and calcium). In HZN+S, small amounts of polyphenols (about 13 mg, three-fold higher than in HZN-S) were also detected. Moreover, the HZN+S exhibited a higher phenolic content (3.9 mg GAE/g), and total antioxidant capacity (18.2 μmol TE/g) than HZN-S (0.8 mg GAE/g and 2.2 μmol TE/g, respectively).

229 *3.2. Main sample features*

230 Of the 90 hyperlipidemic children and adolescents initially screened for the study, 24 subjects did not
231 meet the eligibility criteria. The remaining 66 eligible children agreed to participate to the study. Six
232 participants (4 females) dropped out from the study for personal reasons, not related to the study.
233 Thus, 60 hyperlipidemic children and adolescents (M/F, 34/26) successfully completed the 8-week
234 intervention and were included in the data analysis (**Figure 1)**. The study was performed between
235 January 2015 and October 2015.

236 Main features of the three groups of patients at baseline and after treatment are reported in
237 **Table 2**.

238 Age ranged from 6.7 to 17.5 years, with a mean age ± SD of 11.6 ± 2.6 years. Mean serum
239 lipid levels exceeded the 90^{th} age and sex related percentiles, with the exclusion of HDL-C values,
240 which were in the normal range. Body weight was in the normal range, except for five subjects
241 presenting with mild overweight, BMI remained unchanged throughout the treatment period. Blood
242 pressure levels were in the normal range at baseline and during the entire study period. HZN
243 consumption was appreciated and well tolerated by all patients.

244 The mean ± SD of z-scores for weight were: 0.27±1.10 in controls, 0.51±1.29 in HZN+S and
245 0.22±1.04 in HZN-S groups; z-scores for height were: 0.13±1.13 in control, 0.67±1.41 in HZN+S
246 and 0.29±1.09 in HZN-S groups. Finally, z-scores for BMI were: 0.25±1.11 in control, 0.46±1.36 in
247 HZN+S and 0.12±1.14 in HZN-S groups.

248 Energy and nutrient intake at baseline and during the study period are reported in **Table 3.**
249 The intake of HZNs significantly ($p<0.05$) increased energy, total fat, MUFA and PUFA intake and
250 reduced carbohydrates intake (**Table 3**). No differences were observed in the control group.

251

252 *3.3. Effect of hazelnuts on serum lipid profile*

253 For each group, the serum lipoprotein concentrations before and after each treatment are reported in
254 **Table 4**.

Following the dietary interventions, a significant effect of *time* on serum LDL-C ($p<0.001$), HDL/LDL ratio ($p<0.001$) and non-HDL-C ($p=0.001$), but not of *treatment* and *time x treatment* interaction was detected by the two-way ANOVA. P*ost-hoc* comparisons (LSD test) showed that both HZN+S and HZN-S treatments significantly reduced the concentrations of LDL-C, and increased HDL-C/LDL-C ratio, while no effect was observed in the control group (**Table 4)**. HZN-S only reduced the level of non-HDL-C (**Table 4**).

3.4. Effect of hazelnuts on FA composition of erythrocyte phospholipids

The FA composition of phospholipids in erythrocyte at baseline and at the end of treatments are reported in **Table 4**. Two-way ANOVA showed a significant effect of *time* and *time x treatment* interaction on total MUFAs ($p<0.001$ and $p=0.008$, respectively), and MUFAs/SFAs ratio ($p=0.015$ and $p=0.002$ respectively) in erythrocytes, whose levels were significantly higher in patients receiving HZN after treatment as compared to baseline, and to the control group. An effect of *time* was observed for oleic acid ($p=0.004$) and palmitoleic acid ($p=0.015$), whose levels significantly increased after HZN consumption; and of *time x treatment* interaction for linoleic acid ($p=0.043$), whose levels decreased following HZN-S consumption.

In the control group, an increase of SFA (*time x treatment* interaction, $p=0.036$) and a decrease of total PUFA n-6 (*time* effect, $p=0.019$) in the erythrocyte phospholipids were detected following 8 weeks of dietary advices. Moreover, a *time x treatment* interaction was observed for the levels of margaric acid ($p=0.021$) that significantly increased, and of eicosenoic acid ($p=0.030$), that significantly decreased. A *time* effect was observed for dihomo-γ-linolenic acid levels ($p=0.004$), that significantly decreased compared to baseline. Finally, no significant correlation was found between MUFA increase in erythrocyte phospholipids and serum lipid response to HZN treatments (data not shown).

4. Discussion

Nutrition plays a major role in CV prevention. In particular, dietary patterns in childhood impact on metabolic profile and CV health in adulthood. This concept is relevant when applied to the general population, and, even more, to children affected by primary hyperlipidemias, whose CV risk is definitely higher. A healthy diet rich in vegetables and fiber demonstrated to reduce the CV risk later in life [20]. Fat intake is also critical: the STRIP study, a prospective intervention study involving children, proved that reducing the intake of saturated fat was effective in reducing LDL-C concentrations [21].

To our knowledge, this is the first study evaluating the effects of HZNs intake on lipid profile and fatty acid composition of RBCs in children and adolescents affected by primary hyperlipidemia. LDL-C is considered at present the best biomarker of CV risk, and different cut-off levels are recognized as target levels, depending on age and comorbidities [22].

Although in the HZN groups we observed a significant reduction over time in serum LDL-C levels to 130 mg/dL, which represents the target for children with primary hyperlipidemia [3], these changes were not significantly different from those showed in the control group.

Non-HDL-C is considered as a marker of circulating atherogenic lipoproteins, especially when TG levels exceed 400 mg/dL, a condition limiting the LDL-C significance if calculated by the Friedewald formula, and correlates with CV risk [22].

In our study, we reported a significant *time-effect* reduction for non-HDL-C levels in the HZN-S group, possibly suggesting the contribution of HZN in the management of hyperlipidemia, when added to an appropriate dietary regimen; however, non-HDL-C concentration did not differ significantly from the other two groups.

The HDL-C/LDL-C ratio, considered a marker of the risk of CV events was also significantly increased in the present study, although HDL-C levels did not change.

Our results are in line with the observations reported in several dietary intervention studies performed with HZNs in healthy and/or hyperlipidemic adults. For example, Orem et al. [19], in a

307 double-control sandwich model intervention study including 21 hypercholesterolemic adults, found
308 that a 4-week intervention with HZNs (49-86 g/day raw hazelnuts divided in two portions within the
309 day) significantly reduced total cholesterol, triacylglycerol and LDL-C, while increasing HDL-C. In
310 a randomized crossover study, 4-week consumption of ground, sliced or whole HZNs (30 g/day
311 substituting high saturated fat snacks) improved lipoprotein profile in 48 mildly hypercholesterolemic
312 adult subjects [23]. In both studies, patients followed isocaloric diets during the whole
313 experimentation [19;23]. In a well-controlled two-period's study conducted in 15 young adults with
314 mildly hypercholesterolemia and hypertrygliceridemia, the supplementation of STEP I diet with
315 HZNs (40 g/day raw hazelnuts consumed as snack for 8-weeks) increased HDL-C concentrations
316 while decreasing VLDL-C, triacylglycerol, apolipoprotein B concentrations. In addition, a trend
317 toward lower levels of total and LDL-C, as well as TC/HDL-C and LDL-C/HDL-C ratios was
318 documented [24].
319 In our study, children maintained their usual dietary program, but increased their energy intake when
320 allocated to the HZN interventions (by about 100 and 150 kcal after HZN+S and HZN-S,
321 respectively) and this adds more significance to the detected improvement in lipid profile.
322 In another study [25], a single 4-week intervention with HZNs (1 g/kg/day; 49-86 g/day of
323 unpeeled raw hazelnuts) significantly improved plasma lipid and lipoproteins profile in a group of 21
324 normolipidemic subjects. Similarly, Durak et al. [26] found decreased plasma levels of total and LDL-
325 C and increased HDL-C and TG levels in a group of 30 healthy subjects consuming HZNs for 30
326 days (1 g/kg/day) in addition to their normal daily diets. Despite the strong limitations of these studies,
327 including the absence of the randomization, control group, and the type of intervention developed,
328 these data are in line with our results.
329 In our study, no significant effect of HZN interventions on TG levels was observed. This
330 finding is not surprising since TG levels were in the normal range for most of the subjects and in
331 agreement with Banel & Hu [27] documenting a significant reduction in TG levels only in patients
332 with serum levels >150 mg/dL. A 12-week intervention with 30- and 60-g/day of HZNs in

overweight/obese individuals with normal lipid profile produced a reduction of total and LDL-cholesterol, but no effect on HDL-C and TG levels [28]. On the contrary, a recent meta-analysis [29] demonstrated a positive effect of nuts also on TG levels in subject not affected by CVD.

Based on currently available data, the effect of nut intake on lipid profile and related markers seems to depend on several nut characteristics, i.e. type, daily dose, preparation, and nutritional composition of nut cultivars [30-31]. In most of the trials performed in adults, the administered daily doses of HZN was 0.5 g/kg body weight daily (range 28-30 to 60-70 g per day), being the greatest effects on LDL–C reduction observed for doses >60 g/day [29]. The characteristics of the subjects enrolled is also relevant. Indeed, the cardiometabolic health benefits associated with nut consumption were mainly described in studies conducted in adults reporting a favorable effect on plasma lipid profile, making the comparison of data difficult in case of pediatric population. In addition to the present study, only two studies considered the efficacy of nuts in reducing CV risk in children and adolescents [32-33], but none of them included hyperlipidemic pediatric patients. Scarce data exist on the impact of factors associated with nut consumption - e.g. time of the day, use of whole single vs. divided multiple daily portions, and combination with other foods -, on lipid profile and FAs composition. Moreover, nuts types present important differences in their fat and non-fat constituents [30-31]. According to our data, similar effects on lipid levels were obtained with unpeeled and peeled HZNs. Unpeeled HZNs provides a higher amount of polyphenols. The contribution of polyphenols, as well as of other bioactives (e.g. phytosterols), in the management of dyslipidemia is widely debated. Growing evidence suggests that polyphenol-rich foods could have a potential lowering effect of on cholesterol and postprandial triglycerides [6]; however, studies ascertaining the impact of nut polyphenols in the modulation of lipid profile are scarce. A study performed in atherosclerosis-susceptible mice showed that the consumption of unpeeled walnuts (containing polyphenols), but not walnut oil (only PUFAs), reduced atherosclerotic plaques and decreased the levels of circulating and hepatic lipids [34]. Moreover, an extract derived from HZN skin (rich in fiber, phytosterols and polyphenols) had lipid-lowering blood effects, decreasing the circulating levels of total and LDL-C,

triglycerides and non-esterified free fatty acids in hamsters fed with a high fat diet for 8 weeks [35]. In our study, the intake of both HZNs was able to reduce LDL-C and to improve HDL/LDL-C ratio independently by the polyphenol content.

Another aim of this study was to investigate the effect of HZN intervention in the modulation of FA composition of erythrocyte phospholipids. The structural properties and function of cell membranes appear to be modified in dyslipidemic subjects, since the identification of a correlation between altered FA composition in erythrocyte and adult or pediatric dyslipidemia [9, 36-37]. The role of diet in the modulation of erythrocyte membrane composition has been evaluated using different food products [14, 38-39]. Rajaram et al. [40] reported an increase of PUFA, linoleic acid, and α-linolenic acid in the RBC membranes of normal and mildly hyperlipidemic subjects after 4-week supplementation with walnuts (40 g/day). Similarly, English walnuts intake (30 g/day, 30 days), a good source of PUFAs, improved erythrocyte PUFA in a group of healthy subjects [38]. In our study, we found that 8-week HZN intervention increased the levels of MUFAs, oleic acid and MUFAs/SFAs ratio in HZN treated patients, while no effect was observed in the control group. Since HZNs provide mainly oleic acid and MUFA, our results support the hypothesis of beneficial effects associated with HZN-intervention in the modulation of FA composition of erythrocyte phospholipids. Although no differences in 8 weeks were observed in the control group, we cannot exclude that the increase in MUFA levels observed following HZNs intake depends on endogenous synthesis more than diet [41]. Finally, we failed to demonstrate a correlation between the increase in RBC MUFA levels and serum lipid concentrations after the intake of HZNs possibly because of the small samples size (data not shown). A significant decrease in the levels of erythrocyte palmitoleic and linoleic acid was observed only in patients consuming peeled HZN, although these findings remains unexplained. A reduction of erythrocyte linoleic acid could be postulated as the consequence of *ex-novo* synthesis of arachidonic acid, the major n-6 fatty acid contained in erythrocyte membrane. However, the synthesis rates of arachidonic acid have been reported to be less than 1% [39] and no significant change in its content was observed following HZN interventions. A significant increase in the levels

385 of erythrocyte total SFAs, in particular margaric acid, and a decrease of eicosenoic acid and total
386 PUFAs n-6 was found in control subjects at the end of the study period. The long chain-PUFA omega-
387 6 dihomo-γ-linolenic acid is the immediate precursor of arachidonic acid and it was found that change
388 in dairy intake of this fatty acid can increase plasma pentadecanoic and margaric acids in healthy
389 people [42]. However, based on the analysis of food diaries, no modifications of nutrient intake or
390 eating behaviors was found. On the contrary, HZN consumption increased the total fat intake, with
391 specific regard to MUFAs and PUFAs, while decreasing carbohydrate intake, without significantly
392 affecting total energy intake. Furthermore, we found an overall under-reporting of energy intake as
393 potential limitation of the study, and a moderate deviation from dietary recommendations. In
394 particular, protein and fat intake tended to be higher than suggested. BMI did not significantly change
395 over the intervention, in agreement with results reported in a recent meta-analysis of controlled trials
396 [29].

397 A low omega-3 index (<4%), defined as the sum of eicosapentaenoic and docosahexaenoic
398 acids contained within phospholipids of erythrocyte membranes, is considered a marker of CV risk
399 [43] and it was found [44] in obese children and in the same group of hyperlipidemic children and
400 adolescents characterized in our previous study [37]. As expected for the typical HZN lipid
401 composition, HZN treatment did not significantly improve this parameter.

402 Despite the relatively small sample of pediatric patients enrolled, the promising results
403 obtained in the present study are pivotal for future dietary interventions on this target population.
404 In conclusion, this is the first intervention trial assessing the effects of HZN-enriched diet in children
405 affected by primary hyperlipidemia. Based on our results, HZN interventions increased MUFA and
406 reduce MUFA/SFA ratio in erythrocyte phospholipids in respect to control treatment. Moreover,
407 regular HZN intake seems to reduce serum LDL-C levels over time, even if the levels did not differ
408 significantly from those observed in the control group. Although preliminary, these data provide new
409 information on the potential impact of HZN consumption, in combination with specific dietary

guidelines, in the management of hyperlipidemia since childhood. Future studies are needed to better define HZN impact on these and other markers of CV risk in the same target population.

Authors' contributions

Valeria Deon and Cristian Del Bo' performed the analysis of FA composition of RBC membranes, the statistical analysis, contributed in the analysis of HZN chemical composition, and wrote the manuscript draft. Federica Guaraldi and Francesca Abello enrolled subjects, analyzed serum lipid profile and food diaries. Simona Belviso characterized the composition of hazelnuts. Marisa Porrini contributed to data interpretation and critically revised the manuscript. Patrizia Riso and Ornella Guardamagna designed the study, obtained study funding, and revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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436 **References**

437 1. Juonala M, Viikari JSA, Kähönen M, Solakivi T, Helenius H, Jula A, et al. Childhood levels of
438 serum apolipoproteins B and AI predict carotid intima-media thickness and brachial endothelial
439 function in adulthood: the cardiovascular risk in young Finns study. J Am Coll Cardiol
440 2008;52:293-299.

441 2. Gidding SS, Dennison BA, Birch LL, Daniels SR, Gilman MW, Lichtenstein AH, et al. Dietary
442 recommendations for children and adolescents. Circulation 2005;112:2061-2075.

443 3. Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children
444 and Adolescents: Summary Report. Pediatrics 2011;128:213-256.

445 4. Nettleton JA, Lovegrove JA, Mensink RP, Schwab U. Dietary Fatty Acids: Is it Time to Change
446 the Recommendations? Ann Nutr Metab 2016;68:249-57.

447 5. Ros E. Health Benefits of Nut Consumption. Nutrients 2010;2:652-682

448 6. Sánchez-González C, Ciudad C, Noé V, Izquierdo-Pulido M. Health benefits of walnut
449 polyphenols: An exploration beyond their lipid profile. Crit Rev Food Sci Nutr 2015;29:0.

450 7. O'Neil CE, Keast DR, Nicklas TA, Fulgoni VL 3rd. Out-of-hand nut consumption is associated
451 with improved nutrient intake and health risk markers in US children and adults: National Health
452 and Nutrition Examination Survey 1999-2004. Nutr Res 2012;32:185-94.

453 8. Sarkkinen, ES, Agren JJ, Ahola I, Ovaskainen ML, Uusitupa MI. Fatty acid composition of serum
454 cholesterol esters, and erythrocyte and platelet membranes as indicators of long-term adherence to
455 fat-modified diets. Am J Clin Nutr 1994;59:364-370.

456 9. Kuratko CN, Salem N Jr. Biomarkers of DHA status. Prostaglandins Leukot Essent Fatty Acids.
457 2009;81:111-118.

458 10. Brigandi SA, Shao H, Qian SY, Shen Y, Wu BL, Kang JX. Autistic children exhibit decreased
459 levels of essential Fatty acids in red blood cells. Int J Mol Sci. 2015;16:10061-76.

460 11. Novgorodtseva TP, Kantur TA, Karaman YK, Antonyuk MV, Zhukova NV. Modification of fatty
461 acids composition in erythrocytes lipids in arterial hypertension associated with dyslipidemia.
462 Lipids Health Dis 2011;10:18.

463 12. Guardamagna O, Abello F, Saracco P, Baracco V, Rolfo E, Pirro M. Endothelial activation,
464 inflammation and premature atherosclerosis in children with familial dyslipidemia.
465 Atherosclerosis 2009;207:471–475.

466 13. Ficarra A, Lo Fiego DP, Minelli G, Antonelli A. Ultra fast analysis of subcutaneous pork fat.
467 Food Chem 2010;121:809–814.

468 14. Del Bo' C, Simonetti P, Gardana C, Riso P, Lucchini G, Ciappellano S. Horse meat consumption
469 affects iron status, lipid profile and fatty acid composition of red blood cells in healthy volunteers.
470 Int J Food Sci Nutr 2013;64:147-154.

471 15. Ghirardello D, Bertolino M, Belviso S, Dal Bello B, Giordano M, Rolle L, et al. Phenolic
472 composition, antioxidant capacity and hexanal content of hazelnuts (Corylus avellana L.) as
473 affected by different storage conditions. Postharvest Biol. Technol 2015;112:95-104.

474 16. Cacciari E, Milani S, Balsamo A, Spada E, Bona G, Cavallo L, Cerutti F, Gargantini L, Greggio
475 N, Tonini G, Cicognani A. Italian cross-sectional growth charts for height, weight and BMI (2 to
476 20 yr). J Endocrinol Invest. 2006;29:581-593.

477 17. Knopfholz J, Disserol CC, Pierin AJ, Schirr FL, Streisky L, Takito LL, Massucheto Ledesma P,
478 Faria-Neto JR, Olandoski M, da Cunha CL, Bandeira AM. Validation of the friedewald formula in
479 patients with metabolic syndrome. Cholesterol. 2014;2014:261878.

480 18. Simonetti P, Ciappellano S, Gardana C, Bramati L, Pietta P. Procyanidins from Vitis vinifera
481 seeds: in vivo effects on oxidative stress. J Agric Food Chem 2002;50:6217-6221.

19. Orem A, Yucesan FB, Orem C, Akcan B, Kural BV, Alasalvar C, Shahidi F. Hazelnut-enriched diet improves cardiovascular risk biomarkers beyond a lipid-lowering effect in hypercholesterolemic subjects. J Clin Lipidol 2013;7:123-31.

20. Mikkilä V, Räsänen L, Raitakari OT, Pietinen P, Viikari J. Consistent dietary patterns identified from childhood to adulthood: the cardiovascular risk in Young Finns Study. Br J Nutr 2005;93:923–31.

21. Niinikoski H, Pahkala K, Ala-Korpela M, Viikari, J, Rönnemaa T, Lagström H, et al. Effect of repeated dietary counseling on serum lipoproteins from infancy to adulthood. Pediatrics 2012;129:704–13.

22. Reiner Ž, Catapano AL, De Backer G, Graham I, Taskinen MR, Wiklund O, et al. Clinical Practice Guidelines Committee of the Spanish Society of Cardiology. ESC/EAS Guidelines for the management of dyslipidaemias. Rev Esp Cardiol 2011;64:1160-1168.

23. Tey SL, Brown RC, Chisholm AW, Delahunty CM, Gray AR, Williams SM. Effects of different forms of hazelnuts on blood lipids and α-tocopherol concentrations in mildly hypercholesterolemic individuals. Eur J Clin Nutr 2011;65:117-24.

24. Mercanligil SM, Arslan P, Alasalvar C, Okut E, Akgül E, Pinar A, et al. Effects of hazelnut-enriched diet on plasma cholesterol and lipoprotein profiles in hypercholesterolemic adult men. Eur J Clin Nutr 2007;61:212-20.

25. Yücesan FB, Orem A, Kural BV, Orem C, Turan I. Hazelnut consumption decreases the susceptibility of LDL to oxidation, plasma oxidized LDL level and increases the ratio of large/small LDL in normolipidemic healthy subjects. Anadulu Kardiyoloji Dergisi 2010;10:28-35.

26.Durak İ, Köksal İ, Kaçmaz M, Büyükkoçak S, Çimen BM, Öztürk HS. Hazelnut supplementation enhances plasma antioxidant potential and lowers plasma cholesterol levels. Clin Chim Acta 1999;284:113-115.

506 27. Banel DK, Hu FB. Effects of walnut consumption on blood lipids and other cardiovascular risk
507 factors: a meta-analysis and systematic review. Am J Clin Nutr 2009;90:56-63

508 28. Tey SL, Gray AR, Chisholm AW, Delahunty CM, Brown RC. The dose of hazelnuts influences
509 acceptance and diet quality but not inflammatory markers and body composition in overweight
510 and obese individuals. J Nutr 2013;143:1254-62.

511 29. Del Gobbo LC, Falk MC, Feldman R. Effects of tree nuts on blood lipids, apolipoproteins, and
512 blood pressure: systematic review, meta-analysis, and dose-response of 61 controlled intervention
513 trials. Am J Clin Nutr 2015;102:1347–56.

514 30. Maguire LS, O'Sullivan SM, Galvin K, O'connor TP, O'brien NM. Fatty acid profile, tocopherol,
515 squalene and phytosterol content of walnuts, almonds, peanuts, hazelnuts and the macadamia
516 nut. Int J Food Sci Nutr 2004;55:171-178

517 31. Alasalvar C, Shahidi F, Ohshima T, Wanasundara U, Yurttas HC, Liyanapathirana CM,
518 Rodrigues FB. Turkish Tombul hazelnut (*Corylus avellana* L.). 2. Lipid characteristics and
519 oxidative stability. J Agric Food Chem 2003;51:3797-3805.

520 32 Maranhão PA, Kraemer-Aguiar LG, de Oliveira CL, Kuschnir MC, Vieira YR, Souza MG, Koury
521 JC, Bouskela E. Brazil nuts intake improves lipid profile, oxidative stress and microvascular
522 function in obese adolescents: a randomized controlled trial. Nutr Metab (Lond). 2011;8(1):32.

523 33. O'Neil CE, Keast DR, Nicklas TA, Fulgoni VL 3rd. Out-of-hand nut consumption is associated
524 with improved nutrient intake and health risk markers in US children and adults: National Health
525 and Nutrition Examination Survey 1999-2004. Nutr Res. 2012;32:185-194.

526 34. Nergiz-Ünal R, Kuijpers MJ, de Witt SM, Heeneman S, Feijge MA, Caraballo SCG, et al.
527 Atheroprotective effect of dietary walnut intake in ApoE-deficient mice: involvement of lipids
528 and coagulation factors. Thrombosis research 2013;131:411-417.

529 35. Caimari A, Puiggròs F, Suárez M, Crescenti A, Laos S, Ruiz JA, et al. The intake of a hazelnut
530 skin extract improves the plasma lipid profile and reduces the lithocholic/deoxycholic bile acid
531 faecal ratio, a risk factor for colon cancer, in hamsters fed a high-fat diet. Food Chem
532 2015;167:138-144.

533 36. Vaya A, Martinez Triguero M, Reganon E, Vila V, Martinez Sales V, Sola E, Hernandez Mijares
534 A. Erythrocyte membrane composition in patients with primary hypercholesterolemia. Clin
535 Hemorheol Microcirc 2008;40:289-294.

536 37. Deon V, Del Bo' C, Guaraldi F, Gargari G, Bosusco A, Simonetti P, Riso P, Guardamagna O.
537 Serum lipid profile and fatty acid composition of erythrocyte phospholipids in children and
538 adolescents with primary hyperlipidemia. Int J Food Sci Nutr. 2017;68:339-348.

539 38. Fitschen PJ, Rolfhus KR, Winfrey MR, Allen BK, Manzy M, Maher MA. Cardiovascular effects
540 of consumption of black versus English walnuts. J Med Food 2011;14:890-898.

541 39. Harris WS, Pottala JV, Varvel SA, Borowski JJ, Ward JN, McConnell JP. Erythrocyte omega-3
542 fatty acids increase and linoleic acid decreases with age: observations from 160,000 patients.
543 Prostaglandins Leukot Essent Fatty Acids 2013;88:257-263.

544 40. Rajaram S, Haddad EH, Mejia A, Sabaté J. Walnuts and fatty fish influence different serum lipid
545 fractions in normal to mildly hyperlipidemic individuals: a randomized controlled study. Am J
546 Clin Nutr 2009;89:1657-1663.

547 41. Ferreri C, Masi A, Sansone A, Giacometti G, Larocca AV, Menounou G, Scanferlato R, Tortorella
548 S, Rota D, Conti M, Deplano S, Louka M, Maranini AR, Salati A, Sunda V, Chatgilialoglu C.
549 Fatty acids in membranes as homeostatic, metabolic and nutritional biomarkers: recent
550 advancements in analytics and diagnostics. Diagnostics (Basel). 2016;7(1).

551 42. Aslibekyan S, Campos H, Baylin A. Biomarkers of dairy intake and the risk of heart disease. Nutr
552 Metab Cardiovasc Dis 2012;22:1039-1045.

553 43. Von Schacky C. Omega-3 index and sudden cardiac death. Nutrients 2010;2:375-388.

554 44. Burrows T, Collins CE, Garg ML. Omega-3 index, obesity and insulin resistance in children. Int

555 J Pediatr Obes 2011;6:532-539.

556 **Table 1.** Characterization of fat and bioactives composition of hazelnut *Corylus avellana* L. 'Tonda Gentile delle Langhe'.

557

	HZN+S	HZN-S
Total fats (%)	48.5	52.9
Fatty acids (%)		
14:0 (myristic acid)	0.03	0.03
16:0 (palmitic acid)	5.85	6.02
16:1 cis-9 (palmitoleic acid)	0.23	0.22
17:0 (margaric acid)	0.05	0.05
18:0 (stearic acid)	2.46	2.7
18:1 trans-9 (elaidic acid)	0.02	0.02
18:1 cis-9 (oleic acid)	83.94	84.52
18:2 cis-9, cis-12 (linoleic acid)	6.93	5.97
20:0 (arachidic acid)	0.13	0.13
20:1 cis-13 (paullinic acid)	0.13	0.12
18:3 cis-6, cis-9, cis-12 (γ-linolenic acid)	0.1	0.1
22:0 (behenic acid)	0.02	0.02
20:4 cis-5, cis-8, cis-11, cis-14 (arachidonic acid)	0.03	0.03
Sterols (%)		
Cholesterol	0.1	0.1
Campesterol	4.2	4.1
Campestenol	0.2	0.2
Stigmasterol	0.9	0.9
Delta-7-campesterol	-	0.1
Delta-5.23-stigmastadienol	0.4	0.2
Chlerosterol	0.9	1
β-Sitosterol	81.4	82
Sitostanol	2.4	2
Delta-5-avenasterol	4.9	5.5
Delta-5.24-stigmastadienol	0.7	0.9
Delta-7-stigmastenol	2.7	1.9
Delta-7-avenasterol	1.2	1.1
Total sterols (mg/100g)	147.4	147.4
Tocopherols and tocotrienols (mg/100 g)		
α-Tocopherol	22.9	32.3
β-Tocopherol	0.5	0.6
γ-Tocopherol	1.1	0.6
Total tocopherols	24.4	33.5

Minerals (mg/100 g)		
Magnesium	141.0	142.0
Potassium	597.0	595.0
Calcium	119.0	118.0
Manganese	1.75	1.6
Iron	3.4	3.1
Zinc	1.8	1.8
Phosphorus	418.0	424.0
Copper	1.4	1.5
Polyphenols (mg/100 g)		
Gallic acid	2.9	1.9
Procyanidin B1	1.7	1.2
Epigallocatechin	1.3	-
Procyanidin B2	2.8	-
Epigallocatechin gallate	1.7	-
Gallocatechin gallate	1.9	1.3
Epicatechin gallate	0.4	-
Total phenolic content (mg GAE/g)	3.9	0.8
Total antioxidant capacity (μmol TE/g)	18.2	2.2

HZN+S: hazelnut with skin; HZN-S: hazelnuts without skin. The phenolic content (Folin–Ciocalteu method) was expressed as mg of gallic acid equivalents (GAE) per g of sample. The antioxidant capacity (radical scavenging activity) is expressed as μmoles of trolox equivalent (TE) per g of sample.

562 **Table 2.** Main sample features at baseline and after 8 weeks of intervention.
563

Variables	Control (*n*=18)		HZN+S (*n*=22)		HZN-S (*n*=20)		*T* Effect	*t* Effect	*T x t* Interaction
	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8	*p*	*p*	*p*
Age (years)	12.2 ± 2.3	12.4 ± 2.3	10.8 ± 2.5	11.0 ± 2.5	11.8 ± 2.8	12.0 ± 2.8	0.196	<0.001	0.003
Sex (male/female)	13/5		12/10		9/11				
Weight (kg)	49.5 ± 16.5	50.0 ± 16.6	44.4 ± 15.3	45.0 ± 15.3	47.8 ± 16.6	48.4 ± 16.5	0.595	0.001	0.952
Height (cm)	151.8 ± 15.6	152.9 ± 16.0	145.8 ± 14.6	146.6 ± 14.5	151.2 ± 17.2	151.9 ± 17.0	0.402	< 0.001	0.397
BMI (kg/m^2)	20.9 ± 3.9	20.8 ± 4.0	20.4 ± 4.0	20.3 ± 4.0	20.3 ± 3.7	20.3 ± 3.5	0.880	0.847	0.915
SBP (mmHg)	106.8 ± 9.8	109.0 ± 7.5	103 ± 9.9	105.2 ± 9.3	102.8 ± 10.3	102.5 ± 10.3	0.155	0.252	0.610
DBP (mmHg)	68.0 ± 5.1	67.1 ± 6.9	65.6 ± 6.6	66.5 ± 7.0	65.1 ± 9.3	66.3 ± 7.4	0.600	0.703	0.687

564

565 BMI: body mass index; HZN+S: group treated with hazelnut with skin; HZN-S: group treated with hazelnuts without skin; T, *treatment* effect; t, *time*
566 effect; T x t, *treatment x time* interaction. Values are expressed as mean ± SD.

567

568 **Table 3**. Daily energy and nutrient intakes assessed by patient food diaries, before and after 8 weeks of treatment.
569

Variables	Control (*n*=18)		HZN+S (*n*=22)		HZN-S (*n*=20)		*T* Effect	*t* Effect	*T x t Interaction*
	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8	*p*	*p*	*p*
Energy (kcal)	1126.5 ± 281	1163.5 ± 14	1093.2 ± 194.7	1199.3 ± 180.1*	1241.0 ± 210.2	1358.7 ± 211.0*	0.026	0.002	0.432
Protein (% of energy)	17.0 ± 2.6	16.4 ± 2.9	17.5 ± 2.9	16.9 ± 2.6	16.5 ± 2.4	16.3 ± 2.3	0.612	0.095	0.822
Carbohydrate (% of energy)	50.7 ± 4.3	50.3 ± 3.9	51.2 ± 3.8	46.9 ± 3.6*	52.6 ± 3.9	48.6 ± 4.2*	0.413	<0.001	0.008
Total fat (% of energy)	32.5 ± 3.0	33.3 ± 3.1	31.2 ± 2.9	36.2 ± 1.9*	30.9 ± 2.5	35.2 ± 2.8*	0.609	<0.001	<0.001
SFA (% of energy)	10.1 ± 2.6	10.0 ± 2.2	9.4 ± 1.4	9.6 ± 1.6	8.9 ± 1.9	8.6 ± 1.9	0.102	0.618	0.722
MUFA (% of energy)	14.7 ± 1.9	15.5 ± 3.0	13.1 ± 3.3	17.8 ± 6.5*	17.9 ± 2.5	19.0 ± 2.1*	0.466	<0.001	0.037
PUFA (% of energy)	3.5 ± 0.9	3.3 ± 0.5	4.1 ± 2.3	7.6 ± 3.3*	3.3 ± 1.4	6.8 ± 3.5*	0.002	<0.001	0.003
ω-3 (% of energy)	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.5	1.3 ± 0.6*	0.5 ± 0.3	1.0 ± 0.7*	0.008	<0.001	0.044
ω-6 (% of energy)	2.2 ± 0.8	2.1 ± 0.4	2.8 ± 2.0	6.0 ± 2.8*	2.1 ± 1.2	5.4 ± 2.9*	<0.001	<0.001	0.002
Fibers (g)	9.0± 2.6	9.9 ± 2.5	10.1 ± 2.1	10.5 ± 2.9	11.9 ± 4.8	14.1± 5.3	0.011	0.143	0.221
Cholesterol (mg)	137.5 ± 35.9	138.8 ± 59.1	129.9 ± 39.9	123.6 ± 39.3	133.6 ± 44.8	128.8 ± 45.2	0.739	0.594	0.881

570 HZN+S: group treated with hazelnut with skin; HZN-S: group treated with hazelnuts without skin; MUFAs: monounsaturated fatty acids; PUFAs:
571 polyunsaturated fatty acids; SFAs: saturated fatty acids; T, *treatment* effect; t, *time* effect; T x t, *treatment x time* interaction. Values are expressed as
572 mean ± SD. * Significantly different as compared to baseline ($p \leq 0.05$).
573

574 **Table 4.** Serum lipid profile and fatty acid composition of erythrocyte phospholipids at baseline and after 8-weeks of each treatment.
575

Lipid profile	Control (*n*=18)		HZN+S (*n*=22)		HZN-S (*n*=20)		*T* Effect	t Effect	*T x t Interaction*
	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8	*p*	*p*	*p*
Serum lipids (mg/dl)									
TC	210.3 ± 50.0	204.1 ± 44.9	215.8 ± 42.0	210.5 ± 41.8	221.6 ± 55.6	212.3 ± 52.3	0.815	0.013	0.820
TG	76.5 (35-185)	77.5 (38-165)	67.0 (32 - 194)	58.5 (37 - 159)	61.5 (46-300)	70 (41-264)	0.400	0.190	0.669
HDL-C	55.4 ± 14.9	55.5 ± 12.2	62.0 ± 13.6	63.2 ± 14.3	61.0 ± 16.2	62.4 ± 16.9	0.250	0.393	0.871
LDL-C	136.7 ± 45.2	131.9 ± 45.4	141.9 ± 46.8	132.7 ± 44.1*	141.4 ± 57.3	132.6 ± 55.3*	0.978	< 0.001	0.604
HDL/LDL ratio	0.45 ± 0.24	0.49 ± 0.31	0.49 ± 0.19	0.53 ± 0.21*	0.50 ± 0.24	0.55 ± 0.27*	0.763	< 0.001	0.853
HDL/TG ratio	0.76 ± 0.41	0.82 ± 0.41	1.03 ± 0.50	1.06 ± 0.52	0.9 ± 0.5	0.9 ± 0.5	0.210	0.256	0.967
Non-HDL-C	154.9 ± 47.5	148.6 ± 46.9	153.8 ± 46.5	147.3 ± 46.7	162.4 ± 59.4	151.7 ± 56.1*	0.911	0.001	0.675
Phospolipid FA composition of erythrocytes (%)									
Total SFAs	48.52 ± 2.79	49.85 ± 5.06*	49.23 ± 2.23	48.81 ± 3.01	49.73 ± 1.61	48.97 ± 2.05	0.877	0.880	0.036
Total MUFAs	18.57 ± 1.07	18.49 ± 1.52	18.3 ± 0.75	19.10 ± 0.94*	18.41 ± 0.95	19.23 ± 1.01*	0.576	< 0.001	0.008
Total PUFAs	32.91 ± 2.75	31.67 ± 6.10	32.47 ± 2.12	32.09 ± 3.03	31.85 ± 1.97	31.80 ± 2.10	0.743	0.080	0.329
MUFAs/SFAs ratio	0.38 ± 0.04	0.37 ± 0.03	0.37 ± 0.03	0.39 ± 0.04*	0.37 ± 0.02	0.39 ± 0.03*	0.861	0.015	0.002
PUFAs/SFAs ratio	0.68 ± 0.10	0.64 ± 0.15	0.66 ± 0.07	0.66 ± 0.12	0.64 ± 0.06	0.65 ± 0.07	0.763	0.321	0.144
Total PUFAs n-3	5.16 ± 1.28	4.79 ± 1.53	4.73 ± 0.77	4.60 ± 0.78	4.82 ± 0.91	4.93 ± 0.93	0.556	0.138	0.097
Total PUFAs n-6	25.90 ± 1.26	25.19 ± 4.46	26.08 ± 1.88	25.8 ± 2.19	25.58 ± 1.38	25.30 ± 1.48	0.545	0.019	0.546
PUFAs n-3/n-6	0.20 ± 0.05	0.19 ± 0.06	0.18 ± 0.04	0.18 ± 0.04	0.19 ± 0.04	0.20 ± 0.04	0.557	0.572	0.077
Total LC-PUFAs n-3	5.08 ± 1.28	4.7 ± 1.52	4.64 ± 0.78	4.52 ± 0.77	4.72 ± 0.90	4.85 ± 0.93	0.568	0.161	0.079
Total LC-PUFAs n-6	14.89 ± 1.47	14.23 ± 3.58	15.02 ± 1.64	14.73 ± 1.92	14.69 ± 1.32	14.95 ± 1.62	0.791	0.248	0.183
LC-PUFAs n-3/n-6	0.34 ± 0.08	0.33 ± 0.08	0.32 ± 0.08	0.31 ± 0.07	0.32 ± 0.07	0.33 ± 0.07	0.658	0.509	0.507
Omega-3 index	3.94 ± 1.09	3.66 ± 1.24	3.57 ± 0.74	3.49 ± 0.70	3.63 ± 0.79	3.76 ± 0.83	0.602	0.253	0.070
Saturated fatty acids (%)									
14:0 (myristic acid)	0.38 ± 0.07	0.40 ± 0.24	0.39 ± 0.09	0.39 ± 0.08	0.42 ± 0.08	0.39 ± 0.08	0.733	0.526	0.272
15:0 (pentadecanoic acid)	0.16 ± 0.03	0.16 ± 0.04	0.16 ± 0.03	0.16 ± 0.03	0.16 ± 0.03	0.16 ± 0.03	0.819	0.853	0.555
16:0 (palmitic acid)	23.83 ± 1.07	24.40 ± 2.80	24.01 ± 1.43	24.08 ± 1.72	24.17 ± 1.50	23.63 ± 1.28	0.846	0.888	0.148
17:0 (margaric acid)	0.46 ± 0.13	0.51 ± 0.34*	0.38 ± 0.19	0.41 ± 0.16	0.47 ± 0.14	0.43 ± 0.12	0.174	0.370	0.021
18:0 (stearic acid)	15.02 ± 1.53	15.17 ± 1.63	15.56 ± 1.02	15.21 ± 1.66	15.95 ± 1.36	15.77 ± 1.28	0.211	0.440	0.482
20:0 (arachidic acid)	0.63 ± 0.25	0.60 ± 0.09	0.56 ± 0.05	0.57 ± 0.07	0.55 ± 0.07	0.55 ± 0.07	0.107	0.706	0.863
22:0 (behenic acid)	2.07 ± 0.36	2.19 ± 0.26	2.06 ± 0.22	1.99 ± 0.35	2.03 ± 0.25	2.04 ± 0.26	0.445	0.532	0.080
23:0 (tricosanoic acid)	0.30 ± 0.05	0.32 ± 0.07	0.33 ± 0.06	0.32 ± 0.07	0.31 ± 0.05	0.33 ± 0.12	0.751	0.504	0.581
24:0 (lignoceric acid)	5.68 ± 0.81	6.09 ± 0.85	5.78 ± 0.81	5.69 ± 1.11	5.65 ± 0.85	5.68 ± 0.84	0.712	0.239	0.137
Monounsaturated fatty acids (%)									

16:1n-9 (hypogeic acid)	0.11 ± 0.02	0.11 ± 0.03	0.10 ± 0.02	0.11 ± 0.04	0.11 ± 0.03	0.10 ± 0.02	0.850	0.831	0.084
16:1n-7 (palmitoleic acid)	0.22 ± 0.06	0.22 ± 0.16	0.24 ± 0.09	0.23 ± 0.08	0.26 ± 0.07	0.24 ± 0.07*	0.479	0.015	0.184
18:1n-9 (oleic acid)	10.86 ± 1.09	10.88 ± 1.39	10.89 ± 0.79	11.31 ± 1.06*	10.93 ± 0.85	11.48 ± 0.95*	0.536	0.004	0.166
18:1n-7 (vaccenic acid)	1.08 ± 0.07	1.05 ± 0.08	1.06 ± 0.11	1.08 ± 0.13	1.09 ± 0.11	1.08 ± 0.07	0.744	0.685	0.196
20:1n-9 (eicosenoic acid)	0.23 ± 0.13	0.19 ± 0.07*	0.19 ± 0.02	0.20 ± 0.03	0.19 ± 0.02	0.20 ± 0.03	0.426	0.459	0.030
24:1n-9 (nervonic acid)	6.06 ± 1.21	6.05 ± 0.88	5.82 ± 0.80	6.16 ± 1.10	5.83 ± 0.79	6.12 ± 0.78	0.958	0.082	0.426
Polyunsaturated ω-6 fatty acids (%)									
18:2n-6 (linoleic acid)	10.68 ± 1.12	10.69 ± 1.52	10.78 ± 0.99	10.79 ± 1.16	10.59 ± 1.06	10.06 ± 0.97*	0.312	0.088	0.043
18:3n-6 (γ-linolenic acid)	0.06 ± 0.02	0.06 ± 0.03	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.03	0.688	0.530	0.943
20:2n-6 (eicosadienoic acid)	0.27 ± 0.10	0.23 ± 0.04	0.22 ± 0.03	0.23 ± 0.03	0.24 ± 0.03	0.23 ± 0.04	0.220	0.090	0.190
20:3n-6 (dihomo-γ-linolenic acid)	1.91 ± 0.43	1.82 ± 0.48	1.87 ± 0.37	1.81 ± 0.39	1.94 ± 0.38	1.88 ± 0.41	0.842	0.004	0.813
20:4n-6 (arachidonic acid)	11.11 ± 1.01	10.62 ± 2.69	11.18 ± 1.41	10.93 ± 1.31	10.89 ± 0.98	11.09 ± 1.15	0.865	0.183	0.130
22:4n-6 (adrenic acid)	1.88 ± 0.57	1.78 ± 0.61	1.97 ± 0.49	2.00 ± 0.77	1.86 ± 0.42	1.98 ± 0.60	0.673	0.788	0.447
Polyunsaturated ω-3 fatty acids (%)									
18:3n-3 (α-linolenic acid)	0.08 ± 0.03	0.08 ± 0.04	0.08 ± 0.02	0.08 ± 0.03	0.10 ± 0.04	0.09 ± 0.03	0.142	0.158	0.461
20:5n-3 (eicosapentaenoic acid)	0.35 ± 0.13	0.33 ± 0.16	0.36 ± 0.22	0.33 ± 0.14	0.34 ± 0.17	0.34 ± 0.17	1.000	0.133	0.666
22:5n-3 (docosapentaenoic acid)	1.14 ± 0.27	1.05 ± 0.35	1.07 ± 0.14	1.03 ± 0.19	1.09 ± 0.16	1.09 ± 0.15	0.684	0.068	0.291
22:6n-3 (docosahexaenoic acid)	3.58 ± 0.99	3.33 ± 1.17	3.22 ± 0.60	3.17 ± 0.59	3.29 ± 0.68	3.42 ± 0.71	0.506	0.363	0.064

576 FA. fatty acid; HDL-C: high-density lipoprotein cholesterol; HZN+S: group treated with hazelnut with skin; HZN-S: group treated with hazelnuts
577 without skin; LC-PUFAs: long chain polyunsaturated fatty acids (C ≥20. double bonds ≥3); LDL-C: low-density lipoprotein cholesterol; MUFAs:
578 monounsaturated fatty acids; omega-3 index: sum of eicosapentaenoic acid + docosahexaenoic acid; PUFAs: polyunsaturated fatty acids; SFAs:
579 saturated fatty acids; T. *treatment* effect; t. *time* effect; T x t. *treatment x time* interaction; TC: total cholesterol; TG: triglycerides.
580 Values are expressed as mean ± SD or median (min-max). * Significantly different as compared to baseline ($p < 0.05$).
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582 **Figure 1.** Study flow-chart showing the process of patient selection and enrollment. allocation to the three study groups. and rate of patients completing
583 the study. HZN+S: group treated with hazelnuts with skin; HZN-S: group treated with hazelnuts without skin; control: no treatment group.
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