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Role of bioactive-rich diet in the modulation of risk factors for chronic-degenerative diseases: *in vivo* and *ex vivo* approaches

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ABSTRACT 6

RIASSUNTO 8

1 INTRODUCTION 10

1.1 CHRONIC DEGENERATIVE DISEASES: THE DEMAND FOR PREVENTIVE STRATEGIES 10

1.2 CARDIOVASCULAR DISEASES AND ROLE OF DIETARY FAT 12

1.2.1 Nuts consumption and cardiovascular diseases risk factors 14

1.3 HYPERLIPIDEMIA AND DIETARY GUIDELINES SINCE PEDIATRIC AGE 17

1.4 IMMUNE FUNCTION AND THE ROLE OF NUTRITION 22

1.4.1 Immunomodulatory effect of LC-PUFA 24

1.5 REFERENCES 26

2 AIM OF THE STUDY 33

PART I: IN VIVO STUDIES 34

3 DEVOLOPMENT OF DIETARY INTERVENTION STUDIES IN CHILDREN AND ADOLESCENT WITH PRIMARY HYPERLIPIDEMIA 35

3.1 CHARACTERISATION OF SERUM LIPID PROFILE AND FATTY ACID COMPOSITION OF ERYTHROCYTE PHOSPHOLIPIDS 36

3.1.1 Subjects and Methods 36

Subject enrollment and study design 36

Physical evaluation 37

Blood samples collection and separation 37

Analysis of serum lipid profile 37

Analysis of FA composition of RBC phospholipids 37

Statistical analysis 38

3.1.2 Results 39

Subjects characteristics 39

Serum lipid profile and RBC phospholipid composition 39

Correlation between RBC phospholipid composition and serum lipid profile 42

Food diary analysis and correlation between dietary factors, RBC phospholipid FAs and serum lipids 42

3.2 EFFECT OF REGULAR HAZELNUT INTAKE ON MARKERS OF OXIDATIVE STRESS, SERUM LIPID PROFILE AND FATTY ACID COMPOSITION OF ERYTHROCYTE PHOSPHOLIPIDS 44

3.2.1 Subjects and Methods 44

Study design 44

Hazelnut preparation 44

Fat and bioactive composition of hazelnuts 46

Chemicals 46

Blood sample collection, separation and storage 46

Evaluation of DNA damage through comet assay 47

Analysis of oxidized-LDL 48

Analysis of serum lipids and FA composition of phospholipids in RBCs 48

Statistical analysis 48

3.2.2 Results 49

Characterization of hazelnuts 49

Subjects characteristics 50
Effect of hazelnuts on endogenous and oxidatively-induced DNA damage 51
Effect of hazelnuts on oxidized LDL levels 53
Effect of hazelnuts on serum lipid profile 54
Effect of hazelnuts on fatty acid composition of phospholipids in RBC membranes 54
Correlation between serum lipid profile and markers of oxidative stress 57
3.3 EFFECT OF HEMPSEED OIL SUPPLEMENTATION ON SERUM LIPIDS PROFILE AND FATTY ACID COMPOSITION OF ERYTHROCYTE PHOSPHOLIPIDS 58
3.3.1 Subjects and Methods 59
Study design 59
Statistical analysis 60
3.3.2 Results 61
Subject characteristics 61
Effect of hempseed oil supplementation on serum lipid profile and RBC phospholipid composition 62
3.4 DISCUSSION 64
3.5 REFERENCES 70
PART II: EX VIVO STUDY 76
4 IMMUNOMODULATORY EFFECTS OF LC-PUFA AND VITAMIN D3 ON EX VIVO INNATE IMMUNE CELLS 77
4.1 MATERIAL AND METHODS 78
Chemicals 78
Collection and preparation of blood samples 78
Evaluation of lytic activity of NK cells 78
Phagocytic activity and intensity 79
Statistical analysis 79
4.2 RESULTS 80
Effect of LC-PUFAs with vitamin D on NK cell activity against K562 target cells 80
Effect of LC-PUFAs with vitamin D on phagocytic activity of monocytes and granulocytes 82
4.3 DISCUSSION 83
4.4 REFERENCES 85
5 CONCLUSIONS 87
6 PRODUCTS 88
6.1 COPIES OF PAPERS PUBLISHED 88
6.2 TITLES OF PAPERS SUBMITTED 91
6.3 COPIES OF ABSTRACT OF ORAL COMMUNICATIONS AND POSTERS 92
6.4 AWARD 107
7 ACKNOWLEDGEMENTS 108

ABSTRACT

The rapid increase in prevalence of chronic-degenerative diseases is probably the major global health problem nowadays. Several studies have emphasized the role that dietary patterns rich in specific foods or their bioactive compounds may play in the reduction of disease risk. In fact, nutritional intervention is considered one of the most significant, easily achieved and affordable primary prevention strategy. Recently, increasing interest has been focused on personalized dietary interventions accurately designed to meet specific nutritional needs of target groups of population. The personalized approach seems to be more effective to achieve a defined outcome, especially in targeted subgroups of population considered “at higher risk” to develop chronic diseases. Based on these premises, the aim of the present Ph.D. thesis was to evaluate the effect of specific foods and their bioactives in the modulation of risk factors for chronic-degenerative diseases in “at risk” groups of population, thorough both *in vivo* and *ex vivo* approaches.

The first part (*in vivo* approach) was focused on the impact of two different dietary interventions in a population of Italian children and adolescents affected by primary hyperlipidemia and thus considered at “higher risk” to develop cardiovascular (CV) events later in life. Firstly, the study population was characterized for serum lipid profile and fatty acid (FA) composition of red blood cell (RBC) phospholipids, showing differences according to sex and diagnosis of hyperlipidemia. Moreover, in the hyperlipidemic pediatric patients we observed an overall low omega 3 index (eicosapentaenoic acid, EPA + docosahexaenoic acid, DHA: <4%), an emerging risk factor for cardiovascular diseases (CVD). Secondly, in the same population of hyperlipidemic pediatric patients, it was investigated the effect of regular intake of a specific food (hazelnuts, HZN) or food supplement (hempseed oil, HSO) rich in unsaturated fats and other bioactives, in the modulation of different CVD biomarkers related to hyperlipidemia. We documented that both dietary treatments were effective in the management of primary hyperlipidemia at pediatric age. In particular, we showed that 8-week of hazelnuts intake, consumed with skin (HZN+S) or without skin (peeled, HZN-S), significantly improve the serum lipid profile and had a favorable impact on FAs composition of RBC membranes. In addition, HZN treatments were able to reduce the levels of DNA damage, a marker of oxidative stress, even if the concentrations of plasma oxidized LDL (ox-LDL) did not change following the HZN intervention. The effect of HSO supplementation on lipid profile of hyperlipidemic children was evaluated through a pilot study. Even if preliminary, the results from this study showed an enhancement of the omega-3 index and the RBC phospholipid composition following the intake of HSO for 8 weeks.

The second part of the Ph.D thesis was performed at the Department of Physiology and Biochemistry of Nutrition of Max Rubner-Institut (Karlsruhe, Germany) and devoted to *ex vivo* approaches. Considering that chronic diseases are often associated to a compromised immune response, the objective of this part of research was to investigate the potential immunomodulatory properties of different concentrations of long-chain polyunsaturated fatty acids (LC-PUFA) in conjunction with standard dose of vitamin D3 on *ex vivo* immune cells from healthy volunteers. The effect of these bioactives was evaluated on two mechanisms crucially involved in the innate immune response that specifically act against cancer cells as well as infectious agents: the natural killer (NK) cell activity and the phagocytosis. The findings showed that high dose of LC-PUFA, independently by the presence of the vitamin D, negatively affect the lytic activity of NK cell of target cancer cells, with a dose-dependent response. On the contrary, an enhancement of phagocytic activity of monocytes was observed only when LC-PUFA were combined with vitamin D, suggesting a potential synergic and immunomodulatory role of vitamin D.

In conclusion, through this Ph.D thesis it was possible to increase knowledge on the potential benefits of bioactives rich-diet in the modulation of different risk factors for chronic-degenerative diseases. This aspect is particulary important in at risk target groups of population for whom appropriate and personalized nutritional interventions are considered the primary prevention approach. Future studies on largest population groups aimed to clarify the specific mechanisms of action involved are needed to provide additional demonstration that confirm our results.

RIASSUNTO

Il rapido aumento dell'incidenza delle malattie cronico-degenerative è probabilmente uno dei maggiori problemi di sanità pubblica attuali. Sono numerosi gli studi che supportano il ruolo che determinati modelli alimentari ricchi in specifici alimenti o composti bioattivi hanno nella riduzione del rischio di sviluppare malattie. L'intervento di tipo nutrizionale è sempre più considerato una strategia di prevenzione primaria che risulta tra le più efficaci, convenienti e facilmente perseguibili. Recentemente, un interesse crescente si sta sempre più focalizzando su interventi dietetici personalizzati accuratamente studiati per soddisfare le specifiche esigenze nutrizionali di gruppi di popolazione target. Infatti, un approccio nutrizionale personalizzato sembra essere molto più efficace nell'ottenimento di uno specifico risultato, soprattutto in quei gruppi di popolazione target considerati più a rischio di sviluppare malattie croniche.
Sulla base di queste premesse, lo scopo della presente tesi di dottorato è stato quello di valutare, attraverso approcci *in vivo* ed *ex vivo,* l'effetto del consumo di specifici alimenti e dei loro composti bioattivi nella modulazione di alcuni fattori di rischio per le malattie cronico-degenerative, in gruppi di popolazione "a rischio".

La prima parte della tesi (approccio *in vivo*) ha avuto l'obiettivo di valutare l'impatto di due diversi interventi dietetici in una popolazione di bambini ed adolescenti italiani affetti da iperlipidemia primaria, e quindi considerati a maggior rischio di possibili eventi cardiovascolari futuri. In primo luogo, la popolazione pediatrica in studio è stata caratterizzata per il profilo lipidico sierico e per la composizione degli acidi grassi dei fosfolipidi delle membrane eritrocitarie, mostrando delle differenze in base al sesso e al tipo specifico di iperlipidemia. Inoltre, in questi soggetti è stata osservato un basso indice omega 3 (acido eicosapentaenoico, EPA + acido docosaesaenoico, DHA: <4%), un marker che è stato suggerito essere associato a un elevato rischio di sviluppare malattie cardiovascolari. In secondo luogo, nella stessa popolazione di pazienti pediatrici iperlipidemici è stato studiato l'effetto del consumo regolare di uno specifico alimento (nocciole, HZN) o un supplemento alimentare (olio di semi di canapa, HSO), entrambi ricchi in acidi grassi insaturi e altri bioattivi, nella modulazione di diversi biomarker di rischio cardiovascolare collegati all'iperlipidemia. Entrambi gli interventi dietetici sono risultati efficaci nella gestione dell'iperlipidemia pediatrica. In particolare, abbiamo dimostrato che l'assunzione per 8 settimane di nocciole, consumate con la cuticola (HZN+S) oppure pelate (HZN-S), migliorava significativamente il profilo lipidico sierico e aveva un impatto favorevole anche sulla composizione degli acidi grassi delle membrane eritrocitarie. Inoltre, i trattamenti dietetici con le nocciole sono stati in grado di ridurre i livelli di danno al DNA, un marker di stress ossidativo. Tuttavia, le concentazioni plasmatiche di LDL ossidate (ox-LDL) non hanno subito alcun cambiamento in seguito all'intervento con le nocciole. L'effetto della supplementazione con olio di semi di canapa nella modulazione del profilo lipidico in bambini iperlipidemici è stato valutato attraverso uno studio pilota. Anche se preliminari, i risultati di questo studio hanno mostrato un miglioramento dell'indice omega 3 e della composizione degli acidi grassi delle membrane eritrocitarie in seguito all'assunzione dell'olio di semi di canapa per 8 settimane.

La seconda parte della tesi di dottorato è stata svolta su modelli *ex vivo* presso il Dipartimento di Fisiologia e Biochimica della Nutrizione del Max Rubner-Institut (Karlsruhe, Germania). Considerando che le malattie cronico-degenerative sono spesso associate ad una risposta immunitaria compromessa, l'obiettivo di questa parte di ricerca è stato quello di studiare il potenziale effetto immunomodulatorio di diverse concentrazioni di acidi grassi polinsaturi a lunga catena (LC-PUFA), in combinazione con dosi standard di vitamina D3, in cellule immunitarie *ex vivo* prelevate da volontari sani. L'effetto di questi composti bioattivi è stato valutato su due

meccanismi implicati nella risposta immunitaria innata, che agiscono specificatamente contro le cellule tumorali e gli agenti patogeni: l'attivita delle cellule natural killer (NK) e la fagocitosi. I risultati ottenuti hanno mostrato che gli LC-PUFA ad elevate dosi, indipendentemente dalla presenza o meno della vitamina D, interferiscono negativamente sulla capacità delle cellule NK di lisare e contrastare le cellule tumorali target, mostrando una risposta dose-dipendente. Al contrario, è stato osservato un miglioramento dell'attività fagocitaria dei monociti quando gli LC-PUFA erano coincubati con la vitamina D, suggerendo un potenziale effetto sinergico e immunomodulatorio della vitamina D.

In conclusione, attraverso questa tesi di dottorato è stato possibile aumentare le conoscenze sul potenziale effetto di diete ricche in composti bioattivi nella modulazione di alcuni fattori di rischio per le malattie cronico-degenerative. Questo aspetto risulta particolarmente importante soprattutto nelle popolazioni target considerate più a rischio e per le quali un intervento nutrizionale appropriato e personalizzato viene considerato l'approccio di prevenzione primario. Studi futuri su gruppi di popolazione più ampi e volti a valutare i meccanismi d'azione specifici sono necessari al fine di fornire delle dimostrazioni aggiuntive che confermino i nostri risultati.

1 INTRODUCTION

1.1 CHRONIC DEGENERATIVE DISEASES: THE DEMAND FOR PREVENTIVE STRATEGIES

Chronic degenerative diseases are onset of noncontagious and long duration illness resulted from a variety of risk factors, mainly related to lifestyle and environmental conditions (World Health Organization 2005). In the last years, the global rising of all chronic diseases, including cardiovascular diseases (CVD), chronic respiratory diseases, autoimmune and inflammatory diseases, diabetes and cancers, make current strategies for their management unsustainable. According to the World Health Organization (WHO), in 2012 approximately 68% of all deaths worldwide were ascribed to chronic diseases, among which the 46.2% were principally caused by CVD (WHO 2014). In spite of its public health importance, the growing incidence and prevalence of chronic diseases generally result in significant losses to society, in terms of increase in human suffering and loss of living quality. In addition, the cumulative effects of diseases create a strong impact on the health-care system, becoming an important public issue with several indirect economic impacts (Yach et al. 2004). There is clear evidence that preventive interventions can reduce the burden of morbidity, disability and premature mortality. Moreover, effective actions need strong and long-term public strategies that have a significant public health impact and, at the same time, are affordable and feasible to implement.

In this regard, nutritional intervention is considered one of the most important and easily achieved primary prevention strategy to reduce risk factors for chronic degenerative diseases. Despite dietary patterns vary across the world, according to traditionally based and locally available food, there are some shared characteristics underlying healthy dietary habits. In particular, scientific evidence support that disease burden could be considerably reduced among the overall population through preventive measures directed to improve dietary choices, including: the increase of fruits and vegetables consumption up to the recommended 5 servings a day, the selection of whole grains, the replacement of saturated and trans fatty acids with unsaturated fatty acids, the reduction of sugar and sweetened beverages consumption, and the limitation of salt intake (FAO/WHO 2003; Saeed et al. 2014; Sofi & Dinu 2016). Most of these interventions have been targeted to the populations using 'one size fits all' public health recommendations (e.g. "eat at least 5 portions of fruits and vegetables daily"). However, in most cases the effect size achieved in such interventions was usually relatively modest, especially in the longer term (Celis-Morales et al. 2015). Moreover, the global burden of premature death and chronic ill-health continues to rise, emphasizing the need for more effective strategies.

The above population-wide preventive strategies may be also combined with targeted approaches in order to improve health gains. In fact, while it is necessary to achieve most of the population with strong general nutritional guidelines that have considerable potential to enhance public health in the complex, it is important to avoid inappropriately applying nutritional guidelines to population subgroups that may differ (i.e. genetically). It is noteworthy that a personalized nutritional intervention tailored to meet specific nutritional needs for a target population (e.g. people with similar risk factors/diseases or same age range) can be more effective to achieve a positive outcome. Moreover, in some cases the efficacy of appropriate nutritional intervention depends on when they begin (i.e. the life stage). For example, in subgroups of population "at higher risk" to develop CVD, such as children with primary hyperlipidemia, the treatment of risk factors should start as early as possible in childhood to prevent premature events later in life (Catapano et al. 2011; Weintraub et al. 2011). It is clear that effective intervention need strong and long-term public strategies that are able to early detect

chronic diseases, improve health care and prevent the risk factor exposure starting from early life and continuing with interventions for adults and the elderly. Furthermore, since most evidence on the relationship between diet/nutrition and chronic diseases is based on observational studies, there is specific demand for research aimed to identify the effect of personalized nutritional interventions designed to reduce specific risk factors and ameliorate well-being in target population groups.

1.2 CARDIOVASCULAR DISEASES AND ROLE OF DIETARY FAT

In recent years, several studies have emphasized the role that several foods or dietary bioactive compounds play in the development and treatment of CVD. The CVDs are disorders of the heart or blood vessels supplying the heart (coronary heart disease, CHD), brain (cerebrovascular disease), arms or legs (peripheral arterial disease). They could be caused by blood clots (deep vein thrombosis and pulmonary embolism), or are due to heart muscle and valves damage from rheumatic fever caused by bacteria infection (rheumatic heart disease), or are present at birth (congenital heart disease). The main pathological process that leads to blood vessels damage is the atherosclerosis, an inflammatory process affecting medium-and large-sized blood vessels (Mendis et al. 2011). Major risk factors for CVDs and atherosclerosis, such as hyperlipidemia, hypertension, obesity and diabetes, could be potentially preventable and controlled by adopting healthy diet. Modulation of dietary fat intake is considered the cornerstone in the dietary management of CVD. The first evidence of the correlation between CVD and dietary fat intake came from the Seven Countries Study. This study showed that death rates from CHD during 10 and 15 years of follow-up across 16 cohorts were positively associated with dietary intake of saturated fatty acids (SFA) and inversely associated with dietary intake of monounsaturated fatty acids (MUFA), mainly oleic acid. Furthermore, the variation in CHD death rates observed among different geographical regions was explained by differences in the amount of SFA and MUFA consumed. A strong associations between the replacement of dietary SFA with unsaturated fatty acids and lowered serum total cholesterol (TC) levels was also found (Keys 1965; Keys et al. 1986).

The early results of the Seven Countries Study and the consequent explosion of epidemiological and clinical trials, lead to the international dietary guidelines for CV prevention to specify the amount and type of dietary fatty acids to be consumed. Even if a lack of consensus exists regarding the effect of dietary fatty acid on CVD risk, the focus was on the importance of SFAs intake reduction due to their negative effect on low-density lipoprotein cholesterol (LDL-C) levels. According to the most recent American Heart Association guidelines, the intake of dietary SFA should be limited to 5-6% of total calories to lower LDL-C levels (Eckel et al. 2013). The long-held dogma that saturated fats *per se* cause CVD has lately been challenged by research findings. In fact, recent studies examining the association of dietary SFA with CVD morbidity and/or mortality have revealed inconclusive results, reporting either direct, inverse, or even no association (Hammad et al. 2016; Praagman et al. 2016; Siri-Tarino & Krauss 2016). The relationship between SFAs and CVD is complex, with numerous factors influencing their relative contributions to CVD risk. A critical aspect is what macronutrients should be suggested to replace SFA. Systematic review and meta-analysis of randomized controlled trials replacing SFA with polyunsaturated fatty acids (PUFA) found that the incidence of CVD events decreased by 19%, with 10% reduction in CVD risk with each 5% (of energy) replacement of SFA (Mozaffarian et al. 2010). Moreover, the substitution of SFAs with PUFAs has been associated with significant decreased in TC and LDL-C levels, and a decrease in the TC:HDL-C ratio (Mensink et al. 2003; Siri-Tarino & Krauss 2016). Similarly, when MUFA were used as replacements for SFA, reductions in plasma TC, LDL-C, and high-density lipoprotein cholesterol (HDL-C) levels were observed, even if the association of this substitution with CVD risk seems less clear (Siri-Tarino et al. 2010). In contrast, SFAs replacement with refined carbohydrates and added sugars has been associated with higher levels of triglyceride (TG) and small LDL particles and no effect on CVD (Siri-Tarino et al. 2010; Siri-Tarino & Krauss 2016). Considering these observations, currently the European Society of Cardiology Guidelines on CVD prevention suggest to limit SFA intake to a maximum of 10% of energy by replacing it with PUFAs (Perk et al. 2012). The cardioprotective effect of PUFAs are generally attributed to a reduction in serum TC and LDL-C, which slows the progress of atherosclerosis and delays or prevents the onset of CVD and

cerebrovascular disease (Abdelhamid et al. 2016). The main sources of PUFA are plant and fish oils, with fish being rich in n-3 PUFA and plant oils rich in n-6 PUFA. A large number of studies highlighted the potential importance of restricting n-6 PUFA up to 10% of energy and trying to set the n-6/n-3 PUFA ratio as lower as possible, along with a particular emphasis on consuming adequate amounts of essential fatty acids (Hammad et al. 2016).

Another important aspect when considering the most recent nutritional recommendations for CVD prevention regards to the shift of attention from the macronutrient composition of the diet to the intake of healthy foods or dietary patterns. Among the numerous dietary sources of unsaturated fats that could replace the energy deriving from SFAs, particular interest has been placed on vegetable oils, main sources of MUFA (e.g., extra virgin olive oil) and of good amount of PUFA. Since epidemiological evidence suggests a cardioprotective role of the plant sources of n-3 PUFA α-linolenic acid (ALA), products such as flaxseed, hempseed oil or canola oil have been considered as interesting healthy alternatives. Increased consumption of vegetable oils rich in MUFA and PUFA may improve the fatty acid imbalance typical of modern Western diets, high in SFA, and in the n-6/n-3 fatty acid ratio. The Mediterranean diet is an excellent example of the association between the adherence to a dietary pattern characterized by high unsaturated fat intake and the low prevalence of CVD. In particular, this dietary pattern is characterized by high intake of MUFA (16–29% MUFA out of 33-40% of energy from total fat), and low SFA (<8% of total fats), with extravirgin olive oil being the predominant source of fat (Hammad et al. 2016). The cardioprotective effect of the Mediterranean diet have been ascribed to its richness in food bioactives, such as the oleic acid and ALA, but also to antioxidants and polyphenols, that have been found to exert specific actions on the CV system, particularly on blood pressure, coagulation activity and endothelial function (Torres et al. 2015).

The recent clinical trials of the “PREvención con DIeta MEDiterránea” (PREDIMED) study and the Lyon Diet Heart Study have robustly demonstrated the protective effects of diet rich in unsaturated fats, associated with the adherence to a Mediterranean-type dietary pattern, in primary and secondary prevention of CVD. The primary end point of PREDIMED study, a multicenter, 5-year randomized clinical trial, was to evaluate the effect of nuts or extra virgin olive oil consumption on major CV events (myocardial infarction, stroke and cardiovascular death) in 7447 adults with high risk but no CVD, adhering to a Mediterranean diet. Participants were randomized to three diet groups: two Mediterranean diets supplemented with either extravirgin olive oil or mixed Mediterranean nuts, and a control diet (advice to reduce dietary fat). After a median follow-up of 4.8 years, this study confirmed that, among persons at high CV risk, an energy-unrestricted Mediterranean diet supplemented with both extravirgin olive oil or nuts resulted in a substantial reduction in the risk of major CV events among high-risk persons, in comparison with the control diet (Estruch et al. 2013). This is consistent with epidemiologic studies that showed a 26% reduced risk of all-cause mortality associated with extravirgin olive oil consumption (Buckland et al. 2012). On the basis of its MUFA content that had consistently demonstrated to benefit plasma lipid profile in clinical trials, the olive oil consumption (23 g/day) was issued with a health claim by U.S. Food and Drug Administration. More recently, the European Food Safety Authority released a health claim about the role of extravirgin olive oil polyphenols (5 mg/day) in protecting LDL from oxidation (EFSA Panel on Dietetic Products Nutrition and Allergies 2011). In addition to the MUFA contribution, in the PREDIMED study it was demonstrated that even the intake of dietary ALA, supplied mainly by walnuts and extra virgin olive oil, was inversely correlated to all-cause and CVD mortality (Sala-Vila et al. 2016). The cardioprotective role of ALA intake, in the context of Mediterranean diet, was also investigated in the Lyon Diet Heart Study (de Lorgeril et al. 1994; de Lorgeril et al. 1999). This study was a randomized clinical trial designed to evaluate the effectiveness of a Mediterranean-type diet high in ALA on measures of CHD recurrence rate, after a first myocardial infarction.

A total of 605 subjects who survived a myocardial infarction within 6 months of enrollment were randomly assigned to a control diet or to a Mediterranean-type diet where the use of olive oil was substituted with a canola oil–based margarine as the predominant fat. Compared with olive oil, this margarine contained a similar quantity of SFA (15%), less oleic acid (48% vs. 75%), and slightly greater amounts of linoleic acid (16.4% vs. 8.6%) and ALA (4.8% vs. 0.6%). After a follow-up of 27 months, the risk of cardiac death and nonfatal MI decreased by >60% in the intervention group. Although there were no differences in lipids and lipoproteins between the experimental and control groups, patients adhering to the Mediterranean-style diet had a 50–70% lower risk of recurrent heart disease after 46 months (de Lorgeril et al. 1994; de Lorgeril et al. 1999). Other evidence from epidemiologic data demonstrated a beneficial role of ALA for the primary and secondary prevention of CVD that may be comparable to CVD benefits described for animal sources of n-3 PUFA (Hammad et al. 2016). Even limited, emerging research focused the attention on the potential cardioprotective effect of hempseed oil consumption, being a high source of both the essential fatty acids, i.e. the n-3 ALA and the n-6 linoleic acid (LA) (Rodriguez-Leyva & Pierce 2010). In the whole, these data suggest that specific dietary pattern could be more appropriate than the single nutrient approach for predicting CVD risk. Despite dietary guidelines have advanced considerably through the “replacement of SFA with unsaturated fat message” instead of recommending decreasing SFAs alone, there is still a lack of evidence from well-controlled clinical trials showing the optimal diet to maximally reduce CVD risk.

1.2.1 Nuts consumption and cardiovascular diseases risk factors

There are many interesting foods rich in unsaturated fatty acids and other bioactives that can replace dietary SFAs, in order to reduce CVD risk factors. Growing evidence support the importance to increase the consumption of nuts, to improve blood lipid profile and reduce risk factors for CVD. Nuts are nutrient-dense foods with one seed becoming hard at maturity and a thick, hard pericarp (Stern 1991). The most popular edible tree nuts are walnuts, hazelnuts, almonds, pistachios and macadamias, including peanuts that belong to the legume bean family but have a nutrient profile similar to other tree nuts. The healthy benefits of nuts derive from their high content of bioactives, in particular unsaturated fats (nearly one-half of the total fat content), mostly MUFAs. A sizeable proportion of PUFAs (predominantly LA) are present in walnuts, Brazil nuts and pine nuts, and walnuts are also a rich source of the plant n-3 ALA. Nuts contain also high-quality vegetable protein and often have a high content of the amino acid L-arginine, which is the substrate for endothelium-derived NO synthesis, a main regulator of vascular tone and blood pressure. Moreover, other bioactives with potential health benefits could be found in nuts (**Table 1.1**), such as vitamin E, minerals (potassium, copper, selenium, calcium, and magnesium), phytosterols, fibre and polyphenols (especially in the nut skin) (Alasalvar & Bolling 2015; Ros 2015). The research proving the association between daily unsalted nut consumption and reduced risk factors for CHD, is so strong that in 2003 the U.S. Food and Drug Administration (FDA) issued a health claim reporting that the consumption of 1.5 oz/day (approximately 42.5 g/day) of nuts, in particular walnuts, as part of a heart-healthy diet (low in saturated fat and cholesterol) can reduce the risk of heart disease (US Food and Drug Administration. Qualified Health Claims: Letter of Enforcement Discretion – Nuts and Coronary Heart Disease 2003). Subsequently, in 2011 the European Food Safety Authority (EFSA) claimed the positive effect of consumption of 30 g/day of walnuts, in the context of a balanced diet, as a contribute to the improvement of endothelium-dependent vasodilation in the general population (EFSA Panel on Dietetic Products 2011). The consumption of nuts is now recommended in the guidelines on lifestyle management to reduce CV risk by the American Heart Association/American College of Cardiology (Eckel et al. 2013).

Table 1.1 *Average composition in macro- and micronutrients and selected phytochemicals of 100 g of nuts. Adapted from Ros (2015)*

Nuts (100 g)	Energy (kJ)	Protein (g)	Fibre (g)	Fat (g)	SFA (g)	MUFA (g)	PUFA (g)	LA (g)	ALA (g)	PS (mg)	Na (mg)	K (mg)	Ca (mg)	Total PP (mg)
Almonds	2418	21.3	8.8	50.6	3.9	32.2	12.2	12.2	0.00	120	1	728	248	287
Brazil nuts	2743	14.3	8.5	66.4	15.1	24.5	20.6	20.5	0.05	NR	3	659	160	244
Cashews	2314	18.2	5.9	46.4	9.2	27.3	7.8	7.7	0.15	158	12	660	37	233
Hazelnuts	2629	15	10.4	60.8	4.5	45.7	7.9	7.8	0.09	96	0	680	114	687
Macadamia	3004	7.9	6.0	75.8	12.1	58.9	1.5	1.3	0.21	116	5	368	85	126
Peanuts	2220	25.8	8.5	49.2	6.8	24.4	15.6	15.6	0.00	220	18	705	92	396
Pecans	2889	9.2	8.4	72	6.2	40.8	21.6	20.6	1.00	102	0	410	70	1816
Pine nuts	2816	13.7	3.7	68.4	4.9	18.8	34.1	33.2	0.16	141	2	597	16	58
Pistachios	2332	20.6	9.0	44.4	5.4	23.3	13.5	13.2	0.25	214	1	1025	107	1420
Walnuts	2738	15.2	6.4	65.2	6.1	8.9	47.2	38.1	9.08	72	2	441	98	1558

Notes: LA, linoleic acid; ALA, α-linolenic acid; PS, plant sterols; NR, not reported; Na, sodium; K, potassium; Ca, calcium; PP, polyphenols.

The scientific evidence behind the recognition of nuts as “heart-healthy” foods derive from both epidemiological and clinical research suggesting a consistent inverse association between the frequency of nut intake and development of CHD risks and mortality. In fact, a recent report of two large prospective cohorts of 76464 women from the Nurses’ Health Study and 42 498 men form the Health Professionals Follow-up Study, with 24 and 30 years of follow-up respectively, showed a reduced CHD mortality for individuals of both sexes consuming nuts seven or more times per week. Remarkably, rates of death from cancer, CVD, heart and respiratory diseases were inversely related to frequency of nut consumption (Bao et al. 2013). Similar findings were also obtained from the elaboration of food frequency questionnaires from three large cohorts, including 71764 of Americans, African and European descent enrolled in the Southern Community Cohort Study and 134 265 Chineses from both Shanghai Women's Health Study and Shanghai Men's Health Study (Luu et al. 2015). The results, recently published in JAMA, demonstrated that consumption of nuts (including peanuts) was associated with decreased overall and CVD mortality even across different ethnic groups (blacks, whites and Asians) and among individuals from low socioeconomic status groups (Luu et al. 2015). Intervention studies have also provided new data suggesting health benefits of nut consumption. As previously discussed, findings from the PREDIMED demonstrated a beneficial effect of Mediterranean diets supplemented with mixed Mediterranean nuts (30 g/day: 15 g walnuts, 7.5 g almonds and 7.5 g hazelnuts) in the reduction of the rates of major CV events (Estruch et al. 2013). Moreover, compared to controls, the nut-supplemented group had reduced risk of cardiovascular and cancer mortality and those who consumed >3 servings nuts /week had the lowest rates of total mortality risk (Guasch-Ferré et al. 2013). Concurring with these findings, a recent 2-year vascular imaging study in a PREDIMED subcohort detected an anti-atherosclerotic effect of the Mediterranean diets versus the control diet, namely delayed progression of carotid intimamedia thickness and plaque, which was more pronounced with the nut-supplemented diet (Sala-Vila et al. 2014). Overall, a recent meta-analysis on 23 prospective studies, including a total of 179885 partecipants, concluded that the consumption of 1 serving of nuts/d is associated with reduced risk of coronary artery disease and hypertension, but not with stroke or type 2 diabetes (Zhou et al. 2014).

The beneficial effects of nuts were also evaluated in several short-term randomized clinical trials, mainly aimed to compared the effects of nut-enriched versus nut-free diets on the blood lipid profile in healthy or in hyperlipidemic subjects. A recent meta-analysis summarizing findings from 61 trials indicates that tree nuts intake (1 serving of 28.4 g nuts/day) has consistent lipid-lowering effect reducing levels of TC, LDL-C, apolipoprotein B, and TG. Moreover, the

major determinant of cholesterol lowering appeared to be nut dose rather than nut type, with stronger effects observed in trials providing ≥60 g nuts/d (Del Gobbo et al. 2015). Even if biomarker of oxidation were secondary outcomes in most of nut-supplementation studies, some evidence support the emerging role of nuts in reducing oxidative stress (López-Uriarte et al. 2009). The favorable impact of nuts on lipid profile and oxidative stress appear attributable to their high content in bioactive compounds with antioxidant and lipid-lowering properties contained in both nut kernel and skin (i.e. vitamin E, polyphenols, phytosterols) (López-Uriarte et al. 2009; Alasalvar & Bolling 2015). Although whole nuts may be susceptible to oxidation because of their PUFA content – which is particularly high in walnuts – the potential antioxidant activity of the polyphenols, vitamin E, and other antioxidants contained in nuts and particularly in the skin, may counteract the pro-oxidant effects of fat, thus preventing potentially adverse effects of oxidation (López-Uriarte et al. 2009).

Overall, because of their well-established effect in the reduction of coronary artery disease (Zhou et al. 2014), the consumption of whole nuts could be suggested as an excellent choice within heart-healthy snack foods especially for people with high CVD risk such as children with primary hyperlipidemia.

1.3 HYPERLIPIDEMIA AND DIETARY GUIDELINES SINCE PEDIATRIC AGE

Among the classical risk factors for CVD, hyperlipidemia is considered the major determinant of the atherosclerotic progression and it is related to early CV events, especially in pediatric patients when inherited disorders occur (Groner et al. 2006; Haney et al. 2007). Hyperlipidemias are disorders of lipoprotein metabolism characterized by elevation in serum TC, LDL-C or TG concentrations. (Haney et al. 2007). These disorders, can occur as a result of a genetic defect in lipid metabolism pathways (primary or familial hyperlipidemia) or secondary to other diseases. It is estimated that the primary forms affect 0.5% to 2% of the population and up to 20% of survivors of premature myocardial infarction (Juonala et al. 2008). Through the congenital hyperlipidemias, the familial combined hyperlipidemia (FCHL) and the familial hypercholesterolemia (FH) are the two most common forms, estimated to affect more than 20 million people worldwide (Feldman et al. 2015; Kavey 2015). While the FCHL is an autosomal multigenic inherited lipid disorder characterized by moderate-to-severe elevation in TG and non-HDL-C, minimal elevation in LDL-C, and reduced HDL-C, the FH resulted in strongest manifestation. In fact, FH is caused by an autosomal dominant mutation in the genes encoding LDL receptor, PCSK9 or apolipoprotein B and results in normal levels of TG, reduced HDL-C but elevated LDL-C levels that exceed 400 mg/dL in case of homozygous form (Sniderman et al. 2014; Feldman et al. 2015), evidences of xanthomas by age 5 and vascular disease before age 20 (Haney et al. 2007).

The atherosclerotic process begins early in life and may be present throughout the lifetime. For example, signs of fatty streaks, representing the earliest sign of atherosclerosis, have been observed already in fetal aortas and in children above 3 years of age (Narverud et al. 2014). Thus, compared with adult hyperlipidemic patients, children and adolescents affected by primary hyperlipidemia show a particularly higher risk to develop CVD later in life, mainly due to the chronic exposure to elevated LDL-C accumulating in the intima-media of large muscular arteries (Durrington 2003; Narverud et al. 2014). Several markers of atherosclerotic development are frequently reported in children with primary hyperlipidemia, including markers of inflammation, endothelial function, atherogenic lipids and related mediators (Narverud et al. 2014). In addition, in the early phase of atherosclerosis, systemic signs of enhanced oxidative stress have been detected. Increased oxidative stress results from the imbalance between an excessive reactive oxygen species (ROS) and/or deficiency of antioxidant defense mechanisms, and it is considered the main cause of cellular damages, endothelial dysfunction and atherosclerosis (Lum & Roebuck 2001). Despite the scarce data available, hyperlipidemias, and in particular the primary forms, seem to be associated with increased markers of oxidative stress, evaluated by measuring oxidative DNA damage (DNA strand breaks in lymphocytes, urinary 8-hydroxy-2'-deoxyguanosine concentrations), oxidative lipid damage (oxidized LDL, urine isoprostanes and malondialdehyde) and non-enzymatic or enzymatic antioxidants (Botto et al. 2002; Harangi et al. 2002; Real et al. 2010; Ho et al. 2013; Pedro et al. 2013; Da Silva Pereira et al. 2013; Cortes et al. 2014; Narverud et al. 2014; Tangvarasittichai 2015). Moreover, a significant decrease of endothelial function found in hyperlipidemic children has been strongly correlated to prolonged exposure to ROS, which can inhibit endothelium nitric oxide release or synthase (Pignatelli et al. 2009; Loffredo et al. 2013). Consistent with this is the demonstration that, in patients at risk of atherosclerosis, oxidative stress negatively correlated with endothelial function and antioxidant treatment was able to restore arterial dilatation (Cangemi et al. 2007; Del Bo' et al. 2014). Although research in this field is growing, further studies are required to examine the utility of the most promising oxidative biomarkers to predict prognosis or response to treatment.

Considering the evidence reported, it is clear that early diagnosis and treatment of hyperlipidemic children should be started as soon as possible to attenuate development of the

potential ongoing early atherosclerotic process and correlated risks of CVDs. Even if in the pediatric population the serum cholesterol concentrations is strongly influenced by age, gender, ethnicity and pubertal status, lowering of TC and LDL-C below the 75th percentile, corresponding to <170 and <110 mg/dl respectively, is considered the target therapy in hyperlipidemic children (Daniels & Greer 2008). However, there is still some reluctance to start an aggressive lipid-lowering drug therapy in pediatric age. In this context, healthy lifestyle, which includes appropriate dietary pattern in agreement with the expert panel guidelines for CV health and risk reduction (Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents 2011), physical activity and weight loss in case of excessive body weight, is the cornerstone in the treatment of pediatric hyperlipidemia and represents an important target for CVD prevention (Haney et al. 2007; Catapano et al. 2011). Although the emphasis on reducing saturated fat, trans-fat, and cholesterol is to lower LDL-C, there is provocative evidence that other dietary constituents can reduce atherosclerosis in a manner independent of total cholesterol levels. In fact, in recent years several studies have emphasized the role of several food bioactive compounds in the CVD and atherosclerosis risk and progression. The effect of some bioactives such as PUFAs, phytosterols, vitamins and polyphenols was demonstrated to reduce or attenuate the atherosclerotic lesions by preventing the oxidation of LDL particles, reducing the inflammatory response, leukocyte migration, adhesion moleculelevels and blood pressure (Torres et al. 2015). Therefore, there is a need to establish appropriate recommendations on the intake of foods rich in these bioactives.

Pediatric guidelines for the management of cholesterol in pediatric patients were firstly introduced in 1992 by the National Cholesterol Education Program (NCEP) Pediatric Panel Report (1992), then revised in 1998 by the American Academy of Pediatrics (1998). At that time, two different dietary regimens (STEP-I and STEP-II) were suggested to all children over the age of 2 years as part of a population-based approach to achieve lower cholesterol levels to acceptable range and thus, reduce CV risks. No restriction of fat or cholesterol was recommended for infants <2 years when rapid growth and development require high energy intake. In particular, for all healthy children (2-18 years) the STEP-I diet was promoted as follow: total fat over several days ≤30% of total energy intake and no less than 20% of total energy intake; saturated fatty acids (SFA) <10% of total energy intakes; and dietary cholesterol <300 mg per day. While, for children with high LDL–C levels or for those where a 3-month STEP-I diet did not result in a lower LDL–C level, the Panel suggested to follow STEP-II dietary guidelines, consisting of: no more than 30% and no less than 20% of total energy from fat; <7% of total calories from SFA; ≤10% of calories from PUFA; and no more than 200 mg per day of cholesterol (National Cholesterol Education Program 1992; American Academy of Pediatrics 1998). Scientific evidence supported the effectiveness of this approach in the reduction of the development of atherosclerosis in childhood and adolescence. In particular, a decreased level of plasma TC and LDL-C by <7–9% and 10–20% were found respectively after STEP-I and STEP-II diet, compared with the average American diet (Yu-Poth et al. 1999).

Despite the NCEP guidelines provided the rationale for new dietary prevention efforts initiated early in life, guidelines for other nutrients needed at pediatric age were not addressed. For this reason, in 2011 the National Heart, Lung and Blood Institute (NHLBI) developed new guidelines based on the previous NCEP recommendations and Dietary Guidelines for Americans (2010), to create the CHILD-1 (Cardiovascular Health Integrated Lifestyle Diet–1), evidence-based recommendations for dietary changes to reduce CV risk in pediatric patients. CHILD-1 not only is built on the recommendations for achieving nutrient adequacy in growing children, but also added evidence regarding the efficacy of specific dietary and lifestyle changes in reducing CV risks. Because the focus of these guidelines was on

CV risk reduction, the review specifically evaluated dietary fatty acid and energy components as major contributors to hypercholesterolemia and obesity, as well as dietary composition and micronutrients as they affect hypertension. In accordance with previous NCEP, also the NHLBI developed two-step regimens, the CHILD-1 and CHILD-2, providing different recommendations on the basis of severity of lipid diseases, as reported in **Table 1.2**. In fact, the CHILD-1 represents the first stage in dietary change for children with a positive family history of early cardiovascular disease, obesity, primary hypertension or high-risk medical conditions that might ultimately require more intensive dietary change. The CHILD-1 guidelines confirmed the usefulness of a normocaloric diet administration not exceeding 25–30% of calories from fat, 8–10% from saturated fats, and 20% from MUFA and PUFA, avoiding trans fatty acids and providing not more than 300 mg per day of cholesterol and consuming adequate amounts of fiber. Moreover, specific dietary suggestions and supportive actions were provided for each child age range. For children with identified hypercholesterolemia and elevated lipid levels aged 2-21 years, a more stringent CHILD-2 diet with SFA limited at ≤7% of calories and dietary cholesterol to 200 mg/day has been suggested. In addition, CHILD-2 was subdivided in two different regimens (CHILD-2 TG or CHILD-2 LDL) developed specifically for children older than 2 years with elevated TG or LDL-C levels. In particular, in children with an elevated TG level, reduction of simple carbohydrate intake in favor of complex carbohydrates, a reduced SFA intake, an increased intake of omega-3 from fish, physical activity and weight loss were suggested in order to decreased TG levels. While, in children with FH and abnormal LDL-C levels, a low-fat, low SFA CHILD-2 LDL diet was suggested, underlying the importance to replace the usual fat sources with plant sterols and/or plant stanol esters (>2 g/day) and to increase the intake of cereal enriched with water-soluble fiber psyllium (6 g/day for children 2–12 years and 12 g/d for those ≥12 years). For all children, other life-style modifications have been recommended, including 1 h/d of moderate-to-vigorous physical activity and the limitation of sedentary time (<2 h/day of TV/video) (Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents: Summary Report 2011).

In Europe, the evidence that diet is the main stay of treatment for hyperlipidaemia in childhood was also reported in the Guidelines for the management of dyslipidaemias (Catapano et al. 2011), developed by the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS) These guidelines provided a review of evidence and recommendation for helping physicians in their management of diagnosis and treatment of dyslipidemia. However, despite the document provides a recommendation section on critical lifestyle and dietary modifications that should be adopted to control and prevent CVD since pediatric age, the guidelines were directed to the overall population of dyslipidaemic patients and not specifically to children at different age (Catapano et al. 2011).

Table 1.2 *Evidence-Based Recommendations for Diet and Nutrition: comparison between CHILD-1, CHILD-2 LDL and CHILD-2 TG diet (Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents: Summary Report 2011).*

Age	CHILD-1	CHILD-2 LDL	CHILD-2 TG
Birth–6 months	• Infants should be exclusively breastfed (no supplemental formula or other foods) until the age of 6 months		
6 – 12 months	• Continue breastfeeding until at least 12 mo of age while gradually adding solids; transition to iron fortified formula until 12 months if reducing breastfeeding • Fat intake in infants <12 months of age should not be restricted without medical indication • Limit other drinks to 100% fruit juice (≤4 oz/d); no sweetened beverages; encourage water		
12 – 24 months	• Transition to reduced-fat (2% to fat-free) unflavored cow's milk • Limit/avoid sugar-sweetened beverage intake; encourage water • Transition to table food with: - Total fat: 30% of daily kcal/EER - SFA fat: 8%–10% of daily kcal/EER - MUFA and PUFA >20% of daily kcal/EER - Cholesterol: 300 mg/day - Avoid trans fat as much as possible *Supportive actions:* • The fat content of cow's milk to introduce at 12–24 month of age should be decided together by parents and health care providers on the basis of the child's growth, appetite, intake of other nutrient-dense foods, intake of other sources of fat, and potential risk for obesity and CVD • 100% fruit juice (from a cup), ≤ 4 oz/d • Limit sodium intake • Consider DASH-type diet rich in fruits, vegetables, whole grains, and low-fat/fat-free milk and milk products and lower in sugar		
2 – 10 years	• Primary beverage: fat-free unflavored milk • Limit/avoid sugar-sweetened beverages; encourage water • Encourage high dietary fiber intake from foods • Fat content: - Total fat 25%–30% of daily kcal/EER - SFA: 8%–10% of daily kcal/EER - MUFA and PUFA: >20% of daily kcal/EER - Cholesterol: 300 mg/d - Avoid trans fats as much as possible *Supportive actions:* • Teach portions based on EER for age/gender/age • Encourage moderately increased energy intake during periods of rapid growth and support DASH eating plan • Encourage dietary fiber from foods (age +5 g/die) • Limit naturally sweetened juice (no added sugar) to 4 oz/d • Limit sodium intake	X X X • Fat content: - Total fats: 25–30% of daily kcal/EER - SFA: <7% of daily kcal/EER - MUFA: 10% of daily kcal/EER - Cholesterol: <200 mg/day - Avoid trans fat as much as possible *Supportive actions:* • Children with FH >2 year: replacement of usual fat sources with plant sterols (>2 g/day) • Plant stanol esters as part of a regular diet • The water-soluble fiber psyllium can be added to a low-fat, low-SFA diet (6 g/day for children 2–12 y of age and 12 g/d for those ≥12 y of age	X X X • Fat content: - Total fats: 25–30% of daily kcal/EER - SFA: <7% of daily kcal/EER - MUFA: 10% of daily kcal/EER - Cholesterol: <200 mg/day - Avoid trans fat as much as possible *Supportive actions:* • Replace simple with complex carbohydrates • No sugar-sweetened beverages • Increase dietary fish to increase PUFA ω-3 fatty acids

	• As for all children, 1 h/d of moderate-to-vigorous physical activity and <2 h/day of sedentary screen	• 1 h/d of moderate-to-vigorous physical activity and <2 h/day of sedentary screen time	• 1 h/d of moderate-to-vigorous physical activity and <2 h/day of sedentary screen time
11 – 21 years	• Primary beverage: fat-free unflavored milk • Limit/avoid sugar-sweetened beverages; encourage water • Encourage high dietary fiber intake from foods • Fat content: - Total fat: 25%–30% of daily kcal/EER - SFA: 8%–10% of daily kcal/EER - MUFA and PUFA: >20% of daily kcal/EER - Cholesterol: 300 mg/d - Avoid trans fat as much as possible *Supportive actions:* • Teach portions based on EER for age/gender/activity • Encourage moderately increased energy intake during periods of rapid growth and/or regular • Moderate-to-vigorous physical activity • Advocate dietary fiber: goal of 14 g/1000 kcale • Limit naturally sweetened juice (no added sugar) to 4–6 oz/d • Limit sodium intake • Encourage healthy eating habits: breakfast every day, eating meals as a family, limiting fast-food meals • Support DASH-style eating plan	X X X • Fat content: - Total fats: 25–30% of daily kcal/EER - SFA: <7% of daily kcal/EER - MUFA: 10% of daily kcal/EER - Cholesterol: <200 mg/day - Avoid trans fat as much as possible *Supportive actions:* As for age 2-10 years	X X X • Fat content: - Total fats: 25–30% of daily kcal/EER - SFA: <7% of daily kcal/EER - MUFA: 10% of daily kcal/EER - Cholesterol: <200 mg/day - Avoid trans fat as much as possible *Supportive actions:* As for age 2-10 years

Notes: CHILD-1, Cardiovascular Health Integrated Lifestyle Diet 1; CHILD-2 LDL, CHILD-2 for children with elevated LDL-C; CHILD-2 TG, CHILD-2 for children with elevated triglycerides; EER, indicates estimated energy requirement; FH, familiar hypercholesterolemia; MUFA, mono-unsaturated fat; PUFA, polyunsaturated fat; SFA, saturated fat.

1.4 IMMUNE FUNCTION AND THE ROLE OF NUTRITION

A common denominator playing a role in the prevention of both chronic diseases and infections is a healthy and well-functioning immune system. This may be particularly critical for specific at risk target population groups such as children and the elderly.

The immune system consists of a complex network of specialized cells organized to counteract the infection of harmful microorganisms or other non-self compounds. It is generally divided into two different systems on the base of the speed and specificity of its response: the innate and adaptive immunity. The innate immune response consists in a rapid, non-specific, non-anticipatory and non-clonal response that plays a crucial role in the early first-line defense against intracellular growth microorganisms or malignant cells, through several mechanisms including the phagocytosis, the respiratory burst, release of proinflammatory cytokines or the natural killer (NK) cell activity. Moreover, the innate immune system has also a key role in the initiation and subsequent course of the acquired immunity, which is a more specialized and anticipatory response that becoming prominent after several days as antigen-specific T and B cells have undergone clonal expansion. Although innate and adaptive immune systems have been traditionally viewed as two separate entities, it has now become very clear that these are integrated and interconnected systems (Watson et al. 2010; Noakes & Michaelis 2013).

An excessive or inappropriate innate immune response (i.e. a low NK activity) decrease the ability to counteract pathogenic organisms or cancer cells. Moreover, deviation in the programming of immune cells may lead to allergic reactions against harmless environmental factors or may result in an autoimmune response that is characterized by an immunological attack directed at the host's tissues and followed by inflammatory processes in the affected organs (Nathan 2002; Schleinitz et al. 2010; Kiliç et al. 2013). Even if the inflammation is a protective response of the body against damaging insults, infectious agents and injuries that initiates healing, its chronification compromises even these healing processes. In fact, chronic inflammation can affect nearly all organs and tissues and persistence of an inflammatory environment seem to mark the development of onset of chronic degenerative conditions, including obesity, CVD, diabetes, autoimmune and neurodegenerative conditions (Kiliç et al. 2013; Ricordi et al. 2015).

It is now widely recognized that diet and nutrient status are important factors contributing to immunocompetence. Dietary macro- and micronutrients are essential for the development, maintenance, and optimal functioning of the immune system. Immune competence can be easily dysregulated as a result of changes of nutritional status. Both deficiency and excess of a number of nutrients adversely affect the numbers and activity of immune cells (Kelley 2001; Watson et al. 2010). For example, malnutrition, due to insufficient intake of nutrients, is a striking example of the critical role that nutrition plays in innate immune responses. On the other hand, over-nutrition and high intake of specific nutrients are associated with chronic inflammatory diseases, underlying the fact that nutrients must be present at appropriate levels to ensure proper immune function (Field et al. 2002; Watson et al. 2010; Calder & Parveen 2013). For example, evidence suggests that macronutrient excess can promote inflammatory responses in adipocytes and macrophages, in part through the activation of stress responses. Acute *in vitro* induction of hyperglycemia in healthy individuals and stimulation of monocytes with high glucose levels induces IL-6, IL-18 and TNF-α production (Esposito et al., 2002; Morohoshi et al., 1996). Similarly, excess extracellular and intracellular free fatty acids and triglycerides trigger endoplasmic reticulum stress responses or toll like receptor activation in adipocytes and macrophages (Ozcan et al., 2004, 2009; Schaeffler et al., 2009; Shi et al., 2006). In this context, it is noteworthy that certain dietary lipids are essential in the modulation of several immune functions. For example, n-3 long chain PUFAs (LC-PUFAs) is widely recommended to reduce inflammation in immune/inflammatory chronic diseases such as atherosclerosis, CVD, diabetes

and arthritis, and may be also beneficial in treating infectious diseases (Husson et al. 2016). However, although LC-PUFA have been applied in the resolution of diseases characterized by an overactivation of immune system due mainly to their anti-inflammatory properties, high amounts of PUFA could be responsible for an immunosuppressive state and for an increase in the susceptibility to infectious microorganisms.

Recently, substantial research has also focused on the contribution of specific micronutrients to an optimum functioning of the immune system. Deficiencies in specific minerals or vitamins can cause an increased susceptibility to infection, leading to frequent and chronic infections controlled only through a nutritional rehabilitation treatment (Field et al. 2002; Afacan et al. 2012). As an example, zinc is essential for the activity of many enzymes implicated in the immune activity and its deficiency, that affect one third of the world's population, is a major risk factor for many diseased states that involve the immune systems such as pneumonia, diarrhea, malaria, HIV, etc. (Field et al. 2002; Caulfield et al. 2004; Gruber & Rink 2013) and has been associated with increased chronic inflammation and impaired innate immune activity (Wirth et al. 1989; Sheikh et al. 2010; Bao et al. 2010; Besecker et al. 2011). Similarly, there is much evidence that poor selenium intake impairs the initiation and regulation of inflammation and immune response leading to an ineffective activity against infection, and the benefits of increasing selenium intake through supplementation are clear (Huang et al. 2013). Regarding vitamins, the potential immunoregulation of vitamin D has attracted a lot of attention since it was discovered that all immuno cells express the vitamin D receptor (VDR) and, in most cell types, activation induces further VDR expression. In particular, experimental evidence has shown that some immune cells (NKT cells and CD8αα T cells) do require adequate vitamin D status and VDR signaling to develop and function normally. In addition, the evidence suggests that 1,25$(OH)_2$-vitamin D and the VDR are important inhibitors of autoimmunity mediated by Th1 and Th17 cells (Harvey & Cantorna 2013). Even if vitamin D would be effective in autoimmune disease, it is unclear what dose, how often and/or what the 25(OH)-vitamin D blood levels should be achieved. Moreover, it is recognized that vitamins (i.e. A, B6, B9, B12, C, D, E) and minerals (i.e. iron, zinc, copper, and selenium) work in synergy to support the protective activities of the immune cells. Finally, all these micronutrients, with the exception of vitamin C and iron, are essential for antibody production (Field et al. 2002; Watson et al. 2010; Calder & Parveen 2013). Despite the importance of adequate nutrient intake, the current evidence is not sufficient to make a definite dietary recommendation for prevention or therapy, even in those with existing immune disease. In fact, many promising results have been achieved in *in vitro* experiments. Thus, *in vivo* approaches are needed to have results transferable to practical therapeutic options. Moreover, future investigations are need for a better understanding on the molecular mechanisms of action of nutrients on the immune system and the dose that could be considered safe for the overall metabolism. Currently, the achievement of an adequate nutrient intake is considered the best practice for promoting an optimal immune system function and prevent the risk of chronic degenerative diseases.

1.4.1 Immunomodulatory effect of LC-PUFA

Dietary LC-PUFAs have been shown to influence immune system via different mechanisms, including the reduction of lymphocyte proliferation, and cytokine synthesis, the increase phagocytic activity or modification of NK cell activity (Watson et al. 2010). LC-PUFAs can influence the inflammatory response in either pro- or anti-inflammatory mediators. In particular, since the n-6 LC-PUFA arachidonic acid (ARA) is the precursor of eicosanoids, known to have a central role in inflammation, and since ARA metabolism is a long recognized target for anti-inflammatory therapies, it has generally been assumed that n-6 LC-PUFA must, almost by definition, promote inflammation (Calder 2009). However, the mechanisms that participate in these processes are more complex and still poorly understood, since some ARA-derived eicosanoids have both pro- and anti-inflammatory roles (e.g. prostaglandin E2) and some others may be very important in resolving inflammation (e.g. lipoxin A4) (Calder 2009; Calder & Parveen 2013).

On the contrary, there is evidence from both experimental models and clinical studies that n-3 LC-PUFAs have anti-inflammatory and immunomodulatory effects, considered beneficial in treating infectious, autoimmune and inflammatory chronic diseases (Calder 2006; Watson et al. 2010; Husson et al. 2016). Different mechanisms have been proposed. Extensive data demonstrated that increased consumption of n-3 LC-PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), results in increased proportions of those fatty acids in inflammatory cell phospholipids, partly at the expense of ARA (Calder 2009). Since the same enzymes metabolize both n-3 and n-6 PUFAs, n-3 LC-PUFAs competitively inhibit the production of ARA, and thus ARA-derived proinflammatory eicosanoids, with a final anti-inflammatory effect. Moreover, high intake of n-3 LC-PUFA from fish oils, decreases the production of inflammatory cytokines, other inflammatory agents (e.g. ROS) and the expression of adhesion molecules (Calder 2006; Calder 2009).

Most often, the idea that n-3 LC-PUFAs diminish inflammatory cell functions is interpreted in a favorable way, with the conclusion that they are anti-inflammatory and thus will be beneficial to health. However, because these cells are among the cellular components of the immune system, a reduction in their activity could also compromise the immune response against microorganisms (McMurray et al. 2011; Husson et al. 2016). In fact, inflammatory cells recognize bacteria in a nonspecific way and destroy them by phagocytosis or via the production of superoxide and related ROS in the respiratory burst. Incorporation of n-3 LC-PUFAs into the cell membrane may change the membrane fluidity (Kelley 2001; Shaikh et al. 2015), with a consequent alteration in activity of cell's actin skeleton that is accompanied by a reduction in phagocytosis of microorganisms by macrophages and antigen-presenting cells, phagolysosome formation, and NK cell activity (Thies et al. 2001; Rees et al. 2006; Mukaro et al. 2008; McMurray et al. 2011). The second consequence of the changed membrane fluidity is the inhibition of a clustering of surface proteins, which is involved in numerous signalling pathways in immune cell interactions (Bonilla et al. 2010).

On the basis of current data, although n-3 LC-PUFAs are widely used in the treatment of diseases characterized by an overactivation of immune system (e.g. chronic inflammatory diseases), their intake could be both beneficial and deleterious in the prevention and control of infectious diseases (Anderson & Fritsche 2002; Watson et al. 2010; Husson et al. 2016). In fact, in healthy humans, the supplementation with EPA and DHA at daily dose of 0.5 g/day improves the outcome of infections caused by opportunistic extracellular pathogens that induce a strong inflammatory response (Husson et al. 2016). Thus, the beneficial effects of n-3 LC-PUFAs may result from their anti-inflammatory properties, which limit tissue damage associated with the pathogen and the inflammatory response.

Conversely, high amount of n-3 LC-PUFA could be responsible for an immunosuppressive state and for an increase in the susceptibility to infectious microorganisms. In fact, the anti-inflammatory properties of n-3 LC-PUFA supplementation demonstrated to be detrimental in respiratory, systemic and ocular infections with intracellular pathogens (e.g. *M. tuberculosis*, *Influenza A virus*, *Salmonella spp.*, *L. monocytogenes*, *Herpes simplex virus*), which need a strong inflammatory and immune cell responses to eradicate infected cells (Husson et al. 2016). This effect is particularly important to consider especially in immunocompromised patients and in infants and elderly, who are the major targets for n-3 LC-PUFA supplementation and, at the same time, the main groups at risk of sepsis.

Overall, the review of published studies highlights that the effect of fatty acids on immune system function are conflicting due to multiple factors, including the type and amount of fatty acids consumed with the diet, the different supplementations and the target subjects enrolled in the study. Moreover, for obvious reasons, the altered resistance to infectious microorganisms has been analyzed especially in animal models or *in vitro*, using different microorganism to stimulate the host immune response. Therefore, it is pivotal to define the optimal n-3 LC-PUFA doses, timing of intake, and the characteristics of patients who might benefit from n-3 LC-PUFA supplementation.

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2 AIM OF THE STUDY

The aim of the present Ph.D. thesis was to evaluate the effect of specific foods and food bioactives in the modulation of risk factors for chronic-degenerative diseases in “at risk” groups of population, thorough both *in vivo* and *ex vivo* approaches. Thus, the research project has been subdived in two part, as described below.

PART I

In the first part we developed two different dietary interventions in a population of Italian children and adolescents affected by primary hyperlipidemia, a risk factor for future CV events. In particular, it was examined whether regular intake of food sources of unsaturated fats and other bioactives, i.e. hazelnuts and hempseed oil, were effective in the improvement of CV risk related biomarkers. In this context, three different activities were scheduled:

- Characterization of FA composition of erythrocyte phospholipids to provide the first experimental data in this target population and to ascertain potential association with serum lipids and dietary factors;
- Development of a dietary intervention study to investigate the effect of regular hazelnuts consumption in the modulation of markers of oxidative stress (DNA damage and ox-LDL) and lipid profile (serum lipids and FAs composition erythrocyte phospholipids);
- Development of a dietary intervention study to evaluate the impact of a supplementation with hempseed oil rich in ALA on serum lipid profile and fatty acid composition of erythrocyte phospholipids.

PART II

The second part of the project was devoted to *ex vivo* investigations carried out at the Department of Physiology and Biochemistry of Nutrition of the Max Rubner-Institut (Karlsruhe, Germany). In particular, two different studies were developed on *ex vivo* immune cells from healthy subjects to investigated the immunomodulatory effect of LC-PUFAs (EPA and ARA) with vitamin D in the modulation of two mechanisms crucially involved in the innate immune response:

- The activity of NK cells against target human cancer cell line (K562);
- The phagocytic activity of monocytes and granulocytes against infectious agents (opsonised *Escherichia coli*).

PART I: *IN VIVO* STUDIES

3 DEVOLOPMENT OF DIETARY INTERVENTION STUDIES IN CHILDREN AND ADOLESCENT WITH PRIMARY HYPERLIPIDEMIA

3.1 CHARACTERISATION OF SERUM LIPID PROFILE AND FATTY ACID COMPOSITION OF ERYTHROCYTE PHOSPHOLIPIDS

3.1.1 SUBJECTS AND METHODS

Subject enrollment 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. Patients included in the study were aged 11 ± 3 (mean ± SD) years old and were affected by different types of hyperlipidemia: familial hypercholesterolemia (FH), familial combined hyperlipidemia (FCHL) or polygenic hypercholesterolemia (PHC). Diagnostic criteria of primary hyperlipidemia were based on accepted international standards (Guardamagna et al. 2011). FH was diagnosed in presence of 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 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 >90th 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.

Exclusion criteria were: secondary hyperlipidemia; obesity (body mass index (BMI) ≥ 90th 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.

At recruitment, children were on diet therapy and were selected only 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 through a weekly food diary during the study period. Dietary recommendations were based on the cardiovascular health integrated lifestyle diet (CHILD-1) for children with identified dyslipidemia, as in the American Academy of Pediatrics (Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents: Summary Report 2011). The essential features were: 55% of daily energy from carbohydrate, 15% from protein, 30% from total fat (SFA <10%, MUFA and PUFA 20% of total energy), dietary cholesterol <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).

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. Fasting blood samples were drawn by venipuncture for the analysis of the lipid profile and the fatty acid (FA) composition of red blood cell (RBC) phospholipids.

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 study purpose and protocol were exhaustively explained to all participants and their parents, who signed an informed consent before study enrollment. The trial was registered under ISRCTN.com (identifier no. ISRCTN12261900).

Physical evaluation

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.

Blood samples 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.

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

Analysis of FA composition of RBC phospholipids

FAs extraction from RBC phospholipid was performed in accordance to the method previously described by Simonetti et al. (2002). Briefly, 1 ml of distilled water, 300 mg of RBC, 2.5 ml of butylated hydroxitoluene (110 µg/ml in methanol), and 5 ml of chloroform were transferred into 10 ml plastic tubes. The mixtures were shaken on vortex for 3 min and centrifuged at 1200 g x for 10 min. The chloroform phase was transferred into 10 ml plastic tubes. An aliquot of 2.5 ml chloroform was added to the tubes containing RBCs to perform the second extraction by vortexing for 3 min, and, after centrifugation at 1200 g x for 10 min, the supernatant was transferred into the corresponding tube. The chloroform phase was eluted on a silica cartridge (Sep-Pack PlusSilica, Waters), which was then evaporated under nitrogen steam. The silica phase was transferred into pyrex glass tubes, and 2.5 ml of a toluene/methanol (1:4 v/v) mixture and 200 µl of acetyl chloride were added. After 1 h in an oven at 100°C, 5 ml of K_2CO_3 (6% w/v in water) was added to the silica. After centrifugation at 1200 g x 10 min, the supernatant was transferred into amber glass vials, dried under nitrogen and resuspended in 100 µl of hexane before gas chromatography analysis.

The analysis of FA methyl esters extracts was performed by gas chromatography, as described by Ackman (1986) partly modified. Separations were performed with a 30 m 0.32 mm i.d. Omegawax 320 capillary column, under these conditions: initial isotherm, 140°C for 5 min; temperature gradient, 2°C/min to 210°C; final isotherm, 210°C for 20 min. The injector temperature was 250°C. Injection volume was 1 µl with a split ratio of 1/100, and the flame ionization detector temperature was 250°C. Carrier and makeup gas were hydrogen and nitrogen, respectively. FA 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 $p \leq 0.05$; data are presented as a mean and standard deviation (SD).

3.1.2 RESULTS

The results reported in this section have been recently published (Deon et al. 2016).

Subjects characteristics

The main subject features are summarized in **Table 3.1** and reported in our publication (Deon et al. 2016). The cohort of children and adolescents included 54 hyperlipidemic subjects aged between 6-17 years old: 14 patients were affected by FH (7 F), 21 by FCHL (12 F) and 19 by PHC (7 F). Five subjects were slightly overweight and the mean of blood pressure levels were in the normal range.

Table 3.1 *Subject characteristics (n = 54).*

Parameter	Value
Age, years	11 ± 3
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.

Serum lipid profile and RBC phospholipid composition

Serum lipid profile and FA composition of RBC phospholipids were analysed in all patients and classified according to sex and clinical diagnosis (**Table 3.2**).

Mean of serum lipid parameters exceeded the 90th percentiles (age and sex related), except for HDL-C, showing normal concentrations. The RBC phospholipid composition was SFA>PUFA>MUFA and all patients showed a low omega-3 index value (≤ 4%), that is associated with a high risk of mortality for CVD (Harris & Von Schacky 2004).

No significant serum lipid concentration difference was observed between males and females, while sex-related differences were detected in the phospholipid composition of RBCs. In fact, females showed significantly higher proportion of the SFA stearic acid and lower concentration of the n-6 PUFA dihomo-γ-linolenic acid (DGLA) in RBCs phospholipids (*p*= 0.031 and 0.050, respectively). Moreover, we found a trend toward lower level of MUFA and MUFA/SFAs ratio in RBCs of females with respect to males (*p*= 0.052 and 0.056, respectively).

A further analysis considering the type of primary 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, that is due to the different diagnosis considered. Finally, significantly lower levels of MUFA vaccenic acid and the n-6 PUFA γ-linolenic acids in RBC phospholipids were detected in FH as compared to PHC children (*p*= 0.036 and 0.027, respectively).

Table 3.2 *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			Hyperlipidemia			
		Male	Female		FH	FCHL	PHC	
	n = 54	n = 28	n = 26	*p* value	n = 14	n = 21	n = 19	*p* value
Serum lipids (mg/dl)								
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
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
RBC phospholipid FAs (%)								
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
Saturated								
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
Monounsaturated								
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
n-6 Polyunsaturated								
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 (γ-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 (DGLA)	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 (ARA)	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
n-3 Polyunsaturated								
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 (DPA)	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
22:5n-3 (DPA)	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 (DHA)	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; DGLA, dihomo-γ-linolenic acid; ARA: arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA: docosahexaenoic acid.
Values are reported as mean ± SD. [a,b] Data with different letters are significantly different ($p \leq 0.05$).

Correlation between RBC phospholipid composition and serum lipid profile

Correlations between serum lipid levels and RBC phospholipid composition are reported in **Table 3.3**. 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.

Table 3.3 *R-value of correlation between RBC phospholipid FAs and serum lipid concentrations (n= 54).*

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

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.
*Significantly different by Kendall and Spearman correlations for multiple comparisons (p ≤0.05).

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

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 ± 2.8% protein, 58.6 ± 6.1% carbohydrate and 24.7 ± 6.4% fat (saturated: 7.4 ± 2.1%, monounsaturated: 10.3 ± 4.8%, polyunsaturated: 2.6 ± 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 3.4**. 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)

Table 3.4 *R-value of correlation between dietary factors, RBC phospholipid FAs and serum lipids (n= 23).*

	Dietary factors										
	Energy	Protein	CHO	Total Fat	SFAs	MUFAs	PUFAs	PUFAs n-3	PUFAs n-6	Cholesterol	Fiber
Serum lipids											
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
RBC phospholipid FAs											
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: CHO, carbohydrates; SFAs, saturated fatty acids; MUFAs monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids;TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LC-PUFAs, long chain polyunsaturated fatty acids; omega-3 index, sum of EPA + DHA. *Significantly different by Kendall and Spearman correlations for multiple comparisons ($p \leq 0.05$).

3.2 EFFECT OF REGULAR HAZELNUT INTAKE ON MARKERS OF OXIDATIVE STRESS, SERUM LIPID PROFILE AND FATTY ACID COMPOSITION OF ERYTHROCYTE PHOSPHOLIPIDS

3.2.1 SUBJECTS AND METHODS

Study design

A total of 66 children and adolescents with primary hyperlipidemia (31 females; mean age: 11.6 ± 2.6 years) were selected as previously reported (see **Study 3.1**). The process of patient selection and allocation to the different study groups is depicted in **Figure 3.1**.

The intervention study was an 8-week randomized, single blind, controlled, three-arm, parallel-group (**Figure 3.1**). Patients were assigned to their treatment groups according to a randomization list obtained through the investigating center database. Patients were allocated to receive the different treatments by a pediatrician who was not involved in the study and did not participate to sample analysis. Each patient was randomized to one of the three treatment groups with a 1:1:1 ratio (22 subjects per group): group 1 was treated with one daily portion of hazelnuts with skin (HZN+S); group 2 with one daily portion of hazelnuts withot skin (HZN-S); while group 3 (control) received no treatment. At the beginning of the study, patients assigned to treatment groups received a hazelnut (HZN) supply sufficient for the complete duration of treatment, portioned into pre-weighed packages, and were asked to consume one portion per day for 8 consecutive weeks. The control group was advised to follow a nut-free diet for the subsequent 8 weeks. At enrollment, all children and their parents received nutritional recommendations based on CHILD1 dietary guidelines, as report in the previous study (Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents: Summary Report 2011).

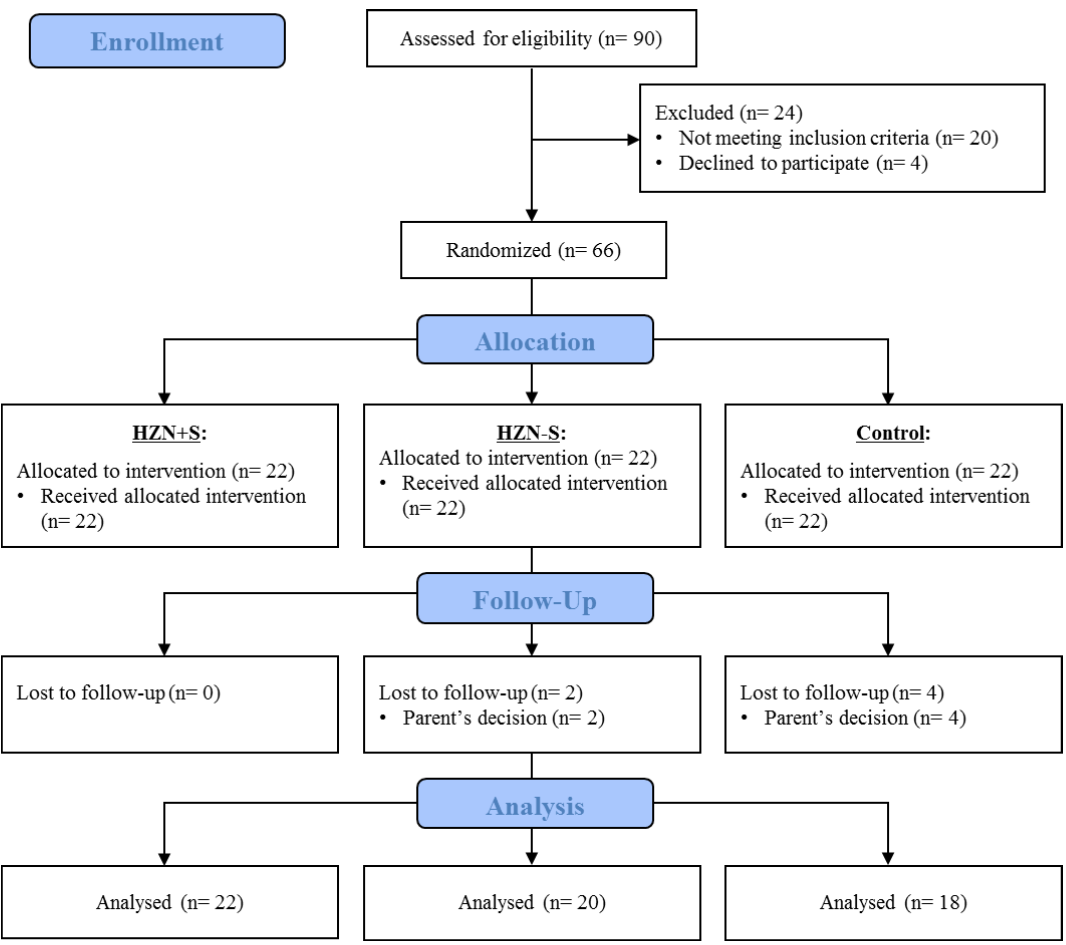
For the duration of the study, all participants were encouraged to maintain the same dietary and lifestyle habits (as assessed before enrollment) and to refrain from consuming any other kind of nuts (a list of prohibited foods was provided). In order to check the compliance to the dietary instructions, subjects and their families were asked to fill in weekly food diaries, before and after enrollment in the study. A nutritionist provided detailed instructions to patients and their parents on how to record food intake. Moreover, patients and their parents were periodically interviewed to verify their compliance to dietary recommendations and actual intake of nuts. In order to estimate the average daily energy and nutrient intake, dietary records were analyzed with MètaDieta® Software using the Italian Food Composition Tables (Carnovale & Marletta 2000).

At baseline and at the end of the study, each patient underwent a medical examination after an overnight fast, during which blood samples and the physical parameters (including height, weight, BMI, blood pressure) were obtained. Blood samples was used for the analysis of serum lipid profile, the RBC phospholipid composition, the DNA damage and oxidized LDL-C (ox-LDL) levels. Participants in the HZN groups were asked to give back any uneaten HZN package at the last visit. Compliance was assessed by weighing the eventual packages returned, and by analysing weekly food diaries filled at baseline and during the study.

Hazelnut preparation

Italian roasted HZNs *Corylus avellana L.*, (cultivar 'Tonda Gentile delle Langhe', from Piedmont, Italy), were provided peeled (HZN-S) or unpeleed (HZN+S), in pre-weighed vacuum packed portions. The amount of HZNs per portion was calculated on the basis of the doses that are generally advised to adults, adjusted on children body weight (0.43 g/kg of body weight on average, corresponding to portions of 15 to 30 g).

Figure 3.1 *CONSORT study flow-chart showing the process of patient selection and enrollment, allocation to the three study groups, and rate of patients completing the study.*



Notes: HZN+S: group treated with hazelnuts with skin; HZN-S: group treated with hazelnuts without skin.

Fat and bioactive composition of hazelnuts

HZNs composition in terms of total fat, sterols and tocopherols content was determined using standardized methods. The HZN oil was extracted using a cold-pressing method. The recovered oil was clarified and stored at -18°C until analyses. Fatty acids composition was obtained through the analysis of derived methyl esters by gas-chromatography, following the method described by Ficarra et al. (2010). The fatty acid concentration (expressed as mg of fatty acid/g of oil) was calculated according to AOAC 963.22 method. The content of minerals was measured by inductively coupled plasma mass spectroscopy (ICP-MS; Varian 820 ICP-MS) (Del Bo' et al. 2013). Polyphenol compounds were extracted following the method suggested by Ghirardello et al. (2016). The total phenolic content was assessed by spectrophotometry by means of the modified Folin–Ciocalteu method, and was expressed as mg of gallic acid equivalents (GAE) per g of sample. Polyphenol composition was determined using a Thermo-Finnigan Spectra-System HPLC equipped with a Finnigan Surveyor PDA Plus detector. The separation was achieved at 22 °C on a C18 RP Lichrospher 250 × 4.6 mm, 5-μm column equipped with a C18 RP Lichrospher 5-μm guard column. The mobile phase was trifluoroacetic acid/ultrapure water (solvent A) and methanol (solvent B); the flow rate was 0.8 mL min-1, and the injection volume was 20 μL. The PDA spectra were recorded over a wavelength (λ) range of 200 to 600 nm, and quantification was performed recording the peak area at a maximum λ of each compound. Identification was achieved by comparing the retention times and spectra of our samples with those of authentic standards. In addition, the antioxidant capacity (radical scavenging activity) of the HZN extracts was evaluated according to method of Ghirardello et al. (2016). The radical scavenging activity was measured by the discoloration of the purple-coloured methanol solution of the 2,2-diphenyl-1-picrylhydrazyl radical recorded at 515 nm. The results were expressed as μmoles of Trolox equivalent (TE) per g of sample.

Chemicals

Normal-melting-point (NMP) agarose, low-melting-point (LMP) agarose, EDTA, Na_2EDTA, Tris–acetate–EDTA buffer, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), RPMI-1640 medium, Histopaque 1077, fetal bovine serum (FBS), hydrogen peroxide (H_2O_2), ethidium bromide, N-lauroylsarcosine sodium salt, Triton X-100, Trizma base, sodium hydroxide (NaOH), bovine serum albumin (BSA) and sodium chloride (NaCl) were obtained from Sigma–Aldrich (St Louis, MO, USA). Formamidopyrimidine-DNA glycosylase (FPG) was kindly provided by Prof. Andrew Collins from the University of Oslo.

Blood sample collection, separation and storage

Fasting blood samples were collected into vacutainers containing lithium heparin as anticoagulant [(to obtain plasma, RBCs and peripheral blood mononuclear cells (PBMCs)] or silicon (to obtain serum). Plasma, serum and RBCs were separated as previously reported (see **study 3.1**).

PBMCs were isolated from heparin whole blood by density gradient using Histopaque 1077. A total of 100 μl of whole blood was gently mixed with 900 μl of cold RPMI medium in microfuge tubes. Then, 100 μl Histopaque 1077 was carefully added to the bottom of the tube and centrifuged at 200 ×g for 4 min at room temperature. The PBMCs were removed and washed with PBS, and centrifuged for 10 s at 5000 ×g at room temperature to pellet the cells. The supernatant was poured off and the pellet resuspended in 50 μl of PBS and used immediately for the determination of *ex-vivo* resistance to H_2O_2-induced DNA strand breaks. A different batch of isolated PBMC was diluted into an appropriate freezing medium composed of 50% FBS, 40% RPMI and 10% DMSO as a cryoprotectant and stored at -80°C for the subsequent determination of endogenous DNA damage.

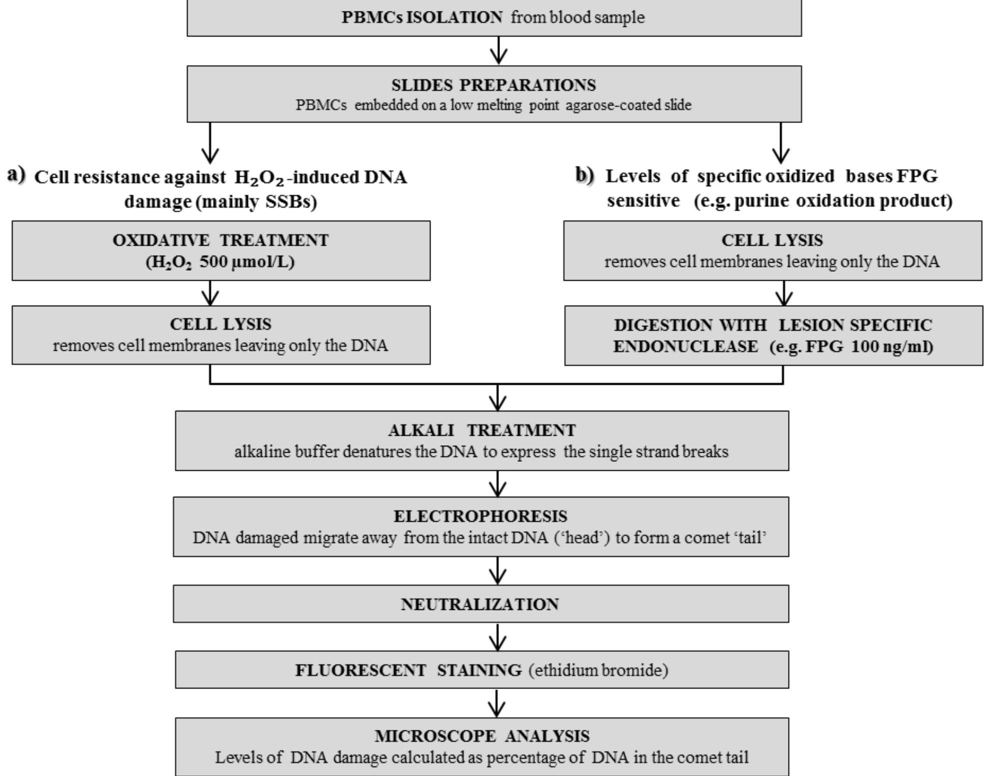
Evaluation of DNA damage through comet assay

The alkaline comet assay was performed for the determination of *ex-vivo* resistance to H_2O_2-induced DNA strand breaks and the determination of endogenous DNA damage as previously reported (Collins et al. 1996; Riso et al. 1999; Guarnieri et al. 2008). The main steps of comet assay are summarized in **Figure 3.2**.

Briefly, PBMCs suspension was mixed with LMP agarose (1.5% wt/vol) in Tris–acetate–EDTA buffer (pH 7.4, at 37°C) and pipetted onto a fully frosted microscope slide (Richardsons Supply Co. Ltd, London, UK) already precoated with NMP agarose (1% wt/vol in Tris–acetate–EDTA buffer). Each slide provided two gels containing ~10^4 cells.

Cell resistance against oxidatively induced DNA damage was measured by treating the cells with H_2O_2. Two slides prepared for each subject: one slide was treated with H_2O_2 (500 µmol/l in PBS) for 5 min at room temperature in the dark, whereas the other one slide was treated for 5 min with a solution of PBS alone (without H_2O_2; control slide). Following the oxidative treatment, slides were immersed in a lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, 1% N-lauroylsarcosine sarcosine sodium salt, pH 10 with NaOH; 1% Triton X-100 and 1% DMSO) for 1 h at 4°C in the dark. Slides were then transferred in a horizontal electrophoresis tank containing an alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA) and kept for 40 min at 4°C. Then, electrophoresis was carried out (25 V, 300 mA, 20 min) in the same alkaline solution at 4 °C in the dark. The samples were subsequently washed in a neutralizing buffer (0.4 M Tris–HCl, pH 7.5) for 15 min at 4°C in the dark, stained with ethidium bromide (2 µg/ml) for 20 min, washed in PBS, drained and coverslipped.

Figure 3.2 *Comet assay steps for the evaluation of cell resistance against H_2O_2 insult (**a**) and endogenous DNA damage (**b**) in PBMCs.*



The detection of endogenous oxidized DNA bases was performed in cryopreserved PBMCs, as previously reported (Collins et al. 1996; Riso et al. 2009) by means of the enzyme formamidopyrimidine DNA glycosylase (FPG), able to detect the oxidized purines (mainly 8-oxo-7,8-dihydroguanine). For this present study, cryopreserved PBMCs were used. In brief, cryopreserved PBMCs were rapidly thawed at 37°C and washed with fresh RPMI medium and cold PBS. Cell suspension was embedded in LMP agarose (1.5% wt/vol in Tris–acetate–EDTA buffer at pH 7.4, 37°C) and pipetted on fully frosted slides previously precoated with NMP agarose (1% wt/vol in Tris–acetate–EDTA buffer). After the lysis phase (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, 1% N-lauroylsarcosine sarcosine sodium salt, pH 10 with NaOH; 1% Triton X-100 and 1% DMSO, for 1 h at 4°C in the dark), slides were washed three times in a buffer (40 mM HEPES, 0.1 M KCl and 0.5 mM EDTA, pH 8.0 with KOH), for 5 min each. Then, one slide was treated with a solution of FPG enzyme (100 ng/ml in enzyme buffer: 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/ml bovine serum albumin, pH 8.0 with KOH), while the other slide (control) with buffer without FPG. Incubation was performed at 37°C for 45 min. The slides were then transferred to electrophoresis buffer and processed as previously described (see oxidatively induced DNA damage).

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

Analysis of oxidized-LDL

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

Analysis of serum lipids and FA composition of phospholipids in RBCs

Serum lipid concentrations (TC, HDL-C, TG, LDL-C and non-HDL-C) and RBCs phospholipids fatty acids analysis were performed as previously described in detail (see **study 3.1**).

Statistical analysis

Sample size was calculated taking into account the expected variation in the primary endpoints considered. In particular, 16 subjects per group were estimated to be sufficient to detect significant differences in DNA damage after HZN interventions, with a power of 80% and p=0.05. All data are presented as mean ± standard deviation (SD).

One-way ANOVA was used to compare the three groups (HZN+S, HZN-S and control group) at baseline. Two-way ANOVA was used to compare the effect of dietary treatment (HZN+S, HZN-S or control group) and time (baseline vs. 8 weeks) on the levels of DNA damage and ox-LDL. Differences were considered significant at $p \leq 0.05$; post-hoc analysis of the differences between treatments was assessed by the Least Significant Difference test considering $p \leq 0.05$ for statistical significance. Regression analysis was used to verify correlations between the variables under study at baseline and percent changes observed between pre-to-post intervention in the three groups. Statistical analysis was performed using STATISTICA software (Statsoft Inc, Tulsa, OK, USA).

3.2.2 RESULTS

Characterization of hazelnuts

The fat and bioactives composition of 100 g of HZN+S and HZN-S are reported in **Table 3.5**. 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).

Table 3.5 *Characterization of fat and bioactives composition of hazelnut Corylus avellana* L. *'Tonda Gentile delle Langhe'.*

	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

Notes: 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.

Subjects characteristics

Of the 90 hyperlipidemic children and adolescents initially screened for the study, 24 subjects did not meet the eligibility criteria. The remaining 66 eligible children agreed to participate to the experimentation. Six participants (4 females) dropped out from the study due to personal reasons, not related to the study. Thus, 60 hyperlipidemic children and adolescents (M/F, 34/26) successfully completed the 8-week intervention and were included in the data analysis (**Figure 3.1**). The study was performed between January and October 2015.

Main features of the three groups of patients at baseline and after treatment are reported in **Table 3.6**. Age ranged from 6.7 to 17.5 years, with a mean age ± SD of 11.6 ± 2.6 years. Mean serum lipid levels exceeded the 90th age and sex related percentiles, with the exclusion of HDL-C values, which were in the normal range. Body weight was in the normal range, except for five subjects presenting mild overweight, BMI remained unchanged throughout the treatment period. Blood pressure levels were in the normal range at baseline and during the entire study period. HZN consumption was appreciated and well tolerated by all patients.

Table 3.6 *Main sample features at baseline and after 8 weeks of intervention.*

Variables	**Control (*n*=18)**		**HZN+S (*n*=22)**		**HZN-S (*n*=20)**	
	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8
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
Sex (M/F)	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*
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*
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
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
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

Notes: BMI, body mass index; HZN+S, group treated with hazelnut with skin; HZN-S, group treated with hazelnuts without skin.
Values are expressed as mean ± SD. * Significantly different as compared to baseline (p≤ 0.05).

Energy and nutrient intake at baseline and during the study period are reported in **Table 3.7**. A significant increase of total fat (*p* <0.001), MUFA (*p* <0.05), and PUFA (*p* <0.001) intake and a reduction of carbohydrates (*p* <0.01), but not of total energy intake was registered following HZN treatments. No differences between baseline and the end of the study were observed in the control group.

Table 3.7 *Daily energy and nutrient intakes assessed by patient food diaries, before and after 8 weeks of treatment.*

Variables	Control (*n*=18)		HZN+S (*n*=22)		HZN-S (*n*=20)	
	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8
Energy (kcal)	1126.5 ± 281	1163.5 ± 14	1093.2 ± 194.7	1199.3 ± 180.1	1241.0 ± 210.2	1358.7 ± 211.0
Protein (% E)	17.0 ± 2.6	16.4 ± 2.9	17.5 ± 2.9	16.9 ± 2.6	16.5 ± 2.4	16.3 ± 2.3
CHO (% E)	50.7 ± 4.3	50.3 ± 3.9	51.2 ± 3.8	46.9 ± 3.6*	52.6 ± 3.9	48.6 ± 4.2*
Total fat (% E)	32.5 ± 3.0	33.3 ± 3.1	31.2 ± 2.9	36.2 ± 1.9*	30.9 ± 2.5	35.2 ± 2.8*
SFA (% E)	10.1 ± 2.6	10.0 ± 2.2	9.4 ± 1.4	9.6 ± 1.6	8.9 ± 1.9	8.6 ± 1.9
MUFA (% E)	14.7 ± 1.9	15.5 ± 3.0	13.1 ± 3.3	17.8 ± 6.5*	7.9 ± 2.5	9.0 ± 2.1*
PUFA (% E)	3.5 ± 0.9	3.3 ± 0.5	4.1 ± 2.3	7.6 ± 3.3*	3.3 ± 1.4	6.8 ± 3.5*
ω-3 (% E)	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.5	1.3 ± 0.6*	0.5 ± 0.3	1.0 ± 0.7*
ω-6 (% E)	2.2 ± 0.8	2.1 ± 0.4	2.8 ± 2.0	6.0 ± 2.8*	2.1 ± 1.2	5.4 ± 2.9*
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
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

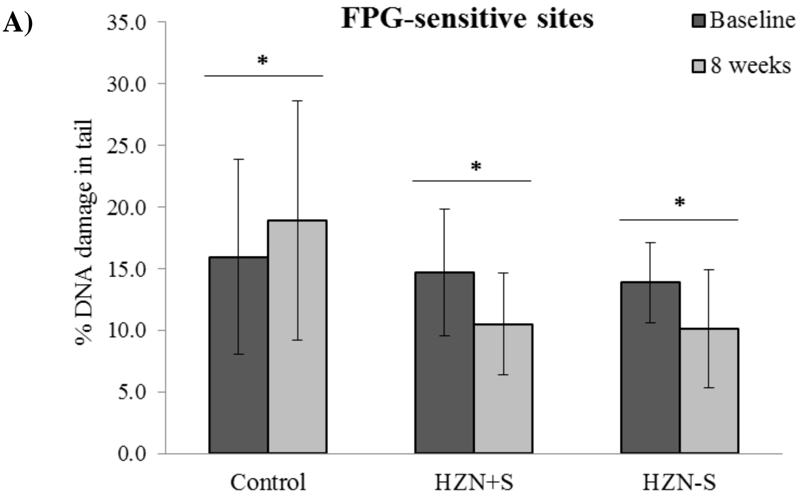
Notes: HZN+S, group treated with hazelnut with skin; HZN-S, group treated with hazelnuts without skin; CHO, carbohydrate; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids. Values are expressed as mean ± SD. * Significantly different as compared to baseline ($p \leq 0.05$).

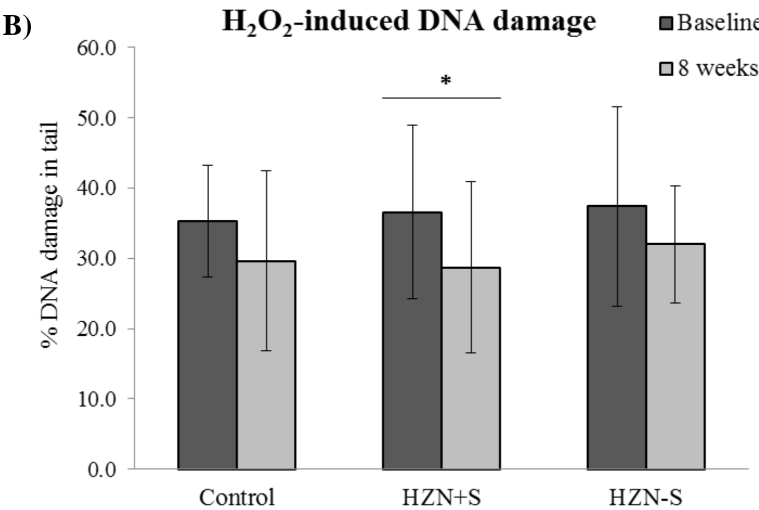
Effect of hazelnuts on endogenous and oxidatively-induced DNA damage

At baseline, hyperlipidemic children showed a mean percentage of endogenous and oxidatively induced DNA damage of 14.8 ± 5.6%, and 36.4 ± 11.7%, respectively. The regular intake of hazelnuts was associated with the reduction (time x treatment interaction, *p*= 0.0006) of the level of endogenous DNA damage by 18.9% (from 14.7 ± 5.1% at baseline, to 10.5 ± 4.1% after 8 weeks; *p*= 0.0029) and 22.5% (from 13.9 ± 3.3% at baseline, to 10.1 ± 4.7% after 8 weeks; *p*= 0.007), respectively after HZN+S and HZN-S intake (Figure 2a). On the contrary, an increase of 26.8% (from 15.7 ± 7.9 at baseline, to 18.9 ± 9.7 after 8-weeks; p= 0.04) in FPG-sensitive sites was registered in the control group at the end of the study period (**Figure 3.3-A**).

With regard to cell protection against oxidative stress, HZN+S treatment significantly decreased (time effect, *p*= 0.002) the level of oxidatively-induced DNA strand breaks by 16% (from 36.6 ± 12.4 at baseline, to 28.7 ± 12.2% after 8 weeks; *p*= 0.02) (**Figure 3.3-B**).

Figure 3.3 *Levels of endogenous (**A**) and oxidatively-induced (**B**) DNA damage, evaluated at baseline and after 8 weeks of treatment, in the control, HZN+S and HZN-S groups. * Significantly different as compared to baseline (p< 0.05).*

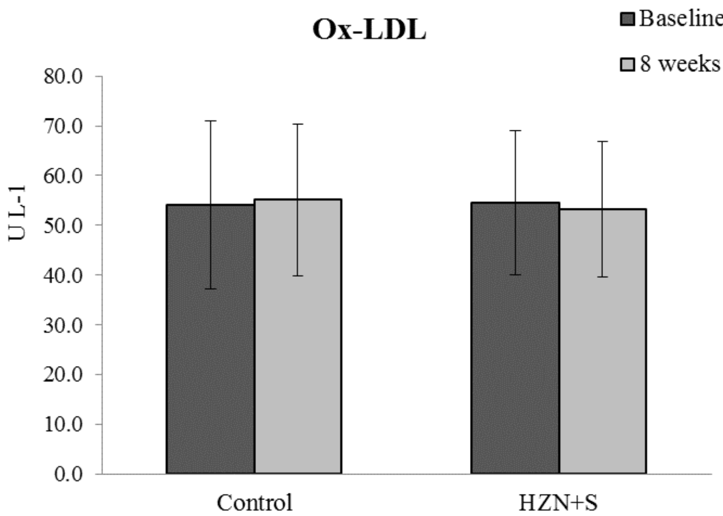




Effect of hazelnuts on oxidized LDL levels

Mean baseline ox-LDL levels in hyperlipidemic children were 54.4 ± 15.4 U/L, and did not significantly change in the control or HZN+S groups after intervention (**Figure 3.4**).

Figure 3.4 *Levels ox-LDL, evaluated at baseline and after 8 weeks of treatment, in the control and HZN+S groups. * Significantly different as compared to baseline (p< 0.05).*



Effect of hazelnuts on serum lipid profile

The serum lipoprotein concentrations are reported in **Table 3.8**.
The two-way ANOVA applied, considering time and treatments as dependent factors, revealed a significant effect of HZN treatment on serum LDL-C, HDL/LDL ratio and non-HDL-C ($p \leq 0.001$). In particular, both HZN+S and HZN-S significantly reduced the concentrations of LDL-C (-6.3%, p=0.005; and -6.0%, p=0.01, respectively), while increasing HDL-C/LDL-C ratio (+10.8%, p=0.007; and +10.2%, p=0.008, respectively), with respect to baseline. In addition, HZN-S reduced non-HDL-C (-6.2%, p=0.008). Serum lipid composition did not change over the study period.

Effect of hazelnuts on fatty acid composition of phospholipids in RBC membranes

The FA composition of phospholipids in RBC membranes at baseline and at the end of treatment are reported in **Table 3.9**.

The two-way ANOVA revealed a significant time x treatment interaction in terms of total MUFAs, oleic acid and MUFAs/SFAs ratio ($p \leq 0.01$), being the levels of MUFA and MUFA/SFA ratio after HZN were significantly different from those registered in the control group (p<0.01 and p=0.005, respectively). In particular, compared to baseline, both HZN+S and HZN-S treatments increased the level of MUFAs (+4.5%, p=0.0001; and +4.5%, p=0.0002), oleic acid (+4.0%, p=0.02; and HZN-S: +5.2%, p=0.004) and MUFAs/SFAs ratio (+5.7%, p=0.003; and +6.4%, p=0.002, respectively). The levels of palmitoleic and linoleic acids also decreased (-9.8%, p=0.004; -4.8%, p=0.002, respectively) after 8-week of HZN-S consumption, while no effects were observed in the HZN+S group.

The significant time x treatment interaction revealed in the control group differently from HZN treatments, time x treatment interaction revealed a significant increase of SFAs (+2.9%, p =0.04) and a decrease of PUFA n-6 (-2.7%, p =0.04) was evident at the end of the study, as compared to baseline. In particular, the margaric acid levels increased (+14.2%, p =0.05), while eicosenoic acid and dihomo-γ-linolenic acid decreased (-10.2%, p =0.01; -3.3%, p=0.04, respectively).

Table 3.8 *Serum lipid profile at baseline and after 8-weeks of each treatment.*

Serum lipid profile (mg/dl)	Control (*n*=18)		HZN+S (*n*=22)		HZN-S (*n*=20)	
	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8
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
TG	76.5 (35-185)	77.5 (38-165)	67.0 (32 - 194)	58.5 (37 - 159)	61.5 (46-300)	70 (41-264)
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
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*
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*
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
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*

Notes: HDL-C: high-density lipoprotein cholesterol; TC: total cholesterol; TG: triglycerides; LDL-C: low-density lipoprotein cholesterol; MUFAs: monounsaturated fatty acids; Values are expressed as mean ± SD or median (min-max). * Significantly different as compared to baseline ($p < 0.05$).

Table 3.9 *Fatty acid composition of phospholipids in RBC membranes before and after each treatment.*

RBC phospholipid composition (%)	Control (*n*=18)		HZN+S (*n*=22)		HZN-S (*n*=20)	
	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8
Total SFAs	48.52 ± 2.79[a]	49.85 ± 5.06*[b]	49.23 ± 2.23[ab]	48.81 ± 3.01[ab]	49.73 ± 1.61[b]	48.97 ± 2.05[ab]
Total MUFAs	18.57 ± 1.07[a]	18.49 ± 1.52[a]	18.3 ± 0.75[a]	19.10 ± 0.94*[b]	18.41 ± 0.95[a]	19.23 ± 1.01*[b]
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
MUFAs/SFAs ratio	0.38 ± 0.04[ab]	0.37 ± 0.03[a]	0.37 ± 0.03[a]	0.39 ± 0.04*[b]	0.37 ± 0.02[a]	0.39 ± 0.03*[b]
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
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
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
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
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
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
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
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
SFAs (%)						
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
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

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
17:0 (margaric acid)	0.46 ± 0.13[a]	0.51 ± 0.34*[b]	0.38 ± 0.19[c]	0.41 ± 0.16[cd]	0.47 ± 0.14[ab]	0.43 ± 0.12[ad]
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
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
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
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
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
MUFAs (%)						
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
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*
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*
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
20:1n-9 (eicosenoic acid)	0.23 ± 0.13[a]	0.19 ± 0.07*[b]	0.19 ± 0.02[b]	0.2 ± 0.03[ab]	0.19 ± 0.02[b]	0.20 ± 0.03[ab]
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
ω-6 PUFAs (%)						
18:2n-6 (linoleic acid)	10.68 ± 1.12[a]	10.69 ± 1.52[a]	10.78 ± 0.99[a]	10.79 ± 1.16[a]	10.59 ± 1.06[a]	10.06 ± 0.97*[b]
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
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
20:3n-6 (DGLA)	1.91 ± 0.43	1.82 ± 0.48*	1.87 ± 0.37	1.81 ± 0.39	1.94 ± 0.38	1.88 ± 0.41
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
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
ω-3 PUFAs (%)						
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
20:5n-3 (EPA)	0.35 ± 0.13	0.33 ± 0.16	0.36 ± 0.22	0.33 ± 0.14	0.34 ± 0.17	0.34 ± 0.17
22:5n-3 (DPA)	1.14 ± 0.27	1.05 ± 0.35	1.07 ± 0.14	1.03 ± 0.19	1.09 ± 0.16	1.09 ± 0.15
22:6n-3 (DHA)	3.58 ± 0.99	3.33 ± 1.17	3.22 ± 0.60	3.17 ± 0.59	3.29 ± 0.68	3.42 ± 0.71

Notes: DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LC-PUFAs, long chain polyunsaturated fatty acids (C ≥20, double bonds ≥3); MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; ω-6, omega-6; ω-3, omega-3; RBC, red blood cell; SFAs, saturated fatty acids. Omega-3 index: sum of EPA + DHA. Values are expressed as mean ± SD.
* Significantly different as compared to baseline ($p < 0.05$). Data with different letters within the same row differ significantly ($p < 0.05$).

Correlation between serum lipid profile and markers of oxidative stress

At baseline, a positive correlation between LDL-C concentrations and H_2O_2-induced DNA damaged (r= 0.34, *p*=0.04) was detected. Furthermore, serum TC, LDL-C and non-HDL-C levels were directly related to ox-LDL (r= 0.84, *p*<0.001; r= 0.85, *p* <0.001; r= 0.87, *p* <0.001, respectively) in the subgroup of subjects analyzed (n=40).

Interestingly, the analysis of correlation performed on percentage changes (post-to-pre intervention) registered for the variables under study confirmed a direct association between ox-LDL concentrations and the levels of TC, LDL-C and non-HDL in both control (r= 0.57, p =0.014; r= 0.73, *p*=0.001; r= 0.72, *p*= 0.001, respectively) and HZN+S group (r= 0.60, p= 0.004; r= 0.56, *p*= 0.008; r= 0.69, *p*< 0.001, respectively).

Moreover, an inverse correlation between the percentage variation in endogenous DNA damage and changes in TC, HDL-C and non-HDL concentrations (r= -0.67, p= 0.002; r= -0.64, p= 0.004; r= -0.54, *p*= 0.020, respectively) were observed following HZN-S treatment.

3.3 EFFECT OF HEMPSEED OIL SUPPLEMENTATION ON SERUM LIPIDS PROFILE AND FATTY ACID COMPOSITION OF ERYTHROCYTE PHOSPHOLIPIDS

3.3.1 SUBJECTS AND METHODS

Study design

The study was an 8-week randomized, single blind, controlled, two-arm parallel-group. A total of 36 hyperlipidemic children and adolescents were selected as previously reported (see **Study 3.1**) and randomly assigned to 1 of the 2 treatment groups of 18 participants each. Patients assigned to the first group (control) were asked to maintained their diet based on CHILD1 guidelines throughout the 8 week of study period (without dietary supplements). Whereas, subjects assigned to group supplemented with hempseed oil (HSO) additionally receive 3 g/day of HSO supplement (4 capsules), providing 700 mg/die of ALA and other bioactives. In **Table 3.10** is reportented the nutritional composition of the study supplement, according to the manufacturer's specification. The study supplement AlfaLife®, produced by hempseed *Cannabis sativa* L., was prepared as gelatine-soft gel capsules and was kindly provided by Freia Farmaceutici Srl (Milan, Italy).

All individuals involved in the trial, including doctors, research staff and parents of children, were aware of the specific product administered. At the beginning of the study, patients allocated to HSO group received a supply of supplement sufficient for the complete duration of treatment. For the duration of the experiments, all participants were encouraged to maintain the same dietary and lifestyle habits (as assessed before enrollment) and to abstain from consuming any other kind of supplements and ALA-rich products during the intervention (a list of prohibited foods was provided). In order to check the compliance to the dietary instructions, subjects and their families were asked to fill in weekly food diaries, before and after enrollment in the study. A nutritionist provided detailed instructions to patients and their parents on how to record food intake. Daily energy and nutrient intake were analyzed with MètaDieta® Software using the Italian Food Composition Tables (Carnovale & Marletta 2000). Compliance was also assessed by counting the eventual capsules returned.

At beginning and at the end of the intervention period, each patient underwent a medical examination after an overnight fast, during which blood samples and the physical parameters (including height, weight, BMI and blood pressure) were obtained. The analysis of serum lipid concentrations (TC, HDL-C, TG, LDL-C and non-HDL-C) and FA composition of RBC phospholipids were performed as previously reported (see **Study 3.1**). Participants in the HSO group were asked to give back any left-over capsules at the last visit.

Table 3.10 *Nutritional composition of HSO supplement, according to the manufacturer specifications.*

Hampseed oil supplement	100 g*	Daily dose (4 capsules, 3 g)
Energy, kcal	896	27
Carbohydrates, g	-	-
Protein, g	-	-
Total Fats, g	> 99.5	3
Total SFAs, g	10.4	0.3
Total MUFAs, g	11.8	0.4
Oleic acid, g	9.0	0.3
Total PUFAs, g	75.5	2.3
ALA	23.0	0.7
EPA	-	-
DHA	-	-
Stearidonic acid	1.1	0.03
LA	48.3	1.4
γ-linolenic acid	3.1	0.1
Cholesterol, g	-	-
Vitamin A, UI	9.5	0.3
Vitamin E, UI	14.9	0.4
Niacin (B3), mg	< 0.001	< 0.0001
Calcium, mg	9.8	0.3
Phosphorus, mg	0.013	< 0.001
Iron, mg	0.004	< 0.001
Magnesium, mg	0.004	< 0.001

Notes: ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HSO, group treated with hempseed oil; LA, linoleic acid; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids.

Statistical analysis

Sample size was calculated from previous studies in order to detect significant differences in the serum lipid concentration and FA composition of RBC phospholipids (Del Bo' et al. 2013; Orem et al. 2013). In particular, 18 subjects per group were estimated sufficient to demonstrate a 5% variation of LDL-C concentration after the supplementation with a p value of 0.05 and a power of 80%. Analysis of difference was applied to compared the effect of treatments (HSO vs control group) on lipid profile. Since baseline levels of FAs in RBCs in control subjects differed from HSO group, the analysis was carried out on percentage changes (obtained as differences between post- to pre-treatment) calculated for each variable considered. Data are reported as mean ± standard deviation (SD) or by mean changes (described as mean of single variation), with 95% confidence interval (CI). STATISTICA software (Statsoft Inc., Tulsa, OK, USA) was used for statistical analysis of data. Significance of difference was set as $p \leq 0.05$.

3.3.2 RESULTS

Subject characteristics

All families of eligible children agreed to participate in the study. A total of 36 hyperlipidemic subjects (23/13, M/F) aged between 6-15 years old (mean age 11.8 ± 2.4) successfully completed the 8-week intervention and were included in the data analysis: 2 patients were affected by FH, 9 by FCHL and 25 by PHC. The supplementation was well tolerated by almost all participants in the HSO group.

Characteristic of patients at baseline and after the study period are reported in **Table 3.11**. Blood pressure levels and BMI were in the normal range (except for eight subjects slightly overweight) and remained unchanged throughout the treatment period.

Energy and nutrient intake at baseline and during the study period did not changed, as reported in **Table 3.12**.

Table 3.11 *Baseline characteristics of subjects in the two study groups.*

Variables	Control (*n*=18)		HSO (*n*=18)	
	Baseline	Week 8	Baseline	Week 8
Age (years)	12.2 ± 2.3	12.4 ± 2.3	11.3 ± 2.4	11.5 ± 2.4
Sex (male/female)	13/5		10/8	
Weight (kg)	49.5 ± 16.5	50.0 ± 16.6	48.8 ± 16.7	49.4 ± 16.3
Height (cm)	151.8 ± 15.6	152.9 ± 16.0	149.5 ± 14.6	150.8 ± 14.4
BMI (kg/m^2)	20.9 ± 3.9	20.8 ± 4.0	21.2 ± 4.2	21.2 ± 4.1
SBP (mmHg)	106.8 ± 9.8	109.0 ± 7.5	108.3 ± 12.4	105.0 ± 9.2
DBP (mmHg)	68.0 ± 5.1	67.1 ± 6.9	66.1 ± 11.4	62.8 ± 10.0

Notes: BMI, body mass index. DBP, diastolic blood pressure; HSO, hempseed oil group; SBP, sistolic blood pressure. Values are reported as mean ± SD.

Table 3.12 *Daily energy and nutrient intake during the intervention period in the control and HSO group.*

Variables	Control (*n*=18)			HSO (*n*=18)		
	Baseline	Week 8	*p*	Baseline	Week 8	*p*
Energy (kcal)	1126.5 ± 281	1163.5 ± 14	0.675	1062.6 ± 221.9	1058 ± 181.7	0.828
Protein (% of E)	17.0 ± 2.6	16.4 ± 2.9	0.633	15.7 ± 2.1	15.1 ± 2.4	0.500
Carbohydrate (% of E)	50.7 ± 4.3	50.3 ± 3.9	0.781	53.8 ± 4.5	53.1 ± 3.4	0.608
Total fat (% of E)	32.5 ± 3.0	33.3 ± 3.1	0.512	30.5 ± 4.79	31.9 ± 4.38	0.395
SFA (% of E)	10.1 ± 2.6	10.0 ± 2.2	0.870	8.9 ± 1.7	9.6 ± 2.3	0.352
MUFA (% of E)	14.7 ± 1.9	15.5 ± 3.0	0.473	13.8 ± 3.31	15 ± 3.3	0.306
PUFA (% of E)	3.5 ± 0.9	3.3 ± 0.5	0.594	3.4 ± 0.82	3.3 ± 0.5	0.515
ω-3 (% of E)	0.6 ± 0.1	0.6 ± 0.1	0.958	0.6 ± 0.13	0.6 ± 0.12	0.703
ω-6 (% of E)	2.2 ± 0.8	2.1 ± 0.4	0.612	2.4 ± 0.76	2.2 ± 0.4	0.44
Fibers (g)	9.0± 2.6	9.9 ± 2.5	0.380	10.7 ± 2.8	9.8 ± 2.8	0.497
Cholesterol (mg)	137.5 ± 35.9	138.8 ± 59.1	0.949	137.6 ± 75.6	128.9 ± 44.8	0.759

Notes: CHO, carbohydrate; HSO, group treated with hempseed oil; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids.
Values are expressed as mean ± SD. * Significantly different as compared to baseline ($p \leq 0.05$).

Effect of hempseed oil supplementation on serum lipid profile and RBC phospholipid composition

The serum lipid concentrations and FA composition of phospholipids in RBCs at baseline and mean changes after treatments are reported in **Table 3.13** and **Table 3.14**, respectively. For each parameter, post-treatment variations, with respect to baseline, were considered for the statistical analysis.

At baseline, TC, LDL-C and non-HDL-C levels exceeded the 90th percentile for age and sex, while HDL-C values were in the normal range. Compared with control group, the analysis of differences revealed a significant effect of HSO treatment in the reduction of HDL/LDL ratio ($p<0.0001$), while other serum lipid levels did not change over the study period (**Table 3.14**).

In RBC phospholipids, 8-week of HSO supplementation led to a significant ($p<0.001$) decrease of total SFA and MUFA levels and an increase ($p<0.0005$) of total PUFA, n-3 and n-6 PUFA, and n-3 and n-6 LC-PUFA levels, when compared to control group. Moreover, in the HSO group a significant improvement ($p<0.001$) of omega-3 index was observed after HSO treatment compared to control, even if the value remained lower than the cut-off level (<4%). The completed list of variation of the single FA concentration in RBCs are reported in **Table 3.14**. Notably, despite the level of ALA in RBCs did not differ between the two study groups, a significant increase of RBC EPA and DHA, possibly derived from the enzymatic convertion of ALA, was observed after HSO supplementation.

Table 3.13 *Serum lipid profile evaluated at baseline and post-treatment variation observed after 8-weeks of treatments in both control and HSO groups.*

Serum lipids (mg/dl)	**Control (n=18)**		**HSO (*n*=18)**	
	Baseline	Δ (95% CI)	Baseline	Δ (95% CI)
TC	210.3 ± 50.0	-6.2 (-19.7; 7.2)	207 ± 14.9	-4.5 (-13.6; 4.6)
TG	76.5 (35-185)	-6.3 (-24.1; 11.6)	86.4 ± 44.0	16.0 (-3.6; 35.6)
HDL-C	55.4 ± 14.9	0.1 (-3.3; 3.5)	58.2 ± 11.9	-1.8 (-5.2; 1.7)
LDL-C	136.7 ± 45.2	-4.8 (-13.6; 4.0)	136.2 ± 18.7	-10.3 (-21.4; 0.7)
HDL/LDL	0.5 ± 0.2	0.04 (-0.01; 0.08)^{a}	3.7 ± 0.8	-3.25 (-3.73; -2.78)^{b}
Non-HDL-C	154.9 ± 47.5	-6.3 (-17.6; 4.9)	153.4 ± 23.1	3.4 (-31.2; 37.9)

Notes: HDL-C, high-density lipoprotein cholesterol; HSO, group treated with hempseed oil; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.
Values are expressed as mean ± SD at baseline and as changes (Δ) calculated considering post-treatment variations (calculated as differences between post- to pre-treatment), with 95% confidence interval (CI min; max). Data with different letters within the same row differ significantly (p<0.05).

Table 3.14 *FA composition of RBC phospholipids evaluated at baseline and post-treatment variation observed after 8-weeks of treatments in control and HSO groups.*

FA composition of	Control (*n*=18)		HSO (*n*=18)	
RBC phospholipids (%)	Baseline	Δ (95% CI)	Baseline	Δ (95% CI)
Total SFAs	48.52 ± 2.79	+3.10 (0.50; 5.70)[a]	59.59 ± 7.33	-5.02 (-8.97; -1.07)[b]
Total MUFAs	18.57 ± 1.07	+0.40 (-0.30; 1.09)[a]	21.91 ± 2.09	-2.12 (-3.23; -1.01)[b]
Total PUFAs	32.91 ± 2.75	-3.50 (-6.62; -0.37)[a]	18.50 ± 8.25	+7.14 (2.48; 11.81)[b]
Total PUFAs n-3	5.16 ± 1.28	-0.83 (-1.51; -0.16)[a]	1.68 ± 1.73	+1.57 (0.60; 2.55)[b]
Total PUFAs n-6	25.90 ± 1.26	-2.40 (-4.69; -0.10)[a]	15.26 ± 5.92	+5.39 (1.82; 8.96)[b]
PUFAs n-3/n-6	0.20 ± 0.05	-0.02 (-0.04; 0.00)[a]	0.09 ± 0.06	+0.05 (0.02; 0.09)[b]
Total LC-PUFAs n-3	5.08 ± 1.28	-0.83 (-1.50; -0.16)[a]	1.57 ± 1.73	+1.57 (0.60; 2.53)[b]
Total LC-PUFAs n-6	14.89 ± 1.47	-2.08 (-3.93; -0.24)[a]	6.77 ± 4.60	+4.08 (1.39; 6.76)[b]
LC-PUFAs n-3/n-6	0.34 ± 0.08	-0.02 (-0.04; 0.01)[a]	0.19 ± 0.08	+0.08 (0.04; 0.13)[b]
Omega-3 index	3.94 ± 1.09	-0.65 (-1.19; -0.10)[a]	1.12 ± 1.24	+1.18 (0.47; 1.88)[b]
SFAs				
14:0 (myristic acid)	0.38 ± 0.07	+0.10 (-0.02; 0.23)[a]	0.53 ± 0.16	-0.07 (-0.16; 0.03)[b]
15:0 (pentadecanoic acid)	0.16 ± 0.03	+0.02 (0.00; 0.04)[a]	0.20 ± 0.05	-0.03 (-0.06; 0.00)[b]
16:0 (palmitic acid)	23.83 ± 1.07	+1.58 (0.10; 3.07)[a]	29.54 ± 3.96	-3.27 (-5.73; -0.80)[b]
17:0 (margaric acid)	0.46 ± 0.13	+0.16 (0.02; 0.31)[a]	0.61 ± 0.18	-0.10 (-0.22; 0.02)[b]
18:0 (stearic acid)	15.02 ± 1.53	+0.34 (-0.19; 0.87)	17.17 ± 2.72	-0.40 (-1.92; 1.12)
20:0 (arachidic acid)	0.63 ± 0.25	+0.02 (-0.11; 0.16)	0.77 ± 0.14	-0.11 (-0.19; -0.02)
22:0 (behenic acid)	2.07 ± 0.36	+0.22 (0.02; 0.41)[a]	2.77 ± 0.51	-0.45 (-0.67; -0.22)[b]
23:0 (tricosanoic acid)	0.30 ± 0.05	+0.04 (0.00; 0.08)[a]	0.40 ± 0.06	-0.06 (-0.09; -0.03)[b]
24:0 (lignoceric acid)	5.68 ± 0.81	+0.61 (0.13; 1.10)[a]	7.58 ± 2.33	-0.54 (-1.49; 0.41)[b]
MUFAs				
16:1n-9 (hypogeic acid)	0.11 ± 0.02	+0.003 (-0.012; 0.018)	0.12 ± 0.04	-0.01 (-0.03; 0.01)
16:1n-7 (palmitoleic acid)	0.22 ± 0.06	+0.05 (-0.05; 0.15)[a]	0.25 ± 0.08	-0.02 (-0.04; 0.00)[b]
18:1n-9 (oleic acid)	10.86 ± 1.09	+0.20 (-0.23; 0.63)[a]	12.67 ± 1.47	-1.14 (-1.90; -0.38)[b]
18:1n-7 (vaccenic acid)	1.08 ± 0.07	-0.02 (-0.07; 0.03)	1.16 ± 0.17	-0.10 (-0.15; -0.04)
20:1n-9 (eicosenoic acid)	0.23 ± 0.13	-0.03 (-0.10; 0.05)	0.26 ± 0.07	-0.03 (-0.06; 0.00)
24:1n-9 (nervonic acid)	6.06 ± 1.21	0.19 (-0.39; 0.77)	7.45 ± 1.10	-0.83 (-1.30; -0.35)
n-6 PUFAs				
18:2n-6 (LA)	10.68 ± 1.12	-0.26 (-0.96; 0.44)[a]	8.22 ± 1.72	+1.28 (0.26; 2.29)[b]
18:3n-6 (γ-linolenic acid)	0.06 ± 0.02	+0.002 (-0.013; 0.017)	0.07 ± 0.03	+0.01 (-0.02; 0.05)
20:2n-6 (EDA)	0.27 ± 0.10	-0.04 (-0.10; 0.05)	0.20 ± 0.08	+0.03 (-0.01; 0.07)
20:3n-6 (DGLA)	1.91 ± 0.43	-0.19 (-0.37; 0.02)[a]	0.94 ± 0.52	+0.43 (0.13; 0.72)[b]
20:4n-6 (ARA)	11.11 ± 1.01	-1.60 (-3.03; -0.17)[a]	4.80 ± 3.38	+3.06 (1.05; 5.07)[b]
22:4n-6 (adrenic acid)	1.88 ± 0.57	-0.31 (-0.63; 0.01)[a]	1.03 ± 0.90	+0.59 (0.13; 1.05)[b]
n-3 PUFAs				
18:3n-3 (ALA)	0.08 ± 0.03	-0.003 (-0.022; 0.015)	0.10 ± 0.04	+0.01 (-0.02; 0.04)
20:5n-3 (EPA)	0.35 ± 0.13	-0.18 (-0.32; 0.04)[a]	0.18 ± 0.12	+0.11 (0.03; 0.18)[b]
22:5n-3 (DPA)	1.14 ± 0.27	-0.02 (-0.08; 0.04)[a]	0.45 ± 0.50	+0.39 (0.12; 0.66)[b]
22:6n-3 (DHA)	3.58 ± 0.99	-0.63 (-1.15; -0.10)[a]	0.94 ± 1.13	+1.07 (0.43; 1.71)[b]

Notes: ALA, α-linolenic acid; ARA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EDA, eicosadienoic acid; EPA, eicosapentaenoic acid; HSO, group treated with hempseed oil; LA, linoleic acid; LC-PUFAs, long chain polyunsaturated fatty acids (C ≥20, double bonds ≥3); MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; n-6 PUFAs, omega-6 PUFAs; n-3 PUFAs, omega-3 PUFAs; RBC, red blood cell; SFAs, saturated fatty acids. Omega-3 index: sum of EPA + DHA.

Values are expressed as mean ± SD at baseline and as changes (Δ) calculated considering post-treatment variations (calculated as differences between post- to pre-treatment), with 95% confidence interval (CI min; max). Data with different letters within the same row differ significantly ($p<0.05$).

3.4 DISCUSSION

A proper nutritional intervention is considered the primary strategy to prevent the onset of chronic degenerative diseases. This concept is relevant when applied to the general population, and, even more, to children affected by primary hyperlipidemias whose future CV risk is relatively high. In fact, hyperlipidemia is a well established risk factor for atherosclerosis, conceivably associated with an increased oxidative stress (Nourooz-Zadeh et al. 2001; Botto et al. 2002; Harangi et al. 2002; Real et al. 2010; Da Silva Pereira et al. 2013; Tangvarasittichai 2015) already from the pediatric age (Martino et al. 2008; Kliemann et al. 2012; Loffredo et al. 2013). Thus intervention treatments must start since childhood to improve cardiovascular prognosis.

To the best of our knowledge, for the first time it was documented that specific dietary interventions with HZNs (with or without skin) or hempseed oil, sources of unsaturated fats and other bioactives, are effective in the management of pediatric hyperlipidemia, improving serum lipid profile and RBCs phospholipid composition, but also oxidative stress markers as demonstrated through the HZN intervention. In addition, our study is the first providing reference value on the FA composition of RBC phospholipids in a group of Italian children and adolescents with primary hyperlipidemia.

In our study, hyperlipidemic children and adolescents were characterized for serum lipid profile and RBC phospholipid composition, showing an overall low omega 3 index and differences according to sex and patient diagnosis of primary hyperlipidemia. The FA composition of RBCs phospholipid 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 children. In our characterisation, children affected by FH showed significantly lower levels of MUFA, vaccenic acid and n-6 PUFA γ-linolenic acid in RBCs, with respect to patients affected by other genetic forms of primary hyperlipidemia (FCH or PCH). 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 increased cholesterol and SFA concentrations in erythrocytes, that were related to the atherosclerotic condition (Taylor 1994; Lausada et al. 2007; Vayá et al. 2009; Novgorodtseva et al. 2011). Moreover, in the present study we observed sex-related differences in RBC phospholipids among hyperlipidemic children, showing a significantly greater proportion of the SFA stearic acid, and lower proportion of n-6 PUFA (in particular DGLA) in females. Lower DGLA values were also observed in serum glycerophospholipids of healthy young girls when compared with boys (Glaser et al. 2010). In addition, in females we found a trend towards lower levels of MUFA and MUFA/SFA ratio in RBCs with respect to males. Venäläinen et al. (2016) reported that a higher plasma level of SFA myristic acid, MUFA palmitoleic acid and reduced concentrations of n-6 PUFA (linoleic acid) was correlated with increased cardiometabolic risk in children. However, since no similar studies were conducted in hyperlipidemic children, the comparison of our data with other from literature is extremely difficult.

Correlation analysis showed a positive association between 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; Fernandez et al. 2005; Mukuddem-Peterson et al. 2005).

Finally, as previously anticipated, in the whole sample of hyperlipidemic children and adolescent we registered a low mean omega-3 index (EPA+ DHA <4%), a factor that contribute to classify them as at future high risk for CVD, according to Harris & Von Schacky (2004). In the pediatric population, only one other study examined the omega-3 index and concluded that a greater

proportion of obese children had a lower index compared with non-obese children (Burrows et al. 2011). However, in an observational study conducted on 16000 patients it was documented a tendency to rise omega-3 index through the lifespan, with an increase by about 1.5 percentage points across the decades (Harris et al. 2013). Since EPA and DHA derive from essential fatty acids, our results suggest a likely reduced synthesis due to the lipid metabolism disorder. However, is noteworthy that our patients did not regularly consume LC-PUFA rich food sources, confirming the importance of the quality of dietary FAs intake. In this regard, the use of whole dietary strategies and/or specific supplements (e.g. with PUFA, MUFA or other food bioactives) could be fundamental to improve this index above all in at risk subjects such as hyperlipidemic children.

Dietary treatment design to replace the intake of SFA and cholesterol with unsaturated fatty acids are considered the primary approach in the management of hyperlipidemia and CVD prevention (Gidding et al. 2005), especially at pediatric age. Clinical and epidemiological studies in hyperlipidemic and healthy adults have consistently demonstrated the favorable effects of nuts in the reduction of coronary artery disease (Zhou et al. 2014) and in the overall health improvement (Alexiadou & Katsilambros 2011; Ros 2015; Grosso & Estruch 2016).

HZNs are among the most popular tree nuts consumed worldwide, and the second richest source of MUFAs - mainly oleic acid- vitamin E and other phytochemicals with remarkable cholesterol lowering effect (i.e. phytosterols) and antioxidant properties (i.e. polyphenols, vitamin E) (López-Uriarte et al. 2009; Bolling et al. 2011). In our study, Italian hazelnuts *Corylus avellana* L. 'Tonda Gentile delle Langhe' were administer peeled (HZN-S) or unpeeled (HZN+S) to evaluate potential differences in the antioxidant properties associated with hazelnut skin. Indeed, a portion of HZN+S or HZN-S provides similar amounts of MUFAs, phytosterols, α-tocopherol and minerals, but different amount of polyphenols (mostly gallic acid), which are mainly present in the skin. Although generally considered a byproduct of peeled HZNs, skins are an interesting edible source of polyphenol compounds with antioxidant properties (Del Rio et al. 2011). Moreover, aavailable evidence suggests a lowering effect of polyphenol-rich foods on cholesterol and postprandial triglycerides (Sanchez-Gonzalez et al. 2015) even if, studies ascertaining the impact of nut polyphenols in the modulation of lipid profile are scarce.

The primary aim of our intervention study was to assess the effect of regular intake of HZN+S or HZN-S in the modulation of DNA damage and ox-LDL levels, both markers of oxidative stress, in children and adolescents with primary hyperlipidemia. In particular, after HZN consumption we observed a reduced level of endogenous (FPG-sensitive site) and H_2O_2-induced DNA strand breaks, while we could not demonstrate any effect of HZN+S on ox-LDLs, whose levels remained stable in treated and untreated patients.

To our knowledge, no previous study investigated the impact of HZN intake on DNA damage in patients at risk of oxidative stress, like children with primary hyperlipidemia, nor the potential different impact associated with the consumption of peeled vs. unpeeled hazelnuts. Based on our data, both types of HZNs significantly reduced the levels of endogenous DNA damage, but only HZN+S significantly decreased the oxidatively-induced DNA damage in PBMCs, a suggested marker of antioxidant protection or cell resistance from an *ex vivo* induced oxidative insult. Indeed, it is noteworthy that high amounts of vitamin E, with well-recognized antioxidant properties, are regularly introduced through HZN intake. Moreover, it may be hypothesized that the higher phenolic content and overall antioxidant capacity demonstrated in HZN+S increased treatment efficacy. In fact, the ability of polyphenol-rich foods to increase antioxidant cell protection by reducing DNA oxidized bases and strand breaks have been documented in several *in vivo* studies (Del Bo' et al. 2015; Martini et al. 2016).

Correlation between regular nuts consumption and reduced markers of oxidative DNA damage was also observed in other intervention studies conducted in adults supplemented with almonds (Li et al. 2007; Jia et al. 2009), brazil nuts (Cominetti et al. 2011), or mixed nuts (López-Uriarte et al. 2010). In particular, two studies carried out in smokers demonstrated that daily almond

supplementations for 4 weeks could decrease the oxidative DNA damage mediated by tobacco (Li et al. 2007; Jia et al. 2009). Furhtermore, 8-week intake of selenium-rich Brazil nuts significantly decreased the levels of DNA strand breaks in a group of obese women with wild-type genotype of glutathione peroxidase1 Pro198Leu polymorphism (Cominetti et al. 2011). A significant reduction of DNA damage, evaluated as 8-OH-dG, was also observed after 8-week treatment with mixed nuts, including hazelnuts, in adult patients with metabolic syndrome (López-Uriarte et al. 2010). However, to our knowledge, no other available studies investigated the relationship between HZN consumption and oxidative stress in humans.

Despite HZNs are rich in MUFAs, which have been associated with reduced susceptibility of LDL to oxidation (Berry et al. 1992), we did not observe significant changes in ox-LDL levels after HZN treatments. A possible explanation is that our study was carried in children with primary hyperlipidemia, showing mild LDL-C elevation and instructed to healthy lifestyle recommendations. The unchanged ox-LDL levels were also observed in other studies following the intake of different nuts (Ros et al. 2004; López-Uriarte et al. 2010). As expected, cholesterol values were directly correlated to ox-LDL. Moreover, the LDL-C concentrations were positively associated with the levels of oxidatively-induced DNA damage, suggesting that both markers may be considered of special interest from the clinical point of view, providing an overall indication of the oxidative status secondary to hyperlipidemia.

A secondary aim of this study was to investigate the lipid lowering effects of HZNs as well as potential additional benefit of their skin, in the management of pediatric hyperlipidemia. Our findings showed that 8-week consumption of both HZN+S and HZN-S significantly reduced serum levels of LDL-C by about 6%, when compared to basal values. LDL-C is considered at present the best biomarker of CV risk when applicable, and different cut-off levels are recognized as target levels. This caution applies also to children, in order to avoid the damaging effects related to the hyperlipidemia later in life (Catapano et al. 2011). In particular, serum LDL-C levels decreased towards 130 mg/dL which represents the LDL-C target to be reached in childhood when primary hyperlipidemia occurs, while LDL-C levels of 110 mg/dL or less is suggested for patients with FH (Reiner et al. 2011). Moreover, following HZN interventions, hyperlipidemic children showed an overall improvement of non-HDL-C levels and HDL-C/LDL-C ratio, biomarkers that greatly correlates with the CV risk. Although HDL-C levels did not change, it should be mentioned that HDL-C/LDL-C ratio is more related to the risk of CVD events than HDL-C "*per se*".

Overall, these results are in line with other observations reported in dietary intervention studies in healthy (Durak et al. 1999; Yücesan et al. 2010) and hyperlipidemic adults or young adults (Mercanligil et al. 2007; Tey et al. 2011; Orem et al. 2013), showing an improved plasma lipid and lipoproteins profile following the regular HZNs intake. For example, similarly to our findings, Orem et al. (2013) reported that 4-week intervention with HZN-enriched diet (30 g/day), significantly reduced TC (-7.8%), triacylglycerol (-7.3%) and LDL-C (-6.2%), while increased serum HDL-cholesterol levels (6.1%), in hypercholesterolemic adult volunteers. Furthermore, in a randomized crossover study, the consumption of 30 g/day of ground, sliced or whole HZNs for 4 weeks improved lipoprotein profile in mildly hypercholesterolemic subjects (Tey et al. 2011). Although it should be recognized the good nutritional quality of HZN+S, providing a higher amount of polyphenols, the positive changes observed on lipid levels in our study were comparable following both interventions with HZN+S or HZN-S. The lipid lowering effect of polyphenols is widely debated. A study performed in atherosclerosis-susceptible mice showed that the consumption of whole walnuts (providing PUFAs and polyphenols), but not only walnut oil (containing only PUFAs), reduced atherosclerotic plaques and decreased the levels of circulating and hepatic lipids (Nergiz-Ünal et al. 2013). Caimari et al. (2015) reported, for the first time, that an extract derived from HZN skin and rich in dietary 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. In our experimental conditions, the intake of both HZN+S and HZN-S was able to reduce LDL-C and to improve HDL/LDL-C ratio, independently from the presence of polyphenols. Since the results are difficult

to compare due to the different models used, further investigations are needed in order to ascertain the contribution of polyphenols introduced through nuts in the modulation of lipid profile in human volunteers.

Finally, since adult dyslipidemia was correlated with an alterated RBC FA composition - that was partly confirmed in our previous preliminary observation in pediatric hyperlipidemia - another aim of this study was to evaluete the potential role of HZN-enrich diet in the improvement of RBC phospholipid composition, which is determined by a combination of metabolism and diet. We failed to demonstrate an effect of HZN consumption on the low omega-3 index previously observed in hyperlipidemic pediatric patients, probably due to the low content of PUFAs. However, 8-week HZN intervention increased the levels of MUFAs, oleic acid and MUFAs/SFAs ratio in RBC phospholipids in both the HZN groups. Since HZN fat composition was mainly represented by MUFA and in particular by oleic acid, whose concentration exceeded 80% of total fats, these results support the effectiveness of HZN interventions in the modulation of RBC phospholipid composition. Similar findings were observed in a crossover study by Rajaram et al. (2009), who reported an increased amount of PUFA, linoleic acid, and ALA in the RBC membranes following 4-week supplementation with walnuts (40 g/day) in a group of normal and mildly hyperlipidemic volunteers. Similarly, English walnuts intake (30 g/day, 30 days) increased RBC PUFA levels in healthy participants (Fitschen et al. 2011). Moreover, in our trial, we also observed a significant decrease of palmitoleic and linoleic acid levels in RBCs following the consumption of HZN-S, but not HZN+S, that is apparently without a plausible explanation. A reduction of linoleic acid could be postulated as the consequence of *ex-novo* synthesis of arachidonic acid, the major n-6 fatty acid in the RBC membranes. However, the synthesis rates of arachidonic acid from linoleic acid have been reported to be less than 1% (Harris et al. 2013) and no significant change in its content was observed following HZN interventions. Regarding the control group, at the end of the study period we documented a significant increase in the levels of total SFAs, in particular margaric acid, and a significant decrease of eicosenoic acid and total PUFAs n-6 (in particular DGLA). Even if DGLA is the immediate precursor of arachidonic acid and dairy intake of this fatty acid can increase plasma pentadecanoic and margaric acids (Aslibekyan et al. 2012), based on the analysis of food diaries, we did not document modifications of nutrient intake and of eating behavior (e.g. increase of dairy products) in our controls. On the contrary, the inclusion of HZNs to the dietary protocol increased the total fat intake (with specific regard to MUFA and PUFA) and decreased the carbohydrate intake without significantly affecting total energy intake. Furthermore, it should be underlined that we found an overall underestimation of energy intake and a moderate deviation from general dietary recommendations (CHILD 1), showing higher protein and fat intake than suggested. Moreover, BMI did not change during the intervention, while height and weight increased probably due to the related growth, in agreement with results reported in a recent meta-analysis of controlled trials (Del Gobbo et al. 2015).

Overall, it could be stated that by considering published data, the effect of nuts intake on oxidative stress markers and lipid profile is dependent on several variables – including the daily dose of nuts administered (ranging from 28-30 to 60-70 g/day for HZNs), different preparation (e.g. rosted vs raw, peeled vs. unpeeled, etc.) and changed nutritional composition among different nut cultivars (Alasalvar et al. 2003; Maguire et al. 2004). Another variable is represented by the impact of factors associated with nut consumption, i.e. combination with other foods, intake of one portion vs. multiple portions or time of consumption. 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 intervention trials were performed in children and adolescents to evaluate the efficacy of nuts in reducing CV risk (Maranhão et al. 2011; O'Neil et al. 2012), but none of them included hyperlipidemic patients.

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

hyperlipidemia, this is the first trial documenting the beneficial effect of the supplementation with different form of HZNs (HZN+S or HZN-S), associated with a balanced diet able to improve cell protection fromDNA damage, to reduce serum LDL-C and to increase MUFA RBC phospholipids, in children and adolescents affected by primary hyperlipidemia.

The importance of the development of dietary intervention for the modulation of the above-mentioned biomarkers related to hyperlipidemia is well recognized and there is specific interest also in food supplements which can have a favorable effect. In our pilot trial we ascertained whether dietary supplementation with HSO, composed by over 80% in PUFAs and other nutritional components, was more effective in enhancing the low RBCs omega-3 index and the overall lipid profile in the same population of hyperlipidemic children and adolescents.

The cardioprotective effect of diets rich in PUFA on hyperlipidemia and coronary artery disease (Chiesa et al. 2016; Pan et al. 2009) have been mainly attributed to the marine n-3 LC-PUFAs EPA and DHA from fish oil (Chiesa et al. 2016; Eslick et al. 2009). In fact, strong evidence associated a reduced levels of EPA and DHA in both diet and RBC membranes with higher risk for CVD and early mortality (Harris et al. 2013). Despite ALA, a plant-derive essential n-3 PUFA, is the direct precursor of EPA and DHA and demonstrated a beneficial role for heart diseases prevention, there is a lack of sufficient evidence from large randomized clinical trials on the CVD benefits of ALA vs. EPA and DHA (Fleming and Kris-Etherton 2014; Pan et al. 2012). Moreover, the major experimental data derived from randomized trials were conducted using the flaxseed oil as a source of ALA. Even if HSO has been used in our diet for hundreds of years, surprisingly little research have been published on its physiological effects (Schwab et al. 2006). HSO has an excellent nutritional composition providing large amounts of PUFAs, (over 80%) mostly represented by the two essential PUFAs ,i.e. the n-3 ALA and the n-6 LA (Callaway 2004). Despite its high content of LA, HSO is among the richest food sources of ALA (23 g ALA/100 g HSO, see **Table 3.10**) (Rodriguez-Leyva and Pierce 2010).and the omega-6 to omega-3 ratio (n-6/n-3) in HSO is 2:1.In the present trial, the daily dose of HSO supplementation was specifically calculated for our pediatric patients to meet the Italian nutritional recommendation, which suggest to introduce n-3 PUFA in the amount of 0.5–2% of total energy intake (Società Italiana di Nutrizione Umana 2014). Specifically, a daily intake of ALA corresponding to 0.5% of total calories is sufficient to meet current recommendations for adequate intake (EFSA Scientific Opinion on Dietary Reference Values for Fats (EFSA Panel on Dietetic Products Nutrition and Allergies (NDA) 2010).

Our findings showed that 8-week dietary supplementation with HSO, providing 700 mg ALA/die, did not affect the lipoprotein profile, except for the significant decrease of HDL/LDL ratio due to a slight (not significant) reduction of HDL-C concentration. Similar results, but in healthy subjects, were also obtained in another trial following HSO consumption (Schwab et al. 2006). The lipid lowering effect of increasing dietary ALA intake is controversial and the limited studies available on hyperlipidemics make the comparison difficult. For example, in a recent randomized controlled trail conducted in hypertrigliceridemic subjects, Dittrich et al. (2015) documented a significant reduction of TC and LDL-C after 10-weeks consumption of linseed oil (7.4 g/die of ALA). Accordingly, similar findings were observed after the consumption of 11.4 g ALA/die from camelina oil for 6 weeks in hypercholesterolemic subject (Karvonen et al. 2002). On the contrary, a significant reduction of HDL-C and TC concentration was found after 12 weeks of ALA-enriched diet (15 g ALA/die) from flaxseed oil in subjects with an atherogenic lipoprotein phenotype (Wilkinson et al. 2005). Moreover, authors subtitled that the beneficial ALA effect was not equivalent to CVD benefits demonstrated for dietary EPA and DHA, including fish oil-induced changes in HDL cholesterol and LDL subclasses (Wilkinson et al. 2005). In addition, a review of short term trials (6–12 weeks) in healthy individuals showed no or inconsistent effects of 1.2–3.6 g/die ALA intake on blood lipids and lipoprotein (Geleijnse, De Goede, and Brouwer 2010).

The secondary aim of this study was to evaluate the effect of HSO supplementation on the modulation of RBC phospholipid composition. Differently to what was observed after HZN intake,

the supplementation was able to improve the overall FA composition of RBC phospholipids by increasing the levels of total PUFA, n-3 and n-6 PUFA subclasses and n-3/n-6 PUFA ratio, while significantly reducing RBC levels of SFA and MUFA. The opposite trend was observed in the control group. Moreover, the intervention with HSO significantly raised the omega-3 index, as well as the relative RBC contents of EPA and DHA, even if the index remained below the cut-off level associated with higher risk for CV events (<4%). By considering the modulation of single RBC fatty acids, in respect to control diet, 8-week intake of HSO significantly increased the levels of LA in RBC, mainly due to the high concentration of this fatty acid in the supplement. This result confirms the efficacy of HSO supplementation in the modulation of RBC phospholipids composition. Similar finding was obtained in a study conducted by Schwab et al. (2006), in which the HSO intake increased the proportion of LA in serum cholesteryl esters and TG. Unexpectedly and in contrast with other studies, the increased dietary intake of ALA was not associated with higher content of ALA in RBC phospholipids with respect to baseline and control. One possible explanation is that increased consumption of ALA could modestly stimulate the ALA pathway for its endogenous conversion into the LC-PUFA derivatives EPA, DPA and DHA. In fact, EPA and DHA levels in RBCs were found to be significantly higher after 8-week ALA supplementation (Burdge 2006). However, as noted by Burdge & Calder (2006), the ALA conversion is limited. In addition, it should be noted that the other studies were conducted in adults, testing an effect of higher doses of ALA. In the whole, our results are in line with few others human trials investigating the impact of increased ALA intake on RBC FA composition. In particular, Barceló-Coblijn et al. (2008), in a study conducted on subjects at high CVD risk, showed that 12-week supplementation with two different doses of flaxseed oil, rich in ALA (2.4 or 3.6 g ALA/die) led to a significant increased EPA, DHA and ALA in RBC phospholipids. The authors, by comparing multiple doses and different time-points, concluded that 2 weeks of treatment with 2.4 g ALA/die was sufficient to obtain alterations of n-3 PUFA in RBC phospholipids (Barceló-Coblijn et al. 2008). Comparable findings on the modulation of RBC FAs with flaxseed oil were also observed in patients with atherogenic lipoprotein phenotype treated with 15 g ALA/die for 6 and 12 weeks (Wilkinson et al. 2005), or in a more recent study conducted in hypertriglyceridemic subjects supplemented with 7.4 g ALA/die (Dittrich et al. 2015). On the contrary, the consumption of margarine fortificated with ALA (4.4 g ALA/die) was not associated with an increased RBC EPA level and omega-3 index but with an increased DHA level (Egert et al. 2012). However, the subjects included in this trial were healthy and the RBC contents of EPA and DHA, as well as the omega-3 index, were relatively high, indicating a good n-3 PUFA status (Egert et al. 2012). In the pediatric population, the effect of ALA supplementation was mainly studied in children with neuro-developmental disorder (Raz, Carasso, and Yehuda 2009; Dubnov-Raz et al. 2014). Only one study carried out in obese children concluded that daily ALA supplementation (1000 mg/die, 6-weeks) increased significantly n-3 FA composition of plasma lipids (Lohner et al. 2007).

Despite the differences in RBC FA composition observed between controls and treated group are plausibly attributed to the HSO supplementation, we could not exclude that such differences may result from the different baseline RCB FA concentrations registered in the two groups. For this reason, to normalize data obtained the statistical analysis was carried out on percentage changes between post- to pre-treatment. Besides this possible limitation and the lack of a placebo-controlled group, this pilot intervention is the first documenting the potential beneficial effect (i.e. improvement of RBC lipid profile) of increase intake of ALA through HSO supplementationin children and adolescents affected by primary hyperlipidemia.

3.5 REFERENCES

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PART II: *EX VIVO* STUDY

4 IMMUNOMODULATORY EFFECTS OF LC-PUFA AND VITAMIN D3 ON *EX VIVO* INNATE IMMUNE CELLS

4.1 MATERIAL AND METHODS

Chemicals

Eicosapentaenoic acid (EPA) and arachidonic acid (ARA) in free fatty acid form and vitamin D3 (1α,25-$(OH_2)D_3$) (all from Larodan, Malmö, Sweden) were dissolved in sterile dimethylsulfoxide (DMSO, Sigma-Aldrich, USA) to produce stock solutions of 100 mM (for EPA and ARA) or 10 µM (for vitamin D), stored at -80°C or -20°C respectively.

Collection and preparation of blood samples

Buffy coats and whole blood samples (lithium-heparin) were collected from peripheral blood of healthy donors who gave their written consent. In order to isolate the PBMCs, buffy-coated blood was diluted with phosphate buffered saline (PBS; PAA, Cölbe, Germany) at a ratio of 1:1, layered onto lymphocyte separation medium (LSM) 1077 (1.077 g/ml; PAA; ratio 1:1) and centrifuged at 700 g for 20 min at 20°C. The PBMC interphase was collected, washed three times with PBS, and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% endotoxin-free heat-inactivated fetal bovine serum (FBS) and 1% L-arginine (all reagents from Invitrogen, Karlsruhe, Germany).

PBMCs viability

To assess the impact of high-dose EPA and ARA on cell viability, PBMC (1×10^6 /ml) were incubated without or with 25, 50, 100, 150, or 200 µM of EPA or ARA for 24 h in a 5% CO_2 humidified atmosphere at 37°C. Control cultures contained 0.2% DMSO vehicle, according to the maximal volume in the treatments. Cell viability was analyzed by flow-cytometrically, as described previously (Jaudszus et al. 2013).

Evaluation of lytic activity of NK cells

The lytic activity of NK cells against target human K562 cancer cell line, at two different ratios of PBMC:K562 (25:1 and 12.5:1), was determined by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany), according to the method described by Chang et al. (1993) and calculated as the percentage of dead target cells in the test samples minus the percentage of dead target cells in the control samples without effector cells.

Briefly, isolated PBMCs from buffy coats were suspended in a 6-well plate with complete RPMI medium (2×10^6 cells/ml) and incubated with increasing concentrations of EPA or ARA (25, 50, 100 µM), with or without 40 nM of vitamin D, for 24 h at 37°C and 5% of CO_2. As two controls, PBMCs were also incubated with DMSO or DMSO with vitamin D 40 nM. Meanwhile, K562 target cells were held under standard culture conditions and passaged the day before the assay. To discriminate effector from target cells, K562 cells were fluorescence labeled with dioctadecyloxacarbocyanine (DiO; Molecular Probes, Leiden, Netherlands). After 24 h, PBMCs were pelleted, resuspended in new RPMI medium and transferred into polystyrene tubes together with labeled K562 cells at the effector:target ratios of 25:1 and 12.5:1, respectively. For controls, K562 were also incubated alone. For an accurate viability cell analysis, the propidium iodide (PI) dye solution (0.252 mg/ml; Molecular Probes) was added to all samples. After 75 min of incubation in the dark, the analysis was performed by means of flow cytometry: affected cells will finally appear double stained. NK cell activity was calculated as the difference in the percentages of PI-positive target cells in the presence and in the absence of effector cells.

Phagocytic activity and intensity

Assessment of phagocytic intensity (number of phagocytized *E. coli* expressed as mean fluorescence) in granulocytes and monocytes was based on a flow cytometric method according to O'Gorman (2002). Briefly, aliquots of 100 µl of whole blood (lithium-heparin) were transferred in polystyrene tubes and preincubated with increasing concentrations of EPA or ARA (25, 50, 100 µM), with or without 40 nM of vitamin D, in duplicate, for 24h at 37°C and 5% of CO_2. As controls, two blood samples were incubated with DMSO and DMSO with vitamin D 40 nM, in duplicate.
To measure phagocytosis, 5 mL opsonized BODYPY FL-labeled *E. coli* (Molecular Probes) and 100 mL chilled whole blood were mixed (i.e. 2×10^{12} *E. coli*/L whole blood) and incubated at 37°C or kept on ice (control) for 10 min. The reaction was stopped by adding 100 µL ice-cold quenching solution (10% trypan blue in PBS). After washing with PBS (without Ca and Mg), whole blood cells were fixed with 100 µL FACS lysing solution (BectonDickinson). DNA was stained with 300 µL of PI solution (0.05 mmol/L PBS) and fluorescence was directly measured by flow cytometry (FACSCalibur; BectonDickinson).

Statistical analysis

A linear mixed model was used to estimate the effect of treatment (EPA or ARA without vit D versus EPA and ARA with vit D) and concentrations of EPA or ARA (0, 25, 50 or 100 µM). Beside main effects, two-way interactions (concentration x treatment) were included. A posthoc test was performed for significant two-way interactions and adjustment for multiple testing (Tukey-Kramer) was applied. Normality of the conditional studentized residuals was checked by visual inspection of the QQ-plot. If the residuals did not show normal distribution, the dependent variable was log-transformed. Further, homogeneity of the residuals was checked by visual inspection of the conditional studentized residual versus predicted values plot. All calculations were carried out using SAS 9.4. PROC MIXED and PROC GLIMMIX. Significance of difference was set at $p < 0.05$ and data are expressed as means ± SD.

4.2 RESULTS

Effect of EPA and ARA on PBMCs viability

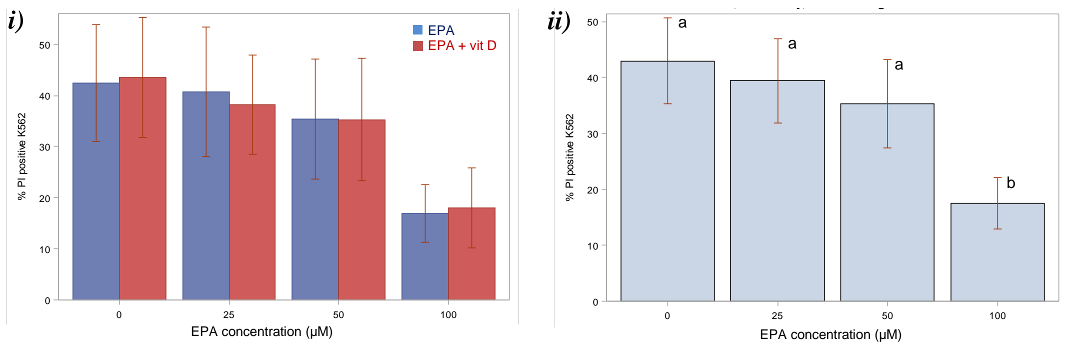
First we tested whether incubation with EPA or ARA at high concentration has an impact on the viability of primary lymphocytes and monocytes over a period of 24 h. Compared with the DMSO control, neither EPA nor ARA showed any cytotoxic effect at 100 µM. A drop in viability was observed only at concentrations higher than 150 µM (data not shown). Based on these data, a maximum fatty acid concentration of 100 µM was used in all subsequent experiments.

Effect of LC-PUFAs with vitamin D on NK cell activity against K562 target cells

The lytic activity of NK cells measured at the two effector:target ratios (25:1; 12.5:1) was not significantly affected by treatment with EPA or ARA incubated alone or in conjunction with vitamin D ($p>0.05$). Moreover, no significant concentration x treatment interaction was found demonstrating a similar trend in the different experimental conditions. On the contrary, a concentration-dependent effect was found ($p<0.0001$) with a significant reduction in NK activity with increasing concentration of both EPA and ARA, independently from the compresence of vitamin D (**Figure 4.1; 4.2**). In particular, in all experimental conditions tested, the highest concentration of EPA and ARA (100 µM) showed a significant reduced ($p<0.001$) NK activity compared to the other concentrations (control, 25 µM, 50 µM). In addition, at effector:target ratio of 12.5:1, the reduced NK activity after the incubation with 50 µM of EPA was significantly different ($p= 0.01$) with respect to control. Furthermore, a dose-dependent response was observed after ARA stimulation: the NK lytic activity significantly decreased with increasing concentration of ARA at both effector:target ratio.

Figure 4.1 *Effect of EPA treatments (EPA or EPA+ vit D) at different concentrations (EPA: 0, 25, 50 or 100 µM) on NK activity (**i**) and relative concentration-dependent effect (**ii**), at effector:target ratio of 25:1 (**A**) and 12.5:1 (**B**). Data with different letters differ significantly ($p\leq0.001$).*

A) Effector:target ratio of 25:1



B) Effector:target ratio of 12.5:1

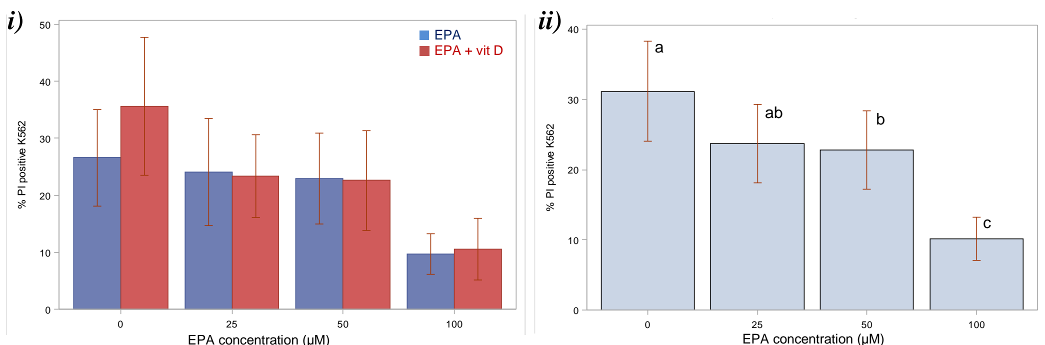
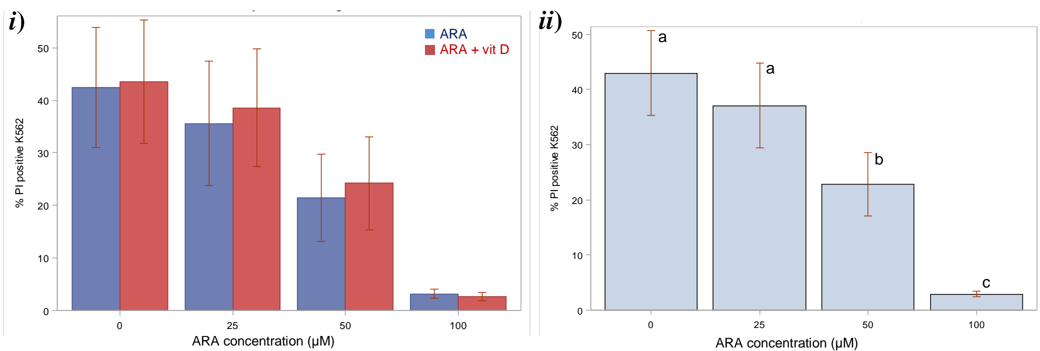
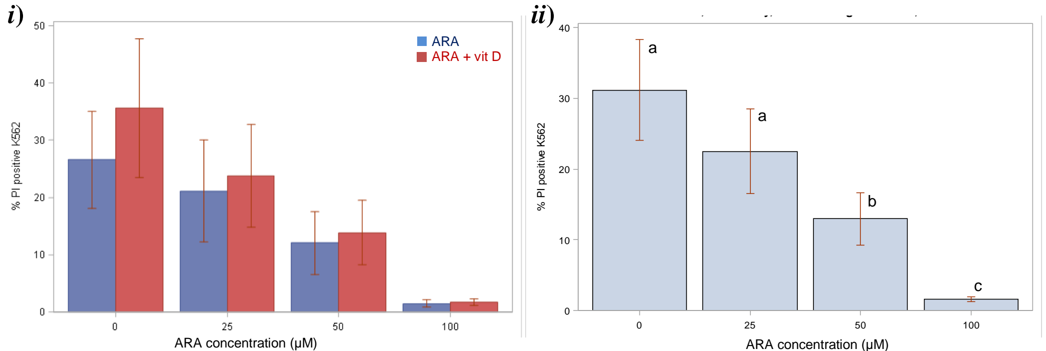


Figure 4.2 *Effect of of ARA treatments (ARA or ARA+ vit D) at different concentrations (ARA: 0, 25, 50 or 100 µM) on NK activity (i) and relative concentration-dependent effect response (ii), at effector:target ratio of 25:1 (**A**) and 12.5:1 (**B**). Data with different letters differ significantly (p≤0.001).*

A) Effector:target ratio of 25:1



B) Effector:target ratio of 12.5:1



Effect of LC-PUFAs with vitamin D on phagocytic activity of monocytes and granulocytes

Compared to the treatment with only fatty acids (EPA or ARA alone), the 24 h co-incubation of EPA or ARA with vitamin D significantly increased the percent of phagocytic activity of monocytes independently from the concentration tested (**Figure 4.3(b)** and **4.4(b)**), showing a significant treatment effect (p= 0.001).
As far as the granulocytes responsiveness to EPA or ARA treatments (with and without vitamin D) is concerned, there was no treatment and concentration effect; a significant interaction between treatment and concentrations was found for ARA (p= 0.05).

Figure 4.3 *Effect of different EPA concentrations (0, 25, 50 or 100 µM) incubated with vitamin D (40 nM) or without (0 nM) (**a**), and relative treatment effect (**b**), on phagocytic activity in monocytes. *, p= 0.001.*

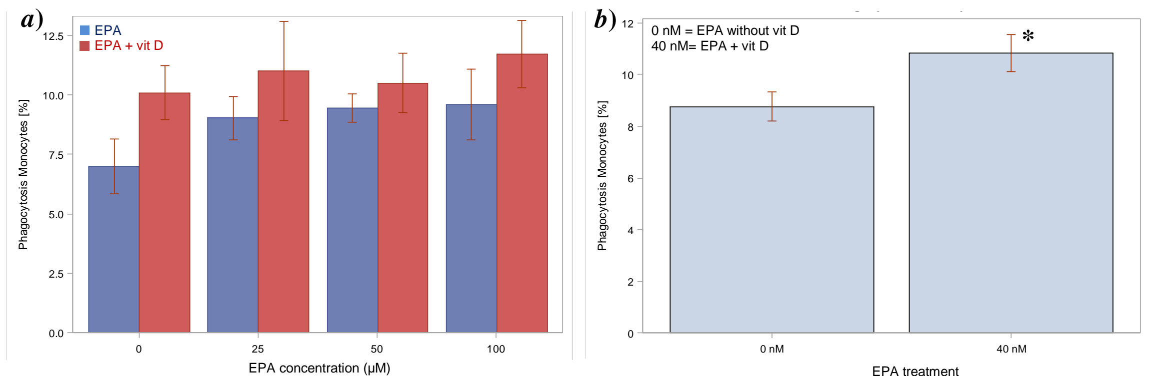
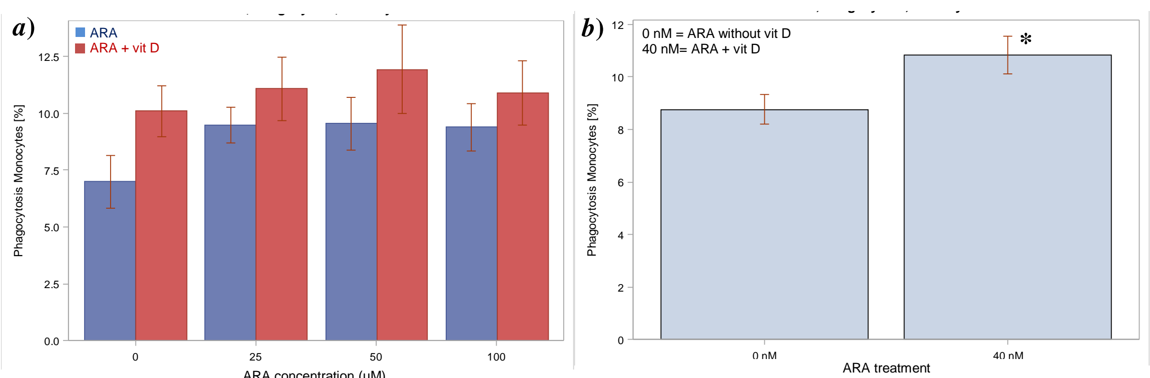


Figure 4.4 *Effect of different ARA concentrations (0, 25, 50 or 100 µM) incubated with vitamin D (40 nM) or without (0 nM) (**a**), and relative treatment effect (**b**), on phagocytic activity in monocytes. *, p= 0.001.*



4.3 DISCUSSION

The optimal functioning of the immune system depends on balanced and adequate nutrition able to affect the immune status and susceptibility to pathogens (Calder 2001; Kelley 2001). Dietary LC-PUFAs, such as ARA, EPA and DHA, are involved in a great number of immune responses, such as lymphocyte proliferation, cytokine synthesis, NK cell activity, phagocytosis and inflammation. While LC-PUFA-rich sources, in particular EPA and DHA, are widely recommended in prevention and attenuation of chronic diseases characterized by inflammation and an overactivation of immune system (Watson et al. 2010; Husson et al. 2016), in some circumstances, high doses of LC-PUFA seem responsible for an increased susceptibility to infectious microorganisms by generating an immunedepressed state (McMurray et al. 2011; Husson et al. 2016). Even if the mechanisms of action are not fully understood, previous investigations underline the importance for clinical trials to define the optimum LC-PUFA doses considered safe for supplemented patients.

In our study, we tested different concentrations of the n-3 fatty acid EPA and the n-6 ARA to evaluate their dose-specific effects in the modulation of the phagocytosis and NK activity, the two main innate immune responses that specifically act against infectious agents as well as cancer cells. In addition, since previous data have demonstrated a beneficial effect of vitamin D3 in enhancing these mechanisms (Lee et al. 2011; Harvey & Cantorna 2013; Min et al. 2013; Djukic et al. 2014), we also investigated the effects of stimulating *ex vivo* innate immune cells only with EPA/ARA at different dose in comparison with their effect in association with vitamin D3. We found that NK activity is negatively affected by increasing concentration of both EPA and ARA, with a significant strong reduction with the highest dose tested of both fatty acid (100 µM). Even if studies reported that 10 to 25 µM are the LC-PUFAs concentrations that approximate the *in vivo* situations, i.e. after ingestion of fish oil (Lamaziere et al. 2015) and 100 µM of *in vitro* LC-PUFA concentration could be considered non-physiological, in our study neither EPA nor ARA showed any cytotoxic effect at 100 µM, when the viability test was performed. A decreased in cell viability was observed only at concentrations higher than 150 µM. Our observations are in line with other LC-PUFAs supplementation studies in humans as well as in animal models. For example, 6-month intake of foods enriched with n-3 LC-PUFA (1 g/day) in healthy humans led to a significantly lower number of circulating NK cells than in controls, that was associated with higher amount of EPA and DHA in erythrocytes (Mukaro et al. 2008). Similarly, in healthy subjects aged <55 years, 12-week supplementation with fish oil (720 mg EPA + 280 mg DHA) resulted in a significant lower NK activity, even if no effect was observed after ARA supplementation (Thies et al. 2001). A reduced NK activity following n-3 PUFA-enrich diet intake, but not after n-6 PUFA diet, was also observed after feeding female rats for 6-week with different fat diets containing a pool of n-3 or n-6 fatty acids (Sasaki et al. 2000). In our study, even if we found a strong significant concentration-effect ($p < 0.0001$) for both fatty acids used, the *ex-vivo* lymphocytes stimulation with ARA generated a more intense dose-dependent response than EPA, with a significantly decreased NK lytic activity with increasing concentration of ARA at both effector:target ratio tested. In accordance with our observations, a recent study conducted in young rats demonstrated that, compared to controls, long-term ARA administration (240 mg/kg body weight) had an inhibitory effect on the tumor cytotoxicity of NK cells evaluated on *ex vivo* rat spleen lymphocytes (Juman et al. 2013). As regard vitamin D, even if recent findings demonstrated the beneficial role of vitamin D3 in increasing the NK activity or in enhancing the immuno-attack of NK cells against malignant cells (Lee et al. 2011; Harvey & Cantorna 2013; Min et al. 2013), we failed to demonstrate that vitamin D3, associated with increasing dose of EPA or ARA could counteract the detrimental effect exerted by these two fatty acid on NK lytic activity.

As far as the phagocytic activity is concerned, here we showed that increasing concentration of LC-PUFAs did not significantly affect the phagocytosis, even at higher dose tested. Similar findings were observed in a placebo-controlled study conducted in 150 healthy subjects, in which

the supplementations with two doses of the n-6 fatty acid ALA (4.5 or 9.5 g /day) or the n-3 EPA+DHA (0.77 or 1.7 g/day) were not sufficient to increase the percentages of blood monocytes or neutrophils engaged in phagocytosis of *Escherichia coli* or the phagocytic activity (Kew et al. 2003). Similarly, 12-week intake of EPA-enriched oil, providing different and higher doses of EPA (1.35, 2.7, or 4.05 g EPA/day), did not influence the neutrophil or monocyte phagocytosis in both young or older men (Rees et al. 2006). On the contrary, in a murine investigation, the *ex vivo* macrophages from mice supplemented for 9 weeks with sea cod oil or flaxseed oil (providing 900 and 2000 mg n-3 PUFA/body weight, respectively), showed a significantly more efficient phagocytosis and intracellular killing of *Streptococcus pneumonia* than controls (Saini et al. 2013). Overall, the impact of an increased intake of LC-PUFAs on human blood monocyte phagocytosis are contradictory, mainly due to the scarce number of placebo-controlled studies in humans and the type of controls and supplements used (e.g. purified fatty acids vs. oils containing a mix of different PUFAs).

Recently, there has been a considerable interest in the role that vitamin D might play in host resistance to infection. Despite we did not observe any differences after EPA or ARA *ex vivo* stimulation, the same treatments with EPA or ARA but associated with 40 nM of vitamin D were able to significantly increased the percent of phagocytic activity of monocytes independently from the concentration tested, showing a strong significant treatment effect. Previous data also reported that vitamin D3 (1×10^{-7} M) was able to enhanced the phagocytic potential also in subjects with a low phagocytic index (less than 20%) (Selvaraj et al. 2004). Moreover, a recent study observed that a vitamin D deficiency condition was associated with decreased rates of phagocytosis and intracellular killing of *E. Coli* K1 in microglial cells in respect to relative control cells. These data suggested a role of vitamin D in the resistance against bacterial infections even in the local immune system of the central nervous system (Djukic et al. 2014). However, at present, there is not adequate information available to understand the mechanism underlying the effect of vitamin D and/or vitamin D deficiency on the innate immune system, and specifically on the ability of a host to counteract infection or the shaping of cancer cells (Bruce et al. 2010; Harvey & Cantorna 2013).

Overall, our results seem to confirm previous observations demonstrating a significant reduction of NK cell lytic activity after the *ex vivo* lymphocytes stimulation with increasing concentration of EPA or ARA, highlighting that caution should be exercised when prescribing high-dose and prolonged supplementation with LC-PUFAs in humans. Differently, the monocyte phagocytosis activity was enhanced by supplementation with vitamin D combined with EPA or ARA, suggesting a specific role of vitamin D, or a synergistic effect with LC-PUFA, in the phagocytosis process. Even if these data could give more information about immunomodulatory effects of LC-PUFAs and vitamin D, additional *in vitro* experimentations are required to define the mechanisms of action of these nutrients, as well as their effect after the interaction with other dietary compounds. Moreover, since current evidence is insufficient to make a definite nutritional recommendation in both physiological and pathological conditions, the use of supplementations in the future will depend on a better understanding of the appropriate dosage by which micronutrient modulate immune responses and inflammatory processes defined through *in vivo* investigations.

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5 CONCLUSIONS

In conclusion, through this PhD thesis it was possible to provide novel results concerning the potential benefits of bioactives rich-diet on the modulation of risk factors for chronic-degenerative diseases. This aspect is particular important in at risk target groups of population for whom appropriate and personalized nutritional interventions are considered the primary prevention approach.

In the first part of the research, we provided the first data on the phospholipid composition of RBCs in a target population of children and adolescents affected by primary hyperlipidemia, one of the major risk factor for CVD. In particular, we documented an overall low omega-3 index, a factor that contribute to classify them as at future high risk of CVD. Specific dietary treatments are fundamental to improve this index and, above all, biomarkers related to hyperlipidemia. Subsequently, in the same target population we demonstrated the effectiveness of dietary treatments designed to replace the intake of SFA with unsaturated fatty acids as a primary intervention for the management of hyperlipidemia since pediatric age. In particular, we firstly showed that HZN-enrich diet could improve serum lipid profile, FA composition of RBC and oxidative stress markers. These positive effects were mainly attributed to the bioactive components contained in the HZN kernel and skin, in particular MUFA, phytosterols and polyphenols. In the subsequent pilot study, the impact of a food supplements consisting of HSO, rich in PUFAs was investigated. In this case, the supplementation was able to enhance the omega-3 index and the RBC phospholipid composition. Overall, these data give strengths to the development of dietary recommendation tailored to meet the specific needs of hyperlipidemic subjects since childhood, when the treatment should effectively start. Future and larger studies are necessary to confirm the beneficial effect of the supplementation with HZN or HSO on hyperlipidemia-related markers, focusing also on the mechanisms of action involved.

In the second part of the research, an *ex vivo* experimentation was performed to improve understanding on the mechanisms of action of specific bioactives suggested as modulators of the innate immune functions, often compromised in several chronic and autoimmune diseases. In particular, our findings showed that elevated dose of LC-PUFA can negatively affect the NK cell lytic activity, underling that the immunomodulatory properties of bioactives could be dose-dependent. Moreover, it is envisaged that caution should be exercised in case of prolonged supplementation. In addition, the results on the phagocytic activity showed that in some cases, it is important to considered the synergistic effect of different bioactives in the modulation of immune function. In fact, the phagocytic activity was significantly enhanced when LC-PUFA were combined with vitamin D. Future studies are needed to confirm our results and increase knowledge on the specific mechanisms of action involved in the immunomodulatory properties of food bioactives, including their interaction and active doses, before they may be considered for preventive and/or therapeutic supplementations.

6 PRODUCTS

6.1 COPIES OF PAPERS PUBLISHED

- **Deon V**, Del Bo' C, Guaraldi F, Gargari G, Bosusco A, Simonetti P, Riso P, Guardamagna G. Characterization of fatty acids composition of erythrocytes membrane, omega-3 index and serum lipid profile of children and adolescent with primary dyslipidemia. *International Journal of Food Sciences and Nutrition 2016.* In press. DOI:10.1080/09637486.2016.1236076.

- Del Bo' C, **Deon V**, Porrini M, Campolo J, Parolini M, Riso P. Intra and inter-day repeatability of peripheral arterial tonometry for the assessment of endothelial function in dietary intervention studies. *Microcirculation 2016, 23:503–511.* DOI: 10.1111/micc.12300.

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RESEARCH ARTICLE

Serum lipid profile and fatty acid composition of erythrocyte phospholipids in children and adolescents with primary hyperlipidemia

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ABSTRACT
This study aimed at characterizing 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 toward 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. Fiber 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.

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KEYWORDS
Children; primary hyperlipidemia; erythrocytes; phospholipids; lipid profile; omega-3 index

Introduction

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

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

Since erythrocytes are incapable of *de novo* phospholipid synthesis, chain elongation or desaturation of FAs, the major pathway to renew the RBC phospholipids is the 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).

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ORIGINAL ARTICLE

WILEY Microcirculation

Intra- and interday repeatability of peripheral arterial function: suitability and potential limitations

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Abstract

Objective: This study aimed to investigate the inter-and intraday repeatability of RHI measured by Endo-PAT in healthy volunteers.

Methods: Interday RHI repeatability was tested in two consecutive days in a group of 31 male subjects. Intraday repeatability was investigated at baseline and after 2 and 4 hour in a group of 16 volunteers. Data were evaluated by analysis of variance. Bland-Altman plot, CV, CR, and ICC were measured.

Results: While interday RHI repeatability was found to be reliable (CV: 6.0%; CR: 0.51; ICC: 0.77), multiple evaluations within the same day significantly ($P<.001$) affected RHI (repeatability of the measurement -CV: 18.8%; CR: 1.26; ICC: 0.48). In particular, a significant increase in RHI occurred at 4 hour compared to 2 hour (+16.8%; $P<.05$) and to baseline (+30.1%; $P<.05$).

Conclusions: RHI showed good interday but poor intraday repeatability. Multiple evaluations increased RHI especially in subjects with endothelial dysfunction who improved or reversed their impairment. These results show the potential limitations of multiple Endo-PAT measurements within the same day and the importance of standardizing the protocols before RHI evaluations.

KEYWORDS
augmentation index, healthy young male, peripheral arterial tonometry, reactive hyperemia index, repeatability

1 | INTRODUCTION

Endo-PAT is a novel non-invasive plethysmographic system developed to measure peripheral arterial function at the level of the fingertips through an index of reactive hyperemia. This index is a ratio of the post-to-pre occlusion PAT amplitude of the tested arm, divided by the post-to-pre occlusion PAT amplitude of the control arm.[1,2] Simultaneously with endothelial function, Endo-PAT can also measure the peripheral AI, which is an established marker of arterial wave reflection.[3] Thus, PAT technology is particularly interesting for the application in clinical research studies, since the measurement of peripheral arterial function and arterial stiffness requires separate equipments. In addition, Endo-PAT has the advantage of providing reliable and reproducible results; it is operator-independent and it records systemic changes on the contralateral arm, allowing for an internal control system.[4,5] Several studies have found a significant correlation between peripheral arterial tonometry and flow-mediated dilation,[6,7] which represents the most popular clinical method to assess endothelial function by means of brachial artery ultrasound scanning.[6,7]

Endothelial nutrition is a new and innovative concept that involves the study of the role of dietary compounds on endothelial function. Preventing the endothelium from becoming dysfunctional by means of nutrients or extra-nutrients that modulate vascular tone and maintain homeostasis of the endothelium, can be of great importance to human

Abbreviations used: AI@75, augmentation index normalized for heart rate of 75 bpm; AI, augmentation index; AMP, adenosine monophosphate; ANOVA, analysis of variance; BMI, body mass index; CR, coefficient of repeatability; CV, coefficient of variation; DBP, diastolic blood pressure; F-RHI, Framingham reactive hyperemia index; HR, heart rate; ICC, intraclass correlation coefficient; LSD, least significant difference; PAT, peripheral arterial tone; RHI, reactive hyperemia index; RH, reactive hyperemia; SBP, systolic blood pressure; SD, standard deviation; SEM, standard error of mean.

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6.2 TITLES OF PAPERS SUBMITTED

- **Deon V**, Del Bo' C, Guaraldi F, Abello F, Belviso S, Porrini M, Riso P, Guardamagna G (2016). Hazelnut intake improves serum lipid profile and fatty acid composition of erythrocyte membranes in children and adolescents with primary hyperlipidemia: a randomized controlled trial. *Submitted to Clinical Nutrition.*

- Guaraldi F*, **Deon V***, Del Bo' C, Vendrame S, Porrini M, Riso P, Guardamagna O. Effect of short-term hazelnut consumption on DNA damage and oxidized-LDL in children and adolescents with primary hyperlipidemia. *Submitted to Atherosclerosis.* ***Equal contribution***.

- Gargari G, **Deon V**, Taverniti V, Gardana C, Denina M, Riso P, Guardamagna O, Guglielmetti S. Intestinal microbial ecosystem of children and adolescents with primary hyperlipidemia: potential role of regular hazelnut intake. *Submitted to Environmental Microbiology*.

- De Benedetti S, Lucchini G, Del Bo' C, **Deon V**, Marocchi A, Penco S, Lunetta C, Gianazza E, Bonomi F, Iametti S. Blood trace metals in a sporadic amyotrophic lateral sclerosis geographical cluster. *Submitted to BioMetals*.

6.3 COPIES OF ABSTRACT OF ORAL COMMUNICATIONS AND POSTERS

- **Deon V**, Del Bo' C, Lanti C, Porrini M, Campolo J, Klimis-Zacas D and Riso P (2014). A single portion of blueberries can affect peripheral arterial tone in young smokers with endothelial dysfunction: preliminary observations and perspectives. 35th National Congress SINU (Italian Society of Human Nutrition), Rome, Italy, 20-21 October, 2014. ISBN 978-88-97843-14-6. *Award for the best scientific oral presentation.*
- **Deon V**, Cagliero P, Del Bo' C, Vendrame S, Porrini M, Riso P and Guardamagna O (2015). Role of a hazelnut intervention on markers of oxidative stress and lipid profile in children with primary dyslipidemia: preliminary observations. 13th National Congress SIPREC (Italian Society for Cardiovascular Prevention), Naples, Italy, 12-14 March, 2015. *Poster presentation.*
- Del Bo' C, **Deon V**, Cagliero P, Vendrame S, Porrini M, Guardamagna O and Riso P (2015). Role of hazelnut consumption on DNA damage and lipid-related markers in children with primary dyslipidemia. Front. Genet. Conference Abstract: ICAW 2015 - 11th International Comet Assay Workshop. doi: 10.3389/conf.fgene.2015.01.00004. *Oral presentation.*
- Del Bo' C, **Deon V**, Lanti C, Porrini M, Campolo J, Klimis-Zacas D and Riso P. Effect of a single portion of blueberries on markers of vascular function and oxidative stress in young smokers: a randomized-controlled trial. 4th International Conference on Foodomics, Cesena, Italy, 8-9 October, 2015. ISBN 978-88-902152-7-8. *Poster presentation.*
- **Deon V**, Del Bo' C, Cagliero P, Vendrame S, Porrini M, Guardamagna O and Riso P (2015). Effect of 8-week hazelnuts consumption on lipid profile, fatty acid composition of erythrocytes membrane and levels of DNA damage in children with primary dyslipidemia. 4th International Conference on Foodomics, Cesena, Italy, 8-9 October, 2015. ISBN 978-88-902152-7-8. *Poster presentation.*
- **Deon V**, Del Bo' C, Cagliero P, Vendrame S, Porrini M, Guardamagna O and Riso P (2015). Can hazelnut intake modulate oxidative stress and lipid-related markers in children with primary dyslipidemia? 12th European Nutrition Conference (FENS), Berlin, Germany, October 20-23, 2015: Abstracts Ann Nutr Metab 2015;67(1):1–601. DOI:10.1159/000440895. *Poster presentation.*
- Del Bo' C, **Deon V**, Porrini M, Campolo J and Riso P (2015). Repeatability of arterial function by EndoPAT2000: a potential tool for studying vasoreactivity of bioactive-rich foods. 12th European Nutrition Conference (FENS), Berlin, Germany, October 20-23, 2015: Abstracts Ann Nutr Metab 2015;67(1):1–601. DOI:10.1159/000440895. *Poster presentation.*
- **Deon V**, Del Bo' C, Cagliero P, Vendrame S, Porrini M, Riso P and Guardamagna O (2015). Effect of hazelnut consumption on lipid profile, fatty acid composition of erythrocyte membranes and oxidative stress markers in children with primary dyslipidemia. 29th National Congress SISA (Italian Atherosclerosis Society), Bologna, Italy, 22-24 November, 2015. Giornale Italiano dell'Arteriosclerosi 2015;6(4):66-120. *Poster presentation.*
- **Deon V**, Del Bo' C, Cagliero P, Vendrame S, Porrini M, Guardamagna O and Riso P (2015). Hazelnut (*Corylus avellana L.*) consumption improves lipid profile and markers of oxidative stress in children with primary dyslipidemia. 36th National Congress SINU (Italian Society of Human Nutrition), Florence, Italy, 2-4 December, 2015. ISBN 978-88-9743-26-9. *Poster presentation.*
- Del Bo' C, **Deon V**, Lanti C, Porrini M, Campolo J, Klimis-Zacas D and Riso P (2015). Effect of a single portion of blueberries (*Vaccinium corymbosum*) on markers of oxidative stress and

inflammation in young smokers. 36th National Congress SINU (Italian Society of Human Nutrition), Florence, Italy, 2-4 December, 2015. ISBN 978-88-9743-26-9. *Poster presentation.*

- **Deon V**, Del Bo' C, Bosusco A, Vendrame S, Porrini M, Simonetti P, Guardamagna O and Riso P (2016). Hazelnut-enriched diet improves lipid profile, fatty acid composition of erythrocytes membrane and markers of oxidative stress in children with primary dyslipidemia: a randomized control trial. 84th EAS (European Atherosclerosis Society) Congress, Innsbruck, Austria, 29 May-1 June 2016. *Poster Presentation.*
- **Deon V**. Effect of bioactive-rich diet on risk factors for chronic-degerative diseases in "at risk" groups of population. 21th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Naples Federico II, Portici, 14-16th September 2016. *Oral presentation*.
- **Deon V**, Del Bo' C, Guaraldi F, Bosusco A, Simonetti P, Porrini M, Guardamagna O and Riso P (2016). Effect of alpha-linolenic acid supplementation on serum lipid profile and fatty acid composition of erythrocyte phospholipids in children and adolescents with primary hyperlipidemia: preliminary observations. 37th National Congress SINU (Italian Society of Human Nutrition), Bologna, Italy, 30 Novembre-2 December, 2016. *Oral presentation.*

35th National Congress SINU (Italian Society of Human Nutrition), 20-21 October 2014 (Rome, Italy)

A single portion of blueberries can affect peripheral arterial tone in young smokers with endothelial dysfunction: preliminary observations and perspectives.

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Background: Cigarette smoke adversely affects vascular function by promoting endothelium injury via reactive oxygen species and nitric oxide dysregulation. Emerging evidence suggests an important role of dietary factors in modulating endothelial function. In particular, blueberries (*Vaccinium corymbosum*) appear to have beneficial effects on peripheral arterial dysfunction induced by acute cigarette smoking in young healthy volunteers.
Objective: The aim of this study is to investigate the possible effect of one portion of blueberries on peripheral arterial function in young smokers with endothelial dysfunction.
Methods: The study involves 16 male smokers with endothelial dysfunction. Subjects are randomized in a 3-armed controlled study with the following 3 experimental conditions: - smoking (S) treatment (one cigarette); - blueberry treatment (300 g of blueberry) + smoking (BS); - control treatment (300 mL of water with glucose and fructose) + smoking (CS). One week of wash-out period is scheduled between each treatment. Blood pressure (BP), heart rate (HR), and peripheral arterial function (reactive hyperemia index, RHI) are measured before and 20 min after smoking by using finger plethysmography method (Endo-PAT2000). Results obtained are elaborated by analysis of variance (ANOVA). Post-hoc analysis of differences between treatments is assessed by the least significant difference (LSD) test with $p \leq 0.05$ as level of statistical significance.
Results and Conclusion: Elaboration of preliminary data on 5 subjects showed that smoking does not affect RHI, BP and HR in subjects with endothelial dysfunction differently from what observed in smokers with normal endothelial function. However, ANOVA revealed a significant difference in the effect of the 3 treatments on RHI (p= 0.01). In particular, BS and CS treatments improved RHI index with respect to S treatment (+30.9 ± 24.2% BS vs S, p= 0.0055; +25.3 ± 27.2% CS vs S, p= 0.014). On the contrary, no effect was observed for systolic and diastolic BP, and HR (p= 0.80, p= 0.75 and p= 0.94, respectively). Further analysis of data on the whole group of subjects will help clarifying the protective effect of blueberry consumption in subjects with endothelial dysfunction.

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13th National Congress SIPREC (Italian Society for Cardiovascular Prevention), 12-14 March 2015 (Naples, Italy)

Role of a hazelnut intervention on markers of oxidative stress and lipid profile in children with primary dyslipidemia: preliminary observations

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Introduction: Several studies suggest that dyslipidemic patients could be more prone to oxidative stress and cardiovascular disease (CVD) risk. Possible strategies to manage dyslipidemia and oxidative stress include dietary interventions.
Aim: The aim is to investigate, in children with primary dyslipidemia, the effect of a hazelnut snacks rich in unsaturated fatty acids and bioactives on markers of oxidative stress and lipid profile.
Methods: An 8-week randomized, controlled, parallel, dietary intervention study has been scheduled. The study involves 60 children (11.8 ± 2.6 years) affected by primary dyslipidemia. Main inclusion criteria were: total cholesterol (TC) and/or triglycerides (TR) value >95° percentile and BMI <95° percentile. Subjects received dietary guidelines and were assigned to the following snack treatment (0.43 g/Kg): 1- hazelnuts with skin, 2- hazelnuts without skin and 3- only the dietary guidelines (control). Before and after the interventions the serum lipid profile and the endogenous and H_2O_2-induced DNA damage (by comet assay) were evaluated.
Results and Conclusion: Results presented are relative to 8 subjects who completed the intervention. Baseline lipid profile were TC: 218.8 ± 53.8 mg/dl, HDL-C: 55.4 ± 15.5 mg/dl, TR: 92.8 (32-194) mg/dl and LDL-C: 149.8 ± 41.3 mg/dl. After the hazelnut treatment, the level of LDL-C was significantly reduced (-11.2%, p=0,020), as compared with control group. At baseline, H_2O_2-induced DNA damage was 39.4 ± 5.6 %. After 8-week hazelnut intake, a DNA damage decrease by 15.3% (not significant) was found in subjects assigned to hazelnut treatment (from 43.7 ± 3.2% to 37.9 ± 6.8%; n=4). No effect was observed in the control treatment (from 35.1 ± 3.8% to 35.1 ± 8.7%; n=4;).
Since the samples analysed are limited (8 out of 60 subjects involved), further elaborations on data of the whole group of children will help understanding the effect of hazelnut consumption on oxidative stress in children with dyslipidemia.

11th International Comet Assay Workshop, 1 - 4 September 2015 (Antwerpen, Belgium)

Role of hazelnut consumption on DNA damage and lipid-related markers in children with primary dyslipidemia.

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Dyslipidemia is a traditional risk factor for atherosclerosis and cardiovascular disease development; it is associated closely th an increase of oxidative stress and a decrease of antioxidative defense mechanisms. Nuts are a rich source of bioactives such as polyunsaturated fatty acids, vitamins, phytosterols and polyphenols. These compounds could play an important role not only in the management of dyslipidemia, but also in the modulation of oxidative stress.
The objective of the present study is to evaluate the effect of hazelnut consumption on markers of oxidative stress, fatty acid composition of erythrocytes and serum lipids, in children with primary dyslipidemia.
Sixty children (11.5 ± 2.5 years) have participated in an 8-week controlled, parallel, dietary intervention study with hazelnuts (0.43 g/kg body weight per day). Subjects received dietary guidelines and were randomized in 3 groups: 1- hazelnuts with skin; 2- hazelnut without skin; 3- control (without hazelnuts).
Before and after intervention, blood samples were collected and used to evaluate the levels of formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites and H_2O_2-induced DNA damage in peripheral blood mononuclear cells (by comet assay), serum lipid profile (by automatic analyzer) and erythrocyte membrane phospholipids composition (by gas chromatography analysis).
Preliminary results on a subgroup (5 subjects receiving hazelnut with skin and 5 controls) show a reduction in the FPG-sensitive sites (from 13.8 ± 3.16% to 7.88 ± 2.98%) and H_2O_2-induced DNA damage (from 44.4 ± 3.1% to 35.7 ± 7.6%) following 8-week hazelnut consumption, while no effect seems occur in the control group.
Hazelnut decreases serum LDL-C level (-11.2%; *p*= 0.01) and seems to affect erythrocyte membrane phospholipids composition compared to baseline, while at this time no difference in triglycerides, total and HDL-cholesterol levels has been documented.
These preliminary results show a tendency towards a decrease in the levels of FPG-sensitive sites, H_2O_2-induced DNA damage and serum LDL-C after an 8-week hazelnut intervention. Data elaboration on the whole group of subjects will help understanding the effect of hazelnut consumption on lipid profile and markers of oxidative stress in children affected by primary dyslipidemia.

Front. Genet. Conference Abstract ICAW 2015 doi: 10.3389/conf.fgene.2015.01.00004.

4th International Conference on Foodomics, 8-9 October 2015 (Cesena, Italy)

Effect of a single portion of blueberries on markers of vascular function and oxidative stress in young smokers: a randomized-controlled trial.

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The consumption of berries (e.g. strawberries, cranberries and blueberries) has been shown to improve vascular function, inflammation and oxidative stress.
The aim of the present study was to investigate the effect of a single blueberry portion on markers of arterial function and oxidative stress in a group of smoker volunteers.
Sixteen healthy male smokers were recruited for a randomized controlled crossover study. Three types of conditions were assessed: 1-smoking treatment (S); 2-control treatment (300 mL of water with sugar + smoking (CS); 3-blueberry treatment (300 g of blueberry + smoking (BS). Each treatment was separated by one week of wash-out period. Peripheral arterial function (reactive hyperemia index, RHI) was measured before and 20 min after smoking. Plasma aminothiol concentrations, endogenous (formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites) and oxidatively induced DNA damage (resistance to H_2O_2-induced DNA damage) in peripheral blood mononuclear cells (PBMCs) were measured at baseline and 20, 60, 90, 120 min and 24 h after smoking. Blueberry consumption, but not the control drink, counteracted the impairment of RHI (-4.4±0.8% BS treatment vs -22.0±1.1% S treatment, $p<0.01$) after acute cigarette smoking. Analysis of variance did not show a significant effect of treatment on the modulation of plasma aminothiol concentrations and the levels of FPG-sensitive sites ($P > 0.05$) and H2O2-induced DNA damage ($P > 0.05$) in PBMCs, but revealed an effect of time for plasma concentrations of total cysteine ($P = 0.007$) and cysteine-glycine ($P = 0.010$) that increased following the three treatments. In conclusion, the consumption of a single blueberry portion counteracted the impairment of endothelial function but failed to modulate markers of oxidative stress. Further studies are necessary to confirm this finding and help clarifying the mechanisms of protection of blueberry against endothelial dysfunction.

ISBN 978-88-902152-7-8

4th International Conference on Foodomics, 8-9 October 2015 (Cesena, Italy)

Effect of 8-week hazelnuts consumption on lipid profile, fatty acid composition of erythrocytes membrane and levels of DNA damage in children with primary dyslipidemia.

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Dyslipidemic subjects are more exposed to oxidative stress and inflammation by increasing their risk to develop atherosclerosis. A dietary approach with nuts could improve dyslipidemia and counteract oxidative stress due to the content of mono- and polyunsaturated fatty acids and bioactive compounds.
The objective of the present study was to evaluate the effect of regular consumption of hazelnuts as a snack on lipid profile, fatty acid composition of red blood cells and DNA oxidative damage in children with primary dyslipidemia.
Sixty child (11.8 ± 2.6 years old) affected by primary dyslipidemia (total cholesterol and/or triglycerides value >95° percentile and BMI <95° percentile) were enrolled in an 8-week randomized, controlled, parallel, dietary intervention study. Subjects were divided into 3 groups of twenty subjects each, with the following conditions: one group consuming 0.43 g/Kg of hazelnuts with skin, one group consuming 0.43 g/Kg of hazelnuts without skin and a control group receiving only the dietary guidelines, without nuts, for 8 weeks. Serum lipid profile, the fatty acid composition of erythrocytes, and the levels of endogenous (formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites) and oxidatively induced DNA damage (resistance to H2O2-induced DNA damage) in peripheral blood mononuclear cells (PBMCs) were evaluated at the beginning and at the end of the intervention.
Preliminary results on 20 subjects shown that hazelnut consumption significantly reduces the levels of total LDL-cholesterol (- 6.37%, p=0.04) and H_2O_2-induced DNA damage (-26.5%, p=0.01), while increases the ratio HDL/LDL-cholesterol (+7.62%, p=0.01) and monounsaturated fatty acid composition of erythrocytes (+4.65%, p=0.006) as compared with control group.
Further elaborations on data of the whole group of subjects will help understanding the effect of hazelnut consumption on markers of oxidative stress, lipid profile and fatty acid composition of erythrocytes membrane in children with dyslipidemia.

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12th European Nutrition Conference (FENS), 20 – 23 October 2015 (Berlin, Germany)

Can hazelnut intake modulate oxidative stress and lipid-related markers in children with primary dyslipidemia?

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Introduction: Regular intake of nuts is suggested to reduce risk factors for cardiovascular disease (CVD) and to improve lipid profile. Dyslipidemic patients seem to be more susceptible to oxidative stress and CVD risk thus, they may benefit from nuts and their bioactives.
Objectives: The aim of this study was to investigate the effect of hazelnuts consumed as snack on oxidative stress markers, erythrocytes fatty acid composition and serum lipids, in children with primary dyslipidemia.
Methods: Sixty children (11.5 ± 2.5 years) with primary dyslipidemia were enrolled into an 8-week controlled, parallel, dietary intervention study with hazelnuts (0.43 g/kg body weight per day). Subjects received dietary guidelines and were randomized in 3 groups: 1- hazelnuts with skin; 2- hazelnut without skin; 3- control (without hazelnuts). Before and after the interventions, lipid profile, endogenous and H_2O_2-induced DNA damage and erythrocyte membrane phospholipids were evaluated.
Results: Preliminary results (n=15 subjects) show that, compared with baseline concentrations, a significant reduction in serum LDL-C level was observed only after hazelnut with skin treatment for 8 weeks (-11.2%; p= 0.01). No difference in triglycerides, total and HDL-cholesterol levels were demonstrated. At baseline, H_2O_2-induced DNA damage was 41.7 ± 10.0%. DNA damage decreased after hazelnut with skin (from 44.4 ± 3.1% to 35.7 ± 7.6%; n=5) as well as following hazelnut without-skin treatments (from 42.4 ± 16.0% to 33.3 ± 4.0%; n=5). No difference was observed in the control group. Moreover, data seem to suggest only a slight modulation of hazelnut treatment on erythrocyte membrane phospholipids composition.
Conclusion: Results on the subsample analysed show a tendency towards a decrease in the levels of H_2O_2-induced DNA damage and serum LDL-C after 8-week of hazelnuts. Further data on the whole group of subjects will help understanding the effect of hazelnut consumption in dyslipidemic children.

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12th European Nutrition Conference (FENS), 20 – 23 October 2015 (Berlin, Germany)

Repeatability of arterial function by EndoPAT2000: a potential tool for studying vasoreactivity of bioactive-rich foods.

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Introduction: EndoPAT2000 is a non invasive pletysmographic method used for the evaluation of arterial function in several epidemiological studies, while its use in human intervention studies is still poor exploited. In this regards, it has been suggested that multiple evaluations performed within the day could improve endothelium-dependent vasodilatation. This could mask/overestimate the effect of intervention with vasoactive dietary compounds in the modulation of arterial function.
Objective: The objective of the present study was to investigate the inter- and intraday repeatability of RHI measured by EndoPAT2000 in healthy volunteers.
Methods: Twenty-two male subjects were recruited for the interday study in which RHI repeatability was tested in two consecutive days at the same time points. Sixteen volunteers were enrolled for the RHI intraday repeatability study measured at baseline, after 2 and 4 h. Data were evaluated by analysis of variance. Agreement between paired RHI was evaluated by Bland–Altman method, coefficient of variation (CV), coefficient of repeatability (CR) and intraclass correlation coefficient (ICC).
Results: The RHI mean did not vary significantly between day 1 and 2 (2.00 ± 0.41 *vs* 1.96 ± 0.33; $p= 0.429$) and showed a good repeatability as documented by CV (6%), CR (0.505), and ICC (0.773).
Multiple evaluations within the day significantly affected RHI ($p=0.0001$) and the repeatability of the measurement. A significant increase occurred at 4 h compared to baseline (+37.5%; $p=0.0001$) and 2 h (+21.5%; $p=0.004$), respectively. In addition, RHI at 4 h showed a low repeatability (CV: 18.8%; CR: 1.26; ICC: 0.48).
Conclusions: In conclusion, RHI showed a good interday repeatability in healthy subjects, while intraday repeatability can be acceptable till 2 h. Further investigations and standardization of the protocols are needed to assess the postprandial effect of vasoactive dietary compounds.

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29th National Congress SISA (Italian Atherosclerosis Society), 22-24 November 2015 (Bologna, Italy)

Effect of hazelnut consumption on lipid profile, fatty acid composition of erythrocyte membranes and oxidative stress markers in children with primary dyslipidemia.

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Dyslipidemic patients are more prone to oxidative stress and inflammation, increasing their risk to develop atherosclerosis. A dietary approach is crucial in the management of dyslipidemia, especially during childhood. Regular intake of nuts, source of mono- and polyunsaturated fatty acids, vitamins and bioactive compounds, could improve lipid profile and counteract oxidative stress.
The aim of the study was to investigate the effect of regular intake of hazelnuts consumed as a snack, on serum lipid levels, fatty acid composition of erythrocyte membranes and levels of DNA damage, as marker of oxidative stress, in children with primary dyslipidemia.
A randomized, controlled, parallel dietary intervention study was scheduled. The study involved 60 children (11.5 ± 2.5 years) affected by primary dyslipidemia, with total cholesterol and/or triglycerides value >95° percentile and BMI <95° percentile). Subjects received dietary guidelines and were divided into 3 groups of twenty subjects each, with the following conditions: one group consuming 0.43 g/Kg of hazelnuts with skin, one group consuming 0.43 g/Kg of hazelnuts without skin and a control group receiving only the dietary guidelines, without nuts, for 8 weeks. Before and after the intervention, blood samples were collected for the evaluation of serum lipid profile, fatty acid composition of erythrocyte membranes (by gas-chromatography), and levels of endogenous and oxidatively induced DNA damage in peripheral blood mononuclear cells (by comet assay).
Preliminary results on 20 subjects shown that hazelnut consumption significantly reduced the levels of total LDL-cholesterol (-6.37%, p=0.04) and H_2O_2-induced DNA damage (-26.5%, p=0.01), while increases the ratio HDL/LDL-cholesterol (+7.62%, p=0.01) and monounsaturated fatty acid composition of erythrocytes (+4.65%, p=0.006), as compared with control group. Further analysis of data on the whole group of subjects will help understanding the potential beneficial effect of hazelnut consumption on oxidative stress markers and lipid profile in children with dyslipidemia.

Giornale Italiano dell'Arteriosclerosi 2015;6(4):66-120.

36th National Congress SINU (Italian Society of Human Nutrition), 2-4 December 2015 (Florence, Italy)

Hazelnut (*Corylus avellana* L.) consumption improves lipid profile and markers of oxidative stress in children with primary dyslipidemia

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Background: Dyslipidemia, a key independent modifiable risk factor for cardiovascular disease, is associated to an increase of inflammation and oxidative stress. A dietary approach is crucial in the management of dyslipidemia, especially during childhood. Regular intake of nuts, source of monounsaturated fatty acids, phytosterols, vitamins (i.e. vitamin E) and polyphenols, could improve lipid profile and counteract oxidative stress.
Objective: The aim of the study was to investigate the effect of the regular intake of hazelnuts (*Corylus avellana* L.*)* consumed as a daily snack, on serum lipid profile and cell protection against DNA damage (endogenous and oxidatively-induced), as marker of oxidative stress, in children with primary dyslipidemia.
Methods: An 8-weeks randomized, controlled, parallel dietary intervention study with hazelnuts (20-30 g based on weight) was scheduled. The study involved 60 children (11.5 ± 2.5 years) affected by primary dyslipidemia, with total cholesterol and/or triglycerides value >95° percentile and BMI <95° percentile. Subjects received dietary guidelines and were randomized into 3 groups: 1- group consuming hazelnuts with skin; 2- group consuming hazelnuts without skin; 3- control group (no hazelnuts) receiving only the dietary guidelines. Before and after intervention, blood samples were collected for the evaluation of serum lipid profile and levels of endogenous and oxidatively-induced DNA damage in peripheral blood mononuclear cells.
Results: The regular intake of hazelnuts, with and without skin, significantly (p<0.05) reduced the levels of serum LDL-cholesterol (-6.6% and -5.5%, respectively) and endogenous DNA damage (-28.6% and -26.9%, respectively) compared to baseline, while no significant effect was observed in the control group. Furthermore, the consumption of hazelnut with skin significantly decreased (-20.3%, p=0.042) the levels of oxidatively-induced DNA damage
Conclusion: Our findings suggest that hazelnut consumption can improve lipid profile and reduce oxidative stress markers in children affected by familiar dyslipidemia.

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36th National Congress SINU (Italian Society of Human Nutrition), 2-4 December 2015 (Florence, Italy)

Effect of a single portion of blueberries (*Vaccinium corymbosum*) on markers of oxidative stress and inflammation in young smokers.

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Background: Blueberries are a rich source of polyphenol-bioactive compounds such as anthocyanins and phenolic acids that have shown to improve vascular function, reduce inflammation and oxidative stress. We previously documented that a portion of blueberries reversed endothelial dysfunction induced by acute cigarette smoking.
Aim: The aim of the present study was to evaluate whether the beneficial effects observed on endothelial function were mediated by modulation of markers of oxidative stress and inflammation in the same group of smoker volunteers.
Methods: Fourteen out of 16 healthy male smokers previously enrolled participated in a 3-arm randomized controlled study. Three types of conditions were assessed: 1-smoking treatment (S); 2-control treatment (300 mL of water with sugar + smoking (CS); 3-blueberry treatment (300 g of blueberry + smoking (BS). Each treatment was separated by one week of wash-out period. Plasma aminothiol concentrations, serum C-reactive protein, endogenous (formamidopyrimidine-DNA glycosylase (FPG)-sensitive sites) and oxidatively induced DNA damage (resistance to H_2O_2-induced DNA damage) in peripheral blood mononuclear cells (PBMCs) were measured at baseline, 20, 60, 90, 120 min and 24 h after smoking. Results were analysed by analysis of variance.
Results: ANOVA did not show an effect of treatment on the modulation of plasma aminothiol concentrations, but revealed a significant effect of time. In particular, we observed a significant increase in total cysteine (P=0.007), cysteine-glycine (P=0.010), but not glutathione (P>0.05) and homocysteine (P>0.05), following acute cigarette smoking in all the three treatments. No significant effect of treatment was also reported for the levels of FPG-sensitive sites and H_2O_2-induced DNA damage in PBMCs, and serum circulating levels of C-reactive protein.
Conclusion: In our experimental conditions, the consumption of a single blueberry portion did not affect markers of oxidative stress and inflammation in young smokers. Further studies are necessary to clarify the mechanisms of protection of blueberry against endothelial dysfunction.

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84th EAS (European Atherosclerosis Society) Congress, 29 May-1 June 2016 (Innsbruck, Austria)

Hazelnut-enriched diet improves lipid profile, fatty acid composition of erythrocytes membrane and markers of oxidative stress in children with primary dyslipidemia: a randomized control trial.

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Aim: Dyslipidemia is the major cause for atherosclerosis and is associated to an increase of oxidative stress. Children affected by primary dyslipidemia may run an additional risk to develop CVD during adulthood. Diet rich in unsaturated fatty acids and bioactives can positively influence the blood lipid profile and the antioxidant status. The aim of the study was to investigate the effect of daily intake of hazelnuts consumed as a snack, on lipid profile, erythrocytes fatty acid composition and markers of oxidative stress in dyslipidemic children.
Methods: An 8-week randomized, controlled, parallel dietary intervention study with hazelnuts (20-30 g based on weight) was scheduled. The study involved 60 children affected by primary dyslipidemia. Subjects received dietary guidelines and were randomized to the following treatment: 1- hazelnuts with skin; 2- hazelnuts without skin; 3- control group (no hazelnuts). Before and after intervention, blood samples were collected for the evaluation of serum lipid levels, fatty acid composition of erythrocytes membrane and levels of endogenous and oxidatively-induced DNA damage in peripheral blood mononuclear cells.
Results: The regular intake of hazelnuts, with and without skin, significantly ($p<0.05$) reduced the levels of serum LDL-cholesterol and endogenous DNA damage, while increased the ratio of MUFA/SFA and MUFA in erythrocytes membrane. Furthermore, the consumption of hazelnut with skin significantly decreased the levels of oxidatively-induced DNA damage. No effect was observed in the control group.
Conclusion: Our findings suggest a beneficial effect of hazelnut-enriched diet in the management of dyslipidemia and oxidative stress in children.

21th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 14-16 September 2016 (Portici, Italy)

Effect of bioactive-rich diet on risk factors for chronic-degerative diseases in “at risk” groups of population.

Deon Valeria

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The aim of the present PhD project was to evaluate the effect of specific foods and food bioactives in the modulation of risk factors for chronic-degenerative diseases in at risk groups of population, thorough the exploitation of in-vivo and ex-vivo approaches. In particular, the first part of the project was focused on a dietary intervention study on children affected by primary hyperlipidemia to evaluate the impact of hazelnut intake on markers of oxidative stress and lipid profile. While in the second part of the project, different food bioactives were investigated for their potential immunomodulatory effect through *ex-vivo* measurements.

37th National Congress SINU (Italian Society of Human Nutrition), 30 November - 2 December 2016 (Bologna, Italy)

Effect of alpha-linolenic acid supplementation on serum lipid profile and fatty acid composition of erythrocyte phospholipids in children and adolescents with primary hyperlipidemia: preliminary observations

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Background: Hyperlipidemia is a modifiable risk factor for atherosclerosis and children affected by primary hyperlipidemia may have an additional risk to develop cardiovascular diseases during adulthood. Diet rich in polyunsaturated fatty acids (PUFA) can positively influence the lipid profile.
Objective: The study aimed to investigate the effect of α-linolenic acid (ALA) supplementation on fatty acid composition of red blood cell (RBC) phospholipids and serum lipid profile in children and adolescents with primary hyperlipidemia.
Methods: An 8-week parallel dietary intervention study was scheduled. Thirty-six hyperlipidemic probands (6-16 years) on diet therapy were subdived into 2 groups: 1- ALA group, receiving 0,7 g/die of ALA supplement; 2- control group, receiving only the dietary guidelines. Before and after the 8-week of treatment, blood samples were collected for the analysis.
Results: Preliminary results (n=20) showed that patients supplemented with ALA significantly reduced the levels of RBC saturated and monounsaturated fatty acids (mean of change from baseline: -7.8% and -2.6%, respectively), while increased the levels of PUFA (+10.4%) and the omega-3 index (+ 1.6%) compared to control group. In particular, ALA supplementation enhanced the RBC levels of n-3 PUFA (+2.2%) and n-6 PUFA (+8.1%). No effect was observed on serum lipid profile.
Conclusion: In conclusion, these preliminary results seem to support the contribution of ALA supplementation in the modulation of RBC phospholipid composition and omega-3 index. Further analysis on the whole group of subjects will help understanding the effect of ALA on RBC phospholipid composition and lipid profile in hyperlipidemic children.

6.4 AWARD

Young Researchers Award for the best scientific paper presented at the National Meeting of Italian Society of Human Nutrition (SINU), Rome, Italy, 20-21 October 2014.

In occasione della Riunione Nazionale SINU "*Nutrizione: perimetri & orizzonti*" - Roma, 20-21 Ottobre 2014

si ringrazia la Dott.ssa **Valeria DEON**

vincitore del premio Giovani Ricercatori SINU per il contributo scientifico selezionato, dal titolo:

A single portion of blueberries can affect peripheral arterial tone in young smokers with endothelial dysfunction: preliminary observations and perspectives

V. Deon, C. Del Bo', C. Lanti, M. Porrini, J. Campolo, D. Klimis-Zacas, P. Riso

Prof. Furio Brighenti, *Presidente*

Dott.ssa Alessandra Fabbri, *Segretario*

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