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 Consumption Enhances Microvascular Reactivity in Young
 Healthy Individuals**

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**Omega-3 Polyunsaturated Fatty Acids Enriched Hen Eggs Consumption Enhances
Microvascular Reactivity in Young Healthy Individuals**

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Abstract

Whilst the beneficial effect of omega-3 polyunsaturated fatty acids (PUFAs) supplementation on cardiovascular (CV) system is well supported in CV patients, the effect of consumption of omega-3 PUFAs enriched functional food in healthy individuals is still not fully elucidated. This study aimed to determine the effect of consumption of omega-3 PUFAs enriched hen eggs on microvascular reactivity (primary outcome), blood pressure (BP) and serum lipid profile in young healthy individuals. Control group (N=16) ate three ordinary hen eggs (277 mg omega-3 PUFAs/day), and OMEGA-3 group (N=20) ate three omega-3 PUFAs enriched eggs containing 259 mg of omega-3 PUFAs/egg daily (ALA 167 mg/egg, EPA 7 mg/egg, DHA 84 mg/egg) for 3 weeks (777 mg omega-3 PUFAs/day). Post-occlusive reactive hyperemia (PORH) in skin microcirculation assessed by laser Doppler flowmetry, serum lipid profile, fasting blood glucose, high-sensitivity C-reactive protein (hsCRP) and arterial BP were measured in all subjects before and after the protocol. PORH was significantly enhanced, and triglycerides, hsCRP and BP were significantly decreased in OMEGA-3 group compared to baseline measurement, while there was no significant difference in Control group after the protocol compared to baseline. This is the first study to demonstrate that consumption of a mixture of omega-3 PUFAs (ALA+EPA+DHA), provided via enriched hen eggs, elicits changes in microvascular reactivity, BP and triglycerides level in healthy subjects that are associated with CV benefits, thus suggesting that daily consumption of omega-3 PUFAs enriched eggs in healthy individuals may potentially contribute to CV risk factors attenuation and disease prevention.

Key words: omega-3 PUFAs, vascular function, microcirculation, laser Doppler flowmetry, serum lipid profile

52 Introduction

53 Nowadays, it is widely accepted that an impaired vascular endothelial function represents
54 one of the main features of cardiovascular (CV) diseases (Ross 1999). Consumption of omega-3
55 polyunsaturated fatty acids (PUFAs) has been shown to reduce CV risk and has a beneficial
56 effect on CV disease progression (Auger et al. 2016). It has been widely investigated whether
57 intake of omega-3 PUFAs may prevent or delay the progression of atherosclerosis via its impact
58 on the initial steps in its pathogenesis, i.e. changes in endothelial and vascular function (Endo
59 and Arita 2016), as opposed to “traditional” CV risk factors such as blood pressure (BP),
60 triglyceride concentrations and/or inflammation. Considerable knowledge on this issue was
61 provided by studies in animal model (Gortan Cappellari et al. 2013; Lopez et al. 2004; Zhang et
62 al. 2013), which demonstrated that omega-3 PUFAs have the potential to improve vascular and
63 endothelial function by: a) increasing bioavailability of nitric oxide (NO) (Gortan Cappellari et
64 al. 2013; Lopez et al. 2004; Zhang et al. 2013); b) reducing oxidative stress level (Gortan
65 Cappellari et al. 2013; Zhang et al. 2013); and/or c) attenuating inflammation (Wang et al. 2011).
66 In order to test knowledge obtained in animal studies, a number of human functional vascular
67 studies were performed that mainly investigated the effect of omega-3 PUFAs (in the form of
68 eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) capsules or walnut-supplemented
69 diet rich in α -linolenic acid (ALA)) on the reactivity of large (conductance) arteries using flow
70 mediated dilation (FMD) of the brachial artery (Siasos et al. 2013; Rizza et al. 2009; Shah et al.
71 2007). There is a paucity of data on the effect of omega-3 PUFAs supplementation on
72 microvascular reactivity which is usually non-invasively tested by the laser Doppler technique.
73 Two recent large meta-analyses that have evaluated the effect of various forms of omega-3
74 PUFAs supplementation on human endothelial function brought conclusions that are not

completely concordant. One meta-analysis reported that omega-3 PUFAs supplementation significantly increased FMD of the brachial artery, and that this effect can be modified by the health status of the participants, or by the dose of omega-3 PUFAs supplementation (Wang et al. 2012). On the contrary, a sensitivity analysis including only double-blinded, placebo-controlled studies indicated that omega-3 PUFAs supplementation did not have a significant effect on vascular endothelial function (Xin et al. 2012). Nevertheless, these analyses were limited by significant heterogeneity in design of relevant studies, including the number of participants, inclusion criteria such as age of participants or whether participants were healthy or diseased, markers of endothelial function that were measured, dose and duration of omega-3 PUFAs supplementation, forms of omega-3 PUFAs that were administered (ALA, EPA, DHA) alone or in combination, and concomitant therapy that was used (Wang et al. 2012; Xin et al. 2012).

Functional food concept has been introduced only recently. This pertains to food of natural origin that contains ingredients with a beneficial effect on human health (Drenjancevic et al. 2017). Food can be considered functional if it is shown to a satisfactory degree that, in addition to appropriate nutritional effects, it also has beneficial effects on one or more target functions of the body, in a way that it is important for improving the health condition and general well-being or reducing risks of disease (Drenjancevic et al. 2017). It is important to emphasize that functional food has to be actual food (not in the form of pills or capsules), and it has to show its effects when consumed in normal daily amounts (Diplock et al. 1999). One of the most common functional poultry products are hen eggs with increased content of desirable omega-3 PUFAs, which are available on the market.

It has been previously described that omega-3 PUFAs enriched functional foods (e.g. hen eggs) may reduce CV risk in CV patients (Make et al. 2003; Lewis et al. 2000, Fraeye et al.

2012). Still, given that in former studies in healthy individuals, omega-3 PUFAs were mostly supplemented in the form of capsules (EPA+DHA) (Siasos et al. 2013; Rizza et al. 2009; Shah et al. 2007), there is a lack of data on the effect of omega-3 PUFAs enriched functional foods on vascular function (both macro- and microvascular) in healthy individuals. Thus, the present study aimed: a) to determine the effect of consumption of omega-3 PUFAs enriched hen eggs on skin microvascular reactivity in response to vascular occlusion (primary outcome); and b) to investigate the effect of consumption of the same eggs on BP, serum lipid values and inflammatory marker (hsCRP) modulation in young healthy lean individuals.

Materials and Methods

Study Population

Eighteen young healthy women and the same number of young healthy men were recruited by way of an advertisement at the Faculty of Medicine, University of Osijek to participate in the present study (Table 1). Eligibility criteria included age range between 18 and 30 years, normal body mass index (BMI), BP values and serum lipid ranges. Also, exclusion criteria included a history of smoking, hypertension, coronary artery disease, diabetes, hyperlipidemia, renal impairment, cerebrovascular and peripheral artery disease. None of the subjects were taking drugs that could affect the endothelium and vascular function. Written informed consent was obtained from each subject. 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 Faculty of Medicine, University of Osijek (class: 602-04/14-08/06, number: 2158-610714-114).

Production of Omega-3 PUFAs Enriched Hen Eggs and Assessment of Fatty Acids Profile of Chicken Feed Mixtures and Edible Part of Eggs

Omega-3 PUFAs enriched hen eggs were produced under a patent of Faculty of Agriculture, Josip Juraj Strossmayer University of Osijek. According to their protocol, common sunflower oil in feed mixtures fed to the laying hens was replaced with a mixture of fish (1.33%), linseed (1.33%), rapeseed (1.33%) and soybean (1%) oil. This replacement resulted in changed fatty acids profile in the laying hen's eggs that had reduced n-6 PUFAs and increased n-3 PUFAs concentration, and thus a very favorable n-6/n-3 PUFAs ratio (2.63:1).

Assessment of fatty acids profile of chicken feed mixtures (n=3) and edible part of eggs (n=10) was performed using gas liquid chromatography. Fat content of homogenized samples was extracted by using the method of Folch et al. (1957). All solvents used were ultrapure-grade by Sigma-Aldrich (Schnelldorf, Germany), and 100 mg/L butylated hydroxytoluene was added to the extraction mixture (chloroform/methanol 2/1 vol/vol) as antioxidant. After this, fatty acid containing lipids were transmethyated by the base-catalyzed sodium-methoxide method of Christie (1982). Gas liquid chromatography was performed on a Shimadzu 2010 apparatus (Kyoto, Japan), equipped with a SP-2380 (Supelco, Bellefonte, USA) type capillary column (30 m x 0.25 mm internal diameter, 0.20 μ m film) and flame ionization detector. To identify individual fatty acids in the chromatogram, a fatty acids standard mixture (Supelco 37 Component FAME Mix, CRM 47885) was used. Analyzed fatty acids are described in Table 2.

Study Protocol

This was a randomized, double-blind, placebo-controlled interventional study. None of the subjects had been taking omega-3 PUFAs enriched functional food nor omega-3 PUFAs

supplementation in the form of capsules prior to enrollment in the present study. Subjects were divided into two groups: control (Control) and experimental (OMEGA-3) group. The experimental group consisted of 20 subjects (10 women and 10 men). Control group consisted of 16 subjects (8 women and 8 men). Neither the researcher nor the subjects knew which group the subjects belong to until the end of the three weeks' dietary protocol (eggs were labeled #1 or #2 before distributed to the Laboratory). During those three weeks the subjects ate three hen eggs per day (total of 63 eggs). Subjects in OMEGA-3 group were given omega-3 PUFAs enriched hen eggs (three per day; around 777 mg of omega-3 PUFAs per day), while subjects in the Control group received standard hen eggs produced on the same farm (three per day; around 277 mg of omega-3 PUFAs per day). Omega-3 PUFAs enriched eggs and standard hen eggs were the same size (M commercial size). Subjects were instructed to boil the eggs for about 10 minutes before consumption. Also, all subjects were instructed to take only the eggs given to them for the purposes of the study (total of 63 eggs) and not to take the other food rich in omega-3 PUFAs, omega-3 PUFAs enriched functional food or any other form of omega-3 PUFAs supplementation during study protocol. The measurements were performed on the first and last day of the protocol.

Clinical Visits

During the study, each subject had two clinical visits. The study protocol was performed in the Laboratory for Clinical and Sports Physiology, Department of Physiology and Immunology at Faculty of Medicine, University of Osijek. All testing occurred in the morning after an overnight fasting. Subjects were instructed not to undertake any strenuous activity during the 24 h preceding the visit and to avoid caffeine intake in the morning before the study visit.

Assessment of Skin Microcirculatory Blood Flow

Microcirculatory blood flow was assessed by laser Doppler flowmetry (LDF) (MoorVMS-LDF, Axminster, UK) in response to vascular occlusion. LDF measurements were performed in a warm room (mean $\pm$ SD temperature = $23.5 \pm 0.5^{\circ}\text{C}$). Data collection started after 30 min of acclimatization to avoid temperature-related changes in blood flow. The subjects were resting in the supine position and the probe and wire were secured in place. The laser probe was attached to the subject's volar forearm skin 13–15 cm from the wrist at the place where basal blood flow was between 5 and 10 perfusion units (PU). LDF measurements were taken under identical conditions, at the same time of the day, and the laser probe was attached to the approximately same place at both study visits.

Microvascular reactivity assessment protocol included a non-invasive test that indicates changes in blood flow during post-occlusive reactive hyperemia (PORH) following release of an occlusion of blood flow (Crakowski et al. 2006). After the 5 min baseline measurement, vascular occlusion was induced by inflating a pneumatic cuff on the upper arm to 30–50 mmHg above the systolic blood pressure (SBP). Measurements were taken before, during, and after release of 1 min (PORH-1), 2 min (PORH-2) and 3 min occlusion (PORH-3), with 10 min interval between each occlusion to allow blood flow to return to baseline level. It is considered that such shorter periods of vascular occlusion are at least partially mediated by endothelium-derived vasoactive metabolites, rather than ischemia products (e.g. adenosine). Microcirculatory blood flow in a given time was expressed in arbitrary PU and determined by software calculating the area under the curve (AUC) during baseline flow, occlusion and reperfusion. Data were analyzed using appropriate software provided by the manufacturer of the LDF device (moorVMS-PC v4.0, Axminster, UK). Because the flow does not reach the value of zero even when perfusion is absent, flow values were expressed as a quotient of a standard comparator – baseline flow. The

final result was expressed as the difference between percentage of flow change during reperfusion and occlusion in relation to baseline (R-O % increase). General procedures for LDF PORH measurements were done according to the protocol already described in our laboratory (Cavka et al. 2013; Cavka et al. 2015).

Anthropometric Measurement

Body mass index (BMI) was calculated as body mass (kg) divided by height (m) squared. Waist and hip circumference were measured and waist-to-hip ratio (WHR) was calculated.

Arterial Blood Pressure Measurement

BP was measured at the beginning of each visit after a 15 min rest in the seated position using an automatic oscillometric monitor (OMRON, Osaka, Japan). The final values of BP were the mean of three repeated measurements. Mean arterial pressure (MAP) was calculated using a formula of the SBP and the diastolic blood pressure (DBP): $MAP = [SBP + 2 (DBP)]/3$.

Biochemical Laboratory Testing

A venous blood sample was taken after 30 min resting in the supine position. Venous blood samples were analyzed for fasting serum lipid profile (total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides), fasting blood glucose (fBG) and high-sensitivity C-reactive protein (hsCRP) using standard laboratory methods at the Department of Clinical Laboratory Diagnostics, University Hospital Osijek.

Statistical Analysis

All results are expressed as mean $\pm$ standard deviation (SD). The sample size required to show a potentially significant effect was calculated based on preliminary data in 10 subjects. To show an expected difference in LDF-measured microvascular blood flow change (R-O %) before and after a specific diet protocol with $\alpha=0.05$ and a statistical power of 80% for paired t-test, the needed sample size is 12 subjects per group. Clinical characteristics and all parameters measured before and after the specific study protocol were compared using a paired t-test. The normality of data distribution was assessed by the Kolmogorov–Smirnov normality test. The Wilcoxon rank-sum test was used when variables were not normally distributed. Student's t-test was used to compare parameters between the experimental groups. When variables were not normally distributed, the Mann–Whitney rank sum test was used. SigmaPlot, version 11.2 (Systat Software, Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Content of Fatty Acids in Chicken Feed Mixture and Edible Part of Eggs

Fatty acids profiles of chicken feed mixture (g/100 g of total fatty acids) and eggs (mg/100 g edible part) are described in Table 2. Each OMEGA-3 egg (average weight 58 g) contained on average 259 mg of omega-3 PUFAs (ALA 167 mg/egg, EPA 7 mg/egg, DHA 84 mg/egg). Each control egg (average weight 58 g) produced on the same farm contained on average 92.4 mg of omega-3 PUFAs.

Characteristics of Study Population

Characteristics of the study population are described in Table 1. There was no difference in participants' age between OMEGA-3 and Control group. Subjects of both sexes from

OMEGA-3 and Control group were lean and had similar anthropometric measures (BMI and WHR) before the study protocol (Table 1). No significant change in BMI and WHR in both OMEGA-3 (BMI, kg/m^2 OMEGA-3 before 23.6 ± 3.2 vs. after 23.6 ± 3.1 , $P=0.979$; WHR OMEGA-3 before 0.77 ± 0.03 vs. after 0.76 ± 0.03 , $P=0.500$) and Control group of subjects (BMI, kg/m^2 Control before 24.6 ± 3.9 vs. after 24.5 ± 4.0 , $P=0.576$; WHR Control before 0.77 ± 0.03 vs. after 0.76 ± 0.03 , $P=0.303$) was induced by consumption of either three omega-3 PUFAs enriched or three ordinary hen eggs over the course of three weeks.

Skin Microvascular Post-Occlusive Reactive Hyperemia (PORH) Response to Consumption of Omega-3 PUFAs Enriched Hen Eggs

PORH following all three vascular occlusion periods (PORH-1, PORH-2 and PORH-3) was significantly increased in OMEGA-3 group after the study protocol compared to before-diet measurement (Figure 1, Panel 1A). There was no change in skin microvascular PORH in the Control group after the diet protocol compared to before-diet LDF measurement (Figure 1, Panel 1B).

Arterial Blood Pressure Response to Consumption of Omega-3 PUFAs Enriched Hen Eggs

BP measurements had demonstrated that all participants were normotensive before the study protocol. There was no difference in BP between two study groups before the study protocol. Table 3 presents BP changes before and after the study protocol in subjects from both study groups. SBP, DBP and MAP were significantly decreased in OMEGA-3 group after the protocol. In Control group, BP was not significantly changed before and after the study protocol.

Biochemical Parameters

Table 4 presents changes in serum lipid profile, fBG and hsCRP values before and after the corresponding study protocol in all subjects. There was no difference in any measured biochemical parameters between the groups before the study protocol. Consumption of three omega-3 PUFAs enriched hen eggs per day for three weeks did not induce a significant difference in cholesterol, HDL cholesterol and LDL cholesterol level in OMEGA-3 group compared to the pre-diet measurement. However, blood triglycerides levels were significantly reduced after the study protocol in OMEGA-3 group compared to values measured before the study. There was no difference in serum lipid profile values measured before and after the study protocol in the Control group. fBG was not significantly changed before or after the specific study protocol in both OMEGA-3 and Control group. hsCRP was significantly reduced in OMEGA-3 group after the protocol compared to pre-diet measurement. There was no significant change in hsCRP values after the study protocol in the Control group compared to values measured before the protocol. However, the OMEGA-3 group, before and after treatment, had lower hsCRP levels than the Control group.

Discussion

The salient findings of the present study are that consumption of three omega-3 PUFAs enriched hen eggs per day (259 mg omega-3 PUFAs/egg: ALA 167 mg/egg, EPA 7 mg/egg, DHA 84 mg/egg; 777 mg omega-3 PUFAs/day) for three weeks: 1) significantly enhanced skin microvascular reactivity to vascular occlusion; 2) significantly decreased arterial BP; 3) significantly decreased blood triglycerides level; and 4) significantly decreased hsCRP level compared to baseline in young healthy population. These findings are in contrast to those pertaining to standard eggs consumed by the control group, which showed no change in

measured parameters after 3 weeks. It should be emphasized that the present study (randomized, double-blind, placebo-controlled interventional study) is one of the few studies to investigate the effect of omega-3 PUFAs supplementation in the form of functional food (omega-3 PUFAs enriched hen eggs) on traditional CV risk factors, and the first one that also investigated its effect on microvascular function in young healthy subjects.

The Effect of Omega-3 PUFAs on Vascular Function

Omega-3 PUFAs alter vascular and endothelial function by incorporating into its membrane phospholipids in which signaling molecules and receptors for endothelial cell function are located (Endo and Arita, 2016). Omega-3 PUFAs may increase NO bioavailability directly by stimulating endothelial nitric oxide synthase (eNOS) gene and protein expression (Gortan Cappellari et al. 2013; Lopez et al. 2004; Zhang et al. 2013), or indirectly by attenuating reactive oxygen species (ROS) and thus decreasing oxidative stress level, as demonstrated in cell cultures and isolated blood vessels (Gortan Cappellari et al. 2013; Zhang et al. 2013). Omega-3 PUFAs may also attenuate cellular and systemic inflammation by reducing soluble cell adhesion molecules (sCAMs), IL-6 and/or CRP level (Wang et al. 2011).

Functional vascular studies, focused on peripheral macrovasculature (e.g. brachial artery FMD), strongly suggested that omega-3 PUFAs supplementation (EPA+DHA, DHA alone or ALA alone) improves vascular and endothelial function in CV disease patients and in patients at high CV risk (Yagi et al. 2015; Tousoulis et al. 2014; Egert et al. 2014; Merino et al. 2014). On the other hand, studies investigating the effect of omega-3 PUFAs supplementation (mostly EPA+DHA capsules) on vascular endothelial function in healthy individuals provided inconsistent results (Siasos et al. 2013; Rizza et al. 2009; Shah et al. 2007). Some authors reported that omega-3 PUFAs supplementation improved brachial artery FMD in healthy

subjects, while others failed to demonstrate such effect (Sanders et al. 2011; Singhal et al. 2013; Skulas-Ray et al. 2011). One study demonstrated that omega-3 PUFAs supplementation (EPA+DHA capsules for 12 months) amounting to less than 1.8 g/day (0.45 and 0.9 g/day) does not significantly improve FMD in healthy adults (Sanders et al. 2011). Supplementation of omega-3 PUFAs in the form of EPA+DHA capsules (1 g in only one dose or 4 g/day during period of 4 weeks) significantly decreased postprandial endothelial dysfunction (Miyoshi et al. 2014; Fahs et al. 2010). On the contrary, a single Mediterranean meal (rich in omega-3 PUFAs) did not alter FMD in healthy men (Lacroix et al. 2016). Two studies reported that omega-3 PUFAs supplementation (Mediterranean diet for 4 weeks or a single fish oil meal) improved postprandial endothelial microvascular reactivity assessed by LDF (Fuentes et al. 2008; Armah et al. 2008). Only one study demonstrated that fish oil supplementation for 8 months increased microvascular reactivity in normal healthy subjects (Khan et al. 2003). In the present study, healthy individuals who consumed three omega-3 PUFAs enriched eggs (ALA+EPA+DHA) for three weeks exhibited improved PORH in skin microcirculation, in all three vascular occlusion periods (Figure 1).

The Effect of Omega-3 PUFAs on Blood Pressure

Omega-3 PUFAs (EPA+DHA rich diet, 700 mg/day for 8 weeks) have a significant role in lowering arterial BP in hypertensive patients (Minihane et al. 2016). The present study is one of very few studies reporting that consumption of omega-3 PUFAs enriched hen eggs for three weeks decreased arterial BP in generally normotensive and healthy young population (Table 3). These results are in concordance with the study by Oh et al., who reported that consumption of 4 omega-3 enriched eggs for 4 weeks decreased BP level in healthy individuals (Oh et al. 1991). Such decrease in BP level was not seen when omega-3 PUFAs were supplemented in the form of

four fish servings per week (~800 mg/serving EPA+DHA for 8 weeks) or fish oil supplementation (2 g/day EPA+DHA for 12 weeks; 1.7 g/day EPA+DHA for 4 weeks) to normotensive individuals (Grieger et al. 2014; Hlais et al. 2013; Root et al. 2013). Omega-3 PUFAs supplementation (EPA+DHA capsules 4 g/day) for 12 weeks diminished increases in mean arterial BP and DBP at the onset of handgrip exercise and therefore reduced CV response to acute physiological stress in young and older healthy subjects, although no change in resting BP after omega-3 PUFAs supplementation was detected (Clark et al. 2016). Furthermore, a beneficial effect of two weeks 3 g/day omega-3 PUFAs supplementation (fish oil or oily fish) on hemodynamic parameters was reported in patients with uncontrolled hypertension and patients older than 45 years, but the same effect was also noticed in normotensive subjects (Cabo et al. 2012). It was suggested that such inconsistency in observed results may reflect genetic variation associated with BP (AlSaleh et al. 2014; Ellulu et al. 2016).

Anti-inflammatory Effect of Omega-3 PUFAs

The present study has shown a significant decrease of hsCRP compared to baseline in the study group consuming omega-3 PUFAs eggs (ALA+EPA+DHA), while such effect was not observed in subjects consuming standard hen eggs (Table 4). This is in agreement with studies showing that omega-3 PUFAs supplementation (fish oil capsules with 300 mg EPA and 200 mg DHA per day for 8 weeks) decreased hsCRP in hypertensive, obese and diabetic patients (Ellulu et al. 2016), as well as in healthy adults (0.85 g EPA + 3.4 g DHA per day for 8 weeks) (Skulas-Ray et al. 2011). On the other hand, some authors did not detect any significant effect of a moderate dose of omega-3 PUFAs (fish oil supplementation 1400 mg EPA+DHA per day for 18 weeks) on CRP and IL-6 in healthy adults (Muldoon et al. 2016), nor was any positive effect of

fish oil omega-3 PUFAs (1.7 g EPA+DHA packets per day for 4 weeks) on arterial health and inflammatory markers observed in young but overweight population (Root et al. 2013).

The Effect of Omega-3 PUFAs on Lipid Profile

It is known that omega-3 PUFAs reduce serum triglyceride concentrations by inhibiting two crucial enzymes involved in hepatic triglycerides biosynthesis, and by increasing very low-density lipoprotein (VLDL) clearance in the peripheral circulation (Jacobson 2008). A large body of evidence reported the clinical relevance of omega-3 PUFAs in the management of all forms of persistent hypertriglyceridemia and high triglycerides in patients with increased CV risk (Zulyniak et al. 2016). The authors of the present study found two studies that investigated the effect of omega-3 PUFAs enriched eggs on serum lipids in healthy individuals. Similarly as in the present study, one study demonstrated that consumption of 4 omega-3 PUFAs enriched eggs per day for 4 weeks significantly decreased mean plasma triglyceride concentration in healthy subjects, while consumption of control eggs had the opposite effect (Oh et al. 1991). Furthermore, another study reported that omega-3 PUFAs enriched eggs (~500 mg DHA + 40 mg EPA + 1 g ALA per egg; 6 eggs/week for 8 weeks) and a walnut-supplemented diet (2.95 g ALA/28.4 g walnuts, 6 times/week) had a beneficial effect on serum lipids compared to standard eggs in healthy lacto-ovo-vegetarians, which is in concordance with the results of the present study (Burns-Whitmore et al. 2014). The results of the present study are also in agreement with others demonstrating the triglyceride-lowering benefits associated with fish oil supplements (EPA+DHA) in children, young and older men (960 mg/day for 16 weeks), as well as in healthy older women (3 g/day for 12 weeks) (Logan & Spriet 2015; Pase et al. 2015; Damsgaard et al. 2013). In contrast, several studies failed to demonstrate a relationship between fish-oil supplementation (2 g/day EPA+DHA for 12 weeks; 1.7 g/day EPA+DHA for 4 weeks) and lipid

profile in healthy individuals (Hlais et al. 2013; Root et al. 2013). This discrepancy could be explained by heterogeneity in design of these studies in terms of participants' age as well as dose, duration and forms of omega-3 PUFAs supplementation.

The strengths of the present study are the following: a) the fact that it is designed as a randomized double-blind placebo-controlled study, b) homogeneity in health status between the intervention and control groups, and c) usage of a natural, cheap and accessible dietary source of omega-3 PUFAs supplementation.

One limitation of this study is the authors' technical inability to measure blood fatty acid profile, since that method is not available at the institutions where the authors work. However, considering the stark and significant differences demonstrated by the results of the present study, as well as the strict instructions that had been given to the subjects in regard of diets, the authors of the present study are confident that the obtained differences are the consequence of the dietary omega-3 PUFAs intake during the study protocol. Another possible limitation of the present study is that consumption of three eggs per day is quite excessive. Since 'functional food should show effects when consumed in normal daily amounts', future studies based on a populations' habitual weekly omega-3 PUFAs rich food intake should be conducted to confirm the findings. Also, it is important to conduct further investigation of the underlying mechanisms involved in CV health background, and to include not only healthy individuals but also CV patients, which would put the results in the context of reduced risk of developing pathology.

In conclusion, the present study provides evidence that omega-3 PUFAs from functional food (omega-3 PUFAs enriched eggs) increased microvascular reactivity in young healthy subjects, and that they increased serum lipid values by decreasing blood triglyceride levels in young healthy individuals. The study has also demonstrated that consumption of omega-3 PUFAs enriched eggs has the potential to decrease BP level and hsCRP in young healthy subjects. The present study is the first one to investigate the effect that the incorporating of n-3 PUFAs enriched eggs in everyday eating habits has on microvascular reactivity. The present study has significant clinical and public health relevance, and it is also important for further investigation of the underlying mechanisms involved in CV health background.

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Conflicts of Interests

The authors declare no conflict of interest.

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Tables

Table 1. Characteristics of study population at baseline

Group	OMEGA-3		Control	
Parameter				
n	20		16	
sex, M/W	10/10		8/8	
age, years	21±1		21±1	
	M	W	M	W
BMI, kg/m ²	24.7±2.3	20.8±1.7	24.3±2.1	20.9±1.8
WHR	0.79±0.03	0.77±0.05	0.81±0.01	0.76±0.04

Results are presented as mean±SD.

n- number of participants; M - men; W - women; BMI - body mass index; WHR - waist-to-hip ratio

Table 2. Fatty acids profile of hens' feeding mixtures and edible part of eggs

Fatty acid	Feeding Mixture (n=3)		Eggs (n=10)	
	Control	OMEGA-3	Control	OMEGA-3
	g/100 g of total fatty acids		mg/100 g egg ¹	
Σ SFA	20.32±0.20	16.38±0.07*	2432.21±79.33	2184.33±44.30*
Σ MUFA	28.47±0.08*	34.20±0.05	2929.57±143.06	2911.51±75.23
Σ n-6 PUFA	46.72±0.15	38.45±0.06*	1442.37±133.95	1343.50±57.56
LA	39.22±1.20	35.65±0.62*	1340.32±131.25	1243.27±57.73
AA	0±0.00*	0.10±0.02	39.84±4.88	42.59±3.25
Σ n-3 PUFA	4.50±0.07*	10.97±0.07	182.14±12.65*	510.35±16.25
ALA	4.09±0.06*	8.33±0.05	81.96±10.19*	329.78±14.30
EPA	0±0.00*	0.88±0.03	4.20±1.07*	13.94±2.17
DHA	0.40±0.07*	1.59±0.06	94.57±9.13*	165.24±12.63
Σ n-6 / Σ n-3 PUFA				

Results are presented as mean ± SD.

n- number of analysis; SD- standard deviation;

Σ SFA- saturated fatty acids (C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C23:0);

Σ MUFA- monounsaturated fatty acids (C14:1, C16:1, C18:1n9t, C18:1n9c, C20:1n9, C22:1n9);

Σ n-6 PUFA- polyunsaturated fatty acids (C18:2n6c, C18:3n6, C20:3n6, C20:4n6, C22:2n6); LA- linoleic acid (C18:2n6c); AA- arachidonic acid (C20:4n6);

Σ n-3 PUFA- polyunsaturated fatty acids (C18:3n3, C20:3n3, C20:5n3, C22:6n3);

ALA- alpha linolenic acid (C18:3n3); EPA- eicosapentaenoic acid (C20:5n3); DHA- docosahexaenoic acid (C22:6n3);

¹edible part; *P<0.05 Control vs. OMEGA-3

Table 3. Arterial blood pressure of study population

group	OMEGA-3		Control	
	before	after 3 weeks	before	after 3 weeks
n	20		16	
SBP, mmHg	118±8	111±8*	120±10	122±8
DBP, mmHg	70±8	67±7*	73±7	75±5
MAP, mmHg	86±7	83±7*	89±8	91±5

Results are presented as mean±SD.
n - number of participants; SBP - systolic blood pressure; DBP - diastolic blood pressure; MAP - mean arterial pressure

* P<0.05 OMEGA-3 before vs. after 3 weeks

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Table 4. Fasting biochemical parameters of study population

group	OMEGA-3		Control	
	before	after 3 weeks	before	after 3 weeks
n	20		16	
cholesterol , mmol/L	4.6±0.8	4.6±0.7	4.2±0.6	4.2±0.7
triglycerides , mmol/L	1.2±0.5	1.0±0.5*	1.0±0.5	0.9±0.3
HDL cholesterol , mmol/L	1.6±0.4	1.6±0.4	1.3±0.2	1.3±0.3
LDL cholesterol , mmol/L	2.7±0.6	2.7±0.5	2.6±0.5	2.5±0.5
fBG , mmol/L	5.4±0.9	5.1±0.7	5.0±0.6	5.2±0.5
hsCRP , mg/L	1.1±1.4	0.8±0.9*	1.8±2.6	1.9±2.9

Results are presented as mean±SD.

n - number of participants; fBG - fasting blood glucose; hsCRP – high-sensitivity C-reactive protein.

* P<0.05 OMEGA-3 before vs. after 3 weeks

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644 **Figure captions**

645 **Figure 1. The effect of consumption of omega-3 PUFAs enriched (1A) and standard hen**
646 **eggs (1B) (three eggs per day for three weeks) on post-occlusive reactive hyperemia**
647 **(PORH) of skin microcirculation in young healthy individuals.** Consumption of omega-3
648 PUFAs enriched hen eggs increased skin microvascular PORH in young healthy individuals
649 when compared to PORH measurement before the study protocol (1A). Consumption of standard
650 hen eggs did not induce any significant change in skin microvascular PORH when compared to
651 PORH measurement before the study protocol in young healthy individuals (1B). Results are
652 presented as mean $\pm$ standard deviation (SD). R-O%- change of microvascular blood flow
653 between reperfusion and occlusion (in relation to baseline). * $P < 0.05$ before vs. after 3 weeks.

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Figure 1

259x354mm (300 x 300 DPI)