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ABSTRACT

This study aimed at characterising the fatty acid (FA) composition of red blood cell (RBC) phospholipids in children and adolescents with primary hyperlipidemia, and to ascertain potential association with serum lipid profile and dietary factors. At this purpose, 54 probands aged 6-17 years were recruited. Subjects showed a low omega-3 index (eicosapentaenoic acid, EPA + docosahexaenoic acid, DHA<4%). Compared to males, females had a trend towards lower levels of total monounsaturated fatty acids (MUFA) and MUFA/saturated fatty acids (SFAs) ratio in RBCs. An inverse relationship between MUFA concentration in RBCs and serum cholesterol or HDL-C/triglycerides ratio was found. Omega-6 polyunsaturated fatty acids (n-6 PUFA) were positively associated to serum HDL-C levels, and inversely to dietary cholesterol. Fibre intake was positively associated with MUFA/SFA ratio. In conclusion, we provide the first experimental data on phospholipid FA composition of RBCs in hyperlipidemic children, showing sex differences and an overall low omega 3-index.

40 **Introduction**

41 Hyperlipidemias are disorders of lipoprotein metabolism characterized by an increase in
42 serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), or
43 triglycerides (TG). These disorders, both primary (monogenic and polygenic inherited
44 forms) and secondary (related to other diseases) forms, are considered a major cause of
45 atherosclerosis and cardiovascular disease (CVD) (Newman et al. 1986; Ahmed et al.
46 1998; Groner et al. 2006; Haney et al. 2007).

47 The atherosclerotic process begins early in life, thus children and adolescents
48 affected by primary hyperlipidemia show a particularly high risk to develop CVD later
49 in life (Durrington 2003), which is mainly secondary to chronic exposure to elevated
50 LDL-C, accumulating in the intima-media of large muscular arteries (Durrington 2003;
51 McGill et al. 2000). Healthy lifestyle, which includes appropriate dietary **pattern in**
52 **agreement with the expert panel guidelines for CV health and risk reduction in children**
53 **and adolescents (Expert panel ..., 2011)**, physical activity and weight loss in case of
54 excessive body weight, is the cornerstone in the treatment of hyperlipidemia and
55 represents an important target for CVD prevention (Haney et al. 2007; Catapano et al.
56 2011). It has been suggested that the lipid composition of RBC membranes can be
57 considered as an additional risk factor in the progression of atherosclerosis and coronary
58 heart disease (Lausada et al. 2007; Tziakas et al. 2010). The fatty acid (FA) composition
59 of red blood cells (RBCs) generally reflects the last three months of dietary fat intake,
60 and it is thought to be a biomarker of the tissue fatty acid status (Sarkkinen et al. 1994;
61 Kuratko et al. 2009; Brigandi et al. 2015).

62 Since erythrocytes are incapable of de novo phospholipid synthesis, chain elongation
63 or desaturation of fatty acids, the major pathway to renew the RBC phospholipids is the
64 direct exchange from plasma lipoproteins to the erythrocyte (Marks et al. 1960;

Farquhar & Ahrens 1963; Reed 1968; Hodson et al. 2008). Thus, the RBC FA composition is postulated to better and earlier reflect the pathology of lipid metabolism, in respect to lipoprotein changes in blood serum, which are affected by recent food consumption (Sarkkinen et al. 1994; Harris & Von Schacky 2004; Novgorodtseva et al. 2011).

Studies conducted in adults found a correlation between altered FA composition in RBCs and coronary heart disease, arterial hypertension, dyslipidemia and other atherosclerosis-related diseases (Engelmann et al. 1992; Taylor 1994; Antoku et al. 2000; Lausada et al. 2007; Vayá et al. 2009; Ristic-Medic et al. 2009; Tziakas et al. 2010; Novgorodtseva et al. 2011; Jacobs et al. 2014). Harris & Von Schacky (2004) demonstrated that a low content of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in the RBC membranes is strongly associated with coronary heart diseases. The total levels of EPA and DHA in RBCs, defined as omega-3 index, was suggested as an additional biomarker of CV risk, and a predictive parameter for morbidity and mortality from CVD (Harris & Von Schacky 2004). Since most of this class of compounds is contained in cell membranes, the index has been also calculated on RBC phospholipids and other cell types; moreover, comparison with data obtained in plasma has been performed (Harris & Thomas 2010; Rice et al. 2016).

Despite the potential impact of knowing the RBC membrane FA composition in patients affected by lipoprotein disorders in terms of risk prediction, studies are still limited and mainly performed in adults. In this regard, trials carried out in children were mainly aimed at characterizing the phospholipid FAs of RBCs in healthy subjects (Laryea et al. 1990; Jakobit et al. 2009; Cortés et al. 2012), or in patients affected by other diseases (Decsi et al. 2002; Vanderjagt et al. 2003; Trebbe et al. 2003; Chen et al. 2004; Bu et al. 2006; Vaisman et al. 2008; Burrows et al. 2011; Perona et al. 2013;

Brigandi et al. 2015; Bueno et al. 2015). Furthermore, data on gender difference in RBC composition in childhood and adolescence have not been adequately investigated.

The aim of the present study is to provide data on the RBC phospholipid FA composition in a population of Italian children and adolescents with primary hyperlipidemia, receiving medical nutrition therapy at least 6 months before the recruitment. Since RBC phospholipid FA composition is influenced by blood lipoprotein profile/concentration changes and diet we also investigated the association with serum biomarkers and dietary parameters.

Materials and methods

Subject enrolment and study design

Fifty-four children and adolescents with primary hyperlipidemia were recruited among pediatric patients cared at the Department of Health Science and Pediatrics of the University of Turin, after a screening for eligibility. Children and adolescents included in the study were 6-17 years old, affected by familial hypercholesterolemia (FH), familial combined hyperlipidemia (FCHL) or polygenic hypercholesterolemia (PHC). Exclusion criteria were: secondary dyslipidemia; obesity (body mass index (BMI) $\geq 90^{\text{th}}$ percentile, age and sex matched); renal, endocrine and liver disorders; or chronic diseases requiring drug treatment (i.e. immunologic, neurologic, or oncohematologic disorders). Participants were not on lipid-lowering treatments (including functional foods) in the previous 3 months and were not smoking. Children on diet therapy were selected after demonstrating appropriate compliance with dietary instructions, provided by a trained nutritionist, in the previous 2 months. Recruited subjects and their families were trained by a nutritionist to adhere to a properly dietary regimen, evaluated by a weekly food diary.

Diagnostic criteria of familial hyperlipidemia were based on accepted international standards (Guardamagna et al. 2011). FH was diagnosed in presence of LDL-C $\geq 95^{\text{th}}$ 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 $>90^{\text{th}}$ age- and sex-specific percentile, with at least one parent affected by hypercholesterolemia, hypertriglyceridemia, or both (IIA, IV, or IIB phenotype, respectively), with concomitant individual and familial lipid phenotype variability. Children with LDL-C levels $>90^{\text{th}}$ percentile and a family history of dominant inherited hypercholesterolemia, but not fulfilling the biochemical international diagnostic criteria of FH or FCHL were diagnosed with PHC.

All subjects enrolled underwent a medical examination in the morning between 8 and 10 a.m. on the day of recruitment. Physical parameters including height, weight and blood pressure were measured. Height and weight were measured to the nearest 0.1 cm and 0.1 kg respectively (Wunder SA.BI. S.r.l. Italy), with the patients wearing hospital gowns and had bare feet. BMI was calculated as body weight in kilograms divided by height squared in meters (kg/m^2). Systolic and diastolic blood pressure was measured with a mercury sphygmomanometer during the medical examination. Fasting blood samples were drawn by venipuncture for the analysis of the lipid profile and the FA composition of RBCs.

Children and their parents had received dietary recommendations based on the cardiovascular health integrated lifestyle diet (CHILD-1) for children with identified dyslipidemia, as in the American Academy of Pediatrics (Expert panel ..., 2011). The essential features were: 55% of daily energy from carbohydrate, 15% from protein, 25-30% from total fat (saturated fat $<10\%$, MUFA and PUFA 20% of total energy), dietary cholesterol <100 mg/1000 kcal and no more than 200 mg/day, and 10-25 g/day of

soluble fibre. In order to examine correct dietary habits, parents of the enrolled children, and probands themselves, were asked to fill in a weekly food diary to be provided at time of the visit. Detailed instructions on how to collect diet records were comprehensively explained by a nutritionist. The nutritional evaluation of macronutrients and FA content of the diet registered was performed with MètaDieta® Software using the Italian Food Composition Tables (Carnovale & Marletta 2000).

The study protocol complied with the principles of the Declaration of Helsinki, and was approved by the ethics committee of the City of Health and Science University Hospital of Turin (EC:CS377). The protocol and the purpose of the study were carefully explained to the participants and to their parents; a written informed consent was obtained from children's parents before enrolment in the study.

Blood sample collection and separation

Venous blood samples of 2.5 ml were drawn into vacutainer tubes containing lithium heparin (to obtain plasma and RBCs) or silicon (to obtain serum). Plasma and serum were separated by centrifugation at 1400 g for 15 min at 4°C. Plasma was stored at -80°C for further analysis, while serum was immediately analysed. The buffy layer of white blood cells was removed using a pipette, and RBCs were washed twice in an equal volume of a physiologic solution (0.9% NaCl, w/v). Two aliquots (0.5 ml) of RBCs were stored at -80°C until the analysis.

Serum lipid profile

Serum levels of TC, HDL-C and TG were directly determined by an automatic biochemical analyzer (Olympus AU2700, Japan), while the LDL-C concentration was

164 estimated using the Friedewald formula ($LDL = TC - (HDL + TG/5)$). Non-high density
165 lipoprotein cholesterol (non-HDL-C) was calculated as TC minus HDL-C.

166 *Analysis of FA composition of RBC phospholipids*

167 Extraction of RBC phospholipids was performed in accordance to the method
168 previously described by Simonetti et al. (2002). Briefly, 1 ml of distilled water, 300 mg
169 of RBC, 2.5 ml of butylated hydroxitoluene (110 µg/ml in methanol), and 5 ml of
170 chloroform were transferred into 10 ml plastic tubes. The mixtures were shaken on
171 vortex for 3 min and centrifuged at 1200 g x for 10 min. The chloroform phase was
172 transferred into 10 ml plastic tubes. An aliquot of 2.5 ml chloroform was added to the
173 tubes containing RBCs to perform the second extraction by vortexing for 3 min, and,
174 after centrifugation at 1200 g x for 10 min, the supernatant was transferred into the
175 corresponding tube. The chloroform phase was eluted on a silica cartridge (Sep-Pack
176 PlusSilica, Waters), which was then evaporated under nitrogen steam. The silica phase
177 was transferred into Pyrex glass tubes, and 2.5 ml of a toluene/methanol (1:4 v/v)
178 mixture and 200 µl of acetyl chloride were added. After 1 h in an oven at 100°C, 5 ml
179 of K₂CO₃ (6% w/v in water) was added to the silica. After centrifugation at 1200 g x 10
180 min, the supernatant was transferred into amber glass vials, dried under nitrogen and
181 resuspended in 100 µl of hexane before gas chromatography analysis.

182 The gas chromatography analysis was performed as described by Ackman (1986),
183 partly modified. Separations were performed with a 30 m 0.32 mm i.d. Omegawax 320
184 capillary column, under these conditions: initial isotherm, 140 °C for 5 min;
185 temperature gradient, 2 °C/min to 210°C; final isotherm, 210°C for 20 min. The injector
186 temperature was 250°C. Injection volume was 1 µl with a split ratio of 1/100, and the
187 flame ionization detector temperature was 250°C. Carrier and makeup gas were

hydrogen and nitrogen, respectively. Fatty acid retention times were obtained by injecting the Omegawax test mix as standard.

Statistical analysis

Statistical analysis was carried out by R statistic software (version 3.1.2). One way repeated-measures analysis of variance (ANOVA) was used to compare the data obtained from subjects stratified by sex and by type of hyperlipidemia; post-hoc analysis of differences between paired data was assessed, when appropriate, by the Least Significant Difference. Differences in serum lipid concentrations, anthropometric data and the proportion of FA composition of RBCs in relation to different lipid disorders was evaluated by non-parametric Wilcoxon-Mann-Whitney test with Benjamini-Hochberg correction. The relationship among variables was assessed by Kendall and Spearman rank, non-parametric correlation tests. This approach was used to achieve the significance level and the trend (direct or inverse) of the data correlation. The level of statistical significance was set at $p<0.05$; data are presented as a mean and standard deviation (SD).

Results

Subjects characteristics

The main features of the study sample are summarized in **Table 1**. The cohort of children and adolescents included 54 hyperlipidemic subjects (26 F), aged 6-17 years old; 14 were affected by FH (7 F), 21 by FCHL (12 F) and 19 by PHC (7 F). Five subjects were slightly overweight; mean blood pressure levels were in the normal range.

Serum lipid profile and RBC phospholipid composition

Serum lipid profile and RBC phospholipid composition of the patients, are reported in

Table 2, and classified according to gender and clinical diagnosis. Mean serum lipid parameters exceeded the 90th percentiles (age and sex related), except for HDL-C, showing normal concentrations. RBC phospholipid composition was SFA>PUFA>MUFA, with low omega-3 index value ($3.76 \pm 1.04\%$).

No significant serum lipid concentration difference was observed between males and females, while it was detected in the phospholipid composition of RBCs, as females showed higher concentrations of stearic acid but lower DGLA concentration ($p= 0.031$ and 0.050 , respectively). Moreover, MUFA content and MUFA/SFAs ratio in RBCs was lower in females than males ($p= 0.052$ and 0.056 , respectively).

A further analysis considering the type of hyperlipidemia revealed that children with FH had higher levels ($p< 0.001$) of serum TC, LDL-C and non-HDL-C, and lower serum HDL/LDL ratio if compared to children with FCHL or PHC. Finally, significantly lower levels of vaccenic acid and γ -linolenic acid were detected in FH as compared to PHC children ($p= 0.036$ and 0.027 , respectively).

Correlation between RBC phospholipid composition and serum lipid profile

Correlations between serum lipid levels and RBC phospholipid composition are reported in **Table 3**. Lower RBC MUFA levels correlated with higher serum TC levels ($p= 0.032$) and HDL/TG ratio ($p= 0.025$), but lower serum TG levels ($p= 0.005$). A positive correlation between serum HDL-C and PUFA n-6 levels ($p= 0.048$) was also detected.

Food diary analysis and correlation between dietary factors, RBC phospholipid FAs and serum lipid profile

Complete and detailed weekly food diaries were obtained from 23 (7 F) out of 54

children, and were used to calculate mean daily energy and macronutrient intake. Results showed a mean daily total energy intake of 1208 ± 151 kcal suggesting a possible overall underestimation of food intake (e.g. in terms of portion size declared). Macronutrients distribution, expressed as percentage of daily energy intake, was: $16.7 \pm 2.8\%$ protein, $58.6 \pm 6.1\%$ carbohydrate and $24.7 \pm 6.4\%$ fat (saturated: $7.4 \pm 2.1\%$, monounsaturated: $10.3 \pm 4.8\%$, polyunsaturated: $2.6 \pm 0.5\%$). Estimated cholesterol intake was 136.2 ± 47.2 mg/day, while fibre intake was 14.0 ± 5.3 g/day, confirming previous data (Guardamagna et al. 2013; Guardamagna et al. 2014). Correlations between dietary factors, the RBC phospholipid composition and serum lipid profile are reported in **Table 4**. An inverse association was found between the dietary intake of cholesterol and n-6 PUFA and n-6 LC-PUFA levels in RBC phospholipids ($p= 0.001$ and 0.017 , respectively). The fibre intake was directly associated with RBC MUFA/SFA ratio ($p= 0.035$).

Discussion

To our knowledge, this is the first study aimed at characterizing the FA composition of RBC phospholipids in a population of Italian children and adolescents with primary hyperlipidemia.

The phospholipid composition of RBCs has been suggested as an additional marker to monitor serum lipoprotein profile changes (Harris et al. 2004; Novgorodtseva et al. 2001) and as an outcome parameter in the management of hyperlipidemia, although no reference values exist neither for healthy nor hyperlipidemic adults or children. Alterations of the erythrocyte lipid matrix have been demonstrated in studies performed in adults affected by dyslipidemia and/or with risk factors for coronary heart diseases (Laryea et al. 1990; Engelmann et al. 1992; Taylor 1994; Antoku et al. 2000; Lausada et

al. 2007; Vajá et al. 2009; Restic-Medic et al. 2009; Novgorodtseva et al. 2011; Jacobs et al. 2014). For instance, Novgorodtseva et al. (2011) described reduced levels of n-3 PUFA, in particular docosapentaenoic acid (22:5n-3) and DHA (22:6n-3), in dyslipidemic patients as compared to normal subjects. Lausada et al. (2007) documented a PUFA decrease and SFA concentrations increase in plasma and RBC membranes of patients with coronary heart disease, as compared to controls.

Similar studies have been performed in children with diabetes, attention-deficit hyperactive disorder, autism, and other various disorders, except for hyperlipidemia, thus, making the comparison of our data with other data from literature extremely difficult (Decsi et al. 2002; Vanderjagt et al. 2003; Trebbe et al. 2003; Chen et al. 2004; Bu et al. 2006; Vaisman et al. 2008; Burrows et al. 2011; Perona et al. 2013; Brigandi et al. 2015; Bueno et al. 2015). Results obtained in subjects with disorders seem to differ from those obtained in the relative controls. As an example, Perona et al. (2013) found higher levels of SFAs, and reduced concentrations of MUFAs and n-6 PUFAs in RBC membranes of obese adolescents as compared to normal weight controls. These results are in line with our observations as hyperlipidemic children and adolescents showed comparable SFA and MUFA levels. However, it should be also underlined that values obtained in different studies and different countries can be significantly affected by the dietary habits, thus direct comparison of results is not always possible.

It is recognized that children affected by hyperlipidemia exhibit an increased risk of atherosclerosis and CVD during adulthood (McGill et al. 2000; Durrington 2003). Recently, the PANIC study evaluated the association between plasma FA composition (in triacylglycerol and phospholipid fractions), the estimated desaturase/elongase activities and the cardiometabolic risk in 384 children (Venäläinen et al. 2016). Authors found a significant positive correlation between FAs metabolism and the

288 cardiometabolic risk score for the pediatric population.

289 The omega-3 index may represent a novel, physiologically relevant and graded risk
290 factor to predict CVD development, thus having a significant clinical utility. A low
291 index ($\leq 4\%$) was associated with a high risk of mortality for CVD and a value $\geq 8\%$ was
292 suggested as a reasonable preliminary target for reducing the risk (Harris & Von
293 Schacky 2004; Von Schacky 2010). In the pediatric population, only one other study
294 examined the omega-3 index and concluded that a greater proportion of obese children
295 had a lower index compared with non-obese children (Burrows et al. 2011). In our
296 study, we documented mean omega-3 index $< 4\%$ in hyperlipidemic children, a factor
297 that contribute to classify them as at future high risk for CVD. Since EPA and DHA
298 derive from essential fatty acids, these results suggest that there is a reduced synthesis
299 or patients did not regularly consume LC-PUFA rich food sources. In this regard, the
300 use of whole dietary strategies and/or specific supplementations (e.g. with PUFA,
301 MUFA or other food bioactives) could be fundamental to improve this index above all
302 in at risk subjects such as hyperlipidemic children.

303 The present analysis on sex-related differences showed that females had a
304 significantly greater proportion of the SFA stearic acid, and lower proportion of n-6
305 PUFA, in particular DGLA. Lower DGLA values were also observed in serum
306 glycerophospholipids of healthy young girls when compared with boys (Glaser et al.
307 2010). Moreover, in females we found a trend towards lower level of MUFA and
308 MUFA/SFA ratio in RBCs with respect to males. Venäläinen et al. (2016) reported that
309 a higher plasma level of SFA myristic acid, MUFA palmitoleic acid and reduced
310 concentrations of n-6 PUFA (linoleic acid) have been correlated with increased
311 cardiometabolic risk in children. Furthermore, higher levels of stearic, palmitic acids
312 and lower levels of linoleic acid in RBC membranes were observed in obese adolescents

(Perona et al. 2013) and in hypercholesterolemic adults (Taylor 1994), as compared to healthy controls. Both sex and gender differences in the pathogenesis, progression and manifestation of atherosclerosis and CVD have been well documented for adults (Maas & Appelman 2010; Mosca et al. 2011; Spence & Pilote 2015), while controversial data have been reported in children (Marelli et al., 2010; Krishnan et al., 2012). In this context, our preliminary findings on sex differences in the pediatric population, associated with higher risk of future coronary diseases, in terms of lipid profile and CVD risks, could be considered by future studies in this field.

Children affected by FH had significantly lower levels of MUFA, vaccenic acid and n-6 PUFA γ -linolenic acid in RBCs, with respect to FCH or PCH patients. These data are consistent with earlier findings in adults demonstrating that subjects with hypercholesterolemia and advanced coronary heart disease exhibited a decreased PUFA level and an increased cholesterol and SFA concentrations in erythrocytes related to the atherosclerotic condition (Taylor 1994; Lausada et al. 2007; Vayá et al. 2009; Novgorodtseva et al. 2011). As far as the pediatric age is concerned, a significantly lower level of vaccenic acid in RBCs was also found in diabetic children, as compared to controls (Decsi et al. 2002).

Correlation analysis performed on the whole study sample (n= 54) showed a positive association between the n-6 PUFA levels in RBCs and serum HDL-C concentrations, which was similar to other observations in young or adults, showing a direct relationship between serum n-6 PUFAs and HDL-C (Ferrucci et al. 2006; Motoyama et al. 2009; Jelenkovic et al. 2014). Furthermore, we observed an inverse correlation between MUFA in RBCs and serum TC concentration and HDL/TG ratio. These results are in agreement with those from previous clinical studies showing that diet rich in MUFA have potential hypocholesterolemic effect (Yu et al. 1995; Gill et al. 2003;

338 Fernandez et al. 2005; Mukuddem-Peterson et al. 2005). In addition, we found a direct
339 correlation between MUFA in RBCs and serum TG. These results are in line with a
340 study conducted in two cohorts of young healthy twins, in which the contribution of
341 genetic and environmental factors at the bases of relationship between FAs and
342 lipoprotein profile have been performed. Results obtained demonstrated the importance
343 of common genetic factors in determining the phenotypic covariation of n-6 PUFAs and
344 MUFAs with TG and VLDL particles (Jelenkovic et al., 2014). The role of metabolic
345 features and genetic factors on lipid profile is widely studied (Xiang et al. 2007;
346 Matthan et al. 2014; Mayneris-Perxachs et al. 2014; Tosi et al. 2014). For example,
347 recent evidences indicate that FA desaturases play an important role in defining blood
348 and tissue lipid profiles. Polymorphisms in the FA desaturase genes FADS1 and
349 FADS2, that encode respectively for the delta-5 and delta-6 desaturases, have been
350 associated with PUFA levels in both serum phospholipids and RBC membranes
351 (Schaeffer et al. 2006; Malerba et al. 2008; Tosi et al. 2014). Since diet could have also
352 contributed, we tried to correlate dietary factors with lipid profile in both serum and
353 RBCs. A correlation between low intake of dietary cholesterol and high level of n-6
354 PUFA and n-6 LC-PUFA in RBCs was found. In addition, the fibre intake was directly
355 associated with the MUFA/SFA ratio. These associations have been obtained on a
356 subgroup of children/adolescents involved (23 out of the 54 subjects) and this can
357 represent a possible limitation of the study. However, these results provide additional
358 evidence supporting that a low-cholesterol and fibre-rich diet could contribute to a more
359 favourable RBC phospholipid FA composition in hyperlipidemic children.

360 Finally, although a limitation of the study is the absence of a normolipidemic control
361 group, our data provides for the first time information about the phospholipid
362 composition of RBCs in a relatively large population of Italian hyperlipidemic children

363 and adolescents.

364

365 **Conclusion**

366 In conclusion, although preliminary, we provided experimental evidence about
367 differences in the phospholipid composition of RBCs according to patient type of
368 hyperlipidemia and sex. The low omega-3 index, **evaluated on phospholipid RBCs**,
369 observed in these children confirms the importance of the quality of dietary FAs intake,
370 particularly in those with primary hyperlipidemia. Further studies should be performed
371 in order to clarify the RBC phospholipid composition differences between
372 hyperlipidemic and normolipidemic children, as well as in relation to sex and diagnosis.
373 Moreover, it would be interesting to study the contribution of specific dietary
374 interventions/regimen on these parameters in children affected by primary
375 hyperlipidemia.

376

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379

380 **Disclosure statement**

381 The authors declare no conflicts of interest regarding this research.

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Table 1. Subject characteristics (n= 54)

Parameter	Value
Age, years (range)	11 ± 3 (6-17)
Sex, M/F	28/26
Weight, kg	47.9 ± 16.4
Height, cm	149.1 ± 16.1
BMI, kg/m ²	20.9 ± 4.0
Systolic blood pressure, mmHg	104.5 ± 10.1
Diastolic blood pressure, mmHg	66.6 ± 7.4

Notes: BMI, body mass index. Values are reported as mean ± SD.

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Table 2. Serum lipid concentrations and **RBC phospholipid FAs composition in** hyperlipidemic children and adolescents (n= 54), according to sex and type of hyperlipidemia

Lipid profile	All subjects	Sex			Dyslipidemia			
	n= 54	Male n= 28	Female n= 26	p-value	FH n=14	FCHL n= 21	PHC n= 19	p-value
<i>Serum lipids (mg/dl)</i>								
TC	232.4 ± 55.6	226.0 ± 56.7	239.2 ± 54.8	0.385	288.2 ± 67.1 ^a	214.2 ± 38.3 ^b	211.4 ± 30.8 ^b	< 0.001
TG	90.9 ± 51.9	94.0 ± 53.4	84.9 ± 50.3	0.658	73.6 ± 31.3	102.3 ± 67.6	91.1 ± 42.1	0.282
HDL-C	58.8 ± 15.4	58.6 ± 15.6	60.2 ± 14.6	0.912	55.0 ± 10.6	57.8 ± 19.6	62.7 ± 12.7	0.340
LDL-C	156.3 ± 58.4	149.1 ± 60.9	163.3 ± 56.8	0.355	219.9 ± 69.5 ^a	137.3 ± 36.5 ^b	130.3 ± 28.0 ^b	< 0.001
HDL/LDL ratio	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.502	0.3 ± 0.1 ^a	0.5 ± 0.2 ^b	0.5 ± 0.2 ^b	< 0.001
HDL/TG ratio	0.9 ± 0.5	0.8 ± 0.5	0.9 ± 0.5	0.537	0.9 ± 0.5	0.8 ± 0.5	0.8 ± 0.4	0.812
Non-HDL-C	173.6 ± 59.4	167.4 ± 61.7	179.0 ± 58.1	0.433	233.2 ± 71.0 ^a	156.5 ± 42.5 ^b	148.6 ± 31.4 ^b	< 0.001
<i>RBC phospholipid FAs (%)</i>								
Total SFAs	48.99 ± 2.23	48.66 ± 2.51	49.44 ± 1.84	0.261	49.23 ± 2.87	49.35 ± 1.53	48.43 ± 2.35	0.389
Total MUFAs	18.45 ± 0.91	18.68 ± 0.84	18.12 ± 0.87	0.052	18.07 ± 0.77	18.59 ± 0.86	18.58 ± 1.02	0.197
Total PUFAs	32.55 ± 2.14	32.65 ± 2.55	32.44 ± 1.67	0.729	32.70 ± 2.81	32.06 ± 1.61	32.99 ± 2.10	0.383
MUFAs/SFAs ratio	0.38 ± 0.03	0.39 ± 0.03	0.37 ± 0.03	0.056	0.37 ± 0.03	0.38 ± 0.02	0.39 ± 0.03	0.296
PUFAs/SFAs ratio	0.67 ± 0.08	0.68 ± 0.09	0.66 ± 0.06	0.452	0.67 ± 0.11	0.65 ± 0.05	0.68 ± 0.08	0.391
PUFAs n-3/n-6	0.19 ± 0.06	0.18 ± 0.03	0.20 ± 0.05	0.758	0.19 ± 0.05	0.20 ± 0.07	0.19 ± 0.04	0.871
LC-PUFAs n-3/n-6	0.33 ± 0.09	0.31 ± 0.05	0.34 ± 0.09	0.633	0.33 ± 0.09	0.34 ± 0.11	0.32 ± 0.08	0.822
Omega-3 index	3.76 ± 1.04	3.54 ± 0.55	3.80 ± 1.08	0.770	3.81 ± 0.87	3.84 ± 1.24	3.62 ± 0.95	0.789
<i>Saturated</i>								
14:0 (myristic acid)	0.41 ± 0.08	0.40 ± 0.09	0.42 ± 0.08	0.336	0.42 ± 0.10	0.4 ± 0.07	0.4 ± 0.08	0.789
15:0 (pentadecanoic acid)	0.16 ± 0.03	0.16 ± 0.03	0.17 ± 0.03	0.152	0.16 ± 0.03	0.16 ± 0.02	0.16 ± 0.03	0.963

16:0 (palmitic acid)	24.05 ± 1.38	24.02 ± 1.52	24.08 ± 1.23	0.864	24.4 ± 1.56	24.06 ± 1.10	23.78 ± 1.52	0.625
17:0 (margaric acid)	0.46 ± 0.17	0.46 ± 0.16	0.45 ± 0.19	0.803	0.42 ± 0.22	0.44 ± 0.19	0.51 ± 0.11	0.387
18:0 (stearic acid)	15.47 ± 1.47	15.06 ± 1.40 ^a	15.92 ± 1.44 ^b	0.031	15.61 ± 2.10	15.68 ± 1.16	15.15 ± 1.24	0.593
20:0 (arachidic acid)	0.56 ± 0.15	0.58 ± 0.20	0.55 ± 0.05	0.579	0.54 ± 0.07	0.55 ± 0.05	0.6 ± 0.23	0.610
22:0 (behenic acid)	1.99 ± 0.28	1.98 ± 1.29	1.99 ± 1.27	0.988	1.94 ± 0.32	2.02 ± 0.24	1.98 ± 0.29	0.607
23:0 (tricosanoic acid)	0.31 ± 0.06	0.31 ± 0.05	0.31 ± 0.06	0.935	0.30 ± 0.07	0.32 ± 0.05	0.31 ± 0.05	0.576
24:0 (lignoceric acid)	5.59 ± 0.75	5.7 ± 0.84	5.46 ± 0.63	0.242	5.45 ± 0.83	5.73 ± 0.63	5.54 ± 0.81	0.583
<i>Monounsaturated</i>								
16:1n-9 (hypogeic acid)	0.11 ± 0.03	0.11 ± 0.01	0.11 ± 0.04	0.524	0.11 ± 0.04	0.11 ± 0.03	0.11 ± 0.01	0.934
16:1n-7 (palmitoleic acid)	0.24 ± 0.07	0.24 ± 0.07	0.23 ± 0.07	0.561	0.23 ± 0.08	0.23 ± 0.08	0.25 ± 0.05	0.795
18:1n-9 (oleic acid)	11.09 ± 0.94	11.16 ± 0.89	11.02 ± 1.01	0.569	11.19 ± 1.16	10.95 ± 0.91	11.18 ± 0.83	0.719
18:1n-7 (vaccenic acid)	1.08 ± 0.11	1.07 ± 0.10	1.09 ± 0.12	0.392	1.02 ± 0.07 ^a	1.08 ± 0.11 ^{ab}	1.12 ± 0.10 ^b	0.036
20:1n-9 (eicosenoic acid)	0.20 ± 0.08	0.21 ± 0.10	0.19 ± 0.02	0.474	0.18 ± 0.02	0.19 ± 0.02	0.22 ± 0.12	0.361
24:1n-9 (nervonic acid)	5.73 ± 0.92	5.9 ± 1.14	5.56 ± 0.56	0.179	5.34 ± 1.11	6.02 ± 0.60	5.70 ± 0.99	0.240
<i>n-6 Polyunsaturated</i>								
18:2n-6 (linoleic acid)	10.70 ± 1.08	10.72 ± 1.06	10.68 ± 1.13	0.916	10.49 ± 0.77	10.78 ± 1.13	10.78 ± 1.24	0.795
18:3n-6 (-linolenic acid)	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.193	0.06 ± 0.02 ^a	0.06 ± 0.02 ^a	0.07 ± 0.02 ^b	0.027
20:2n-6 (eicosadienoic acid)	0.23 ± 0.07	0.24 ± 0.09	0.22 ± 0.05	0.222	0.20 ± 0.06	0.23 ± 0.03	0.26 ± 0.10	0.138
20:3n-6 (dihomo-γ-linolenic acid)	1.91 ± 0.38	2.01 ± 0.46 ^a	1.81 ± 0.24 ^b	0.050	1.75 ± 0.26	1.95 ± 0.37	1.99 ± 0.44	0.137
20:4n-6 (arachidonic acid)	11.05 ± 1.14	10.89 ± 1.29	11.23 ± 0.95	0.270	11.43 ± 1.23	10.84 ± 0.86	11.01 ± 1.33	0.446
22:4n-6 (adrenic acid)	1.93 ± 0.49	1.92 ± 0.53	1.94 ± 0.44	0.867	1.9 ± 0.53	1.76 ± 0.27	2.14 ± 0.58	0.114
Total PUFA n-6	25.89 ± 1.58	26.15 ± 1.37	25.85 ± 1.50	0.815	25.83 ± 1.69	25.62 ± 1.58	26.25 ± 1.51	0.457
Total LC-PUFAs n-6	14.90 ± 1.46	15.07 ± 1.19	14.82 ± 1.64	0.681	15.08 ± 1.65	14.56 ± 1.16	15.14 ± 1.59	0.392
<i>n-3 Polyunsaturated</i>								
18:3n-3 (α-linolenic acid)	0.09 ± 0.04	0.09 ± 0.02	0.09 ± 0.02	0.827	0.09 ± 0.04	0.09 ± 0.04	0.10 ± 0.03	0.291
20:5n-3 (eicosapentaenoic acid)	0.36 ± 0.21	0.38 ± 0.21	0.33 ± 0.22	0.402	0.37 ± 0.29	0.35 ± 0.22	0.36 ± 0.12	0.988

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22:5n-3 (docosapentaenoic acid)	1.10 ± 0.20	1.13 ± 0.21	1.07 ± 0.17	0.207	1.08 ± 0.20	1.04 ± 0.19	1.18 ± 0.19	0.130
22:6n-3 (docosahexaenoic acid)	3.40 ± 0.89	3.41 ± 0.96	3.38 ± 0.82	0.888	3.43 ± 0.67	3.49 ± 1.05	3.26 ± 0.86	0.781
Total PUFAs n-3	4.95 ± 1.17	4.68 ± 0.63	5.02 ± 1.21	0.632	4.98 ± 0.99	4.97 ± 1.39	4.90 ± 1.06	0.976
Total LC-PUFAs n-3	4.86 ± 1.17	4.59 ± 0.63	4.93 ± 1.21	0.637	4.89 ± 0.99	4.88 ± 1.39	4.80 ± 1.06	0.970

Notes: FH, familiar hypercholesterolemia; FCHL, familiar combined hyperlipidemia; PHC, polygenic hypercholesterolemia; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; LC-PUFAs, long chain polyunsaturated fatty acids (C ≥20, double bonds ≥ 3); omega-3 index, sum of EPA + DHA. Values are reported as mean ± SD. ^{a,b} Data with different letters are significantly different (*p*<0.05).

Table 3. Correlation between **RBC phospholipid FAs** and serum lipid concentrations (n= 54)

<i>RBC phospholipid fatty acids</i>	Serum Lipids					
	TC	HDL-C	LDL-C	TG	HDL/LDL	HDL/TG
Total SFAs	0.095	-0.001	0.095	-0.077	-0.051	0.022
Total MUFAs	-0.290*	-0.138	-0.274	0.371*	0.064	-0.308*
Total PUFAs	0.020	0.072	0.021	-0.137	0.030	0.161
MUFAs/SFAs ratio	-0.208	-0.057	-0.210	0.262	0.073	-0.180
PUFAs/SFAs ratio	-0.025	0.029	-0.015	-0.058	0.021	0.084
Total PUFAs n-3	0.104	-0.155	0.151	-0.068	-0.200	0.011
Total PUFAs n-6	0.023	0.275*	-0.028	-0.155	0.198	0.250
PUFAs n-3/n-6	0.085	-0.220	0.151	-0.039	-0.231	-0.043
Total LC-PUFAs n-3	0.105	-0.168	0.159	-0.064	-0.213	0.000
Total LC-PUFAs n-6	0.174	0.207	0.120	-0.197	0.063	0.243
LC-PUFAs n-3/n-6	-0.020	-0.229	0.059	0.006	-0.181	-0.080
Omega-3 index	0.085	-0.203	0.160	-0.080	-0.233	-0.006

Notes: TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SFAs, saturated fatty acids; MUFAs monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; LC-PUFAs, long chain polyunsaturated fatty acids; omega-3 index, sum of EPA + DHA. *Statistical significance after Kendall and Spearman correlations for multiple comparisons ($p < 0.05$).

Table 4. Correlation between dietary factors, RBC phospholipid FAs and serum lipids (n= 23)

	Dietary factors										
	Energy	Protein	Carbohydrate	Total Fat	SFAs	MUFAs	PUFAs	PUFAs n-3	PUFAs n-6	Cholesterol	Fiber
<i>Serum lipids</i>											
TC	-0.265	-0.202	0.302	-0.204	-0.196	-0.221	-0.376	0.011	-0.083	-0.386	-0.210
HDL-C	-0.381	-0.126	0.163	-0.149	0.118	-0.102	-0.034	-0.087	-0.182	-0.254	-0.203
TG	-0.013	-0.101	-0.210	0.249	0.402	0.123	0.181	-0.007	0.099	0.180	0.104
LDL-C	-0.219	-0.243	0.187	-0.079	-0.147	-0.118	-0.339	0.204	-0.027	-0.280	-0.214
HDL/LDL ratio	-0.252	0.012	-0.012	-0.049	0.167	0.017	0.147	-0.081	-0.113	-0.061	-0.074
HDL/TG ratio	-0.289	-0.054	0.282	-0.289	-0.194	-0.147	-0.154	-0.010	-0.145	-0.277	-0.196
<i>RBC phospholipid FAs</i>											
Total SFAs	-0.145	-0.108	0.150	-0.096	-0.297	-0.105	-0.010	-0.350	0.083	0.265	-0.235
Total MUFAs	0.103	-0.137	-0.142	0.123	0.547	0.292	0.409	0.265	0.282	0.360	0.493
Total PUFAs	-0.059	0.174	-0.044	0.049	0.081	-0.047	-0.203	0.277	-0.216	-0.485	-0.012
MUFAs/SFAs ratio	0.193	-0.056	-0.150	0.123	0.504	0.271	0.324	0.340	0.212	0.088	0.525*
PUFAs/SFAs ratio	0.072	0.146	-0.093	0.069	0.164	0.027	-0.108	0.321	-0.128	-0.407	0.131
Total PUFAs n-3	0.360	0.434	-0.397	0.181	-0.039	0.027	0.017	0.105	0.039	0.137	0.135
Total PUFAs n-6	-0.164	-0.105	0.115	0.029	-0.076	-0.054	-0.282	0.201	-0.243	-0.762*	-0.245
PUFAs n-3/n-6	0.363	0.429	-0.409	0.167	-0.059	0.010	0.029	0.078	0.049	0.260	0.150
Total LC-PUFAs n-3	0.360	0.434	-0.397	0.181	-0.039	0.027	0.017	0.105	0.039	0.137	0.135
Total LC-PUFAs n-6	0.002	0.071	-0.201	0.118	0.108	0.051	-0.108	0.225	-0.157	-0.522*	-0.142
LC-PUFAs n-3/n-6	0.368	0.463	-0.390	0.135	-0.083	-0.034	0.007	0.020	0.034	0.390	0.176
Omega-3 index	0.358	0.441	-0.350	0.127	-0.076	-0.032	-0.010	0.081	0.029	0.265	0.154

Notes: TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SFAs, saturated fatty acids; MUFAs monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; LC-PUFAs, long chain polyunsaturated fatty acids; omega-3 index, sum of EPA + DHA. *Statistical significance after Kendall and Spearman correlations for multiple comparisons ($p < 0.05$).