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Post-prandial effects of hazelnut-enriched high fat meal on LDL oxidative status, oxidative and inflammatory gene expression of healthy subjects: a randomized trial

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Abstract. – OBJECTIVE: Postprandial oxidative stress is characterized by an increased susceptibility of the organism towards oxidative damage after consumption of a meal rich in lipids and/or carbohydrates. Micronutrients modulate the immune system and exert a protective action by reducing low-density lipoproteins oxidation (ox-LDL) via induction of antioxidant enzymes.

SUBJECTS AND METHODS: The clinical study was a randomized and cross-over trial, conducted through the CONSORT flowchart. We evaluated the gene expression of 103 genes related to oxidative stress (HOSp) and human inflammasome pathways (HIp), and ox-LDL level at fasting and after 40 g raw "Tonda Gentile delle Langhe" hazelnut consumption, in association with a McDonald's® Meal (McDM) in 22 healthy human volunteers.

RESULTS: Ox-LDL levels significantly increased comparing no dietary treatment (NDT) *vs.* McDM, and decreased comparing McDM *vs.* McDM + H ($p<0.05$). Percentage of significant genes expressed after each dietary treatment were the follows: (A) NDT *vs.* McDM: 3.88% HIp and 17.48% HOSp; (B) NDT *vs.* McDM + H: 17.48% HIp and 23.30% HOSp; (C) McDM *vs.* McDM + H: 17.48% HIp and 33.98% HOSp.

CONCLUSIONS: Hazelnut consumption reduced post prandial risk factors of atherosclerosis, such as ox-LDL, and the expression of inflammation and oxidative stress related genes. Chronic studies on larger population are necessary before definitive conclusions.

Key Words:
Oxidized-LDL, Nutrigenomic, Hazelnut consumption.

Abbreviations list

Alanine transferase = ALT; Appendicular skeletal muscle mass index = ASMMI; Aspartate aminotransferase = AST; Atherogenic Index = AI; Atomic absorption spectroscopy = AAS; Baseline = B; Body mass index = BMI; Cholesterol/Saturated fatty acids Index = CSI; Chronic non-communicable diseases = CNCDs; Coronary artery disease = CAD; C-reactive protein = CRP; Dry weight = DW; Erythrocyte sedimentation rate = ESR; Ferric Reducing Antioxidant Power = FRAP; Fold Change = FC; Food and Drug Administration = FDA; Gallic Acid Equivalent = GAE; Hazelnut Consumption = HC; High Density Lipoproteins = HDL; Human inflammasome pathways = HIp; Human Oxidative Stress pathways = HOSp; Inductively coupled plasma mass spectrometry = ICP-MS; Low-Density Lipoproteins = LDL; McDonald's® Meal = McDM; McDonald's® Meal with Hazelnut = McDM + H; Monounsaturated fat-

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ty acids = MUFAs; Net Acid Excretion = NAE; Nitric Oxide = NO; Nutritional quality index = NQI; Oxidized Low-Density Lipoproteins = ox-LDL; Peroxide Value = PV; Potential Renal Acid Load = PRAL; Saturated fatty acids = SFAs; Thrombogenicity Index = TI; Total Antioxidant Capacity = TAC; Total body bone = TBBone; Total body fat = TBFat; Total body fat percentage = PBF; Total body lean = TBLean; Total Phenolic Content = TPC; Waist/hips ratio = W/H; World Heart Organization = WHO.

Genes abbreviations list

Absent In Melanoma 2 = AIM2; Actin Beta = ACTB; Albumin = ALB; Arachidonate 12-Lipoxygenase = ALOX12; Baculoviral Iap Repeat Containing 3 = BIRC3; B-Cell Ccl/Lymphoma 2 = BCL2; Bcl2 like 1 = BCL2L1; Caspase 1 Apoptosis-Related Cysteine Peptidase (Interleukin 1 Beta Convertase) = CASP1; Caspase 1 Apoptosis-Related Cysteine Peptidase = CASP5; Caspase Recruitment Domain Family Member 6 = CARD6; Cathepsin B = CTSB; Chemokine (Cxc Motif) Ligand 2 = CXCL2; Copper Chaperone For Superoxide Dismutase = CCL5; Cytoglobin = CYGB; 24 Dehydrocholesterol Reductase = DHCR24; Dual Oxidase 1 = DUOX1; Dual Oxidase 2 = DUOX2; Dual Specificity Phosphatase 1 = DUSPO1; Eosinophil Peroxidase = EPX; Epoxide Hydrolase 2 Cytoplasmic = EPHX2; Fas (Tnfrsf6) Asociated Via Death Domain = FADD; Ferritin Heavy Polypeptide 1 = FTH1; Forkhead Box M1 = FOXM1; Glutamate-Cysteine Ligase, Catalytic Subunit = GCLC; Glutathione Peroxidase (Phospholipid Hydroperoxidase) = GPX4; Glutathione Peroxidase (Plasma) = GPX3; Glutathione Peroxidase 1 = GPX1; Glutathione Peroxidase 7 = GPX7; Glutathione Reductase = GSR; Inhibitor of Kappa Light Polypeptide Gene Enhancer In B-Cell Kinase Gamma = IKBKG; Keratin = KRT1; Mediterranean Fever = MEFV; Methionine Sulfoxide Reductase A = MSRA; Myeloperoxidase = MPO; Mpv17 Mitochondrial Inner Membrane Protein = MPV17; NADPH dehydrogenase quinone 1 = NQO1; Neutrophyl Cytosolic Factor 1 = NCF1; Nitric Oxide Synthase 2 Inducible = NOS2 or iNOS; Nrl Family Card Domain Containing 4 = NLRC4; Nudix (Nucleoside Dyphosophate Linked Moiety X) Type Motif 1 = NUDT1; Oxidation Resistance 1 = OXR1; Oxidative Stress Responsive 1 = OXSR1; Pannexin 1 = PANX1; Peroxidasin Omolog (Drosophila) = PXDN; Peroxiredoxin 2 = PRDX2; Peroxiredoxin 4 = PRDX4; Peroxiredoxin 5 = PRDX5; Peroxiredoxin 6 = PRDX6; Phosphatidylinositol 3,4,5 Triphosphate Dependent Rac Exchange Factor 1 = PREX1; Polynuclotide kinase 3' phosphatase = PNKP; Pyd And Card Domain Containing = PYCARD; Ribosomal Protein Large P0 = RPLPO; Seleno Protein P, Plasma, 1 = SEPP1; Serine/Treonine Kinase 25 = STK25; Surfactant Protein D = SFTPD; Superoxide Dismutase 1 Soluble = SOD1; Beta Activated Kinase 1/Map 3k7 Binding Protein 1 = TAB1; Thioredoxin = TXN or TRX; Thioredoxin Reductase 2 = TXNRD2; Thioredoxin Intercating Protein = TXNIP; Tool Interleukine 1 Receptor (Tir) Domain Containing Adaptor Protein) = TIRAP; Tumor Necrosis Factor (Ligand) Superfamily Member 11 = TNFSF11; Tumor Necrosis Factor (Ligand) Superfamily Member 4 = TNFS4; Tumor Necrosis Factor = TNF; Uncoupling Protein 2 (Mitochondrial Proton Carrier) = UCP2.

Introduction

According to the World Health Organization (WHO)[1] the frequency of chronic non-communicable diseases (CNCDs) is rapidly rising, with an increase of 15% globally between 2010 and 2020 (44 million deaths). CNCDs could be defined as "food related" pathologies, as the causes are in part attributable to the quality of food in the diet and to the modulation of certain genes induced by the foods themselves[2]. Food and Drug Administration (FDA) has authorized a health claim that the consumption of nuts leads to a reduced risk of coronary artery disease (CAD)[3].

Effects of almond, peanut, walnut, have been studied extensively[4]. However, there are few trials investigating the effects of hazelnut consumption on lipid profiles in normolipidemic[5] and hypercholesterolemic subjects[6].

Due to their special composition of macro and micronutrients, hazelnuts are distinct from other dried nuts, and the same composition gives it a special flavor and distinctive healthy properties[7,8]. Hazelnut has some cardioprotective compounds including vitamin E (α-tocopherol isomer), phytosterols, vitamin B6, folate, L-arginine, polyphenols and fiber[9]. Moreover, hazelnut is the second richest source of monounsaturated fatty acids (MUFAs) among nuts. It contains approximately 82-83% MUFAs, mainly oleic acid (18:1) and less than 7.7-8.0% saturated fatty acids (SFA). One of the most important features of the hazelnut among tree nuts, it has the highest ratio of unsaturated/saturated fatty acids (10.8-11.9)[10]. Monounsaturated fatty acids have been reported to have hypolipidemic effects[6].

The atherosclerotic processes underlying cardiovascular disease are intimately connected with a state of chronic inflammation, involving a variety of pathological changes such as endothelial cell activation, low-density lipoprotein (LDL) modification, macrophage chemotaxis, and vessel smooth muscle cell migration[11-13].

In industrialized societies, LDL cholesterol concentrations often exceed physiological requirements.

At vasal level, the formation of oxidized low-density lipoprotein (ox-LDL), also observed in healthy subjects[14], convert the native LDL into pathogenic[15], immunogenic and atherogenic particles[16]. This ox-LDL recalls macrophages, with the consequent activation of monocyte-macrophage system and formation of foam-cells, contributing together to the vascular inflammation, lipid deposition, and smooth muscle cell differentiation, to the development of atherosclerosis and cardiovascular disease[12,13].

The chance to identify a new tool among functional foods able to prevent oxidative processes, is a promising therapeutic strategy against atherosclerosis and many other diseases[17,18].

To our knowledge, the postprandial effects of a high fat meal have never been studied about the levels of some typical biomarkers associated with CVD, including oxidative stress, anti-inflammatory and antioxidant activity. Therefore, we set-up a randomized, and crossover dietary treatment trial in healthy human volunteers to investigate the antiatherogenic effects of 40 g raw hazelnut intake, by evaluating the post prandial plasma ox-LDL level, after a McDonald's® Meal (McDM).

As few studies have a nutrigenomic approach to highlight the effects of hazelnut consumption [19,20], we investigated the gene expression of 103 genes related to oxidative stress and inflammation, in the same conditions. We hypothesized that the magnitude of changes in postprandial responses would be less with the McDM associated with hazelnut, compared with the McDM alone.

Subjects and Methods

The Human Intervention Study

Study Design

The primary aim of the present randomized, crossover dietary treatment trial in healthy human volunteers was to assess the effect on the post prandial plasma ox-LDL level in healthy subjects after a McDM, with and without the supplement of 40 g of raw hazelnut. The second aim was to examine the contribution of 40 g of raw hazelnut intake in changes of gene expression levels of 103 genes related to oxidative stress and inflammation, in the same conditions.

The trial study protocol has been registered with ClinicalTrials.gov Id: NCT01890070. The experimental study was conducted through the CONSORT flowchart presented in Figure 1.

The randomized crossover study was divided into no. 2 dietary intervention: (1) McDM: McDMonald's Meal; (2) McDM + H: McDMonald's Meal + 40 g of raw hazelnuts. A washout period of 3 weeks was inserted. The nutritional status was detected at baseline (T1). The blood sample for nutrigenomic and biochemical analysis was

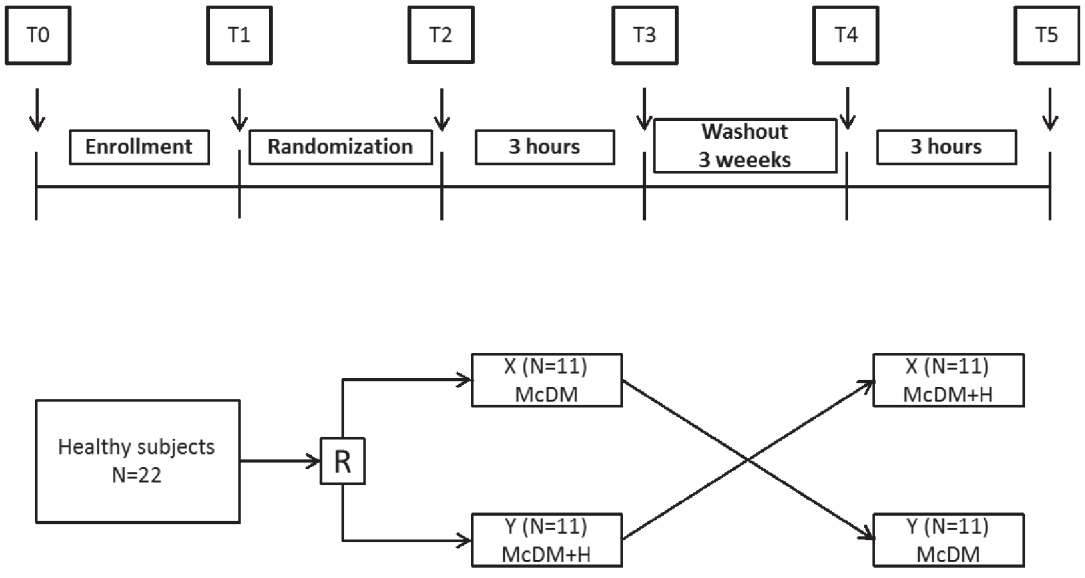


Figure 1. Study design and intervention.

collected at baseline (T1, T2, T4), and 3 hours after meals (T3, T5).

The clinical study was a randomized, and crossover dietary intervention trial. Subjects, who were eligible for the study, were randomly divided into two groups (X and Y). The order of administration was determined using computer-generated random numbers.

The randomization is based on a randomized Latin square to form two possible combinations of the two treatments, and the two groups were balanced (11 subjects/group). Randomization and implementation procedures were performed by a person from the clinical investigation unit not involved in the clinical trial.

At T0, start of the study, healthy volunteers were enrollment. After the enrollment (T1), health and nutritional status were evaluated for all subjects (Baseline).

The clinical evaluation at baseline was based on anthropometric, body composition evaluation and blood analysis.

The study had no. 2 dietary treatment (DT): 1) McDM; 2) McDM + 40 g of raw hazelnut (McDM + H). As first, the group X received the no. 1) DT, and the group Y received the no. 2) DT. After three weeks of washout period, to avoid additive effects on treatments to follow, the DT for group X and Y were reversed.

Fasting (T2, T4), considered as no dietary treatment (NDT), and three hours after each DT (T3, T5), subjects underwent to blood analysis for quantification of ox-LDL, and a genomic evaluation of 103 genes belonging to the pathway of oxidative stress, and inflammation (Figure 2).

Subjects

A total of 25 participants were consecutively recruited from May 2014 to June 2014, within a program of a routine medical check-up at the Section of Clinical Nutrition and Nutrigenomic, at the University of Rome "Tor Vergata".

The following are the eligibility criteria for the study: aged between 18 and 65 years and body mass index (BMI) ≥19 kg/m^2. The exclusion criteria were as follows: pregnancy, active smoking, high blood pressure (≥140/90 mmHg), BMI >30 kg/m^2, acute or chronic diseases, autoimmune diseases HIV/AIDS, cancer, gastrointestinal diseases, vegetarianism, and the use of supplements (e.g. antioxidants, vitamins); use of anti-inflammatory drugs or that can change the oxidative stress. For this reason, subjects who habitually consumed beverages rich in antioxidants such as tea, herbal tea, coffee and cocoa, 500 ml higher amounts, (as tested with the food frequency questionnaires) were excluded.

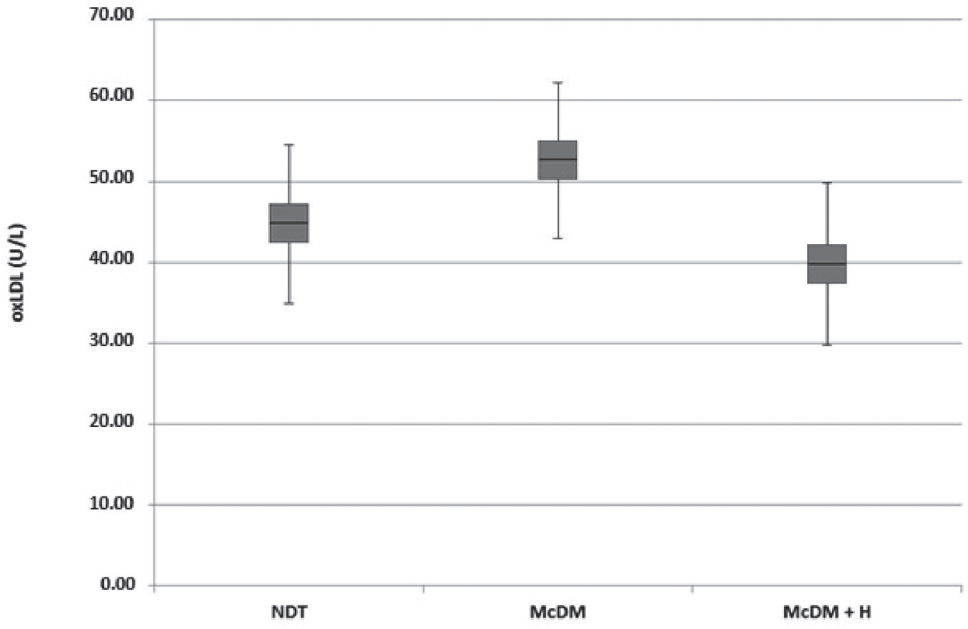


Figure 2. Comparative values of ox-LDL level for each dietary treatment.

Subjects that daily were consuming flavonoid-rich beverages, such as tea, herb tea, coffee, cocoa and fruit juice, more than 500 ml (as estimated from food frequency questionnaire), were also excluded.

Of the 25 subjects enrolled, 3 of them did not meet the inclusion criteria; therefore, 22 participants were resulted eligible for the study. The number of volunteers has been chosen according to previous nutrigenomic study in a clinical trial in which the number of volunteers was 24[20].

Participants were asked to exclude hazelnut and other tree nut types 15-day before the first intervention (run-in period) and during the study. Natural foods rich in antioxidants intake was monitored so that individual diets had similar antioxidant contents throughout the study.

Subjects were asked to maintain their usual lifestyle habits and to report any illness or abnormality presented during the study period.

After each DT, it was evaluated the presence of symptoms due to treatment (e.g. bloating, indigestion, dizziness, or other) by medical staff. The study was completed by all enrolled subjects.

Informed consent was signed by all participants in accordance with principles of the Declaration of Helsinki.

Nutritional status assessment, genomic analysis, settings and data collection were performed at the Section of Clinical Nutrition and Nutrigenomic, Department of Biomedicine and Prevention of the University of Rome "Tor Vergata". Any changes to trial outcomes after the trial commenced occurred.

Anthropometric Measurements

At T1, after a 12-hour overnight fast, all patients underwent assessment of body composition, according to standard methods[21] and were instructed to take off their clothes and shoes before undergoing the measurements.

A flexible steel metric tape to the nearest 0.5 cm was used for waist and hip circumferences, according to International Society for the Advancement of Kin anthropometry protocol taken at the greatest posterior protuberance of the buttocks and as recommended by the National Institute of Health Guidelines. Body weight (kg) was measured to the nearest 0.1 kg, using a balance scale (Invernizzi, Rome, Italy)[22]. Height (m) was measured using a stadiometer to the nearest 0.1 cm (Invernizzi, Rome, Italy). BMI was calculated using the formula: BMI = body weight/height^2 (kg/m^2).

Dual X-ray Absorptiometry (DXA)

At T1, body composition analysis was assessed by DXA (i-DXA, GE Medical Systems, Milwaukee, WI, USA) to evaluate total body fat (TBFat) and total body lean (TBLean), according to the previously described procedure[23], after quality control and calibration. The subjects were instructed on the testing procedure.

The range of coefficient of variation (coefficient of variation = 100 x SD/mean) intra and inter subjects was 1%-5%. The coefficient of variation for bone measurements is less than 1%; the coefficient of variation on this instrument for five subjects scanned six times over a 9-month-period were 2.2% for TBFat, and 1.1% for TBLean.

Total body fat percentage (PBF) was calculated as TBFat mass divided by total mass of all tissues, also considering the total body bone (TBBone), as the follow:

$$PBF = \frac{TBFat}{(TBFat + TBLean + TBBone)} \times 100$$

Analysis of Blood Samples

After 12-hour of fasting, at T1 blood samples were collected in sterile tubes with EDTA (Vacutainer®) (Life Technologies Corporation, Carlsbad, CA, USA) place on ice and centrifuged at 1600 rpm for 1 minutes at 4°C. Blood analyses included aspartate aminotransferase (AST), alanine transferase (ALT), creatinine, fibrinogen and were carried out, according to standard procedures, with ADVIA®1800 Chemistry System (Siemens Aktiengesellschaft, Munich, Germany)[24]. Fasting glucose was measured with glucose oxidase method and automated glucose analyzer (COBAS INTEGRA 400, Roche Diagnostics, Indianapolis, IN, USA). HDL-cholesterol and LDL-cholesterol were analyzed with enzymatic colorimetric techniques (Roche143 Modular P800, Roche Diagnostics, Indianapolis, IN, USA). Serum CRP levels were measured with the high-sensitivity sandwich enzyme immunoassay (Immunodiagnostic). Serum triglycerides were analyzed on the Beckman Synchron LX20 (Beckman Coulter, Brea, CA, USA) automated. All blood samples were analyzed with the same lot of reagents or assay to reduce variability.

Analyses were carried out at the accredited Clinical Chemical Laboratories of the "Nuova Annunziatella" Clinic of Rome, Italy.

Sample Collection and RNA Extraction

PAX gene Blood RNA Tubes (Pre Analytix Qiagen, Hombrechtikon, Switzerland) was used to collected and stabilized blood samples, which were then stored at -80°C. PAX gene Blood miRNA Kit (Pre Analytix Qiagen, Hombrechtikon, Switzerland) was used to purify total RNA of each sample, which was then quantified and assessed for quality by spectrophotometry (Nanodrop, Wilmington, DE, USA) and agarose gel electrophoresis.

Quantitative Real-Time pcr and Data Analysis

Specific RT2 Profiler PCR Arrays (Qiagen, Venlo, The Netherlands) was used for Human Oxidative Stress (PAHS-065ZA, Qiagen, Venlo, The Netherlands) pathway and Human Inflammation (PAHS-097ZA, Qiagen, Venlo, The Netherlands) pathway. qRT-PCR experiments were performed according to manufacturer's instruction (Qiagen, Hombrechtikon, Switzerland).

Fold Change value (FC)=$2^{-\Delta\Delta CT}$ was used to plot relative gene expression[25]. Genes were considered statistically significant with an absolute FC value of >1.5 and p-value < 0.05.

Low-Density Lipoprotein Oxidative Status

After a 12-hour overnight, fast blood samples were collected and stabilized in EDTA, and stored at -80°C until analysis. Circulating ox-LDL level in plasma was measured by enzyme-linked immunosorbent assay using the mAb-4E6 antibody (Mercodia AB, Uppsala, Sweden), according to the customer protocol.

Dietary Treatment Description

Dietary treatment consisted of a high fat meal (McDM), composed by no. 1 Big Tasty Bacon®, and no. 1 small French Fries (meal Kcal composition: 26.8% Kcal from carbohydrates; 18.2% Kcal from protein, which 70% from animal proteins; and 55% Kcal from fat).

The McDM was supplemented with 40 g of Italian hazelnut. The daily raw hazelnuts supplement was provided in pre-weighted packages to each study participant. Subjects consumed the hazelnuts as they were provided. Hazelnuts were consumed as snacks once (40 g) after the McDM.

Hazelnut Sample Description

Nut samples served to participants were from the Italian hazelnut (H, *Corylus avellana L.*) cultivar 'Tonda Gentile delle Langhe' (TGL, syn. "Tonda Gentile Trilobata") and were harvested in 2012 in Piedmont (Italy) in the area of GPI "Nocciola Piemonte".

After harvest, the nuts were dried to about 5% moisture content in the kernel. The nuts, at 10% starting moisture content, were dried in a food dryer with a slow stream of warm air (50°C) for 8 h, After an additional cooling step of 6 h, the samples were shelled and stored for 4 months in storage room with modified atmosphere (1% oxygen, 99% nitrogen) until consumption to maintain the quality of the hazelnuts[26].

Nutritional Characteristics of Hazelnuts

Gravimetric method was used to assess hazelnut moisture content. Hazelnuts protein concentration was obtained by mineralization in sulfuric acid, steam distillation, acid-base titration. Hazelnuts lipid content was measured through solvent extraction[27]. Fatty acid composition was estimated according to UNI EN ISO 12966-2: 2011 + ISO 5508: 1990 methods. For the oil, titratable acidity (% of oleic acid) were determined according to ISO 660: 2009, and peroxide value (PV, determined by iodometric titration and expressed as meq of active O_2/kg of oil) was assessed according to European Official Methods of Analysis (Council Regulation, EEC-N. 2568/91). Oil tocopherol content was determined by HPLC as Kodad et al[28] described. Mineralization in nitric acid, inductively coupled plasma mass spectrometry (ICP-MS) or atomic absorption spectroscopy (AAS)[29] were used to micronutrient analysis.

Total Phenolic Content (TPC) and Total Antioxidant Capacity (TAC)

Total phenolics content of triplicate raw hazelnut kernel extracts was evaluated with the Folin-Ciocalteu procedure[30], and gallic acid was used as standard. At the same time, FRAP (Ferric Reducing Antioxidant Power) assay was used for the total antioxidant capacity estimation[31,32].

Bromatological Composition of Meals

McDM and McDM+H bromatological composition was evaluated with DIETOSYSTEM version 12.00.13 (DS Medica® SRL, Milan, Italy). The following nutritional quality indices[33] were evaluated:

Atherogenic Index (AI) determines the atherogenic risk of a diet. It is calculated as the ratio between saturated and unsaturated fatty acids:

$$IA = \frac{[(4 \times C14:0) + C16:0 + C18:0]}{[\sum MUFA + \sum PUFA - \omega 6 + \sum PUFA - \omega 3]}$$

Thrombogenicity Index (TI) shows the relationship between saturated and unsaturated fat acids; in the calculation is assigned a different weight to the various ω-3 and ω-6 fatty acids on the basis of their anti-thrombogenic power, taking into account also monounsaturated fatty acids:

$$IT = \frac{(C14:0 + C16:0 + C18:0)}{(0.5 \times MUFA) + (0.5 \times PUFA) - \omega 6 + (3 \times PUFA) - \omega 3 + PUFA - \omega 3/PUFA - \omega 6.}$$

Cholesterol/Saturated fatty acids Index (CSI) can be used as a fast and accurate way to assess the content of saturated fat and dietary cholesterol. It expresses the lipid quality of the food and at the same time provides a good indicator for the detection of atherogenic risk. The value of the CSI is expressed on a scale from 1 to 100. The atherogenic potential of food refers to the cholesterol and saturated fat contained in it; lower index suggests a lower probability of incidence of cardiovascular disease:

CSI = (1.01 x g of saturated fat) + (0.05 x mg of cholesterol).

Potential Renal Acid Load (PRAL) is used to calculate the chemical equilibrium between nutritional alkalizing and acidifying substances and the relationship between physiological pH and blood circulation:

PRAL (mEq/d) = 0.49 x protein (g/d) + 0.037 x phosphorous (mg/d) – 0.021 x potassium (mg/d) – 0.026 x magnesium (mg/d) – 0.013 x calcium (mg/d).

Statistical Analysis

A paired *t*-test or a non-parametric Wilcoxon test was performed to evaluate differences before and after nutritional intervention. In all statistical tests performed, the null hypothesis (no effect) was rejected at the 0.05 level of probability.

The differences between ox-LDL level in the intervention arms was calculated as the follow:

Δ% = ((Z-W)/W) x 100, where Δ% is the percentage variation of oxidation of LDL, calculated as ratio of absolute variation to the base value; in particular Z is the value of oxidation LDL after nutritional treatment (McDM, and McDM+H) and W is the value of oxidation LDL at the nutritional of reference (NDT and McDM).

The value used to plot relative gene expression was determined using the expression Fold Change (FC) = $2^{-\Delta\Delta CT}$[25].

Data were normalized and we have considered a significant fold change (p <0.05) at least equal to <-1.50 (down-regulated) or >1.50 (up-regulated).

Results

Nutritional Parameters of Hazelnut

Protein, micronutrient and total lipid content, the fatty acid composition, the oil titratable acidity (expressed as % of oleic acid) and peroxide values are reported in Table I.

Table I. Nutritional characteristic of "Tonda Gentile delle Langhe" hazelnuts.

Parameter	% value
Water (g/100 g)	4.10
Proteins (g/100 g dw)	12.90
Fats (g/100 g dw) Fatty acids (%)	
Saturated	68.44
Myristic acid	0.02
Palmitic acid	6.02
Margaric acid	0.03
Stearic acid	2.54
Arachidic acid	0.1
Behenic acid	0.02
Lignoceric acid	0.01
Unsaturated	
Palmitoleic acid	0.24
Eptadecenoic acid	0.07
Oleic acid	84.65
Linoleic acid	5.98
Linolenic acid	0.07
Eicosenoic acid	0.16
Oil acidity (% oleic acid)	0.19
Oil PV (O_2, meq kg^{-1})	<0.1
α-tocopherol (mg/100 g)	24.1
Potassium (mg/100 g dw)	671.0
Phosphorous (mg/100 g dw)	250.1
Magnesium (mg/100 g dw)	128.90
Calcium (mg/100 g dw)	145.10
Zinc (mg/100 g dw)	2.51
Iron (mg/100 g dw)	3.16
Selenium (mg/100 g dw)	1.00
Total polyphenols (mg/100 g GAE)	84.63
FRAP (mmol Fe^{2+}/kg)	15.85

Dw=dry weight; PV=peroxide value; GAE: Gallic acid equivalent; FRAP: Ferric Reducing Antioxidant Power.

Table II. Bromatological composition of meals.

a) Macronutrients and nutritional quality indices	McDM	McDM + H
Calories (Kcal)	1144.04	1406.03
Water (g)	264.45	266.25
Proteins (g)	51.99	57.51
Animal proteins (g)	40.78	40.78
Vegetal proteins (g)	10.60	16.13
Carbohydrates (g)	81.89	84.33
Simple carbohydrates (g)	11.52	13.16
Complex carbohydrates (g)	62.68	63.40
Fats (g)	69.89	95.53
Saturated fats (g)	21.71	23.38
Lauric acid (g)	0.08	0.08
Myristic acid (g)	17.29	17.59
Palmitic acid (g)	11.81	12.79
Stearic acid (g)	6.14	6.49
Arachidic acids (g)	0.13	0.16
Behenic acid (g)	0.01	0.01
Monounsaturated fats (g)	30.71	45.36
Myristoleic acid (g)	0.23	0.23
Palmitoleic acid (g)	1.01	1.06
Oleic acid (g)	26.72	42.11
Eicosenoic acid (g)	0.79	0.80
Erucic acid (g)	0.18	0.18
Unsaturated fats (g)	13.96	16.04
Linoleic acid (g)	10.49	12.52
Linolenic acid (g)	1.20	1.25
Arachidonic acid (g)	0.32	0.32
Eicosapentaenoic acid (g)	0.09	0.09
Docosanoic acid (g)	0.09	0.09
Dietary fiber (g)	3.68	6.92
Soluble fiber (g)	1.51	1.51
Insoluble fiber (g)	2.15	2.15
AI	1.97	1.42
TI	1.73	1.27
PRAL	632.00	748.00
CSI	31.05	32.73
b) Vitamins	**McDM**	**McDM + H**
Thiamine (mg)	0.62	0.82
Riboflavin (mg)	0.41	0.45
Niacin (mg)	12.86	13.98
Pantothenic acid (mg)	1.08	1.08
Pyridoxine (mg)	1.67	1.91
Folic acid (µg)	79.55	108.35
Cyanocobalamin (µg)	2.12	2.12
Ascorbic acid (µg)	24.55	26.15
Biotin (µg)	8.01	22.01
Retinol (µg)	74.31	86.31
Calciferol (µg)	1.49	1.49
Tocopherol (mg)	4.14	10.14
Menadione (µg)	54.47	54.47

Table continued

Table II. *Continued.* Bromatological composition of meals.

c) Minerals	McDM	McDM + H
Sodium (mg)	1066.19	1070.59
Potassium (mg)	1497.22	1683.62
Iron (mg)	4.52	5.84
Calcium (mg)	191.28	251.28
Phosphorus (mg)	495.00	623.80
Copper (mg)	0.48	1.00
Magnesium (mg)	155.46	219.46
Manganese (mg)	0.26	2.54
Zinc (mg)	9.54	10.34
Selenium (µg)	2.45	3.25
Chrome (µg)	66.25	71.85
Nichel (mg)	43.40	91.40
Iodine (µg)	48.93	49.53
Chlorine (mg)	175.15	179.15
Fluorine (mg)	0.09	0.10
Calcium/phosphorus (mg)	1.15	1.34
Uric acid (mg)	0.00	0.00
Phytic acid (mg)	0.28	0.68
Caffeic acid (mg)	0.00	0.00
Malic acid (mg)	131.06	131.06
Citric acid (mg)	790.00	790.00
Tartaric acid (mg)	12.80	12.80
Oxalic acid (mg)	242.16	309.12

Bromatological Composition of Dietary Treatment and Indices

The macronutrient content of McDM and McDM + H are reported in Table II (a), along with NQI, AI, TI, PRAL and CSI. Also, vitamins and minerals content of each meal are reported in Table II (b) and Table II (c).

Clinical Trial

Of the 25 initial participants initially enrolled, 22 subjects were eligible for the study subjects declined to participate during the first phase, and another one did not meet the inclusion criteria, because he measured a BMI > 30 kg/m^2.

In Table III and Table IV are shown the nutritional status characteristics and blood parameters of subjects, at baseline. The value of obese subjects was obtained by DEXA: total percentage of obese was estimated as a 63.64% of total subjects: 83.33% of females and 40.00% of males. According to BMI, no obese subjects were identified. None subjects were osteoporotic. According to Appendicular Skeletal Muscle Mass Index (ASMMI), 9.09% of total subjects were sarcopenic, of which 20.00% were male subjects. None women were sarcopenic.

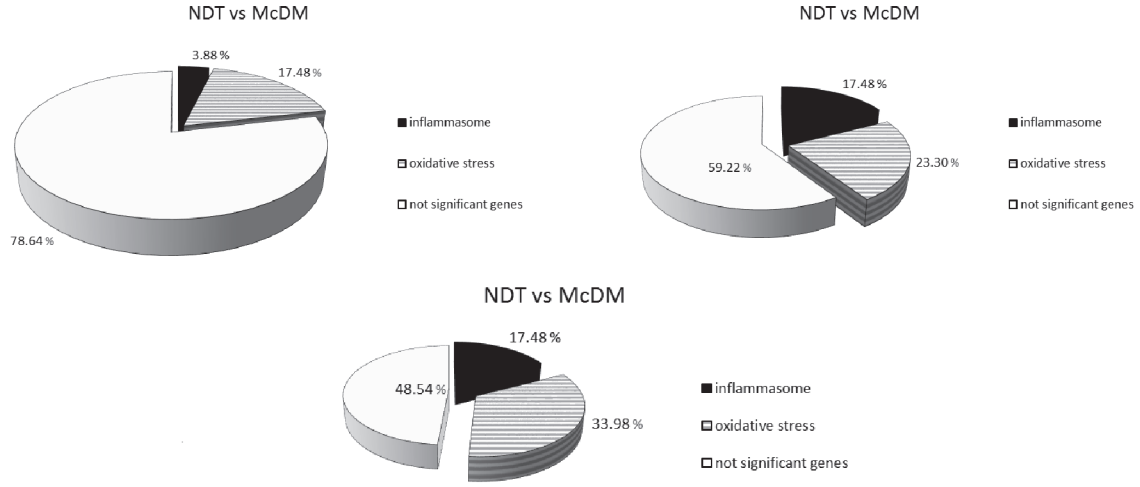


Figure 3. Expression of genomic pathways.

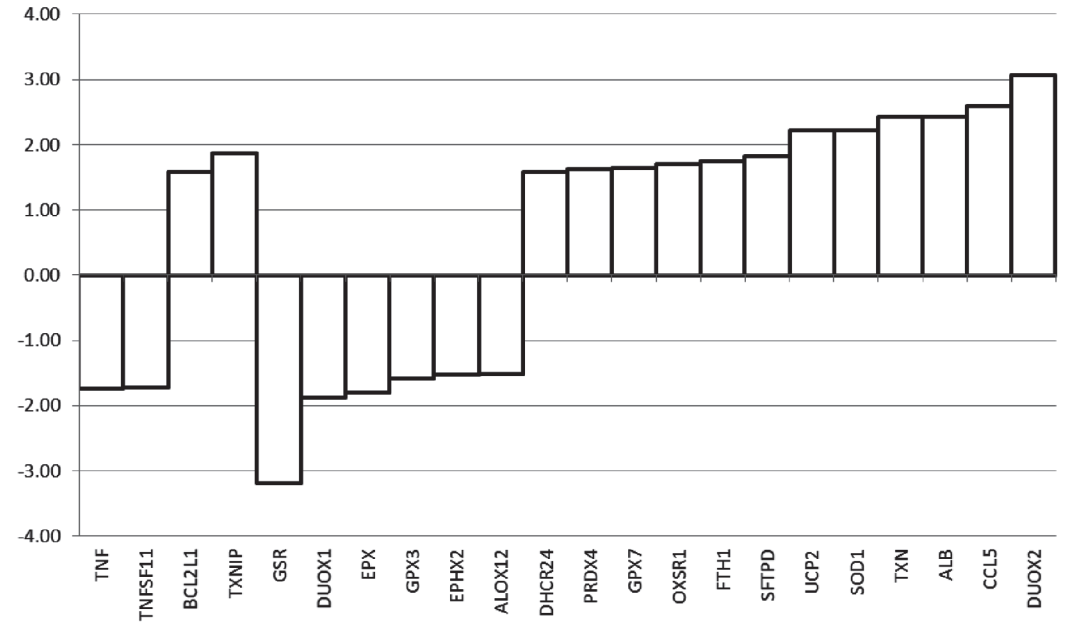
The comparison of ox-LDL level in the nutritional intervention was shown in Figure 2. As the value of NDT on T2 and T4 were comparable, we decided to calculate mean value. A significant increase (p<0.05) of ox-LDL level was highlighted between NTD *vs.* McDM, showing a Δ% = 17.56%. Ox-LDL levels significantly decreased (p<0.5) comparing McDM *vs.* McDM + H (Δ% = -24.43%). No significant value was detected in NDT *vs.* McDM + H (p>0.05).

Moreover, we analyzed a total of 103 genes after each nutritional intervention. In particular we focused on different expression levels of 74 genes of oxidative stress, and 29 of inflammasome (see supplement Table 1-6S). The different levels of fold change were analyzed for the condition: i) NDT *vs.* McDM; ii) NDT *vs.* McDM + H; iii) McDM *vs.* McDM + H.

After data normalization, considering an absolute fold change at least equal to ±1.50 (absolute FC ≥ ±1.50), we identified differential gene expression levels for each condition, as shown in Figure 3.

As reported in Figure 4, comparing NDT *vs.* McDM, the genes with a significant fold change (p <0.05) were: Tumor Necrosis Factor (TNF); Tumor Necrosis Factor (Ligand) Superfamily Member 11 (TNFSF11); Bcl2 like 1 (BCL2L1); Thioredoxin Intercating Protein (TXNIP); Glutathione Reductase (GSR); Dual Oxidase 1(DUOX1); Eosinophil Peroxidase (EPX); Glutathione Peroxidase (Plasma) (GPX3); Epoxide Hydrolase 2 Cytoplasmic, (EPHX2); Arachidonate 12-Lipoxygenase (ALOX12); 24-Dehydrocholesterol Reductase (DHCR24); Peroxiredoxin 4 (PRDX4); Glutathione Peroxidase 7 (GPX7); Oxidative Stress Responsive 1 (OXSR1); Ferritin Heavy Polypeptide 1 (FTH1); Surfactant Protein D (SFTPD); Uncoupling Protein 2 (Mitochondrial Proton Carrier) (UCP2); Superoxide Dismutase 1 Soluble, (SOD1); Thioredoxin (TXN); Albumin (ALB); Copper Chaperone for Superoxide Dismutase (CCL5); Dual Oxidase 2 (DUOX2).

Comparing NDT *vs.* McDM+H (Figure 5), the genes with a significant fold change (p <0.05) were: Pyd and Card Domain Containing (PYCARD); Actin Beta (ACTB); Fas (Tnfrsf6) Associated Via Death Domain (FADD); Cathepsin B (CTSB); TGF Beta Activated Kinase 1/Map 3k7 Binding Protein 1 (TAB1); Baculoviral Iap Repeat Containing 3 (BIRC3); Chemokine (Cxc Motif) Ligand 2 (CXCL2); Mediterranean Fever (MEFV); Nrl Family Card Domain Containing 4 (NLRC4); Caspase 1 Apoptosis Related Cysteine Peptidase (Interleukin 1 Beta Convertase) (CASP1); Caspase Recruitment Domain Family Member 6 (CARD6); Absent In Melanoma 2 (AIM2); Tool Interleukine 1 Receptor (Tir) Domain Containing Adaptor Protein) (TIRAP); Pannexin 1 (PANX1); Inhibitor Of Kappa Light Polypeptide Gene Enhancer In B-Cell Kinase Gamma (IKBKG); Tumor Necrosis Factor (Ligand) Superfamily Member 4 (TNFS4); B-Cell Ccl/Lymphoma 2 (BCL2); BCL2L1; Glutamate-Cysteine Ligase, Catalytic Subunit (GCLC); Oxidation Resistance 1 (OXR1); OXSR1; PRDX4; Dual Specificity Phosphatase 1 (DUSPO1); Myeloperoxidase (MPO); Phosphatidylinositol 3,4,5



NDT: No dietary treatment; McDM: McDonald's® Meal; McDM + H: McDonald's® Meal + 40 g of raw hazelnuts. Different levels of up and down regulation of genes analyzed between NDT vs. McDM. The significant values are expressed as $p<0.05$ for a level of fold change ±1.50. Inflammasome genes: TNF; TNFSF11; BCL2L1; TXNIP; Oxidative Stress genes: GSR; DUOX1; EPX; GPX3; EPHX2; ALOX12; DHCR24; PRDX4; GPX7; OXSR1; FTH1; SFTPD; UCP2; SOD1; TXN; ALB; CCL5; DUOX2.

Figure 4. Gene expression levels between NDT vs. McDM for each genomic pathway.

Triphosphate Dependent Rac Exchange Factor 1 (PREX1); Ribosomal Protein Large P0 (RPLPO); Mpv17 Mitochondrial Inner Membrane Protein (MPV17); Keratin, (KRT1); CCL5; ALB; Cytoglobin (CYGB); Nitric Oxide Synthase 2 Inducible (NOS2); Peroxidasin Omolog (Drosophila)

Table III. Baseline characteristics of healthy volunteers.

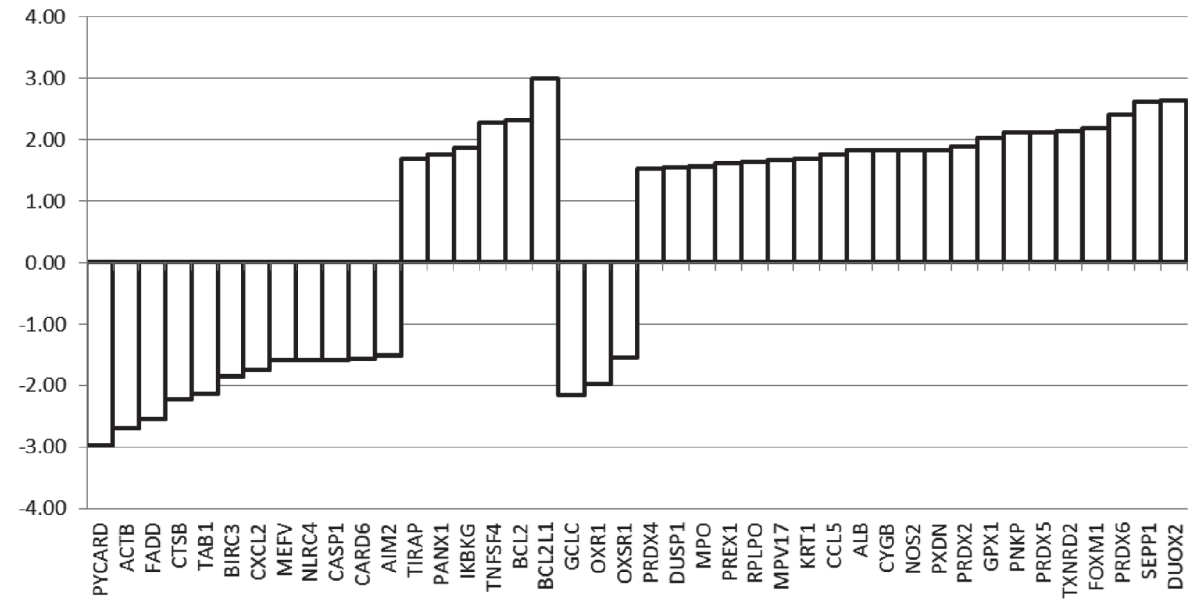
Parameters	Min-Max	Mean ± SD
Age (y)	25.60-46.90	31.23 ± 6.14
Height (cm)	158.00-186.00	170.45 ± 11.07
Weight (kg)	55.00-92.70	67.81 ± 11.35
BMI (kg/m^2)	20.00-27.10	23.28 ± 2.43
Neck (cm)	33.00-43.00	37.44 ± 3.17
Waist (cm)	68.00-86.00	76.44 ± 5.13
Abdomen (cm)	78.00-102.00	88.44 ± 8.56
Hip (cm)	88.00-108.00	97.50 ± 7.49
W/H	0.67-0.88	0.79 ± 0.06
PBF (%)	18.80-40.90	28.83 ± 6.56
TBFat (kg)	13.16-28.35	19.36 ± 5.04
TBLean (kg)	34.94-60.22	45.56 ± 9.33
ASMMI (kg/m^2)	5.69-8.65	7.04 ± 0.93

Results are expressed in mean value ± standard deviation, and minimum and maximum for each parameter. BMI: Body Mass Index; W/H: waist/hips ratio; PBF: percentage of body fat mass; TBFat: total body fat mass; TBLean: total body lean; ASMMI: appendicular skeletal muscle mass index.

Table IV. Baseline blood values of healthy volunteers

Blood parameters	Min-Max	Mean ± SD
Azotemia (mg/dl)	21.00-44.00	32.63 ± 7.69
Creatinine (mg/dl)	0.70-1.14	0.95 ± 0.14
Glycemia (mg/dl)	80.00-98.00	88.50 ± 5.86
Total Cholesterol (mg/dl)	128.00-183.00	161.00 ± 18.08
HDL Cholesterol (mg/dl)	38.00-62.00	46.86 ± 9.17
LDL Cholesterol (mg/dl)	51.00-117.00	89.86 ± 25.54
Triglycerides (mg/dl)	35.00-101.00	59.14 ± 26.82
AST (U/l)	10.00-37.00	23.13 ± 7.88
ALT (U/l)	14.00-31.00	22.50 ± 6.05
PCR (mg/dl)	0.10-1.00	0.34 ± 0.28
ESR (mm/h)	5.00-13.00	8.38 ± 3.50
Fibrinogen (mg/dl)	226.00-300.00	250.83 ± 28.90

Results are expressed in mean value ± standard deviation, and minimum and maximum for each parameter. HDL: High-density lipoprotein; LDL: low-density lipoprotein; AST: aspartate transferase; ALT: alanine transferase; PCR: Reactive C Protein; ESR: erythrocyte sedimentation rate.



NDT: No dietary treatment; McDM: McDonald's® Meal; McDM + H: McDonald's® Meal + 40 g of raw hazelnuts. Different levels of up and down regulation of genes analyzed between NDT vs. McDM. The significant values are expressed as $p<0.05$ for a level of fold change ±1.50. Inflammasome genes: PYCARD; ACTB; FADD; CTSB; TAB1; BIRC3; CXCL2; MEFV; NLRC4; CASP1; CARD6; AIM2; TIRAP; PANX1; IKBKG; TNFS4; BCL2; BCL2L1; Oxidative Stress genes: GCLC; OXR1; OXSR1; PRDX4; DUSPO1; MPO; PREX1; RPLPO; MPV17; KRT1; CCL5; ALB; CYGB; NOS2; PXDN; PRDX2; GPX1; PNKP; PRDX5; TXNRD2; FOXM1; PRDX6; SEPP1; DUOX2.

Figure 5. Gene expression levels between NDT vs. McDM + H for each genomic pathway.

(PXDN); Peroxiredoxin 2 (PRDX2); Glutathione Peroxidase 1(GPX1); Polynucleotide kinase 3' phosphatase (PNKP); Peroxiredoxin 5 (PRDX5); TXNRD2; Forkhead Box M1(FOXM1); Peroxiredoxin 6 (PRDX6); Seleno Protein P, Plasma,1 (SEPP1); DUOX2.

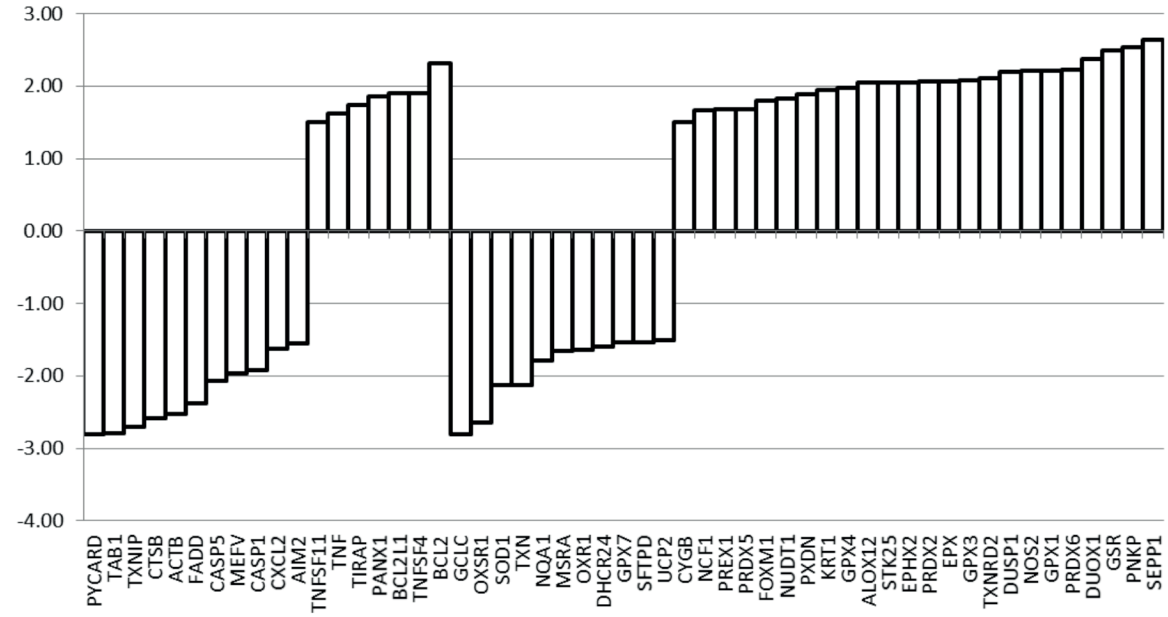
Comparing McDM *vs.* McDM + H (Figure 6) the genes with a significant fold change ($p<0.05$) were: PYCARD; TAB1; Thioredoxin Intercating Protein (TXNIP); CTSB; ACTB; Fas (Tnfrsf6) Asociated Via Death Domain (FADD); Caspase 1 Apoptosis Related Cysteine Peptidase (CASP5); MEFV; CASP1; Chemokine (Cxc Motif) Ligand 2 (CXCL2); Absent In Melanoma 2 (AIM2); TNFSF11; TNF; Tool Interleukine 1 Receptor (Tir) Domain Containing Adaptor Protein) (TIRAP); Pannexin 1 (PANX1); BCL2L1; TNFSF4; BCL2; GCLC; OXSR1; SOD1; TXN; NADPH dehydrogenase quinone 1 (NQO1); Methionine Sulfoxide Reductase A (MSRA); OXR1; DHCR24; GPX7; SFTPD; UCP2; CYGB; Neutrophyl Cytosolic Factor 1 (NCF1); Phosphatidylinositol 3,4,5 Triphosphate Dependent Rac Exchange Factor 1 (PREX1); PRDX5; FOXM1; Nudix (Nucleoside Dyphosophate Linked Moiety X)Type Motif 1 (NUDT1); PXDN; Keratin (KRT1); Glutathione Peroxidase (Phospholipid Hydroperoxidase) (GPX4); ALOX12; Serine/Treonine Kinase 25 (STK25); EPHX2; PRDX2; EPX; GPX3; TXNRD2; DUSPO1; NOS2; GPX1; PRDX6; DUOX1; GSR; PNKP; SEPP1.

Discussion

Dietary patterns have been associated with several cardiovascular diseases (CVD) risk factors such as blood pressure, obesity, serum lipids, and inflammatory markers[34].

After an average of two hours from eating a meal, a persisting for several hours, blood concentrations of glucose and lipids are raised, and there has been observed a remodeling of the structures of macronutrients, such as protein, carbohydrates, nucleic acids and lipids, as a result of damage from oxidative processes[35]. In particular, the conformational change that affects the lipids leads to acute alteration of endothelial function, with atherosclerosis initiation process and progression[36].

Previous studies[37] reported that a simple change of ≈25% of energy load from fat to carbohydrate in a meal significantly improves postprandial pro-athe-



NDT: No dietary treatment; McDM: McDonald's® Meal; McDM + H: McDonald's® Meal + 40 g of raw hazelnuts. Different levels of up and down regulation of genes analyzed between McDM vs. McDM + H. The significant values are expressed as $p<0.05$ for a level of fold change ±1.50. Oxidative Inflammasome genes: PYCARD; TAB1; TXNIP; CTSB; ACTB; FADD; CASP5; MEFV; CASP1; CXCL2; AIM2; TNFSF11; TNF; TIRAP; PANX1; BCL2L1; TNFSF4; BCL2; Stress genes: GCLC; OXSR1; SOD1; TXN; NQA1; MSRA; OXR1; DHCR24; GPX7; SFTPD; UCP2; CYGB; NCF1; PREX1; PRDX5; FOXM1; NUDT1; PXDN; KRT1; GPX4; ALOX12; STK25; EPHX2; PRDX2; EPX; GPX3; TXNRD2; DUSPO1; NOS2; GPX1; PRDX6; DUOX1; GSR; PNKP; SEPP1.

Figure 6. Gene expression levels between McDM vs. McDM + H intake for each genomic pathway.

rogenic factors. Therefore, we investigated whether a high fat meal could be associated with circulating markers of oxidative stress and inflammation, then contributing to increasing of cardio metabolic risk.

Results observed in this exploratory study support the scientific evidence regarding the deleterious impacts of a high fat meal, such as the McDM, which represents a usual meal of Western dietary pattern. In fact, after a McDM, the levels of ox-LDL were significantly higher respect to NDT ($p< 0.05$).

Antioxidants in plasma have a protection role on native LDLs, naturally exposed to oxidants. Some antioxidants are a part of LDL composition. The total plasma antioxidant status results from the interaction of several circulating antioxidants[38].

According to observational researches[39], due to the synergism of different nutrients consumed in a daily diet, it could be difficult to separate out specific effects.

Moreover, micronutrients modulate the immune system and exert a protective action by reducing LDL-cholesterol oxidation via induction of antioxidant enzymes[40].

Monounsaturated fatty acids have been reported to have hypolipidemic effects[41].

Hazelnut composition is particularly important for its fatty acid composition that represents over 60% the dry weight of the hazelnut, a bioactive compound such as tocopherols, vitamins, essential minerals and amino acids, antioxidants, fiber, phytochemicals[42]. Fatty acids composition of hazelnut oil is comparable to olive oil composition[43], and it is rich in MUFA and PUFA (omega 9 and omega 6) fatty acids. Oleic acid, in particular, is present in about 80%, followed by linoleic, palmitic and stearic acid (these account for more than 95% of the total)[44]. The content in proteins, which belong mainly to the group of globulins and albumins, varies from 10% to 18% and are 12.90% in the used hazelnuts. Pala et al[45] reported that 22% of the daily protein requirement may be made by consuming 100 g of nuts a day. Moreover, hazelnuts can be considered as a good source of phenolic compounds. In the hazelnut sample used for the study, the TPC was higher respect to TPC values found in fruits of major species such as apple, pear, nectarine, kiwi fruit and in the kernel of other important nut

species, such as almond and nut pine[46]. The antioxidant capacity was higher of the mentioned species, but not as high as in soft fruits, such as blackberry and blueberry, and in walnut. Contrary to main fruit species, hazelnut can be stored in appropriate conditions for over a year maintaining unaltered the sensory, physical and nutraceutical characteristics, as demonstrated by Ghirardello et al[26]. This makes the product easily available during all the year both for the direct consumption and for the food industry to be used in confectionery, increasing the consumption sources. Although most hazelnut production goes to the industry for processing, the consumption of raw hazelnut is advised due to the content of total antioxidants in the pellicle (episperm), representing over 50% of the antioxidants of the whole kernel, usually removed for processing after roasting. The flavan-3-ols are the main subclass of polyphenols found in the kernel, present in monomeric and polymeric form, and represents more than 95% of total polyphenols of the episperm. In episperm, flavonoids (catechin, epicatechin, quercetin, procyanidins) and dihydrochalcones together account for 3.5% of total polyphenols, and phenolic acids are responsible for less than 1%. The main phenolic acids are gallic and protocatechuic acids[47].

Hazelnuts are rich sources of essential vitamins and antioxidants such as vitamins B1, B2, B6, niacin, thiamin and α-tocopherol. In particular, α-tocopherol is the most active form of vitamin E, and is a powerful antioxidant involved in some chronic diseases risks reduction such as heart disease, type 2 diabetes, hypertension, tumor formation and some negative effects associated with aging. The α-tocopherol also plays an important protecting role against cognitive decline and Alzheimer's disease[48].

Hazelnut is an excellent source of minerals and has a good amount of potassium (500-800 mg/100 g), phosphorus (200-450 mg/100 g), magnesium (150-200 mg/100 g), calcium (250-400 mg/100 g), manganese, iron, zinc and copper, and contains a low-level of sodium. Furthermore, metals such as copper, iron and manganese play an important role in the biosynthesis of linoleic acid[49]. In general, the kernel composition is influenced by the genotype (cultivar), growing area (climate, soil) and agronomic practices[50,51]. For this reason, it is important to assess the quality and origin of the product consumed to optimized the diet and the effect of the hazelnut on human health.

In the present study, a comparison between the bromatological analyses of the two dietary interventions showed no differences regarding energy, proteins, and carbohydrates contents. However, despite the significant increase of fats, a reduction of indexes AI and TI consuming McDM + H ($p<0.05$), confirmed the cardio-protective effect of hazelnut supplementation.

The present study suggests that hazelnut-enriched dietary treatment was associated with an inhibition of oxidation of LDL particles. According to our previous data[20], after the consumption of McDM supplemented with 40 g of hazelnut, we observed a significant decrease of ox-LDL levels ($p<0.05$). The opposite was observed for the McDM alone.

Yücesan et al[52] demonstrated that α-tocopherol is lipid soluble and, for this reason, is able to be transported inside the LDL particle. Moreover, it is able to stop radical chain propagation, with scavenging action in the lipid peroxidation[53].

Moreover, it may be suggested that high MUFA and α-tocopherol content of a hazelnut-enriched diet could be responsible for the decrease in the susceptibility of LDL to oxidation.

As reported by Mercanligil et al[6], high-fat and high-MUFA-rich hazelnut diets can promote a positive lipid profile variation on patients affects by hypercholesterolemia, and for this reason, these diets were better than low-fat diets. However, according to the concentrations of VLDL cholesterol, apolipoprotein B and triacylglycerol, there are positive effects on cardiovascular risk profile.

Ox-LDL values in association with transcriptomics studies, allow us to investigate the effects of several dietary patterns on healthy status.

One of the aims of this work was the study of the interactions between a typical oxidant meal combined with an anti-oxidant food and oxidative stress and inflammatory pathways.

To determine whether hazelnuts consumption have an antioxidant and anti-inflammatory effects on gene expression profile of healthy subjects, we conducted a transcriptomic analysis of 103 genes related to antioxidant defenses and inflammasome pathway, after a high fat meal with or without 40 g of hazelnuts supplementation.

After data normalization, we observed significant differential gene expression levels according to the different dietary treatment ($p<0.05$) (Figures 3, 4, 5). The most relevant results were related to differential gene expression of catalase (CAT), Copper Chaperone For Superoxide Dismutase (CCL5), g-glutamylcysteine ligase (GCLC), Glutathione peroxidase 1 (GPX1), Superoxide Dismutase 1 Soluble (SOD1), Nitric Oxide Synthase

2 Inducible (NOS2), cysteine-aspartic acid protease 5 (CASP5), Thioredoxin (TXN).

As previously demonstrated[54], we confirmed the trend of CAT and CCL5. CCL5 gene is one of several chemokine genes clustered on the q-arm of chromosome 17. CAT gene encodes the anti-oxidant enzyme catalase that plays a central role in the body defense against oxidative stress. In a pro-inflammatory situation, due to the consumption of a high-fat meal, CAT is down-regulated and CCL5 are up-regulated. On the other hand, comparing McDM *vs.* McDM + H we obtained an up-regulation of CAT and a down-regulation of CCL5, due to the effect of hazelnuts association with McDM.

The catalytic subunit of GCLC is involved in the synthesis of GSH, which is implicated in maintaining intracellular redox balance. Our results show a significant down-regulation of both, comparing NDT *vs.* McDM + H, and McDM *vs.* McDM + H. These results demonstrated the post-prandial anti-oxidant effect of hazelnuts, in accordance with the results obtained by Krzywanski et al[55], which highlighted an increase of levels of GCLC after conditions of oxidative stress.

In cells, total glutathione can be free or bound to proteins. Free glutathione is present mainly in its reduced form, which can be converted to the oxidized form during oxidative stress, and can be reverted to the reduced form by the action of the enzyme GSR. This enzyme is encoded in human by GSR gene[56]. GPX1 gene is a member of the glutathione peroxidase family and encodes a selenium-dependent glutathione peroxidase that is one of two isoenzymes responsible for the majority of the glutathione-dependent hydrogen peroxide-reducing activity in the epithelium of the gastrointestinal tract.

We have obtained an up-regulation of both GSR and GPX1 genes, probably due to hazelnuts supplementation to the high fat meal. It suggests that the anti-oxidant effect of hazelnut biochemical compound could be related to an increase of this anti-oxidant enzyme at the transcription level, indicating a protective role against oxidative stress. This finding is supported by Khurana et al[57] that found an up-regulation of GSR and GPX1 genes in mice with diet enriched of blueberry, indicated a protective role of these fruits against oxidative stress.

A particular trend about the gene expression of SOD1 and NOS2 was found. These two genes displayed a trend that was contrary to literature data.

Nitric oxide (NO) has a role in a multitude of physiological activities, involved in the regulation of vascular tone and neurotransmission, suppression of microbes or tumor cells[58,59]. NO is synthesized from an L-arginine substrate, by the inducible NOS (iNOS or NOS2) protein, expressed in response to cytokines, lipopolysaccharide (LPS) and a host of other agents[60].

SOD1 gene encodes a protein that is an isoenzyme that contrast free superoxide radicals in the body and it is the main cellular defense in vascular cells[61].

In our analysis, SOD1 was down-regulated and NOS2 was up-regulated. Our results are confirmed by Goldsteins et al[62] that have demonstrated how the presence of SOD1 activity in the inter-membrane mitochondrial space causes a paradoxical increase of reactive oxygen species production (ROS) upon the mitochondrial stress.

Moreover, we found a down-regulation of the CASP5 comparing McDM *vs.* McDM+H. Caspases are involved in execution phase of cell apoptosis.

Our results support that pro-inflammatory stimuli and stress inducers of endoplasmatic reticulum regulate mRNA synthesis of CASP5 as demonstrated by Bian et al[63].

The comparison between baseline *vs.* McDM shows an up-regulation of CASP5 gene, while the comparison between McDM *vs.* McD + H shows a down-regulation of CASP5 gene. So, it may be suggested that hazelnut supplementation may reduce the expression of CASP5 having a possible protective effect.

Also, we observed an up-regulation after a McDM and a down-regulation after McDM + H of TXN gene, which increased expression, is associated with a better antioxidant defense biomolecules[64,65]. In our study the down-regulation of TXN gene is probably due to the supplementation of hazelnut. However, the interactions between TXN and hazelnut are still unclear.

The small number of participants of our work represents the major limit of this study, though from 8 to 25 subjects represents an acceptable number for genomic studies[54].

However, as previously reported[20], our data highlight the positive effect of hazelnut in association with McDM on ox-LDL, indicating that the antioxidant potential of the nutrients found in hazelnut may be an essential component to combat post-prandial inflammation, and oxidative stress related to the outbreak of chronic non-communicable diseases.

Although more studies are recommended to investigate whether increasing the antioxidant capacity to prevent or reduce oxidation of LDL and related gene expression, our study confirms the need of a long-term lifestyle interventions based on healthy diet, combined with a regular nut consumption, to improve mechanisms of metabolic and cardiovascular diseases, such as oxidative stress, and inflammation[66].

Conclusions

Due to the complexity of the hazelnut composition, it is not easy to know the effect of the single compound or compound category on the overall beneficial action on CVD risk.

Although our data cannot lead to a certain conclusion of the fact that the reduction of oxidative processes induced by 40 g of hazelnut in the post prandial time, it seems reasonable to conclude that these results show the favorable acute effects on some risk factors of atherosclerosis. More research is needed on a larger population after a regular intake of hazelnut, before definitive conclusions can be made.

Acknowledgments

We are indebted to all the subjects who volunteered in the clinical trial. We also thank the entire medical team from the clinical research unit for their technical assistance in conducting the clinical aspects of this study.

Trial Registration

This protocol has been registered with ClinicalTrials.gov Id: NCT01890070.

Conflict of interest

The authors declare no conflicts of interest.

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