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Original Research

Consumption of Hen Eggs Enriched with *n-3* Polyunsaturated Fatty Acids, Selenium, Vitamin E and Lutein Incites Anti-Inflammatory Conditions in Young, Healthy Participants — A Randomized Study

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Abstract

Background: Dietary supplementation with compounds that possess antioxidant and anti-inflammatory properties (*n-3* polyunsaturated fatty acids (PUFAs), selenium, vitamin E, lutein), has been shown to positively correlate with improvements in chronic conditions, although understanding of these combined effects in healthy humans is limited. The study aimed to evaluate the effects of enriched eggs consumption on oxidative status and inflammatory conditions in healthy volunteers. We hypothesized that a three-week diet containing enriched eggs can alter the immune response of healthy adults towards anti-inflammatory conditions. **Methods**: 34 participants consumed 3 hard-boiled hen eggs per day (21 days): Control group—regular hen eggs (*n*-3 PUFAs = 438 mg, selenium = 0.054 mg, lutein = 0.330 mg and vitamin E = 1.785 mg) (*N* = 14); 4Nutri group—hen eggs enriched with 4 nutrients (*n-3* PUFAs = 1026 mg, selenium = 0.06 mg, lutein = 1.85 mg and vitamin E = 3.29 mg) (*N* = 20). Samples were taken before and after the protocol. Serum concentrations of lipid mediators and cytokines were measured with enzyme-linked immunosorbent assay (ELISA) and antibody-based, magnetic bead reagent kits on the Luminex platform, respectively. Serum oxidative stress and antioxidant capacity were measured using standardized methods, while gene expression in peripheral blood mononuclear cells (PBMCs) was measured via real-time PCR. **Results**: Decreased serum levels of pro-inflammatory interleukin 17A (IL-17A) and an increased neuronal nitric oxide synthase (nNOS) expression in the 4Nutri group, together with alteration of metabolites produced via cyclooxygenase (COX) pathways in the Control group, suggest a shift towards anti-inflammatory conditions in participants who consumed enriched hen eggs. **Conclusions**: Present results suggest that the combined action of *n-3* PUFAs and antioxidants may have a protective role in resting, non-inflammatory conditions. **Clinical Trial Registration**: NCT04564690.

Keywords: functional food; IL-17; inflammation; *n-3* PUFAs; nNOS; COX

1. Introduction

Inflammation and/or oxidative stress induce endothelial activation and contribute to its functional impairment, ultimately increasing the risk of developing early atherosclerotic lesions [1,2]. Anti-inflammatory and cardioprotective effects of an *n-3* polyunsaturated fatty acids (PUFAs) supplemented diet have been extensively documented in a wide range of studies such as chronic patients [3–5], pregnant women [6,7] and healthy, young people [8–10].

Lately, efforts are being made in the direction of creating novel approaches for improvement of the quality of life such as metabolic diet typing and functional foods. Functional foods, indistinguishable from conventional foods, are considered to be somewhat enhanced foods that exert therapeutic benefits for human health beyond their nutritive values due to enrichment with bioactive components [11]. Nevertheless, a complete definition is still not fully explained/accepted as well as there is no legal or regulatory status assigned [11–14]. So-called nutraceuticals, from "nutrition" and "pharmaceutical", are described as products derived from food sources that exert certain preventive and therapeutic characteristics in terms of antioxidant and anti-inflammatory action [12–15].

Recently, we have demonstrated that consumption of *n-3* polyunsaturated fatty acids (*n-3* PUFAs) enriched hen eggs altered immune response towards inflammation-resolving conditions through effects on lipid mediators and cytokine microenvironment [8,9], and enhanced microvascular endothelium-dependent vasodilation [10]. In addition to solely *n-3* PUFAs supplements, supplementation with compounds that possess antioxidant and anti-inflammatory

properties, such as selenium, vitamin E and lutein, has been positively correlated with potential improvements in chronic patients' conditions throughout literature, although understanding and evidence in human subjects on this matter are limited [16–18]. The combined effects of these antioxidants and *n-3* PUFAs have not been thoroughly investigated yet in the context of inflammation.

The main objective of the present study was to address the effects of the consumption of novel enriched hen eggs (*n-3* PUFAs, selenium, vitamin E and lutein) [19] on systemic oxidative stress and antioxidant capacity, as well as levels of pro- and anti-inflammatory cytokines in young, healthy individuals without any underlying comorbidities. We hypothesized that dietary intake of eggs enriched with such compounds can induce a decrease in inflammatory markers and oxidative stress levels and act as a tool of protection in future regarding inflammation-derived illnesses.

2. Methods and Materials

2.1 Study Design and Participants

A total of 35 young and healthy volunteers of both sexes, aged between 18–30 years old, were enrolled in this prospective, interventional, randomised study (study registered under ClinicalTrials.gov Identifier: NCT04564690; title: "Effect of Enriched QUARTET® Hen Eggs on Cardiovascular Function in Cardiovascular Patients and Healthy Individuals."). Exclusion criteria for participants were prior history of hypertension, coronary artery disease, diabetes mellitus, renal impairment, cerebrovascular and peripheral artery disorder, hyperlipidaemia and use of oral contraceptives or drugs that could affect endothelium.

Participants were recruited by the researcher and were included using a survey questionnaire to meet the inclusion and exclusion criteria of the study. After recruitment, a simple randomization procedure was performed by an unbiased associate drawing A or B for each subject, i.e., determining the affiliation to a group—A (Control group) or B (4Nutri group). Volunteers who met mentioned criteria were given the codes by the researcher that didn't contain any personal information: ZCI-Q-1-No. or ZCI-Q-2-No. Label ZCI-Q denotes the name of the project within which the study is conducted; number -1- or -2- denotes belonging to the group (control or 4Nutri, depending on the eggs to be consumed), and were assigned by an unbiased associate that performed simple randomization and divided the eggs among participants. No. is the ordinal number of study participants (1, 2, 3…). Labels indicating group affiliation were known only to the associate assigning the intervention, while neither study participants nor the researcher knew to which groups they belonged throughout the duration of the study.

The study was carried out at the Department of Physiology and Immunology, Faculty of Medicine in Osijek, Croatia and included two appointments. All study participants were instructed to eat three hard-boiled hen eggs (L commercial size) per day for three weeks (total of 63 eggs). Participants were divided into Control group (N = 14; W/M = 6/8) and 4Nutri group (N = 20; W/M = 9/11). Control group consumed regular hen eggs (*n-3* PUFAs content ~438 mg/per day; selenium content ~0.05 mg/per day; lutein content ~0.33 mg/per day; vitamin E content ~1.79 mg/per day), while 4Nutri group consumed hen eggs enriched with four nutrients (*n-3* PUFAs content ~1026 mg/per day; selenium content ~0.06 mg/per day; lutein content ~1.85 mg/per day; vitamin E content ~3.29 mg/per day).

Study participants were instructed to follow their usual meal schedule and to avoid any supplements (e.g., *n-3* PUFAs or vitamin E, selenium and lutein). They were advised to consume only the eggs provided for them during the study, preferably in the mornings after boiling them for 10 minutes. Each respondent recorded what he/she consumed over the duration of the study protocol in a diet diary in form of 24 hours recalls, designed and previously published by our research group [20]. Blood samples were taken on the first and last day of the study protocol for the purpose of serum collection and peripheral blood mononuclear cell isolation. A CONSORT diagram is presented in Fig. 1; a CONSORT checklist is presented in **Supplementary Table 1**.

Enrichment of hen eggs was executed by an associate research group from the Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek. Eggs enriched with four nutrients were produced by replacing standard feedstock used in feeding mixtures fed to laying hens, with a mixture of fish oil (0.75%), flaxseed oil (1%), reptile oil (2%) and soy oil (1.25%), 0.5 mg/kg of organic selenium, 200 mg/kg of lutein and 200 mg/kg of vitamin E [19]. The edible part (~60 g) of hen egg enriched with four nutrients (*n-3* PUFAs, selenium, lutein and vitamin E) contained approximately 342 mg of *n-3* PUFAs (~189 mg of α-linolenic acid, ALA; ~19 mg of eicosapentaenoic acid, EPA; ~135 mg of docosahexaenoic acid, DHA), 0.02 mg of selenium, 0.616 mg of lutein and 1.097 mg of vitamin E [19].

As stated above, this study is registered on Clinical Trials as a part of a larger research, therefore there are several primary and secondary outcomes. Primary outcomes for this particular participant group and results are the measurements of biomarkers for oxidative stress and antioxidant capacity, and serum protein concentrations of various pro- and anti-inflammatory cytokines and cell adhesion molecules. Secondary outcomes include measurement of blood pressure and serum lipid profile. A pilot study was performed on a total of 16 participants after the respective dietary protocols (N (Control group) = 8; N (4Nutri group) = 8), considering the outcomes. Effect and sample size were calculated before the recruitment of participants for the main study.

All study participants were legal adults (18 years old) and signed informed consent before their inclusion in the

CONSORT 2010 Flow Diagram

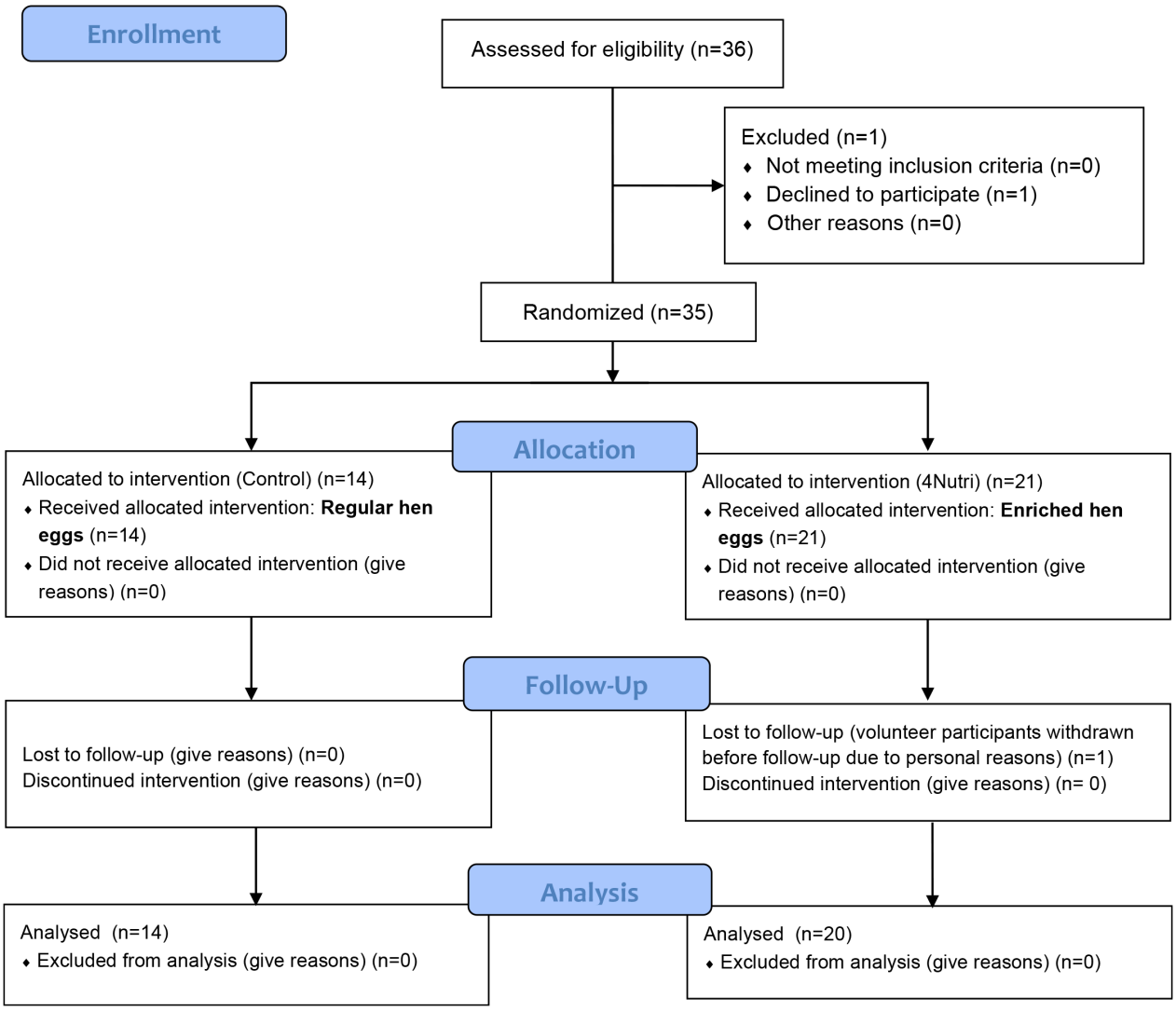

Fig. 1. CONSORT 2010 Flow diagram.

study. The study protocol and procedures conformed to the standards set by the latest revision of the Declaration of Helsinki and were approved by the Ethical Committee of the Science Center of Excellence, Josip Juraj Strossmayer University of Osijek (Class: 602-04/14-08/06; Reg. No.: 2158-610714-114) and Ethics Committee of the Medical Faculty Osijek (Class: 602-04/20-08/07, Reg. No.: 2158-61-07-20147). Participants were all students who volunteered to participate, therefore no selection bias was present in our research. Compensation was provided for respondents in the form of travel expenses. Fresh eggs were delivered from a poultry farm (Marijančanka d.o.o.) to the laboratory and distributed to participants within a week.

2.2 Anthropometry, Hemodynamic and Laboratory Testing

The arterial blood pressure of participants was measured with an automatic oscillometer (HR P-3-1134, OMRON, Osaka, Japan); the average of three measurements in a sitting position after 10 minutes of rest, before the start and after the end of the protocol, was taken as the final value. During both visits, subjects had their Body Mass Index (BMI; kg/m^2), calculated from the ratio of weight in kilograms to a square meter of height, and waist-to-hip ratio (WHR).

Venous blood samples were collected after half an hour of rest in a calm and quiet room, at both appointments. Samples were taken after an overnight fast and were analysed for the following: leukocytes, erythrocytes, haemoglobin, haematocrit, platelets, triglycerides, cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, C reactive protein (CRP) and fasting blood glucose, liver enzymes (AST, ALT, GGT), urea, creatinine, urate, sodium, potassium, total iron and transferrin.

2.3 Measurements of Fatty Acids Profile, Vitamin E, Selenium and Lutein Concentrations in Serum Samples of Study Population

The *serum fatty acid profile* was determined by gas chromatography–tandem mass spectrometry at the BIOCentre's Bioanalytical Laboratory, BIOCentre — incubation centre for biosciences, Zagreb, Croatia (by Thermo Fisher GC Trace 1300 coupled with a TSQ 9000 Triple Quadrupole) with a solution of fatty acid methyl esters (FAME MIX) purchased as 30 mg/mL of the total concentration of fatty acids in methylene chloride (Supelco Inc., Bellefonte, PA, USA) [10,21].

Selenium concentrations in serum samples were determined by inductively coupled plasma mass spectrometry (ICP-MS) (ICP-MS, Agilent 7500a, Agilent Technologies Inc., California, USA) after appropriate sample preparation. This protocol was optimized by partner institutions through project cooperation [22]. Each serum sample on the ICP was analyzed by internal pooled plasma control and reference material NIST 1567b (wheat flour, National Institute of Standards and Technology, Gaithersburg, MD, USA) was used for the control of the analytical method. All samples were analyzed in triplicate.

Lutein concentrations in serum samples were determined according to the protocol by Tzeng *et al*. (2004) [23,24]. Deionized water and 0.01% ascorbic acid (Gram-mol, GRAM-MOL d.o.o., Zagreb, Croatia) dissolved in absolute ethanol (Gram-mol GRAM-MOL d.o.o., Zagreb, Croatia) was added to serum samples and mixed thoroughly. Hexane (Carlo Erba, CARLO ERBA Reagents GmbH, Milano, Italy) was added to the mixture, stirred and centrifuged at 2500 rpm/20 min. The supernatant was separated and evaporated, followed by lutein concentration determination using high-performance liquid chromatography (HPLC). A high-resolution end-capped HPLC column (Hypersil; particle size 5 μm; 4.6 $\times$ 250 mm) and a C18 analytical column (Shim-pack GIST; particle size 5 μm; 250 $\times$ 4.6 mm) were purchased. HPLC LC-30 NEXERA (Shimadzu, Japan, 2018) was used for the determination of concentrations.

Vitamin E concentrations in serum samples were determined according to the protocol by Jargar *et al*. [25]. Serum samples were slowly added to absolute ethanol with gentle shaking to denature the proteins. Sample tubes were shaken for 30 seconds while protected from light. Further, xylene was added to separate the supernatant from proteins. Once again, tubes were mixed thoroughly and centrifuged at 3000 rpm/10 minutes. The supernatant containing xylene was separated in a clean tube and followed by adding 2,2-bipyridyl (Acros Organics, Geel, Belgium, Lot: A0433287) and $FeCl_3$ (Carlo Erba, CARLO ERBA Reagents GmbH, Milano, Italy, Batch no. V1L014201L), resulting in pink colouration. After incubation, the absorbance was measured using a spectrophotometer (PR 3100 TSC Microplate Reader, BioRad Laboratories, Hercules, CA, USA) at 492 nm, and the obtained absorbance was proportional to the serum vitamin E concentration. A five-point calibration curve was also made.

2.4 Isolation and Cryopreservation of Peripheral Blood Mononuclear Cells (PBMCs)

Approximately 16 mL of whole blood was collected in 10 mL vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and diluted with 1$\times$ Phosphate-buffered saline (PBS), 1:1 ratio. Diluted sample was layered on Ficoll-Paque® PLUS centrifugation media (GE Healthcare Bio-Sciences AB, Uppsala, Sweden, Lot:10296933). Samples were centrifuged at 800 G with brake off, for 25 minutes. Mononuclear cells visible as a cloudy ring separating two layers—yellow plasma and colourless media, were collected and rinsed twice in 1$\times$ PBS. Cell count and viability was estimated by staining with 0.4% Trypan blue solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany, Lot: RNBH9907) and using a Bürker-Türk counting chamber. Refrigerated reagents and buffers used in isolation were warmed up to room temperature (RT, ~20–25 °C) prior to isolation. Samples were processed within three hours of collection.

Mixture of dimethyl Sulfoxide (DMSO; Supelco, Merck KGaA, Darmstadt, Germany) and ethylenediaminetetraacetic acid (FBS) was used for cryopreservation purposes (1:9 ratio). Cryovials containing samples were stacked in Mr. Frosty freezing container (Nalgene Labware, Thermo Fisher Scientific, Waltham, MA, USA) containing isopropyl alcohol and placed in a –80 °C freezer for 24 hours before transferring them into labelled containers.

2.5 Measurement of Markers for Oxidative Stress and Antioxidant Capacity

Oxidative stress was measured in the serum of all participants, before and after the respective diet, by spectrophotometric measurement of lipid peroxidation products (Thiobarbituric acid reactive substances, TBARS), while antioxidant capacity was measured in the same manner using the ferric-reducing ability of plasma (FRAP), according to the previously described protocols from our laboratory [26–29]. Serum levels of myeloperoxidase (MPO) protein were measured in all participants, before and after dietary protocol, by commercially available *in vitro* enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) on compact absorbance reader for 96-well microplates (BioRad PR 3100 TSC, Bio-Rad Laboratories, CA, USA). Spectrophotometric antioxidant enzyme activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) were measured before and after respective dietary protocols from serum samples of study participants practising previously described methods by our research group [26,30,31]. The activity of examined enzymes was expressed as U (mg protein) $^{-1}$. Measurements were performed using a Lambda 25 UV-Vis spectrophotometer with UV WinLab 6.0 software package (PerkinElmer for the Better, Waltham, MA, USA).

Total RNA was extracted with TRIzol reagent (Life Technologies, Carlsbad, CA, USA, Lot: 13901902) from homogenised stored PBMC samples of participants according to available protocols previously optimized in our laboratory [26,32]. RNA concentrations and sample purity were determined using a Nanophotometer P300 UV/VIS, IMPLEN. Sample purification and cDNA preparation were performed according to the manufacturer's instructions from Sigma-Aldrich and Applied Biosystems. Determination of gene expression of nitric oxide synthases (NOS); endothelial nitric oxide synthase (eNOS) - sequence: forward: 5′-CAACAGCATCTCCTGCTCAGA-3′ and reverse 5′-CCACTTCCACTCCTCGTAGC-3′; neuronal nitric oxide synthase (nNOS) - sequence: forward: 5′-ATTTATBCCTTTCCAGCC-3′, and reverse 5′-AGGATCCAGGCTTCAGGCTA-3′; inducible nitric oxide synthase (iNOS) - sequence: forward 5′-AGAGCCAGAAGCGCTATCAC-3′, and reverse 5′-TTGAAGTGGTGCACTCAGCA-3′), antioxidative enzymes SOD2 (sequence: forward: 5′-GGCCTACGTGAACCTGA-3′, and revers 5′-CAGGTTTGATGGCTTCCAGC-3′), GPx1 (sequence: forward: 5′-TATCGAGAATGTGGCGTCCC-3′, and reverse 5′-TCTTGGCGTTCTCCTGATGC-3′) and GPx4 (sequence: forward: 5′-TTCCGGTGTAACCAGTTCGG-3′, and revers 5′-GTGGAGAGACGGTGTCCAAA-3′) was performed by real-time quantitative PCR (Bio-Rad CFX96) from stored PBMC samples of participants. Gene expression was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH - sequence: forward 5′-ACAGTCAGCCGCATCTTCTT-3′, and revers 5′-AATTTGCCATGGGTGGAAT-3′) gene.

2.6 Multiplex and Simplex Quantitation of Adhesion Molecules, Pro- and Anti-Inflammatory Cytokines Serum Concentrations

Serum concentrations of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), endothelial-leukocyte adhesion molecule 1, E-selectin (CD62E), type 1 membrane glycoprotein endoglin (CD105), tumour necrosis factor-alpha (TNF-α), pro- and anti-inflammatory cytokines including interleukin 17A (IL-17A), interleukin 6 (IL-6) and interleukin 10 (IL-10), were measured before and after respective diet, using the Invitrogen ProcartaPlex antibody-based, magnetic bead reagent kits (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) and panels for multiplex and simplex protein quantitation on the Luminex 200 instrument platform (Luminex Corp., Austin, TX, USA). Measurements were performed in the Laboratory of Molecular and HLA Diagnostics of the University Hospital Osijek (Osijek, Croatia). Data were analysed by using ProcartaPlex Analyst free software (eBioscience, Affymetrix by Thermo Fisher Scientific, Waltham, MA, USA) and are expressed as a concentration in picograms per millilitre.

2.7 ELISA Assays

Serum concentrations of leukotriene B4 (LTB4) (Cusabio, Houston, TX, USA, LTB4 Cat. No. CSB-E08033h) and B5 (LTB5) (MyBioSource, MyBioSource Inc., CA, USA, LTB5 Cat.No. MBS3800320), prostaglandins E2 (PGE2) and E3 (PGE3) (MyBioSource, MyBioSource Inc., San Diego, CA, USA, PGE2 Cat. No. MBS007171, PGE3 Cat.No. MBS9310830) were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits on compact absorbance reader for 96-well microplates (BioRad PR 3100 TSC, Bio-Rad Laboratories, Hercules, CA, USA).

2.8 Statistical Analyses

Statistical analyses were performed using Graph Pad Prism v6.01 (GraphPad Software, San Diego, CA, USA) and Microsoft Excel 2016 (Microsoft Office 365, Microsoft Corporation, Redmond, WA, USA). GPower v3.1.9.7 software (Heinrich Heine University Düsseldorf, Düsseldorf, Germany) was used for sample size calculation. Cohen's d (Δ / SD) effect size was determined according to the primary outcome of the study. A pilot study was conducted during the preparation of the study. The sample size required to show a potentially significant effect was calculated based on preliminary data collected from a total of 16 subjects. To detect differences in primary outcomes recorded in this study with a significance level of 0.05 and a statistical power of 80% for a paired *t*-test, the required sample size was 14 subjects per group.

The normality of distributions was tested by Shapiro–Wilk test. Data are presented as average $\pm$ standard deviation (SD). Student's *t*-test and Mann–Whitney tests were used for group comparisons, while Paired *t*-test and Wilcoxon rank-sum tests were used to test the differences between the measurements within a group. Correlations between paired datasets were determined by the Spearman rank test. Two-tailed $p < 0.05$ was considered significant.

3. Results

3.1 Nutrient Content in Edible Part of Eggs

The content of *n-3* PUFAs, selenium, lutein and vitamin E in the edible part of regular and enriched hen eggs consummated by study participants is shown in Table 1.

Table 1. Content of fatty acids, selenium, lutein and vitamin E in edible part of hen eggs.

An L-grade egg with an average weight of 68 g has about 60 g of edible portion.		
Parameter	Regular eggs	4Nutri eggs
∑SFA	1566 ± 346	1442 ± 185
∑MUFA	1976 ± 189	2419 ± 139
∑*n*-6 PUFA	1263 ± 148	747 ± 46*
LA	1165 ± 140	702 ± 43
AA	89 ± 9	44 ± 4*
∑*n-3* PUFA	146 ± 20	342 ± 25*
ALA	71 ± 11	189 ± 16*
EPA	n.d.	19 ± 2*
DHA	75 ± 11	135 ± 11*
∑*n*-6/∑*n-3* PUFA	8.71	2.18*
Selenium (mg)	0.0183	0.02305*
Lutein (mg)	0.11	0.616*
Vitamin E (mg)	0.595	1.098*

Results are shown as mean ± standard deviation (SD). ∑SFA, saturated fatty acids; ∑MUFA, monounsaturated fatty acids; ∑n-6 PUFA, polyunsaturated fatty acids; LA, linoleic acid; AA, arachidonic acid; ∑*n-3* PUFA, polyunsaturated fatty acids; ALA, alpha linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Paired *t*-test; significance level *$p < 0.05$ Regular vs. 4Nutri enriched hen eggs.

3.2 Anthropometric, Hemodynamic and Biochemical Characteristics of Study Population

Initial anthropometric and hemodynamic values of all participants enrolled in the study are presented in Table 2. There were no significant differences regarding the age of participants between the control (21.9 ± 2.6 years old) and 4Nutri group (21.4 ± 2 years old). According to the average baseline BMI of both control (24.33 ± 3.6 kg/m^2) and the 4Nutri group (23.49 ± 3.1 kg/m^2), study participants fit in the normal BMI range according to the WHO criteria for European population weight classification (18.5–24.9 kg/m^2).

Participants were lean and normotensive, and there were no differences in these values after consumption of regular nor enriched eggs compared to respective baseline values as there were no significant differences between the two experimental groups. Based on the medical history and biochemical characteristics presented in Table 3, all participants had normal fasting blood glucose, hsCRP, and fasting lipid profile levels. There were no significant differences in measured parameters after both diet protocols when compared to respective baseline values.

Table 2. Effect of control and enriched eggs (with four nutrients) consumption on anthropometric and hemodynamic parameters of study population.

Parameters	Control group		4Nutri group	
N (W/M)	14 (6/8)		20 (9/11)	
Age	21.9 ± 2.6		21.4 ± 2	
	Before	After	Before	After
BMI (kg/m^2)	24.33 ± 3.6	23.96 ± 3.2	23.49 ± 3.1	23.53 ± 3.1
WHR	0.84 ± 0.07	0.84 ± 0.07	0.81 ± 0.09	0.81 ± 0.09
SBP (mmHg)	110 ± 17	108 ± 15	109 ± 11	104 ± 14
DBP (mmHg)	72 ± 12	69 ± 4	78 ± 16	72 ± 6
MAP (mmHg)	85 ± 7	82 ± 7	88 ± 13	84 ± 6

Data are presented as mean ± standard deviation (SD). *N*, number of participants; W, women; M, men; BMI, body mass index; WHR, waist to hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

3.3 Serum Levels of Four Nutrients Following Diet Protocol

Following the three-week dietary protocol, serum levels of EPA, DHA, vitamin E and lutein significantly increased only in the 4Nutri group compared to the baseline levels following the dietary protocol as presented in Table 4.

3.4 Oxidative Stress and Antioxidant Capacity Following Diet Protocol

Three-week consumption of enriched eggs did not significantly alter serum TBARS or FRAP in the 4Nutri group

Table 3. Effect of control and enriched eggs consumption on biochemical parameters of study population.

Parameters	Control group		4Nutri group	
N (W/M)	14 (6/8)		20 (9/11)	
	Before	After	Before	After
Fasting blood glucose (mmol/L)	4.6 ± 0.3	4.6 ± 0.5	4.4 ± 0.4	4.9 ± 0.8*
hsCRP (mg/L)	0.6 ± 0.5	1.1 ± 1.5	1.1 ± 0.9	2.3 ± 0.9
Cholesterol (mmol/L)	4.3 ± 0.8	4.6 ± 1.5	4.5 ± 0.5	4.7 ± 0.7
Triglycerides (mmol/L)	0.8 ± 0.3	0.9 ± 0.3	1.1 ± 0.5	0.9 ± 0.3
HDL cholesterol (mmol/L)	1.5 ± 0.4	1.4 ± 0.4	1.3 ± 0.2	1.3 ± 0.2
LDL cholesterol (mmol/L)	2.5 ± 0.7	2.9 ± 0.8 *	2.8 ± 0.4	2.9 ± 0.7

Results are expressed as average ± standard deviation (SD). AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamic transferase; hsCRP, high-sensitivity C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Paired *t*-test; significance level *$p < 0.05$ before vs. after within the group (Control or 4Nutri group). Reference range, general population.

Table 4. Effect of regular (Control group) and enriched hen eggs (4Nutri group) consumption on concentrations of *n-3* fatty acids, vitamin E, selenium and lutein in serum samples of study population after three-week diet protocol.

Parameter	Control group		4Nutri group	
	Before	After	Before	After
C20:3[cis-11,14,17] 11,14,17-Eicosatrienoic acid	N/F	N/F	N/F	N/F
C20:4[cis-5,8,11,14] Eicosa-5,8,11,14,17-pentaenoic acid	15.1 ± 6.9	14.1 ± 4.6	13.7 ± 2.9	18.6 ± 5.6*†
C22:6[cis-4,7,10,13,16,19] cis-4,7,10,13,16,19-Docosahexaenoic acid	54.0 ± 19.9	89.9 ± 37.7*	65.6 ± 25.1	132.1 ± 64.7*
n-6/*n-3* ratio	11.1	8.3	10.5	6.3*
Vitamin E (μg/mL)	10.27 ± 3.67	10.30 ± 3.687	6.63 ± 3.22	11.26 ± 2.87*
Selenium (μg/L)	62.44 ± 9.37	66.37 ± 10.09	64.88 ± 17.65	68.88 ± 3.082
Lutein (μmol/L)	0.199 ± 0.104	0.199 ± 0.202	0.153 ± 0.073	0.232 ± 0.078*

Results are expressed as mean ± standard deviation (SD). <LOQ - below limit of quantification; N/F- not found. Paired *t*-test; significance level *$p < 0.05$ before vs. after within the group (Control or 4Nutri); Student's *t*-test; significance level †$p < 0.05$ difference between the groups (Control vs. 4Nutri).

Table 5. Effects of regular (Control group) and enriched hen eggs (4Nutri group) consumption on the serum antioxidant activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) after three-week diet protocol.

Parameter	Control group		4Nutri group	
	Before	After	Before	After
SOD U $(mg/P)^{-1}$	7.76 ± 0.56	7.61 ± 0.45	8.04 ± 0.75	7.65 ± 0.53
GPx U $(mg/P)^{-1}$	0.02 ± 0.004	0.02 ± 0.001	0.02 ± 0.002	0.02 ± 0.002
CAT U $(mg/P)^{-1}$	3.91 ± 1.34	4.95 ± 2.95	4.72 ± 1.56	6.48 ± 3.14

Data are presented as mean ± standard deviation (SD). SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase.

compared to respective baseline values, neither did regular eggs in Control group (Fig. 2). MPO serum values were significantly increased in the 4Nutri group after consumption of enriched eggs compared to baseline values (Fig. 3). MPO serum values in the Control group remained unaltered after consumption of regular eggs. Antioxidative enzyme activity was measured for superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). There were no significant differences detected in the Control or 4Nutri group after respective dietary protocols compared to the respective baseline values, although there was a slight trend towards an increase of CAT activity ($p = 0.08$) in the 4Nutri group after dietary protocol (Table 5).

mRNA expression of only nNOS was significantly increased in PBMCs of participants in 4Nutri group after dietary protocol compared to the baseline levels (Fig. 4E). mRNA expression of antioxidative enzymes remain unchanged (Fig. 4A–D, 4F).

3.5 Serum Cytokine Concentrations Following Diet Protocol

Serum concentrations of pro- and anti-inflammatory cytokines (IL-17A, IL-10) and tumour necrosis factor-alpha

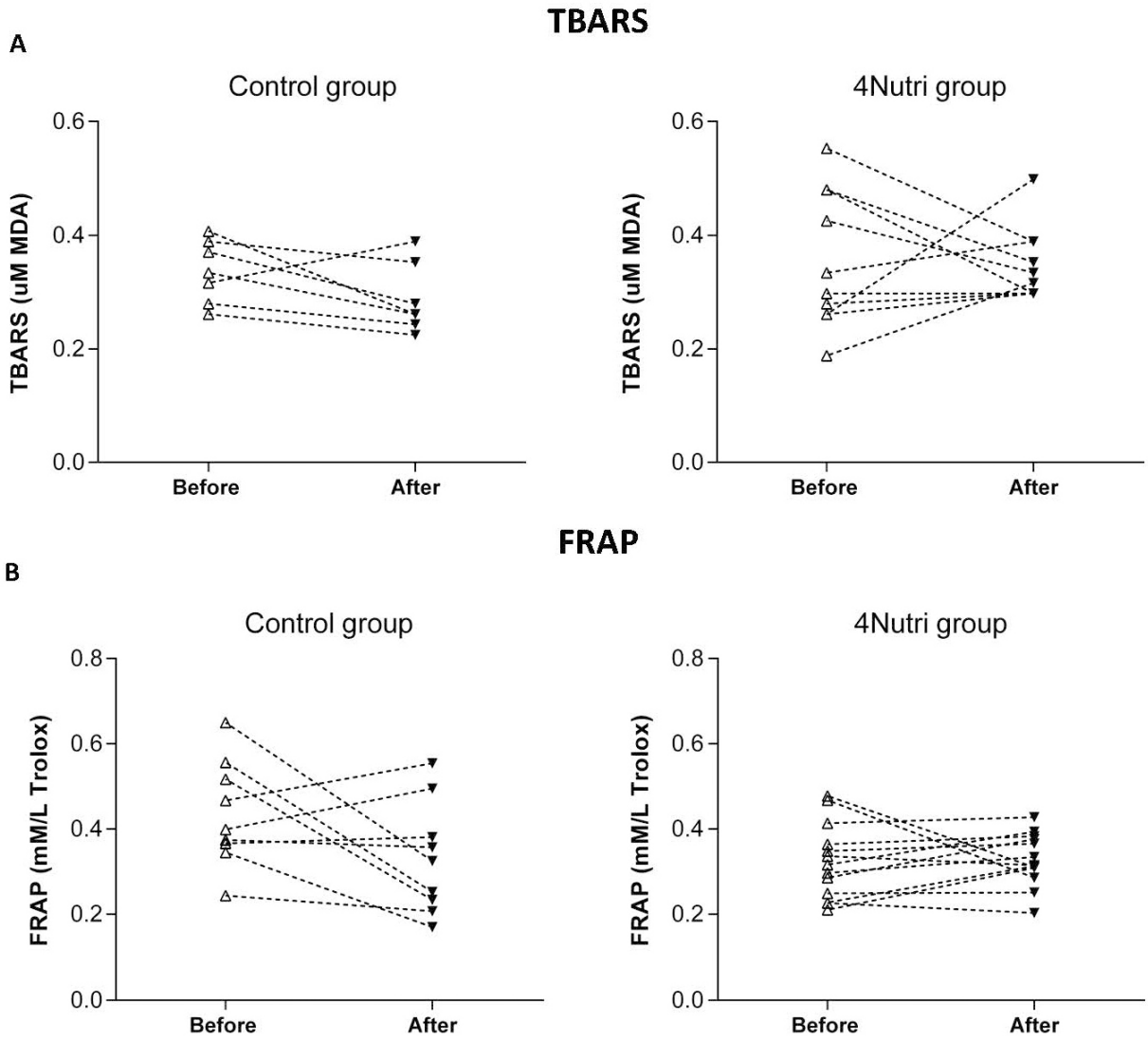

Fig. 2. Effects of regular (Control group) and enriched hen eggs (4Nutri group) consumption on the serum levels of oxidative stress (TBARS) (A) and antioxidant capacity (FRAP) (B) after three-week diet protocol. Effects of regular (Control group) and enriched hen eggs (4Nutri group) consumption on the serum levels of oxidative stress (TBARS) (A) and antioxidant capacity (FRAP) (B) after three-week diet protocol. Both diets didn't significantly alter neither marker after respective diet protocols. Paired *t*-test; significance level $p < 0.05$; before vs. after within the group (Control or 4Nutri).

Table 6. Effects of regular (Control group) and enriched hen eggs (4Nutri group) consumption on the serum concentrations of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), endothelial-leukocyte adhesion molecule 1 E-selectin (CD62E), type 1 membrane glycoprotein endoglin (CD105) after three-week diet protocol.

Parameter	Control group		4Nutri group	
	Before	After	Before	After
ICAM-1 (pg/mL)	2732 ± 1251	2754 ± 1607	3393 ± 1486	3567 ± 1315
VCAM-1 (pg/mL)	5010 ± 1835	5904 ± 1084	3861 ± 2599	5231 ± 2049
E-selectin (pg/mL)	189.41 ± 36.29	201.62 ± 40.78	182.23 ± 41.86	184.46 ± 53.19
Endoglin (pg/mL)	4033 ± 1190	3602 ± 1081	3145 ± 902	3575 ± 1063

Data are presented as mean ± standard deviation (SD). ICAM-1, intracellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1.

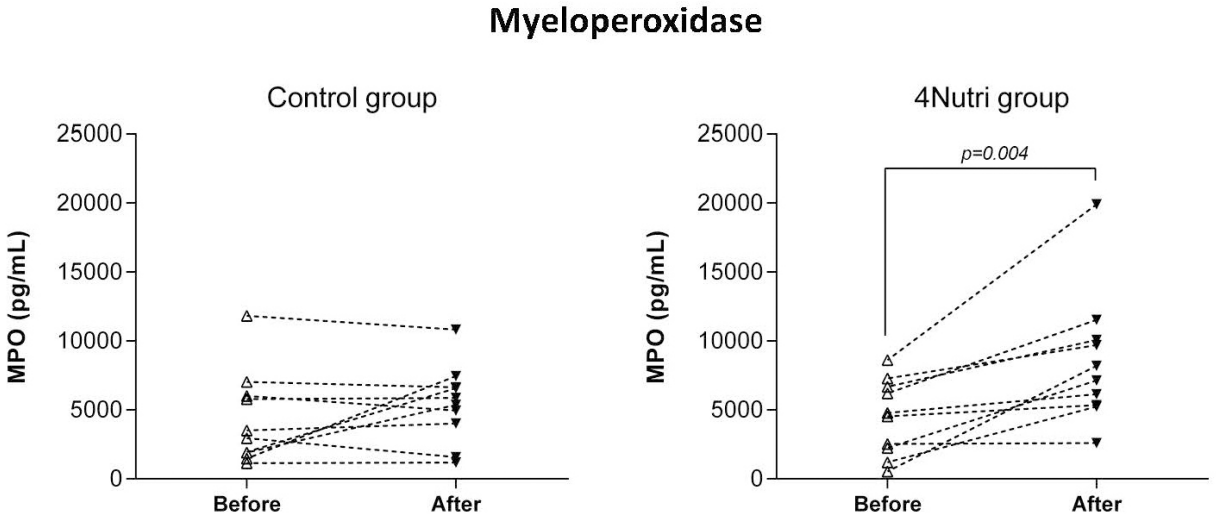

Fig. 3. Effects of regular (Control group) and enriched hen eggs (4Nutri group) consumption on the serum levels of MPO after three-week diet protocol. Paired *t*-test; significance level $p < 0.05$; before vs. after within the group (Control or 4Nutri).

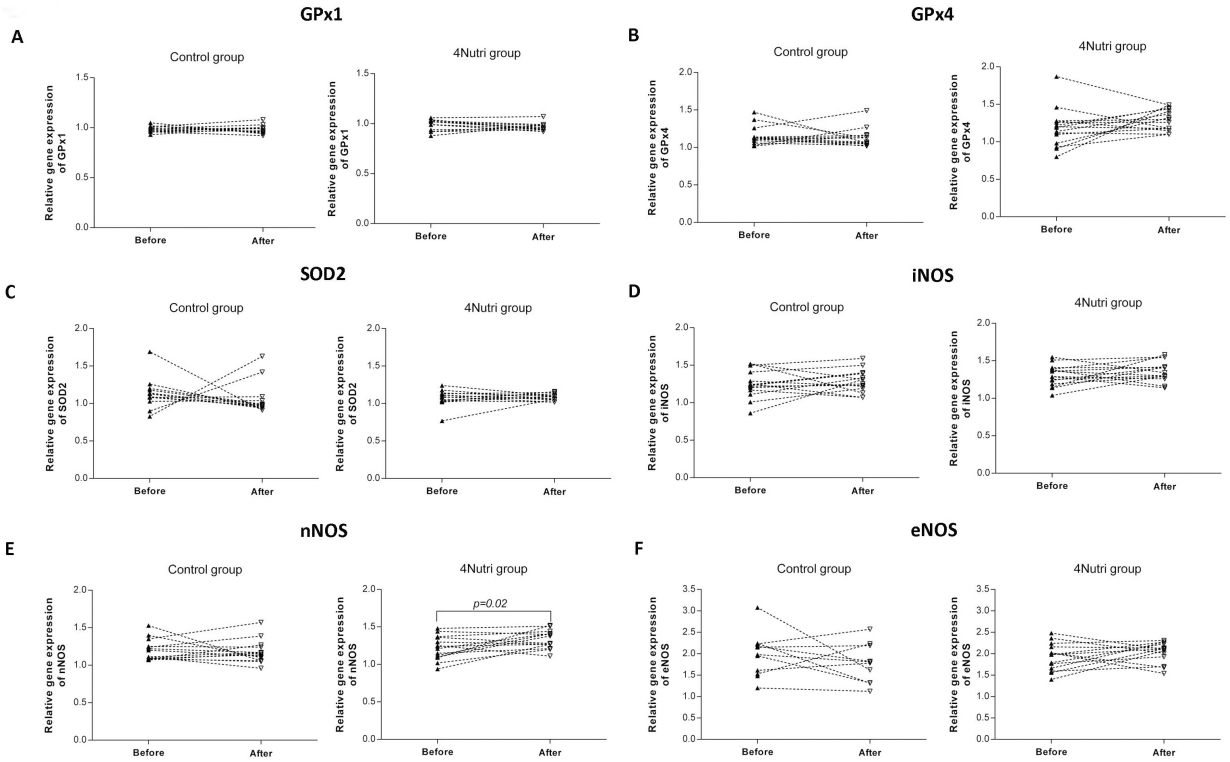

Fig. 4. Effects of regular (Control group) and enriched hen eggs (4Nutri group) consumption on relative gene expression of GPx1 (A), GPx4 (B), SOD2 (C), iNOS (D), nNOS (E) and eNOS (F) in PBMCs after three-week diet protocol. GPx, glutathione peroxidase; SOD, superoxide dismutase; iNOS, inducible nitric oxide synthase; nNOS, neural nitric oxide synthase; eNOS, endothelial nitric oxide synthase. Paired *t*-test; significance level $p < 0.05$; before vs. after within the group (Control or 4Nutri).

in the control and 4Nutri group, before and after dietary protocols, are presented in Fig. 5. There were no significant changes of IL-10 or TNF-α (Fig. 5A,B) observed in any of the groups after respective diet protocols. There was a significant decrease in IL-17A serum concentration after the consumption of eggs enriched with four nutrients compared to the baseline values ($p = 0.031$) (Fig. 5C).

Serum concentrations of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), endothelial-leukocyte adhesion molecule 1 E-selectin (CD62E), type 1 membrane glycoprotein endoglin (CD105) before and after consumption of regular

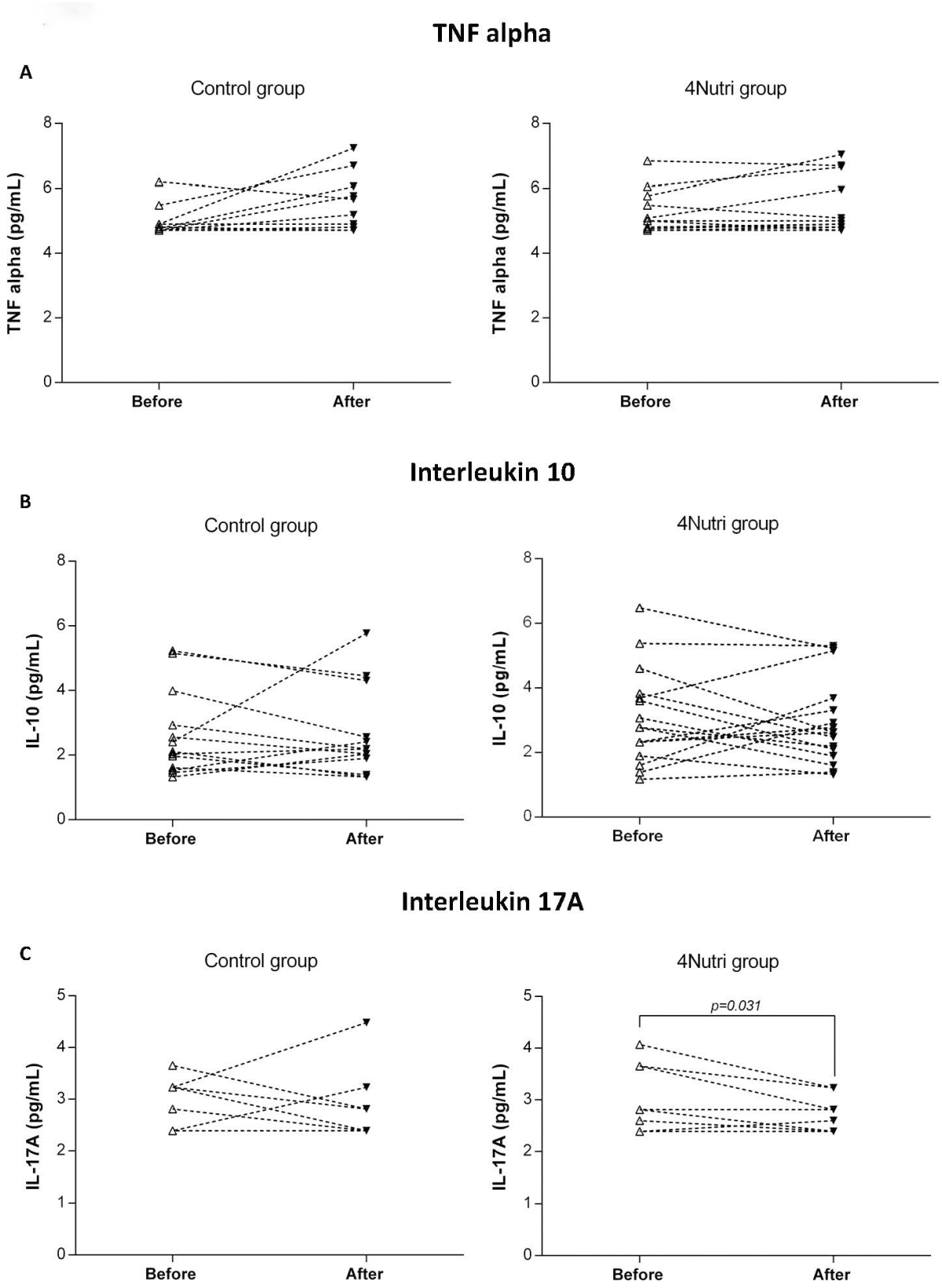

Fig. 5. Effects of regular (Control group) and enriched hen eggs (4Nutri group) consumption on the serum concentrations of tumor necrosis factor alpha (TNF-α) (A), anti- inflammatory cytokine interleukin 10 (IL-10, B) and pro-inflammatory cytokine IL-17A (C) after three-week diet protocol. TNF, tumor necrosis factor; IL, interleukin. Paired *t*-test; significance level $p < 0.05$; before vs. after within the group (Control or 4Nutri).

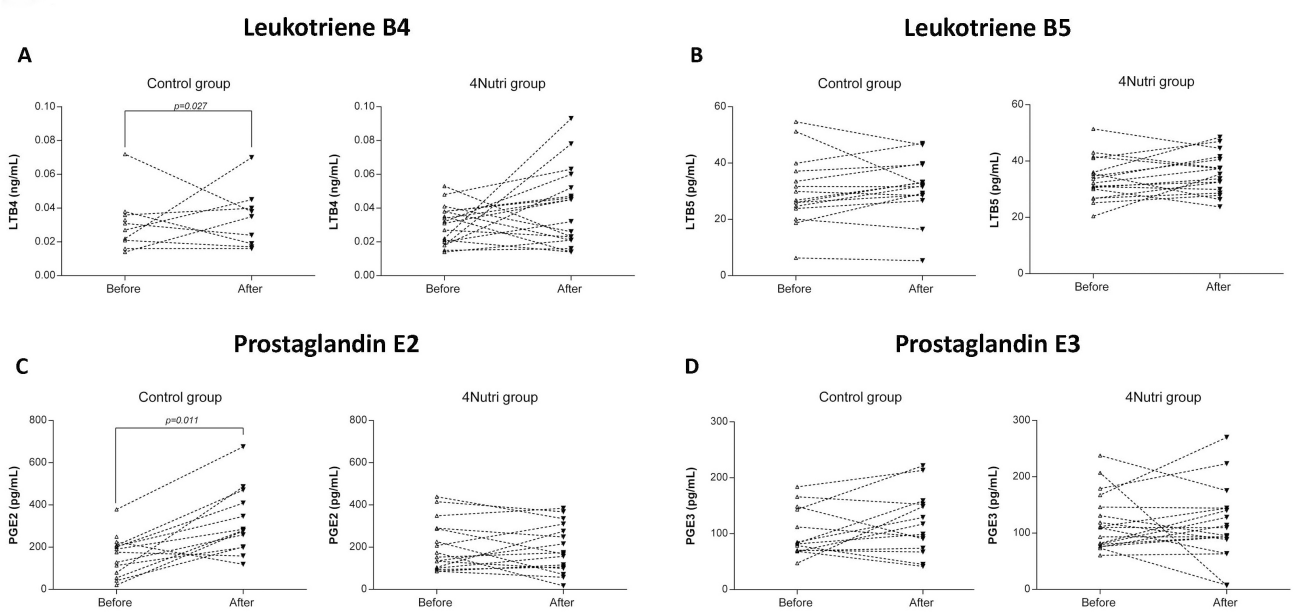

Fig. 6. Effects of regular (Control group) and enriched hen eggs (4Nutri group) consumption on the serum concentrations of lipid mediators LTB4 (A), LTB5 (B), PGE2 (C) and PGE3 (D), after respective dietary protocols. LTB4, leukotriene B4; LTB5, leukotriene B5; PGE2, prostaglandin E2; PG E3, prostaglandin E3. Paired *t*-test; significance level $p < 0.05$; before vs. after within the group (Control or 4Nutri).

eggs and eggs enriched with four nutrients, are presented in Table 6. There were no significant changes observed in either control or 4Nutri group or between groups before or after respective diet protocols or compared to baseline values.

3.6 Changes in Serum Concentrations of Pro- and Anti-Inflammatory Lipid Mediators Originating from n-6 (AA) and n-3 (EPA) Fatty Acids Following Diet Protocol

Serum concentrations of pro-inflammatory eicosanoids (LT B4 and PG E2) from AA, and inflammation-resolving oxylipins (LT B5 and PG E3), before and after the dietary protocols, are shown in Fig. 6. Pro-inflammatory LT B4 serum concentrations significantly increased in the Control group after dietary protocol (Fig. 6A), while anti-inflammatory LT B5 serum concentrations remained unchanged in both groups compared with respective baseline values (Fig. 6B). Serum concentrations of PGE2 (Fig. 6C) significantly increased while PGE3 concentrations remained unchanged (Fig. 6D) following consumption of regular hen eggs. Leukotriene B4/B5 (Fig. 7A) and prostaglandin E2/E3 (Fig. 7B) ratios were calculated and compared across the measurements. Both ratios remained unchanged in the 4Nutri group, while the prostaglandin E2/E3 ratio significantly increased in Control group ($p = 0.008$).

3.7 Correlation Analysis

Correlation analysis was performed to assess the relationship between all measured parameters: biochemical and anthropometric measurements, serum concentrations of PUFAs derived lipid mediators, cytokines, adhesion molecules, oxidative stress parameters and antioxidative enzymes activity/expression. Results are as follows: BMI of study participants correlated positively with triglyceride serum levels in the Control group (r = 0.643; p = 0.0001) and with WHR in the 4Nutri group (r = 0.550; p = 0.0002).

In the Control group, serum MPO concentrations positively correlated with PGE2 serum concentrations (r = 0.646; p = 0.002) and negatively with SOD activity (r = –0.590, p = 0.009). Furthermore, lipid mediators PGE2 and LTB4 concentrations correlated positively after regular eggs consummation (r = 0.546, p = 0.002). There was also a significant positive correlation between serum concentrations of TNF-α and ICAM-1 (r = 0.587; p = 0.02), as well as between E-selectin and LDL-cholesterol (r = 0.643; p = 0.004). There was a strong positive correlation noticed between nNOS and iNOS in the Control group (r = 0.796; $p < 0.0001$).

In the 4Nutri group, serum TNF-α concentrations significantly negatively correlated with ICAM-1 concentration (r = –0.665; p = 0.003) while positively correlated with iNOS expression (r = 0.585; p = 0.007). A significantly negative correlation was observed between serum IL-17A concentrations and endoglin (r = –0.627; p = 0.005), and serum IL-17A and HDL-cholesterol (r = –0.432; p = 0.045), while serum IL-17A positively correlated with antioxidant potential in samples (FRAP) (r = 0.445; p = 0.038). nNOS expression in PBMCs of 4Nutri group negatively correlated

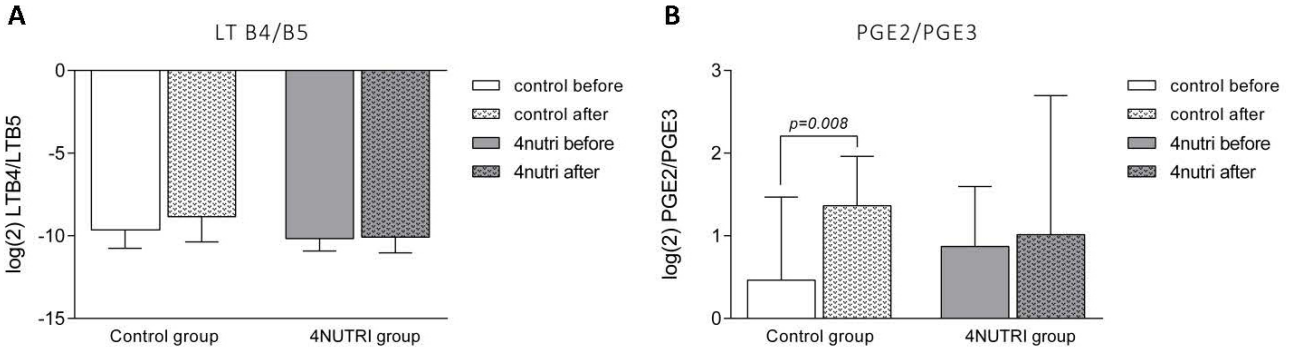

Fig. 7. Effects of regular (Control group) and enriched hen eggs (4Nutri group) consumption on leukotriene B4/B5 (A) and prostaglandin E2/E3 (B) serum ratio after respective dietary protocols. LTB4, leukotriene B4; LTB5, leukotriene B5; PGE2, prostaglandin E2; PGE3, prostaglandin E3. Paired *t*-test; significance level $p < 0.05$; before vs. after within the group (Control or 4Nutri).

with cholesterol (r = –0.458; $p = 0.01$) and LDL-cholesterol serum levels (r = –0.464; $p = 0.009$). Further, ICAM-1 in serum negatively correlated with PGE2 concentration (r = –0.548; $p = 0.02$).

3.8 Dietary Assessment Analysis

The study population in the present study consisted of mainly students. There were no major differences between study groups regarding their daily dietary habits. Participants mainly failed to adhere to recommended guidelines for consuming at least 5 meals/per day (>70% of participants). Approximately 54% of participants consumed three or fewer fruit/vegetable units per day which are below the recommended daily intake of five units. Also, their intake of *n-3* PUFAs-rich food is low. More than 90% of participants consume dairy fermented products. Only a small part of study participants (~30%) include foods rich in *n-3* PUFAs in their weekly diet plan. The results of this dietary assessment are consistent with previously published results by our research group regarding the dietary habits of students [15].

4. Discussion

The present study is the first randomized, controlled, interventional study which investigated the effects of consumption of hen eggs enriched with four nutrients—*n-3* PUFAs (ALA, EPA, DHA), selenium, vitamin E and lutein—on markers of inflammation and oxidative stress in young and healthy adults without any underlying comorbidities. The main findings of the present study suggest that consumption of enriched hen eggs has prevented the increase in serum concentrations of pro-inflammatory oxylipins (PGE2, LTB2), as well as decreasing serum concentrations of pro-inflammatory cytokine IL-17. This was accompanied by a shift in a positive correlation between TNF-α and ICAM-1 in control conditions towards their significant negative correlation after consumption of enriched hen eggs, as well as increased expression of nNOS. Previously, we have demonstrated moderate anti-inflammatory effects of *n-3* PUFAs enriched functional food in acute and chronic cardiovascular patients [33] and its cardioprotective and inflammation-resolving effect in the healthy adult population [8,10], which is further supported by results of other studies on *n-3* PUFAs supplementation [34–36].

The present study extended our research on the possibility of hen eggs serving as a functional food since there are no studies that examined the effects of consumption of combined *n-3* PUFAs and antioxidants in food. The nutrients used to enrich eggs in the present study (*n-3* PUFAs, selenium, vitamin E and lutein) individually have an important impact on the inflammatory status and oxidative stress in the organism. Enriched eggs have a significantly higher content of *n-3* PUFAs, lutein and vitamin E compared to regular eggs [19]. The observed modification of pro-inflammatory and less-inflammatory mediators in the present study can be mainly attributed to the consumption of *n-3* PUFAs. *n-3* PUFAs' anti-inflammatory beneficial potential is mainly manifested through substrate competition with *n-6* PUFAs (i.e., arachidonic acid pathway) in the enzymatic pathways of cyclooxygenases and lipoxygenases [8]. Depending on the fatty acid substrate in excess, binding to the enzyme active site leads to the production of pro- (from *n-6* PUFAs) or anti-inflammatory lipid mediators (from *n-3* PUFAs) [37]. For example, EPA competitively binds with COX-1 and COX-2 and promotes the production of less-inflammatory PGE3 while suppressing AA binding which produces highly inflammatory PGE2 [38]. These lipid mediators have an important role in maintaining the cytokine environment through direct effects on cytokine production and T lymphocyte function [8,39] leading to inflammatory or inflammation-resolving conditions. They also promote microvascular vasodilatation in human skin [40].

Vitamin E (α-Tocopherol), is an essential nutrient that

serves as a potent antioxidant in tissues due to its high biological activity, free radical trapping and hydrogen donating mechanism and is largely dependent on other nutrients intake, especially *n-3* PUFAs [41,42]. PUFAs are essential structural components of the cell membranes since they are incorporated into the lipid layer [43], while vitamin E, also representing a constituent of membranes to a smaller extent, serves as a free radical scavenger, membrane stabilizer and protector of polyunsaturated membrane lipids [44,45]. Vitamin E and its metabolites downregulate pro-inflammatory cytokines and elevate anti-inflammatory response [46,47]. In addition, vitamin E and its metabolites have also been connected with COX inhibition, specific suppression of COX-2 activity [48–51]. Consumption of enriched hen eggs caused a significant increase in serum vitamin E concentration, while no significant difference was found in the Control group or between groups. This goes in line with other studies done on healthy adults. Meydani *et al.* [52] showed that short-term supplementation with vitamin E (800 mg/day) increased serum levels with no negative side effects in older adults. Similarly, Hodis *et al.* [53] showed that α-Tocopherol supplementation significantly increases plasmatic levels of vitamin E in healthy adults with moderately elevated LDL-cholesterol levels. Recently, vitamin E was discussed concerning inflammatory skin diseases, since it was stated that serum vitamin E levels were lower in patients suffering from vitiligo, psoriasis, atopic dermatitis and acne [54].

There is a strong link between ROS, oxidative stress and inflammatory response. In the present study, a decrease in serum levels of IL-17A is mainly associated with the effects of *n-3* PUFAs supplementation, which was earlier described in mice models for colitis [55] and induced arthritis [56], alongside decreased mRNA expression of other pro-inflammatory cytokines. Such effect is confirmed by our previously published results in healthy individuals [10] in which serum pro-inflammatory interferon-gamma (INF-γ) levels decreased, while serum anti-inflammatory IL-10 levels increased following consumption of *n-3* PUFAs enriched eggs. A positive correlation between serum concentrations of TNF-α and ICAM-1 presented in the Control group was lost in the 4Nutri group, suggesting that endothelium was in a resting state. Also, in the 4Nutri group, a significantly negative correlation between ICAM-1 and pro-inflammatory PGE2 was detected. Previously reported decreased levels of pro-inflammatory markers such as IL-1, TNF-α, IL-6 and CRP, might also be partially attributed to other added nutrients, as previously reported for selenium [57,58], vitamin E [46,59,60] and lutein [61] supplementation, although these mechanisms are yet to be investigated as the evidence in support of this hypothesis is still scarce. Additionally, the positive correlation between TNF-α and ICAM-1 in the Control group was shifted towards a significant negative correlation after the consumption of enriched hen eggs. This is an interesting finding, since TNF-α acts as an inflammatory cytokine, and upregulates ICAM-1 expression in acute inflammatory conditions [62,63], as well as cytokines such as IL-1 or IFN-γ [64]. It seems that ICAM-1, which is usually expressed in endothelial cells at low levels, changes its activation pathway in the presence of *n-3* PUFAs, previously elaborated by Goua *et al.* [65]. This may occur also under the influence of nutrients with antioxidant activity such as vitamin E, selenium and lutein due to the connection between ICAM-1 and oxidative stress induced-NF-κB pathway activation [66].

Surprisingly, MPO serum concentration was significantly increased after enriched eggs consumption but unaltered after regular eggs consumption. However, it has been speculated that increased MPO protein levels might not be important *per se* in an inflamed environment, but the subsequent neutrophil recruitment and activity [67]. MPO affects neutrophil functions and cytokine production. It catalyses the production of harmful products only in presence of hydrogen peroxide (H_2O_2) [68]. In the present study, this is hardly the case since TBARS and FRAP have not been changed significantly following dietary protocols. Present results suggest that elevated protein levels of MPO in the 4Nutri group did not lead to a shift towards inflammatory conditions, which is partially confirmed by significantly decreased levels of pro-inflammatory IL-17A in the serum of participants who consumed enriched eggs. Nevertheless, increased MPO protein levels are usually associated with inflammation, although this enzyme also exhibits strong antifungal and antibacterial properties and its deficiency can amplify the inflammatory response [68]. Indeed, MPO can serve as an anti-inflammatory reagent in cases where there is no infection, such as in participants of the present study, whose hsCRP serum levels were in the reference range as there was no inflammation present at the time of enrolment [69].

Nitric oxide (NO), a highly reactive signalling and defence molecule produced by nitric oxide synthase (NOS) plays a crucial role in the pathogenesis of inflammatory response and, depending on the physiological conditions in the environment, it behaves as an anti- or pro-inflammatory mediator [70,71]. Lately studies have shown that nNOS, found predominantly in the nucleus of resting endothelial cells, contributes to endothelium-dependent vasodilatation, has a vascular protective effect, but also has a role in the initiation and early phase of inflammatory response since its absence up-regulates pro-inflammatory cytokines and increases leukocyte recruitment leading to enhanced cytokine-mediated response [62–64,72]. IFN-γ coupled with IL-17 intensifies inflammation and up-regulation of iNOS [73]. Presently, in a group that consumed enriched hen eggs, a moderate anti-inflammatory effect was present due to increased mRNA expression of nNOS and decreased serum levels of IL-17A after enriched eggs consumption. *n-3* PUFAs (especially DHA) and selenium inhibit NO production and iNOS expression through upregulation of cellu-

lar glutathione (GSH) levels accompanied by inhibition of oxidative stress-sensitive NF-κB activation [66,74], while DHA also enhances brain NOS activity [75]. Since our study group consisted of healthy, young people and there is no evidence of inflammation or oxidative stress present, as well as no major antioxidant activity, it seems that nNOS has an important role as a basal NO contributor in the resting, non-inflammatory environment. This is supported by the negative correlation of nNOS with cholesterol/LDL-cholesterol in the 4Nutri group, while it correlated positively with iNOS only in the Control group.

COX pathway is inhibited by supplemental DHA, EPA and ALA [8,37,76], which was confirmed in the current study. Interestingly, there was a significant increase in LTB4 and PGE2 levels but also PGE2/PGE3 ratio after regular eggs consumption. This may be related to increased intake of arachidonic acid via regular eggs. However, there were no significant changes in LTB4 and PGE2 concentrations following the consumption of enriched eggs, suggesting that a favourable ratio of *n-3* and *n-6* PUFAs in these eggs has beneficial anti-inflammatory potential. This justifies functional food in everyday diet.

5. Conclusions

In conclusion, this study population consisted of young, healthy adults without underlying comorbidities; thus no oxidative stress or major anti-inflammatory effects were detected. Decreased serum levels of pro-inflammatory IL-17A and an increased nNOS expression in the 4Nutri group, together with alteration of metabolites produced via COX pathways in the Control group, suggest a shift towards anti-inflammatory conditions in participants who consumed enriched hen eggs, while such changes were not observed in Control group. Present results suggest that the combined action of *n-3* PUFAs and antioxidants play an important role in the nNOS activation pathway and this cooperation may have a protective role in resting, non-inflammatory conditions. The change in nNOS expression was surprising and grants further investigation.

6. Limitations

The limitation of the present study was the duration of the dietary protocol of 21 days when one month is in general a minimum for the intervention duration. Furthermore, MPO activity wasn't measured even though protein levels do not necessarily correspond with MPO activity [77] and can vary greatly between respondents, since several plasma proteins such as ceruloplasmin can reversibly inhibit its activity [78,79]. Furthermore, the study population consisted of young and healthy participants therefore there was no inflammation or impairments that required mitigation in such sense.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

Conceptualisation—PŠ, NK, AS and ID; data curation—PŠ, NK, AM, SM and ZM; formal analysis—PŠ, NK, AM, SM and ZM; funding acquisition—ID; investigation—PŠ, NK, AM and ZM; methodology—PŠ, NK, AM, ZM and SM; project administration—AS and ID; resources—ID; software—PŠ, NK, AM, AS and ZM; supervision—AS and ID; validation—AS and ID; visualisation—PŠ, NK, AS and ID; writing—original draft preparation—PŠ, NK, AM, ZM, SM, AS and ID; writing—review and editing—PŠ, NK, AM, ZM, SM, AS and ID. All authors have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

The study protocol and procedures conformed to the standards set by the latest revision of the Declaration of Helsinki and were approved by the Ethical Committee of the Science Center of Excellence, Josip Juraj Strossmayer University of Osijek (Class: 602-04/14-08/06; Reg. No.: 2158-610714-114) and Ethics Committee of the Medical Faculty Osijek (Class: 602-04/20-08/07, Reg. No.: 2158-61-07-20147).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2712332.

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