UTJECAJ KONZUMACIJE KOKOŠJIH JAJA OBOGAĆENIH OMEGA-3 MASNIM KISELINAMA NA MIKROVASKULARNU REAKTIVNOST I SUSTAVNU UPALU KOD ZDRAVIH MLADIH LJUDI – RNDOMIZIRANA KONTROLIRANA STUDIJA

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Nikolina Kolobarić

INFLUENCE OF OMEGA-3 ENRICHED HEN EGGS CONSUMMATION ON MICROVASCULAR REACTIVITY AND SYSTEMIC INFLAMMATION IN HEALTHY YOUNG PEOPLE – RANDOMIZED CONTROLLED STUDY

Doctoral dissertation

Osijek, 2022.

SVEUČILIŠTE JOSIPA JURJA STROSSMAYERA U OSIJEKU
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Nikolina Kolobarić

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Zahvale

Izrada ove disertacije ne bi bila moguća bez pružene prilike i ukazanog povjerenja od strane predsjednice Zavoda za fiziologiju i imunologiju te voditeljice projekta u okviru kojeg je studija rađena, prof. dr.sc. Ines Drenjančević, ujedno i ko-mentorice rada. Zbog toga sam neizmjerno zahvalna i mogu se samo nadati da sam dano povjerenje i opravdala.

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LIST OF ABBREVATIONS

24HR – 24-hour recall

AA – arachidonic acid

Ach – acetyl choline

AChIR – acetylcholine induced relaxation

ALA – α-linolenic acid

Ang II – angiotensin II

APC – antigen presenting cell

AUC – area under the curve

BH2 – 7,8-dihydrobiopterin

BH4 – tetrahydrobiopterin

BMI – body mass index

BP – blood pressure

COX – cyclooxygenase

CV – cardiovascular

CVD – cardiovascular disease

DA – dietary assessment

DBP – diastolic blood pressure

DHA – docosahexaenoic acid

DMSO – dimethyl sulfoxide

ECW – extracellular water

ED – endothelial dysfunction

EDTA - ethylenediaminetetraacetic acid

eNOS – endothelial nitric oxide synthase

EPA – eicosapentaenoic acid

EPC – endothelial progenitor cell

ET-1 – endothelin-1

FADS1 – fatty acid desaturase 1

FADS2 – fatty acid desaturase 2

FBS – fetal bovine serum

FFM – fat free mass

FFQ – food frequency questionnaire

FMO – fluorescence minus one

FVD – fixable viability dye

HDL-cholesterol - high-density lipoprotein cholesterol

HR – heart rate

hsCRP – high sensitivity C-reactive protein

ICAM-1 – intercellular adhesion molecule 1

ICW – intracellular water

IF – interferon

IL – interleukin

iNOS – inducible nitric oxide synthase

IVD – in vitro diagnostics

LA – linoleic acid

LDF – laser Doppler flowmetry

LDL-cholesterol – low-density lipoprotein cholesterol

LOX – lipoxygenase

LT – leukotriene

MAP – mean arterial pressure

MaR – maresin

MCH – mean corpuscular haemoglobin

MCHC – mean corpuscular haemoglobin volume

MCV – mean corpuscular volume

MD – Mediterranean diet

MDA – malondialdehyde

MPV – mean platelet volume

N – number of participants

NO – nitric oxide

PBMC – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PD – protectin

PF – plasma fluid

PG – prostaglandin

PGI_2 – prostacyclin

PUFA – polyunsaturated fatty acid

RCF – relative centrifugal force

RDW-CV – red cell distribution width

ROS – reactive oxygen species

RPMI - Roswell Park Memorial Institute Medium

RT - room temperature

Rv – resolvin

SBP – systolic blood pressure

SD – standard deviation

SDF-1α – stromal cell-derived factor 1

sLEx – Sialyl Lewisx

SNP – sodium nitroprusside

SPMs – specialized pro-resolving mediators

TAC – total antioxidant capacity

TBW – total body water

Th17 – T helper lymphocyte

TLR4 – toll-like receptor 4

Treg – T regulatory lymphocyte

VCAM-1 – vascular cell adhesion molecule 1

VEGF – vascular endothelial growth factor

W/M – women/men

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1. INTRODUCTION

1.1. Polyunsaturated fatty acids (PUFAs)

1.1.1. Chemical structure and classification

Fatty acids are building components of lipids in living organisms composed of a long carbon chain with carboxylic acid head and methyl terminal group (1–3). Depending on the saturation degree of their chain, fatty acids can be classified as saturated (single carbon bonds) or unsaturated (double carbon bonds) (2). While saturated fatty acids are deemed as "bad", mono- (one double carbon bond) and polyunsaturated (two or more double carbon bonds) fatty acids are considered crucial for normal organism functioning (1,3). Depending on the location of the first double carbon bond, polyunsaturated fatty acids (PUFAs) can be divided into *n-3* and *n-6* PUFAs, nutritionally the most significant ones (4–6). These PUFAs are synthetized from essential alpha-linolenic (ALA) and linoleic acid (LA) precursors, which originate from dietary intake since the human body is unable to produce them due to a lack of necessary enzymes - delta-15 desaturase (Figure 1.1.) (1–3,5–7). The main dietary sources of PUFAs are fish and other marine sources, nuts, flaxseed oil, soybean oil, and canola oil (1,3,5,8,9).

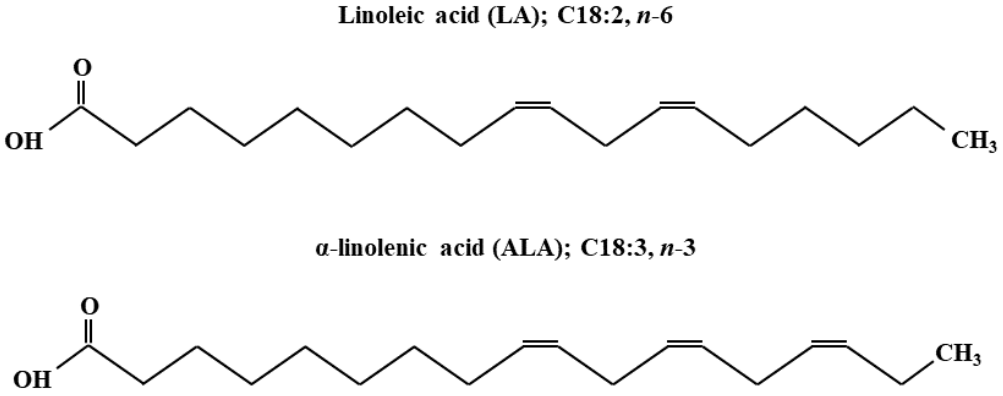


Figure 1.1. Chemical structures of *n-6* and *n-3* polyunsaturated fatty acids (PUFAs) - linoleic acid (LA; C18:2) and alpha-linolenic acid (ALA; 18:3) (source: made by the author of the dissertation).

1.1.2. Metabolic pathways

Regardless of inability to produce them, humans and animals possess the ability to metabolize ingested essential fatty acids (LA and ALA) into long-chain derivatives through actions of elongase and desaturase enzymes, with the latter being rate-limiting for PUFA metabolism (delta-5 and delta-6 desaturases) (Figure 1.2.) (2,5). Overproduction of one fatty acids path will

limit the production of the other (2,5). Such mutual limitation is the result of a competitive relationship between *n-3* and *n-6* PUFAs because the same enzyme - delta-6 desaturase, also known as fatty acid desaturase 2 (FADS2) - catalyses the conversion of ALA and LA in their long-chain metabolites - eicosapentaenoic acid (EPA) and arachidonic acid (AA), respectively (5,7,8,10). Long-chain products of *n-3* and *n-6* PUFA pathways have opposite physiological functions and are notably different, functionally and metabolically (5,7). LA dietary intake is generally higher than ALA intake since modern, Western diets are prevalently richer in *n-6* PUFAs, which leads to increased levels of these derivatives in plasma and cells (9,10).

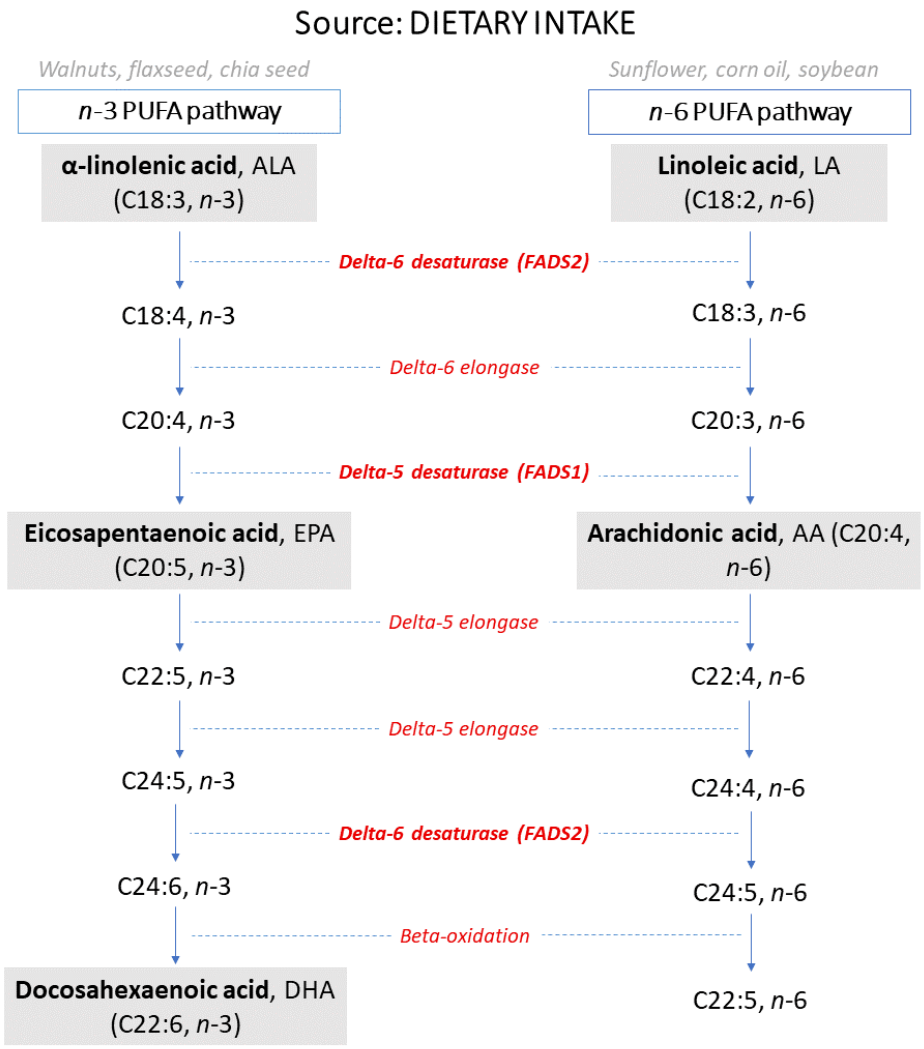


Figure 1.2. Metabolism of long-chain polyunsaturated fatty acids (PUFA). Schematic presentation of PUFAs synthesis from essential fatty acid precursors, alpha-linolenic acid (ALA; *n-3* PUFA pathway) and linoleic acid (LA; *n-6* PUFA pathway) through enzyme derived desaturation and elongation processes. PUFA – polyunsaturated fatty acid; FADS2 – fatty acid desaturase 2; FADS1 – fatty acid desaturase 1 (source: made by the author of the dissertation).

1.1.3. Health benefits

For the last couple of decades, there has been growing clinical end epidemiological evidence on immunomodulatory and cardioprotective effects, dietary intake of *n-3* polyunsaturated fatty acids (PUFAs) has in terms of chronic, inflammation-derived disorders such as asthma, diabetes, cardiovascular (CV) and neurodegenerative diseases (11–14).

Such beneficial impacts of *n-3* PUFAs supplementation, confirmed in several randomised control trials, were observed in *CVD (cardiovascular diesease) patients* manifested as increased myokine irisin, decreased LDL-cholesterol and hsCRP serum levels, elevated gene expression of sirtuins, decreased toll-like receptor 4 (TLR4) expression and blood pressure (15–19); *pregnant women* manifested as a beneficial effect on plasma total antioxidant capacity (TAC), malondialdehyde (MDA), nitric oxide (NO) levels and tissue inflammation (11,20); *neurodegenerative disorders* as a prevention measure especially concerning Alzheimer's disease risk (21,22); *autoimmune diseases* (rheumatoid arthritis, lupus diabetes, multiple sclerosis, inflammatory bowel diseases) manifested as significantly decreased inflammation through alleviated disease symptoms and discomfort, decreased levels of pro-inflammatory markers and improved clinical outcomes (23–28). In animal models (rats, rabbits, mice), previously reported effects of *n-3* PUFAs supplementation included decreased levels of pro-inflammatory, and increased levels of anti-inflammatory cytokines, reduced oxidative stress and enhanced antioxidant capacity, reduced infarct size (29–34).

Several articles addressed potentially harmful effects of long-term supplementation with PUFAs, such as: lipid peroxidation when exposed to oxidative stress (35,36), changes in HDL/LDL-cholesterol ratio (37–39), impaired hemostasis (40,41), decline of glycemic control (42,43) and effects on liver function through increased activity of liver enzymes (44–46). These claims were largely rejected due to later reports that proved no clinical significance. Such adverse effects were mainly associated with treatment in diabetic or hypertensive patients and included high daily intake of PUFAs (2-5g/daily) and were associated with increased intake of *n-6* PUFAs (44). Furthemore, the US Food and Drug Administration (FDA) concluded that *n-3* PUFAs supplementation is safe as long as its intake doesn't exceed 2 g/per day (47,48).

1.2. Functional Food

Lately, efforts are being made in the direction of creating novel approaches for improvement of the quality of life like metabolic diet typing and functional foods. Functional foods are

considered to be somewhat enhanced foods that exert certain therapeutic benefits for human health due to enrichment with bioactive components, although the a complete definition is still not fully explained and/or accepted as well as there is no legal or regulatory status assigned (47,49–51). So-called nutraceuticals, from "nutrition" and "pharmaceutical", are indistinguishable from conventional foods but suggest a preventive and therapeutic role (49,52,53). Such concept has gained popularity over the last decade in individuals anxious to diminish consequences of aging and unhealthy lifestyle, disregarding the genetic factors and overall dietary habits that can significantly alter the bioavailability of specific nutrients (51). *n-3* PUFAs enriched foods have shown a favourable effect on health through improvement of microvascular activity, anti-inflammatory and antioxidant status, lipid metabolism and therefore can potentially serve as a sufficient tool for protection from future inflammation-derived and chronic diseases (4,48,52,54–57).

Nevertheless, it should be clarified that the term functional foods can still be viewed as a marketing tool for stating health claims such as maintenance of well-being and reduction of health-related risks (58). Considering the limitations of the existing evidence base, in terms of short duration of clinical trials and the use of surrogate outcomes, these studies need to be interpreted with caution. High-quality clinical trials, with long-follow up and with hard endpoints are needed to prove beneficial effects of functional foods as interventions for prevention of treatment of various conditions (59,60).

1.3. Inflammation and inflammatory response

Inflammation is a complex biological response of the immune system characterized by the accumulation of plasma proteins and leukocytes following harmful events such as infection and/or cell damage that can potentially lead to disease (61,62). The process itself consists of several steps, including recruitment of cells, inflammatory mediator release, and permeability changes which minimize the damage and restore homeostasis in tissues and organs through mitigation processes (62). The acute inflammatory response is originally a result of the innate immune system, while it also tends to be intensified by a locally acquired immune response. Chronic inflammation, on the other hand, is described as long-lasting inflammation that can be a result of untreated acute inflammation, unsuccessful elimination of pathogens, or various autoimmune disorders (63,64). Failure of the immune system to mitigate and efficiently resolve inflammation to achieve balance consequently leads to the development of chronic diseases such as CVD, arthritis, asthma, psoriasis, and cancer (65).

1.3.1. T lymphocytes and cytokine microenvironment

T lymphocytes originate from haematopoietic cells (bone marrow) and are described as small cells containing a large nucleus with a central role in both innate and adaptive immunity (66,67). They manage cell immunity through activation of phagocytes and promotion of destruction of pathogens/infected cells while also producing cytokines and activating other immune cells (61,66). Once naïve T cells encounter a specific antigen they can interact with antigen-presenting cells (APCs), subsequently differentiate into particular lineages depending on the surface markers, effector cytokines, transcription factors and interact with host cells as effector T cells ($CD4^+$, $CD8^+$, Treg cells) (66,68).

Cytokines are small protein or glycoprotein molecules secreted by various cells such as macrophages, dendritic cells, mastocytes, and others with predominant producers being T cells, acting as mediators in inflammation and immunological reactions, influencing both innate and adaptive immune systems (61,69,70). They can act synergistically or antagonistically, have autocrine, paracrine, or endocrine activity, and while several cells can secrete the same type, a single cytokine can also have a pleiotropic effect on different cell types (71). Depending on their action and the cells in which they were made, several types of cytokines can be recognized such as chemokines, interleukins (IL), growth factors, interferons (IF), and others (70,71). Cytokines act as signalling molecules and humoral regulators of activity, differentiation, proliferation and production of cells, and other cytokines (69,70).

1.3.2. T helper and T regulatory lymphocytes

Based on the cytokine presence in the microenvironment, two main classes of $CD4^+$ effector T lymphocytes are differentiated from naïve T cells: T helper 17 lymphocytes (Th17) and T regulatory lymphocytes (Tregs) (66,68,72). Both Th17 and Tregs are heavily dependent on the presence of TGF-β1 cytokine, which promotes differentiation of both lineages depending on other cytokines, e.g. when accompanied by IL-6 or IL-21, naive cells differentiate into Th17 cells while in the absence of said cytokines it promotes Tregs differentiation (73). These effector T cell populations are further characterized by their specific functions and unique, lineage-specific cytokine secretion (74,75). The interplay between pro-inflammatory Th17 and immunosuppressive Tregs has been in the centre focus of research since their ratio is of crucial importance for homeostasis and overall immunity, with one being the stimulatory participant of generating inflammatory environment and the latter being a suppressor of further pathological processes – activation, proliferation and effector function (66,67).

1.3.3. PUFAs role in inflammatory response through lipid mediator biosynthesis

Process of inflammation tackling and resolving also demands the co-operation of several lipid mediators which originate from essential *n-3* and *n-6* PUFAs (76). Pro-inflammatory prostaglandins (PG) and leukotrienes (LT) are derivatives of long-chain *n-6* PUFA Arachidonic acid (AA), while minimally inflammatory eicosanoids (PG, LT) and inflammation resolving resolvins (Rv), protectins (PD), and maresins (MaR) originate from long-chain *n-3* PUFAs, Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA).

Biosynthesis of these mediators depends on which long-chain PUFA is used as the enzyme substrate for cyclooxygenases (COX) and lipoxygenases (LOX) as well as on inflammatory stimuli present (54,77–79). AA derivatives play a crucial role in triggering and maintaining the inflammation, while EPA and DHA derivatives have a role in terminating the inflammatory response and blocking further cell recruitment while promoting phagocytosis (76,80). Biosynthesis of eicosanoids and specialized pro-resolving mediators is shown in Figure 1.3.

Because of their direct involvement in inflammation and its resolution, the intake ratio between these potent *n-3* and *n-6* PUFAs is of great importance for homeostasis and overall wellbeing (81). The balance between pro- and anti-inflammatory derivatives that are synthetized from fatty acid precursors and their ratio take part in the pathogenesis of a variety of chronic diseases including CVD and cancer (81–84).

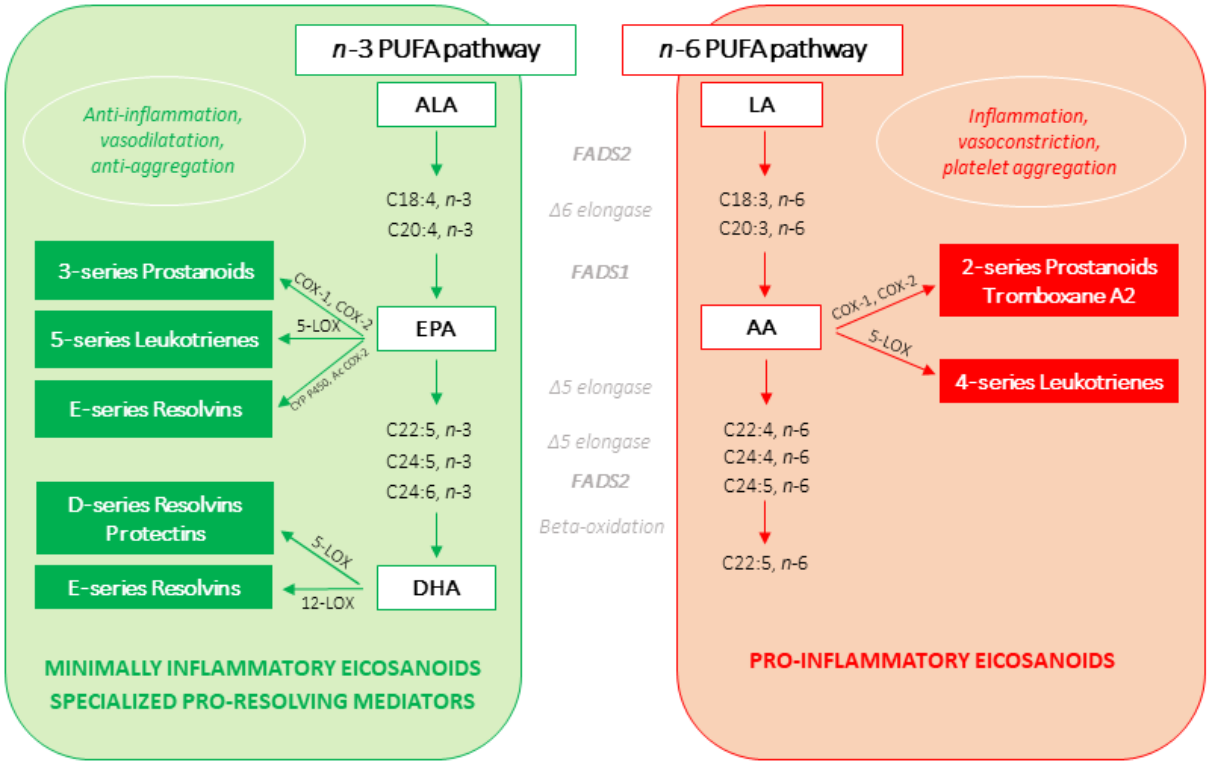


Figure 1.3. Eicosanoid and specialized pro-resolving mediator biosynthesis. Biosynthesis of lipid mediators depends on which long-chain polyunsaturated fatty acid (PUFA) is used as the enzyme substrate for cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. These enzymes catalyse the conversion of *n-3* (EPA, DHA) or *n-6* (AA) PUFAs, depending on which substrate is present in excess, to minimally inflammatory eicosanoids and specialized pro-resolving mediators (SPMs) or pro-inflammatory eicosanoids, respectively (source: made by the author of the dissertation).

1.4. Endothelium and its functional impairment

The endothelium is a metabolically active inner layer of cells that line the inside of the heart and lumen of blood vessels ,and serves as a highly selective barrier between the vascular wall and circulating blood (85). Its complex role involves operating as an active endocrine, paracrine, and autocrine organ necessary for the regulation and preservation of vascular homeostasis (85–87). This is enabled by achieving a balance between the production of vasoconstrictors – angiotensin II (Ang II), endothelin-1 (ET-1), reactive oxygen species (ROS), and vasodilators - nitric oxide (NO), prostacyclin (PGI_2) (87–89). Other than regulating vascular tone, blood flow and coagulation, endothelial cells also have a capacity to produce and secrete various cytokines, chemokines, growth factors, and adhesion molecules, which directly involves them in inflammatory response (88,90–92).

Endothelial dysfunction (ED), which is characterized by a loss of anti-inflammatory and vasodilator properties of the endothelium, is considered as one of the first occurrence in the development of CVDs (88,92). Basically, in an environment affected by increased risk factors (obesity, smoking, physical inactivity), endothelium loses its normal regulatory functions and initiates inflammatory processes accompanied by an increase in vasoconstrictor products (88,91). As a result of this functional impairment, various CVDs develop such as hypertension, atherosclerosis, stroke, obesity, diabetes, thrombosis, and other (91). According to Poredos et al. (93), ED is reversible when risk factors are eliminated and life habits are changed which means that it is possible to reverse vascular function. Endothelial dysfunction (ED), which is characterized by a loss of anti-inflammatory and vasodilator properties of the endothelium, is considered as one of the first occurrence in the development of CVDs (88,92). Basically, in an environment affected by increased risk factors (obesity, smoking, physical inactivity), endothelium loses its normal regulatory functions and initiates inflammatory processes accompanied by an increase in vasoconstrictor products (88,91). As a result of this functional impairment, various CVDs develop such as hypertension, atherosclerosis, stroke, obesity, diabetes, thrombosis, and other (91). According to Poredos et al. (93), ED is reversible when risk factors are eliminated and life habits are changed which means that it is possible to reverse vascular function.

1.4.1. PUFAs effect on endothelial function

PUFAs, precisely *n-3* PUFAs, have the potential to prevent the development and progression of a variety of CVD, including atherosclerosis-related diseases, partly by improving endothelial function as demonstrated in various epidemiological and randomised clinical trials (14,94–98). Some of these vasculo-protective properties include decreased blood pressure (BP) confirmed by the meta-analysis of seventy randomised controlled trials (99,100), decreased formation of atherosclerotic lesions in mice (101), increased anti-inflammatory properties (102), improved endothelium-dependent vasodilation of conduit arteries, and increased antioxidant capacity (103).

Although the mechanisms of endothelial function modulation by *n-3* PUFAs are under-investigated, there are several possible targets: by increasing endothelium NO production (Figure 1.4.a), by reducing reactive oxygen species (ROS) formation or increasing their elimination (104) (Figure 1.4.b), by decreasing vascular or systemic inflammation (Figure 1.4.c) or inducing angiogenesis/neovascularization and mobilization of bone-marrow-derived

endothelial progenitor cells (EPCs) (105) (Figure 1.4.d). *n-3* PUFAs may increase NO either by activation of inducible nitric oxide synthase (iNOS) or endothelial nitric oxide synthase (eNOS) (2,106,107). Although the mechanisms of endothelial function modulation by *n-3* PUFAs are under-investigated, there are several possible targets (summarized in Figure 1.4.): by increasing endothelium NO production (Figure 1.4.a) by reducing reactive oxygen species (ROS) formation or increasing their elimination (104) (Figure 1.4.b), and by decreasing vascular or systemic inflammation (Figure 1.4.c) or inducing angiogenesis/neovascularization and mobilization of bone-marrow-derived endothelial progenitor cells (EPCs) (105) (Figure 1.4.d). *n-3* PUFAs may increase NO either by activation of inducible nitric oxide synthase (iNOS) or endothelial nitric oxide synthase (eNOS) (2,106,107). Moreover, in addition to NO, *n-3* PUFAs can also increase the activity and/or expression of other endothelium-derived vasodilators (108) (Figure 1.4.e). Lastly, endothelial dysfunction is also characterized by endothelial activation and inflammation, which may be attenuated by *n-3* PUFAs anti-inflammatory potential (109–112) (Figure 1.4.c), as suggested by studies in cell culture and animal studies. However, these potential mechanisms underlying the improvement of endothelial dysfunction by *n-3* PUFAs supplementation in the form of functional food have not been fully investigated especially in healthy individuals.

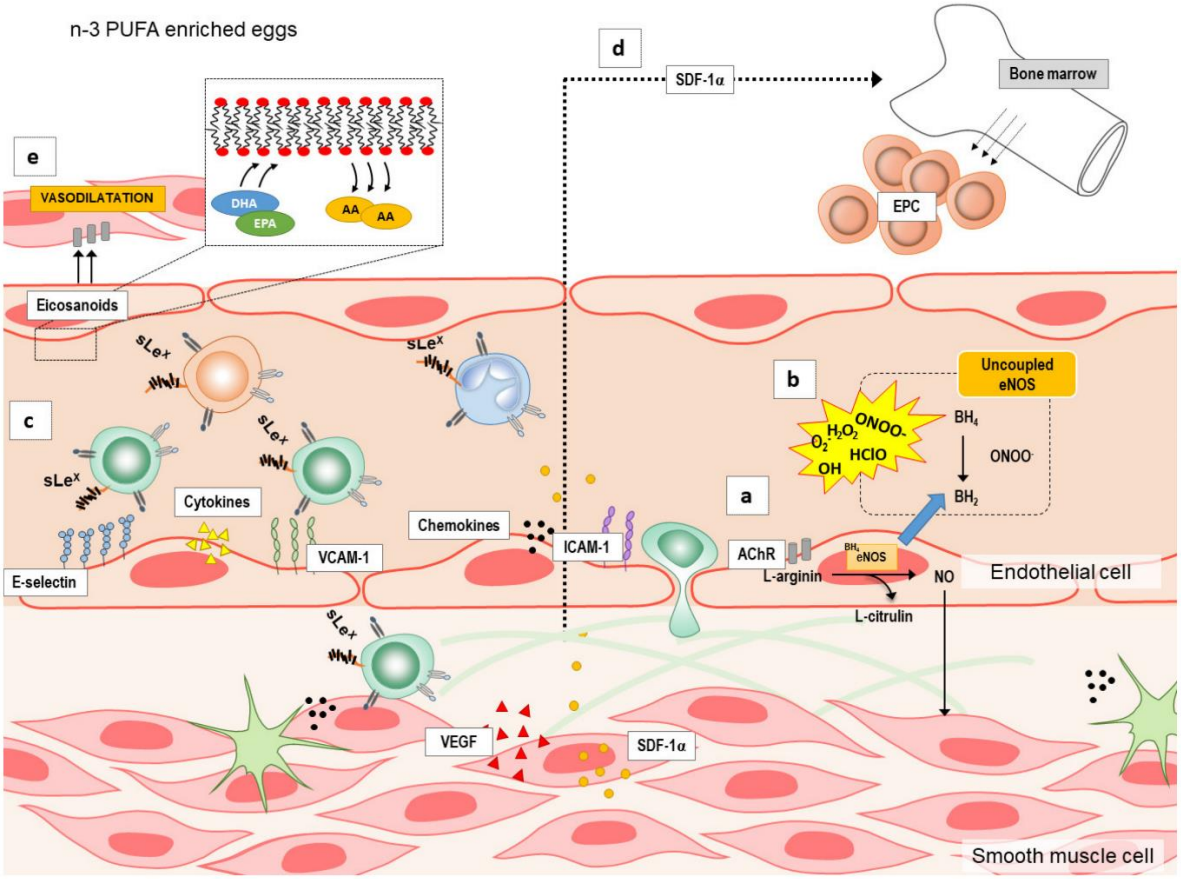


Figure 1.4. The summary of the potential mechanisms by which *n-3* polyunsaturated fatty acids (*n-3* PUFAs) supplementation may modulate endothelial function. Several possible targets by which *n-3* PUFAs supplementation modulate endothelial function are: (a) increasing production of endothelium nitric oxide (NO), (b) reducing formation or increasing elimination of reactive oxygen species (ROS), (c) decreasing vascular or systemic inflammation (e.g., endothelial activation and endothelium–leukocyte interaction), (d) inducing angiogenesis/neovascularization and mobilization of bone-marrow derived endothelial progenitor cells (EPCs), and (e) increasing the expression and/or activity of other endothelium-derived vasodilators (e.g., eicosanoids). A—arachidonic acid; AChIR—acetylcholine-induced relaxation; BH4—tetrahydrobiopterin; BH2—7,8-dihydrobiopterin; DHA—docosahexaenoic acid; eNOS—endothelial nitric oxide synthase; EPA—eicosapentaenoic acid; ICAM—1-intercellular adhesion molecule 1; *n-3* PUFAs—*n-3* polyunsaturated fatty acids; SDF-1α—stromal cell-derived factor 1; sLEx—Sialyl Lewisx; VCAM-1—vascular cell adhesion molecule 1; VEGF—vascular endothelial growth factor (source: obtained from (113) with permission of the author).

1.5. Dietary habits

While optimal nutrition contributes to health, wellbeing, and normal development (114), an unbalanced, nutrient-poor diet accompanied by a sedentary lifestyle and harmful habits, consequently, leads to a greater risk of developing a variety of chronic diseases such as diabetes, CVDs, and cancer (115–117). Higher 'body mass index (BMI) ($\geq$ 25 kg/m^2) and weight problems in youth, usually linked to poor diet, are likely to cause hypertension and other cardiovascular problems together with reduced quality of life in the future (118–120). Further, according to Lupi et al. (2015) (121), there is also a visible change in student/young adults' dietary habits present when living away from family.

Considering the geographical location of Croatia, there are several types of local cuisines present alongside, Turkish (pastries, bread, and coffee), Hungarian (meat stews), Mediterranean (olive oil, fish, nuts), and Slavic (lard, dried meat) influences depending on the region (122). Mediterranean diet (MD), characterized by consumption of whole grains, vegetables and fruits, olive oil, nuts, fish, and low amounts of local dairy products/red meat, is naturally rich in *n-3* PUFAs and therefore has been claimed as a potential tool in the prevention of developing chronic diseases (123–128), although it is necessary to conduct larger number of clinical trials in the future due to paucity of quality evidence (129). Positive influences of MD include cardiovascular protection, metabolic and cognitive benefits, and protection against stroke, obesity, diabetes, cancer, and allergies (127,130–133). On the other hand, consumption of deep-fried/roasted foods, alongside a higher intake of saturated fatty acids and smoked products of

animal origin is typical for our geographical area (134). Jelinić et al. (2009) (122) recorded the highest prevalence of unhealthy dietary habits among the general population (representative sample) in Eastern Croatia. Further, young adults showcase certain specificities in their dietary habits such as irregular meals and higher energy intake, with women showcasing an overall better diet quality than men (135).

Accurate dietary assessment (DA) is essential for gathering data on food consumption, dietary habits, and nutritional status at the population and individual level (136). Alongside measurements of anthropometric and biochemical parameters and clinical examination, DA is a necessary tool in the prediction of chronic disease risk and overall health promotion (115,116,116,137). Food Frequency Questionnaires (FFQ) and 24-h recalls (24HR) are the most frequently used methods in dietary and nutrition research (138,139).

2. HYPOTHESIS

A diet that includes a short-term intake of functional foods enriched with omega-3 fatty acids leads to the alleviation of systemic inflammation through reduction of pro-inflammatory and increase of anti-inflammatory markers followed by improvement of microvascular reactivity.

3. SPECIFIC RESEARCH OBJECTIVES

In the population of young and healthy participants to determine the effect of the three-week consummation of hen eggs enriched with *n-3* PUFAs, in terms of difference between baseline and end-point values, on:

1) Prevalence of pro-inflammatory Th17 and anti-inflammatory T regulatory lymphocytes, cytokine profile of peripheral mononuclear cells (PBMCs) and levels of serum inflammatory parameters;

2) Concentration of anti-inflammatory and pro-inflammatory metabolites of the enzymatic pathway of lipoxygenase and cyclooxygenase;

3) Microvascular reactivity in the skin and endothelium-dependent or endothelium-independent mechanisms of vasodilation.

4. MATERIALS AND METHODS

4.1. Study design and participants

Trial design

This was a randomized, placebo-controlled, double-blind study (part of the clinical trial registered as ID NCT02720250 Omega-3 Fatty Acids Enriched Food and Microvascular Reactivity).

Eligibility criteria for participants

Young, healthy adults of both sexes participated in this study. Exclusion criteria for participants were: smoking, prior history of hypertension, renal or cerebrovascular impairments, coronary artery disease, diabetes mellitus and chronic inflammatory disorders. Volunteers who met the eligibility criteria were included in the study by the researcher and were awarded a label indicating the project, group of respondents ("healthy") and ordinal number.

Setting and location

The research was carried out at the Department of Physiology and Immunology, Faculty of Medicine in Osijek, Croatia.

Interventions

The study protocol lasted for three weeks, and included two appointments. All study participants were instructed to eat three hard-boiled hen eggs (medium commercial size) per day for the duration of the protocol (three weeks). Participants were split into two groups: Control group (N = 21; W/M = 10/11) consumed regular hen eggs (*n-3* PUFAs content ~249 mg/per day), while *n-3* PUFAs group (N = 19; W/M = 10/9) consumed *n-3* PUFAs enriched hen eggs (*n-3* PUFAs content ~1053 mg/per day) (Figure 4.1.).

Enrichment of hen eggs was executed by the associate research group from the Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek by re-placing soybean oil (5%) with the mixture of fish (1.5%) and linseed (3.5%) oil in feed mixtures fed to laying hens. Each enriched egg (edible part ~60 g) contained approximately 351 mg of *n-3* PUFA in total (~230 mg of ALA, ~15 mg of EPA, ~105 mg of DHA). Participants consumed a total of 63 hard-boiled (boiled for 10 minutes) eggs during the study.

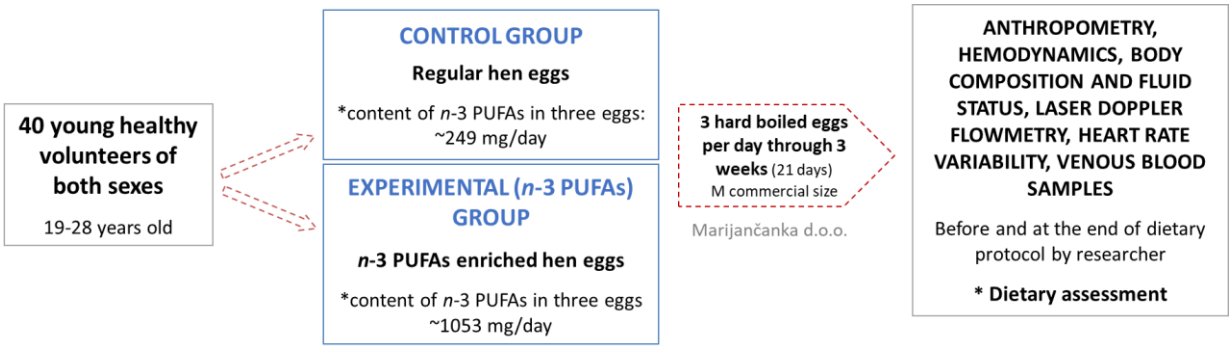


Figure 4.1. Schematic presentation of study design and groups of participants involved as well as methods that are being implemented (source: made by the author of the dissertation).

Study participants kept a diet diary in the form of 24 hour recalls (Annex 1) during study protocol for the purpose of tracking food and beverage intake. They were instructed to follow their usual meal schedule and to avoid taking any supplements that could alter final results, especially other *n-3* PUFA supplements. At each appointment, the blood samples were taken for peripheral blood mononuclear cell isolation and serum collection. Between appointments, participants were contacted several times ($\geq 3\times$) via E-mail and/or phone call (at least once a week) to assure compliance to study protocol.

Outcomes

The primary outcome of the study included a change in the frequency of T lymphocytes–Tregs and Th17–after the dietary protocol, while secondary outcomes included quantification of serum levels of lipid mediators originating from *n-6* (LTB4, PGE2) and *n-3* fatty acids (LTB5, PGE3, RvE1), cytokine secretion by PBMCs following PMA–ionomycin activation, levels of serum inflammatory parameters and microvascular reactivity in the skin including endothelium-dependent or endothelium-independent mechanisms of vasodilation. Preliminary data were gathered from a total of 10 respondents after the same dietary protocols as described above (N(Control) = 5; N (*n-3* PUFA) = 5), considering the primary outcome. The same simple randomisation procedure was performed and given the results obtained, effect and sample size were calculated before the recruitment of participants for the main study.

Sample size

During optimisation of the protocol and design of the research, a pilot study was conducted with a total of 10 respondents. Effect size (Cohen d, Δ/SD) required for a statistical strength of 80% with bilateral $\alpha = 0.05$, paired t-test, before and after the dietary protocol is 1.1 for the Treg lymphocyte population and requires a sample of at least 13 participants per group (GPower

v3.1.9.7). For the estimated, expected arithmetic means of the frequency of $Foxp3^+$ Treg lymphocytes in the peripheral blood of healthy individuals before (16.8%) and after the dietary protocol (9.2%), with corresponding standard deviations of 6.1% and 7.2%, the difference the arithmetic mean is 9% which corresponds to a biologically large effect. The effect size required for a statistical strength of 80% with bilateral $\alpha = 0.05$, paired t-test, before and after the dietary protocol is 0.9 for the Th17 lymphocyte population and requires a sample of a minimum of 19 participants per group. For estimated, expected arithmetic means of the frequency of $IL\text{-}17A^+$ Th17 lymphocytes in the peripheral blood of healthy individuals before (0.72%) and after the dietary protocol (0.49%), with corresponding standard deviations of 0.29% and 0.19%, the difference of arithmetic means is 11%, which corresponds to a biologically large effect.

Randomization – sequence generation, allocation concealment mechanism and implementation

The dates of arrival of the respondents were scheduled in advance by the researcher. Eggs were divided by an unbiased associate according to the pre-arranged schedule of arrivals that did not contain personal data but previously assigned labels. The simple randomization (140) procedure was performed using a coin (letter-1 or head-2) to assign group affiliation by unbiased associate who added the number 1 or 2 in brackets to the previously mentioned label for each individual participant, after the ordinal number, depending on which group it is: 1 - the control group that consumed regular hen eggs; 2 - *n-3* PUFA group that consumed *n-3* PUFAs enriched eggs.

Blinding

Labels indicating group affiliation were known only to the associate assigning the intervention, while study participants nor the researcher knew which group they belonged to during the study. This particular associate did not participate in any of the performed analyses related to this specific study population.

Ethics

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving research study participants were approved by the Ethical Committee of the Faculty of Medicine, University of Osijek (CLASS: 602-04/21-08/07; Reg. No.:2158-61-07-21-49). No selection bias was present in our study. All study participants signed informed consent prior to the inclusion in the study, and there was no compensation provided for their participation. Fresh eggs were delivered from the farm (Marijančanka d.o.o.) to the laboratory once a week and distributed to participants entering the protocol within 7 days.

Dissertation was written according to CONSORT 2010 guidelines for reporting a randomized trial (141) (Annex 2).

4.2. Anthropometry, hemodynamics and laboratory testing

Venous blood samples were taken after the overnight fast on the first and last day of the dietary protocol. Samples were analysed for full blood cell count, iron, transferrin, creatinine, urea, plasma electrolytes (sodium, potassium, calcium), high-sensitivity C-reactive protein (hsCRP), fasting blood glucose and lipid profile (total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides) using standard laboratory methods and operating protocols at the Clinical Department of Laboratory Diagnostics, University Hospital Osijek. All analyses were performed on Olympus instrument using IVD certified reagents and protocols provided by the manufacturer. Sodium, potassium and calcium were measured by potentiometry; hsCRP, transferrin and ferritin (iron) were measured using immunoturbidimetric assays; blood cell count, haematocrit levels, red cell indices (MCV, MCH, MCHC), RDW-CV and MPV were evaluated by impedance spectroscopy; other parameters were measured by spectrophotometry. The lipid profile was measured directly.

BMI was calculated using a participant's body mass (kg) and height (m) measures obtained at each appointment by the researcher (BMI = body mass/height in m^2). BMI was calculated and categorized based on WHO criteria for European population: underweight <18.5 kg/m^2; normal weight 18.5–24.9 kg/m^2; pre-obesity 25.0–29.9 kg/m^2; obesity class I 30.0–34.9 kg/m^2; obesity class II 35.0–39.9 kg/m^2; and obesity class III > 40 kg/m^2. BP Heart rate (HR) and BP were measured at the beginning of each of the two visits, after resting for 15 minutes in a sitting position using an automated oscillometric sphygmomanometer (OMRON M3, OMRON Healthcare Inc.; Osaka, Japan). The final values of HR and BP were the mean of three repeated measurements.

4.3. Body composition and body fluid status measurements

A four-terminal portable impedance analyser (Maltron Bioscan 920-II, Maltron International Ltd.; Rayleigh, Essex, UK) was used for body composition and body fluid status. Empirically derived formulas (the original manufacturer's software) were used to calculate the estimated Fat% (Fat Mass%), Fat Free Mass% (FFM%), Intracellular Water% (ICW%), Extracellular Water% (ECW%), Total Body Water% (TBW%), Body Density (kg/L), Interstitial Fluid (IF) and Plasma Fluid (PF).

4.4. Analysis of serum fatty acids profile

Serum fatty acids profile was analyzed via gas chromatography–tandem mass spectrometry (GC–MS/MS) technique. 37 component fatty acid methyl esters (FAME MIX) stock solution were purchased as 30 mg/mL total concentration of fatty acids in methylene chloride (Supelco Inc.; Bellefonte, PA, USA), and used for the preparation of standard solutions. The sample preparation procedure from Wang et al. [68] was modified and used for analysis. For serum fatty acids profile analysis, the GC-MS/MS system by Thermo Fisher GC Trace 1300 coupled with a TSQ 9000 Triple Quadrupole was used. Serum fatty acids profile analysis was performed at the BIOCentre's Bioanalytical Laboratory, BIOCentre - incubation centre for biosciences, Zagreb, Croatia.

4.5. Peripheral blood mononuclear cells (PBMCs)

4.5.1. Isolation from whole blood

Venous blood samples were collected in 10 ml EDTA containing vacutainer tubes (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Samples were processed within three hours of collection. Refrigerated reagents and buffers used in isolation were warmed up to room temperature (~20 -25°C) (RT).

Approximately 16 mL of collected whole blood was diluted with pre-prepared 1x Phosphate-buffered saline (PBS), 1:1 ratio in 50 mL conical centrifuge tubes. Next, the diluted sample was carefully layered on ~16 mL (depending on the volume of sample) of Ficoll-Paque® PLUS centrifugation media (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) without mixing the layers – tube should be tilted under 45° angles and diluted sample carefully pipetted onto centrifugation media. Tubes with samples were centrifuged for 25 minutes at RCF 800 G with breaks off, at RT (Rotina 380, Hettich GmbH & Co. KG, Tuttlingen, Germany). After the centrifuge camecame to a complete stop, tubes were carefully removed and placed on a rack. Mononuclear cells are visible as a cloudy ring separating two layers - yellow plasma and colourless media (Figure 4.2.).

Cells were collected in 10 mL centrifuge tubes and rinsed twice with 1x PBS (10 min, RCF 800G with breaks on). The supernatant was discharged, and cells were re-suspended in 5 mL of 1x PBS. Cells were stained with 0,4% Trypan blue solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and counted manually using Bürker-Türk counting chamber in order to determine viability (50 µL of cell suspension and 100 µL of staining solution) (142).

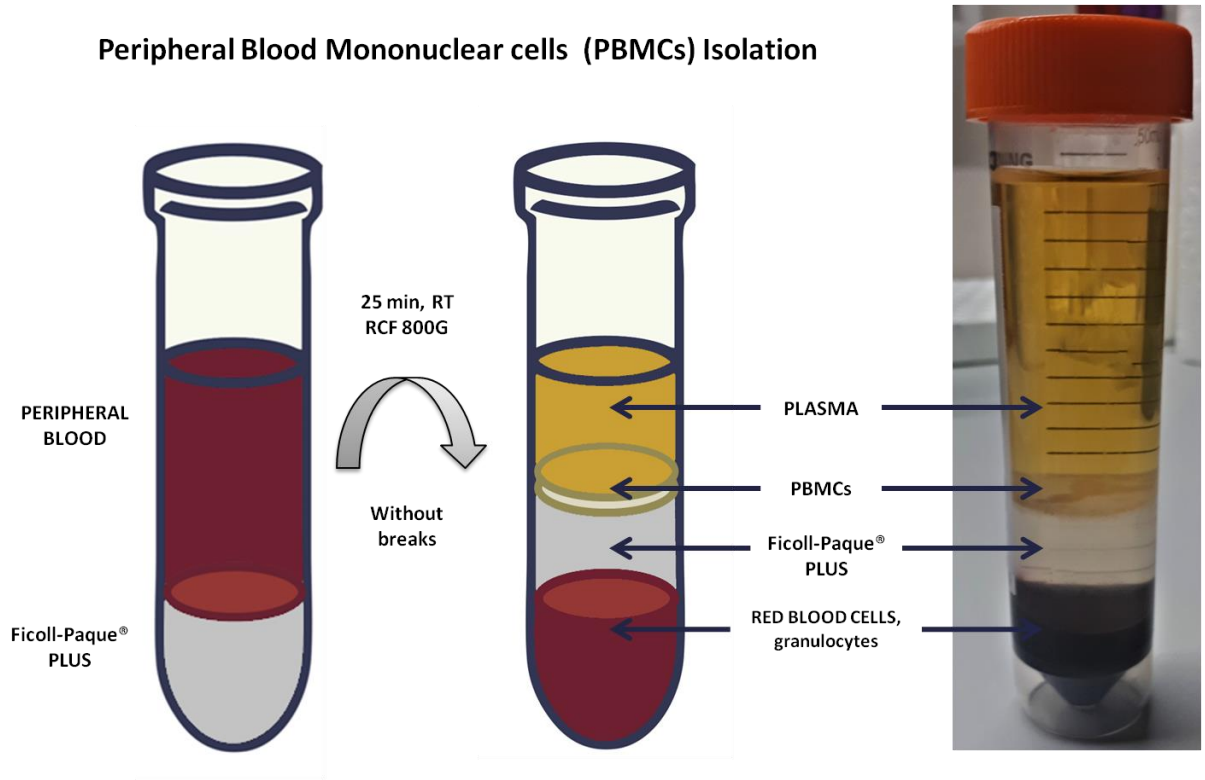


Figure 4.2. Schematic presentation of PBMCs isolation from whole blood using separation medium (source: made by the author of the dissertation).

4.5.2. Cultivation

PBMCs were cultured in RPMI-1640 media with L-glutamine (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), supplemented with the addition of Fetal Bovine Serum (10%) (FBS; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and penicillin-streptomycin antibiotic (1%) (Capricorn Scientific GmbH, Ebsdorfergrund, Germany). Cell suspensions were stored in 24-well plates and placed in an incubator (Shel Lab, CO_2 Series, Sheldon manufacturing Inc, OR, USA) under the following conditions: ~37°C, 5% CO2, >80% humidity level for 24 hours before any further proceedings.

4.5.3. Cryopreservation and thawing

For the purpose of cryopreservation Dimethyl Sulfoxide (DMSO; Supelco, Merck KGaA, Darmstadt, Germany) and FBS were used at a 1:9 ratios. Additionally, for optimal cell preservation, the cryovials were stacked in Mr. Frosty freezing container (Nalgene Labware, Thermo Fisher Scientific, Waltham, MA, USA) containing isopropyl alcohol (Gram-mol, Gram-mol d.o.o., Zagreb, Croatia) and placed in a -80°C freezer for at least 24 hours.

Thawing of samples was carried out with FBS/antibiotics supplemented RPMI-1640 culture media. The mmedia was pre-heated to ~37°C. Cryovials containing cells were carefully dipped into the water bath (~37°C) for roughly 1 minute and removed as soon as the sample detached itself from the cryovial's wall to avoid the potential harmful effects of DMSO exposure on cells. Samples were immediately transferred into pre-prepared 15 mL centrifuge tubes and 10 mL of warm medium was pipetted onto it in drip mode to avoid osmotic shock (1 mL of total medium was used for cryovial rinse). Tubes were centrifuged (5 min, 1500 RPMI, RT). After completion, the supernatant was discharged and 5 mL of medium was added. Cells were re-suspended and once again centrifuged under the same conditions. After centrifugation, the supernatant was discharged. Cells were re-suspended in 1.5 mL of fresh medium, transferred to 24-well plates and kept in an incubator for 24 hours (~37°C, 5% CO2, >80% humidity).

4.5.4. Cell viability

Analyses to identify cell viability and exclude possible bias induced by nonspecific staining of dead/dying cells in our samples, included a) staining cells with 0,4% Trypan blue solution and counting live cells in Bürker-Türk chamber under the light microscope; and b) staining cells with Fixable Viability Dye (FVD) eFluor™ 780 (eBioscience™, Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) which is detectable on a flow cytometer upon excitation with 633 nm red laser. Samples included in the final analysis and calculations had cell viability ≥80 %.

4.5.5. Magnetic cell sorting

After thawing the samples and adjusting the cell numbers to $1{,}2x10^7$, $CD4^+$ T cells were separated using negative magnetic selection utilizing a biotinylated antibody cocktail and streptavidin-coated magnetic beads via commercially available magnetic beads (MagniSortTM Human $CD4^+$ T cell, Enrichment kit; Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA), and following the protocol provided by the manufacturer (Protocol available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/8804-6811.pdf).

Samples were transferred to 5 mL polystyrene round-bottomed tubes (12 x 75 mm; BD Falcon, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), washed twice with 1x PBS (5 min, 800 G) and cells were re-suspended in 200 µL of 1x PBS/3% FBS/10 mM EDTA. After that, 40 µL of Enrichment Antibody Cocktail was added, mixed by pulse vortex (IKA® Vortex GENIUS 3) five times and left for 10 min incubation at RT. After the completion of incubation, 4 mL of 1xPBS/10mM EDTA was added and tubes were centrifuged (800 G/5 min). The

supernatant was discharged, and 200 µL of 1xPBS/3% FBS/10 mM EDTA plus 40 µL of MagniSort Negative Selection Beads (re-suspended thoroughly before adding for optimal performance) were added to samples, mixed five times by pulse vortex and left for incubation (5 min, RT).

After incubation, 1xPBS/10 mM EDTA was added up to 2.5 mL volume and cells were re-suspended. Tubes were, one at a time, inserted into the MagniSort magnet (eBioscience, Affymetrix by Thermo Fisher Scientific, San Diego, CA, USA) until the bottom of the tube touched the working surface through the hole of the magnet and incubated in RT for 5 min. After incubation, the magnet containing the tube was picked and the supernatant was poured in a new 5 mL tube in one continuous movement without shaking or tapping as this may reduce the purity of cells. Tubes containing bound cells are removed from the magnet and discharged. Negatively selected cells are ready for activation.

4.5.6. Activation of $CD4^+$ T lymphocytes

In order to activate $CD4^+$ T cells and promote cytokine production, magnetically sorted cells were shortly (4 hours) stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin. CD4 T-cell activation was carried out in 24-well plates (4 hrs, ~37°C, 5% CO2, >80% humidity level) with commercially available cell stimulation cocktail (500×; eBioscienceTM, Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) at the final concentration of 2 µL/mL (full protocol available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/00-4970.pdf).

Calcium Chloride ($CaCl_2$) was added to the stimulation media to provoke a long-lasting intracellular calcium signalling that would evoke a cellular response (5 µL/mL final concentration).

In order to prevent cytokine secretion and allow assessment of IL-17 producing cells by flow cytometry (intracellular IL-17 staining, detailed protocol given in section 4.7.7.) Brefeldin A Solution (1000×; eBioscience^{TM}, Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) was used as an inhibitor of protein transport to Golgi apparatus with resulting accumulation of proteins in the endoplasmic reticulum (3 µL/mL final concentration; available at https://assets.thermofisher.com/TFS-Assets/LSG/manuals/00-4506.pdf).
Cell stimulation cocktail, Brefeldin A and $CaCl_2$ were added together and at the same time to the cell suspension. After completion of four-hour incubation, 200 µL of 0.1 M EDTA was added and incubated for 15 min at RT in order to stop the reaction.

4.5.7. Flow cytometry

Frequencies of $CD4^+Foxp3^+$ regulatory T lymphocytes and $CD4^+IL\text{-}17A^+$ T helper lymphocytes among isolated peripheral blood mononuclear cells were determined by the flow cytometry method. Sample preparation and staining protocols for intracellular antigens for flow cytometry were modified versions of recommended protocols (available at www.thermofisher.com). For intracellular staining Foxp3 Transcription Factor staining buffer set was used (eBioscience™, Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). In short, prior to cell surface staining, fixation/permeabilization and intracellular/nuclear staining steps, dead cells were irreversibly labelled with FVD and non-specific antibody capturing by Fc receptors was blocked by the addition of Human Fc Block reagent (BD Pharmigen™, BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). After incubation at RT, staining with appropriate antibody mixture was carried out depending on the cell subset of interest. Along with careful sample preparation and optimization of staining protocols, single-stain, fluorescence minus one (FMO), unstained and negative controls were included in our experiments in order to reliably distinguish positive cells from background/negative staining and nonspecific effects.

Fluorescence compensation matrix for multicolour flow cytometry analysis was calculated using BDTM CompBeads Anti-Mouse Ig, κ/Negative Control Compensation Particle Set (BD Biosciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Measurements of stained samples were carried out by BD FACSCanto II cytometer (FACSCanto II, Becton Dickinson, San Jose, CA, USA) equipped with blue Argon 488 nm and Red HeNe 633 nm laser lines. Data analysis and visualisation were performed using the FlowLogic software (Inivai Technologies, Mentone, Australia).

Regulatory T lymphocytes (Tregs)

To assess the frequencies of Treg cells among PBMCs, the following mouse anti-human antibodies mixture for the cell surface staining was used: CD3 FITC (clone: OKT3, eBioscience™, Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA), CD4 PerCP-eFluorTM 710 (clone: SK3, eBioscienceTM), CD127 PE-Cy7 (clone: eBioRDR5, eBioscience™), CD25 APC (clone: BC96, eBioscience™); while Foxp3 PE (clone: 235A/E7, eBioscience™) antibody was used for intracellular staining of cells. The optimal antibody concentration was determined by antibody titration experiment on 1 x 10^6 PBMCs and based on stain-index calculations.

Helper T lymphocytes (Th17)

Th17 lymphocytes' rate among total peripheral blood CD4 lymphocytes were determined using PMA-ionomycin activated CD4 cells and the following mouse anti-human antibodies mixture: CD3 PerCP-eFluor™ 710 (clone: SK7, eBioscience™, Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA), CD4 PE-Cy7 (clone: SK3, eBioscience™), CD196 APC (clone: R6H1, eBioscience™) for cell surface antigens and RORγt PE (clone: AFKJS-9, eBioscience™) and IL-17A FITC (clone: eBio64DEC17, eBioscience™) for intracellular antigens.

4.6. Luminex assay

4.6.1. Collection of supernatants from PMA-Ionomycin treated PBMC cell cultures

After thawing, the PBMCs were allowed to rest and recover overnight in culture media (~37°C, 5% CO_2, >80% humidity level). Before proceeding to the next step, cells were counted and the number of cells was adjusted to 300,000 cells per 200 µL of stimulation media for each sample. Stimulation media consisted of supplemented RPMI-1640 culture media, PMA-Ionomycin and $CaCl_2$ at previously mentioned final concentrations (section 4.7.6.). PBMC activation was carried out in 96-well plates (4 hours, ~37°C, 5% CO_2, >80% humidity level). Following 4-hour incubation period, the collected supernatant was stored at -80° C until analysis.

4.6.2. Multiplex and simplex protein quantitation of pro- and anti-inflammatory cytokines, transforming growth factor and chemokine supernatant concentrations

Supernatant concentrations of pro- and anti-inflammatory cytokines, including interleukin 17A (IL-17A), interleukin 23 (IL-23), interleukin 6 (IL-6), interleukin 10 (IL-10); and transforming growth factor beta 1 (TGF-β1); and the levels of monocyte chemoattractant protein-1 (MCP-1) chemokine were measured using the Invitrogen Procarta-Plex 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 the concentration in picograms per millilitre.

4.7. Measurement of serum pro- and anti-inflammatory cytokines, chemokines, growth factors, and soluble cell adhesion molecules protein concentration

Serum protein concentration of pro-inflammatory cytokines: interferon gamma (INFγ), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 17A (IL-17A), interleukin 23 (IL-23) and interleukin 9 (IL-9); anti-inflammatory cytokines and immunomodulatory cytokines: interleukin 21 (IL-21), interleukin 22 (IL-22), interleukin 10 (IL-10); chemokines: stromal cell-derived factor 1 alpha (SDF-1α); latency associated peptide (LAP), vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor A (VEGF-A), soluble vascular cell adhesion molecule 1 (sVCAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1) were measured with Invitrogen ProcartaPlex antibody-based, magnetic bead reagent kits and panels (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) for multiplex protein quantitation using the Luminex 200 instrument platform (Luminex Corp., TX, USA). Measurements were performed in Laboratory for Immunology and Allergology Diagnostics Osijek University Hospital, Osijek, Croatia. Quantitation was done in ProcartaPlex Analyst free software (eBioscience, Affymetrix by Thermo Fisher Scientific, CA, USA) and expressed as the concentration in picograms per millilitre.

4.8. ELISA assay

Serum concentrations of Leukotriene B4 (LTB4), Leukotriene B5 (LTB5) (Cusabio, Houston, TX, USA), Prostaglandin E2 (PGE2), Prostaglandin E3 (PGE3) (MyBioSource, MyBioSource Inc., San Diego, CA, USA), Resolvin E1 (RvE1) (MyBioSource, MyBioSource Inc., San Diego, CA, USA) were measured by commercially available enzyme-linked immuno-sorbent assay (ELISA) kits on compact absorbance reader for 96-well microplates (BioRad PR 3100 TSC, Bio-Rad Laboratories, CA, USA).

4.9. Assessment of microvascular endothelium-dependent and endothelium-independent vasodilation

Laser Doppler flowmetry (LDF) (MoorVMS-LDF, Axminster, UK) was used to assess an endothelium-independent and endothelium-dependent vasodilation of forearm skin microcirculation by iontophoresis (non-invasive transdermal application of charged substances) of sodium nitroprusside (SNP) and acetylcholine (ACh), respectively. Measurements were performed at both study visits at room temperature (approx. 23.5 °C).

Data collection began after resting, lying face up for 30 minutes. The laser Doppler probe was attached to the skin of the forearm volar surface, 13–15 cm from the wrist, taking into account to place the probe in the same place at each study visit using adhesive discs (doubled sided; provided by the manufacturer). SNP and ACh were iontophoretically-administered through a drug-delivery electrode attached at the site of the LDF probe. The unstimulated basal blood perfusion was recorded for 5 min. After that, either negatively charged 1% SNP solution was applied by means of three pulses of 0.1 mA of negative current for 30 s, followed by a four pulses of 0.2 mA for 30 s, with 90 s between each dose, or the positively charged 1% vasodilator ACh solution was iontophorezed with anodal current applied by means of seven pulses of direct electric current of 0.1 mA for 30 s with 30 s between each dose. The pulsed iontophoretic protocols are adapted to obtain a stable plateau of the maximal LDF response. Microcirculatory blood flow was expressed in arbitrary perfusion units and determined by software calculating the area under the curve (AUC) during baseline flow and during ACh or SNP administration. The result was expressed as a blood flow increase following SNP or ACh administration in relation to basal flow (SNP or ACh blood flow increase) [69,70,71].

4.10. Dietary assessment

Rather than using standardized forms, we adapted our questionnaire to presumed eating habits prevalent in the study area with the possibility of choosing the offered answers in the form of tabular presentation rather than writing down everything by hand, in order to avoid boredom and reluctance of volunteers to participate. The form (section 12. Appendices) is constructed as a table for the sake of simplicity and transparency. The table is divided into four main parts: Breakfast, Lunch, Dinner, and Snack. Each meal involves food and beverages. Meals are divided into Fruits, Vegetables, Dairy Products, Meats, Carbs, Cereals, Beverages, Sweets, and Nuts. Method of preparation and portion sizes were taken into consideration depending on the type of food. The participant fills the table by placing a plus/tick symbol in the places provided in accordance consumption and preparation of food that day. After each meal, there is a section called “Comments” where it is necessary to emphasize whether any dietary supplement was taken that could significantly affect or alter nutrient intake that day. If not present in the table, what was consumed can also be written in this section. Portion sizes are described as: small (1/2 of a cup/slice/piece); medium/normal portion (cup/slice/piece); large (2 cups/slices/pieces).

Nutrient status and energy consumption were determined and analyzed with Nutri Prog—software for nutritionists and dieticians at the Department of Endocrinology, University Hospital Osijek. Participants were divided according to dietary protocol, sex and residence: native—students that are studying in hometown; non-native—students studying out of hometown.

4.11. Statistical analysis

Statistical analyses were performed using Microsoft Excel 2016 (Microsoft Office 365, Microsoft Corporation, Redmond, WA, USA), Graph Pad Prism v6.01 (GraphPad Software, San Diego, CA, USA) and SigmaPlot v11 (Systat Software, Inc., Chicago, IL, USA) software. Cohen's d (Δ / SD) effect size was determined according to the primary outcome of the study - change in the frequency of T lymphocytes, Tregs and Th17. GPower v3.1.9.7 (Heinrich Heine University Düsseldorf, Düsseldorf, Germany) was used for sample size calculation.

The normality of distributions was tested by Shapiro–Wilk test. Data are presented as arithmetic mean (standard deviation, SD). Student 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. Analysis of covariance between dependable variables and covariates was performed in XLSTAT 2021 statistical software for Excel. Results on dietary habits were expressed as the percentage (%) of adherence or failure to adhere to recommended guidelines. The cchi–square test was performed for differences between groups regarding adherence to recommended guidelines. Two-tailed $p < 0.05$ was considered significant.

5. RESULTS

Participant flow

A CONSORT diagram for transparent reporting of trials is presented in Figure 5.1. Forty-four healthy adults of both sexes were recruited according to eligibility criteria for the participation in the study. Forty-two participants were randomized and divided into Control group (*N*=21) and *n-3* PUFAs group (*N*=21). Two participants from *n-3* PUFA were lost to follow-up due to personal reasons, with total number of participants included in final analysis being Control group (*N*=21) and *n-3* PUFA group (*N*=19).

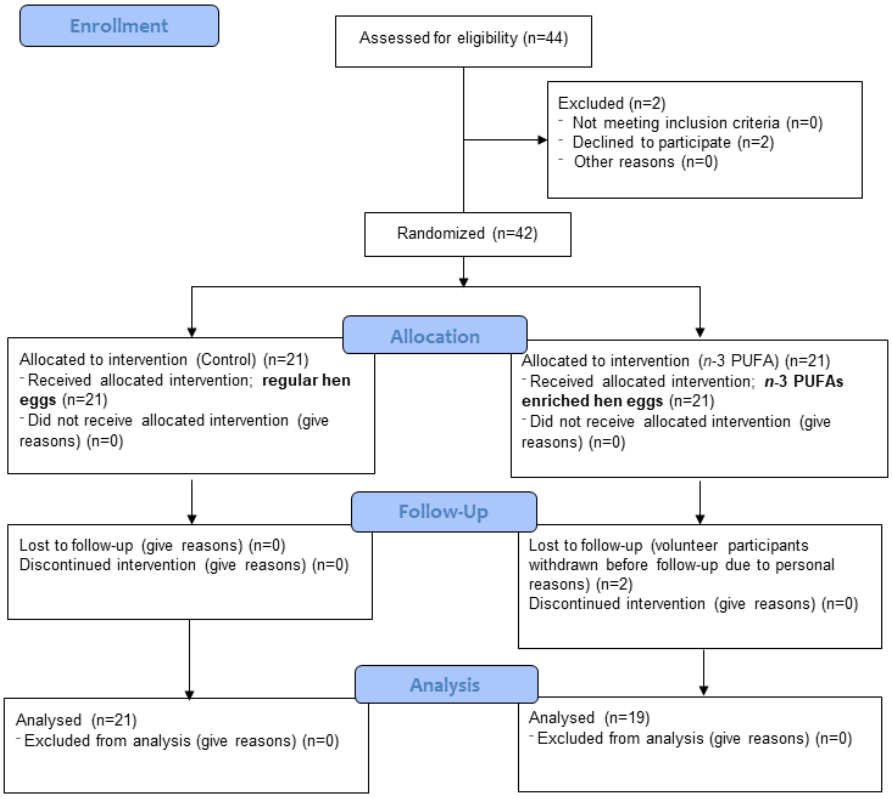


Figure 5.1. CONSORT 2010 flow diagram. Graphically described study recruitment: enrolment, intervention, follow-up, data analysis (source: made by the author of the dissertation).

Recruitment

Participants were recruited during April, 2019. First appointment for all of the randomized participants was held during May, 2019. Follow-up appointment was exactly three weeks after the first one, i.e. the last days of May and first week of July, 2019.

5.1. Baseline anthropometric and biochemical parameters

Participants' baseline anthropometric and biochemical characteristics are presented in Table 5.1. All participants were average built and had normal BP, full blood count, serum electrolytes,

renal function, hsCRP, fasting blood glucose, and lipid levels. There was no significant difference in all measured parameters (e.g., age, BMI, BP, HR, and biochemical parameters) at the moment of entering the study protocol between participants in the Control and *n-3* PUFAs group.

Table 5.1. Baseline clinical characteristics of the study population.

Parameter	Control group	*n-3* PUFA group	*p*
***N* (W/M)**	21 (10/11)	19 (10/9)	-
Age (years)	23.8 (2.79)	23.8 (2.34)	0.954
BMI (kg/m^2)	24.2 (3.01)	22.72 (3.53)	0.168
SBP (mmHg)	117 (10)	115 (11)	0.528
DBP (mmHg)	77 (8)	77 (8)	0.472
MAP (mmHg)	90 (7)	90 (7)	0.987
HR (beats per min)	77 (11)	78 (13)	0.374
Urea (mmol/L)	5.27 (1.32)	5.91 (1.25)	0.183
Creatinine (µmol/L)	78.85 (16.68)	85.67 (18.29)	0.289
Sodium (mmol/L)	138.1 (2.36)	137.92 (1.51)	0.812
Potassium (mmol/L)	4.15 (0.25)	4.24 (0.23)	0.283
Calcium (mmol/L)	2.44 (0.06)	2.41 (0.07)	0.265
Iron (umol/L)	17.55 (5.96)	19.34 (6.09)	0.419
Transferrin (g/L)	2.87 (0.49)	2.78 (0.39)	0.62
Fasting blood glucose (mmol/L)	4.82 (0.57)	4.64 (0.82)	0.474
hsCRP (mg/L)	1.85 (2.41)	1.53 (1.36)	0.672
Cholesterol (mmol/L)	5.26 (0.96)	4.39 (0.74)	0.012*
Triglycerides (mmol/L)	1.09 (0.48)	1.25 (1.08)	0.573
HDL cholesterol (mmol/L)	1.61 (0.37)	1.38 (0.29)	0.071
LDL cholesterol (mmol/L)	3.27 (0.81)	2.71 (0.49)	0.037*
Leukocytes (x10E9/L)	6.17 (1.38)	6.12 (1.44)	0.918
Platelets (x10E9/L)	256 (65.97)	228.83 (36.82)	0.202
Erythrocytes (x10E12/L)	4.77 (0.33)	4.72 (0.39)	0.692
Haemoglobin (g/L)	140.2 (10.99)	142.75 (12.05)	0.544
Haematocrit	0.41 (0.03)	0.41 (0.03)	0.702
MCV (fL)	86.11 (3.73)	88.7 (3.15)	0.054
MCH (pg)	29.45 (1.49)	30.23 (1.12)	0.126
MCHC (g/L)	341.85 (5.58)	340.92 (6.36)	0.667
RDW-CV (%)	13.8 (0.92)	14.08 (0.51)	0.35
MPV (fL)	10.43 (0.49)	10.73 (0.58)	0.129

Results are expressed as arithmetic mean (standard deviation, SD). *N* – Number of participants; W – Women; M – Men; BMI – Body Mass Index; SBP—systolic blood pressure; DBP—diastolic blood pressure; MAP—mean arterial pressure; HR—heart rate; hsCRP – high sensitive C-reactive protein; HDL – high density lipoprotein; LDL – low density lipoprotein. MCV—mean corpuscular volume; MCH—mean corpuscular haemoglobin; MCH—mean corpuscular haemoglobin concentration; RDW-CV—red cell distribution width; MPV—mean platelet volume. Student t-test; significance level $p<0.05$* Control group vs. *n-3* PUFA group. Reference range – general population.

5.2. Frequencies/abundance of peripheral blood Treg and Th17 lymphocytes are reduced following dietary protocols

Represenatative dot plots illustrating gating strategy, exclusion of doublets and dead cells, and T cells gating, prior to Treg lymphocytes analysis, are shown in Figure 5.2. (i-iv).

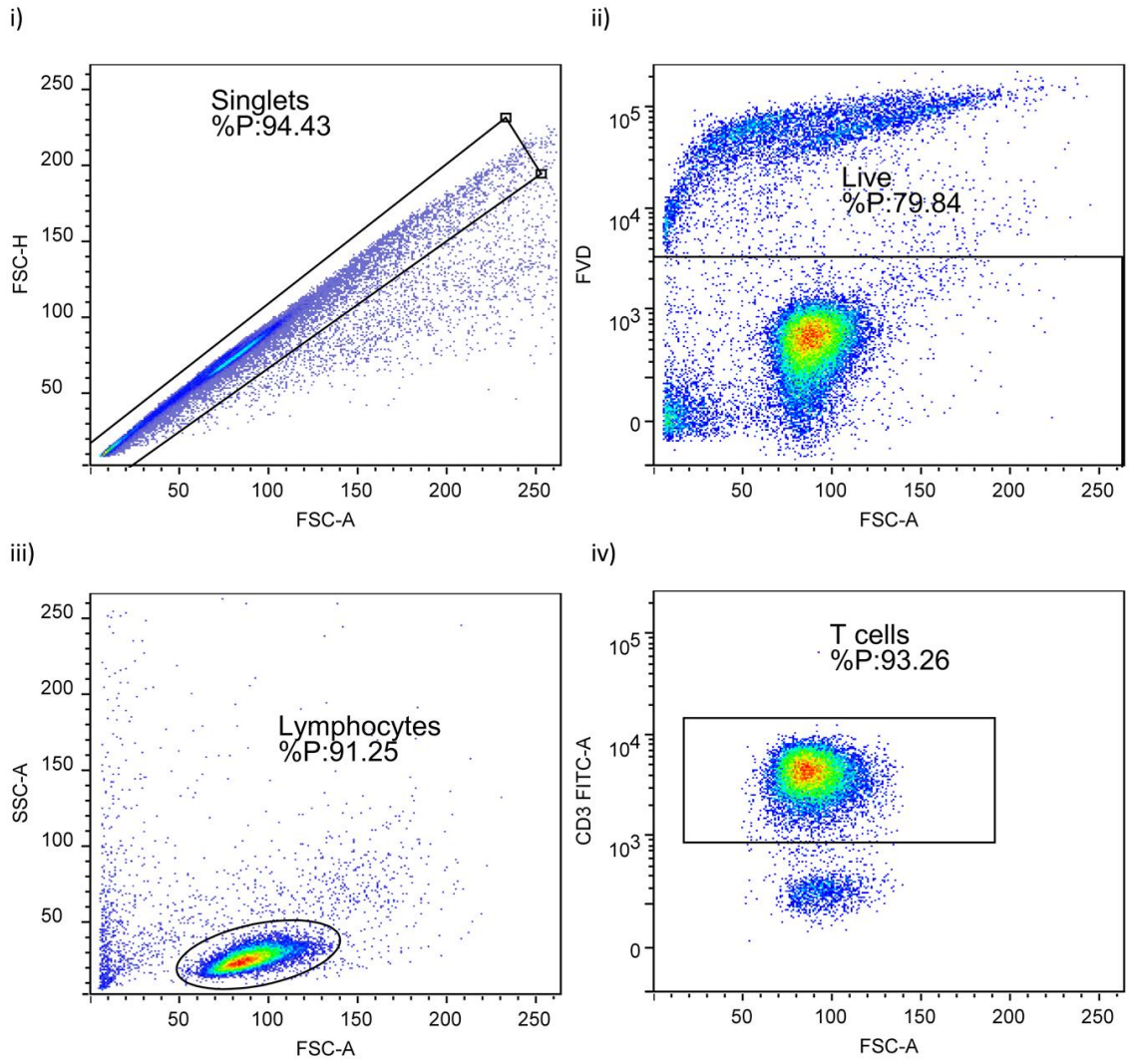


Figure 5.2. Representative dot plots illustrating gating strategy, including the exclusion of doublets using forward scatter area (FSC-A) versus forward scatter width (FSC-W) analysis (i), gating on live cells negative for amine-reactive fixable viability dye (ii), lymphocytes (iii) and $CD3^+$ T cells (iv).

Both dietary protocols resulted in a significant decrease of CD25/Foxp3-expressing peripheral blood lymphocytes within the $CD4^+CD127^+$ subpopulation ($p < 0.001$; Figure 5.3. i, ii). The same differences were observed when frequencies of these cells ($CD25^+Foxp3^+$) were compared within the total peripheral T helper pool ($p < 0.001$ and $p = 0.002$ for the Control group and *n-3* PUFAs group, respectively; Figure 5.3. ii).

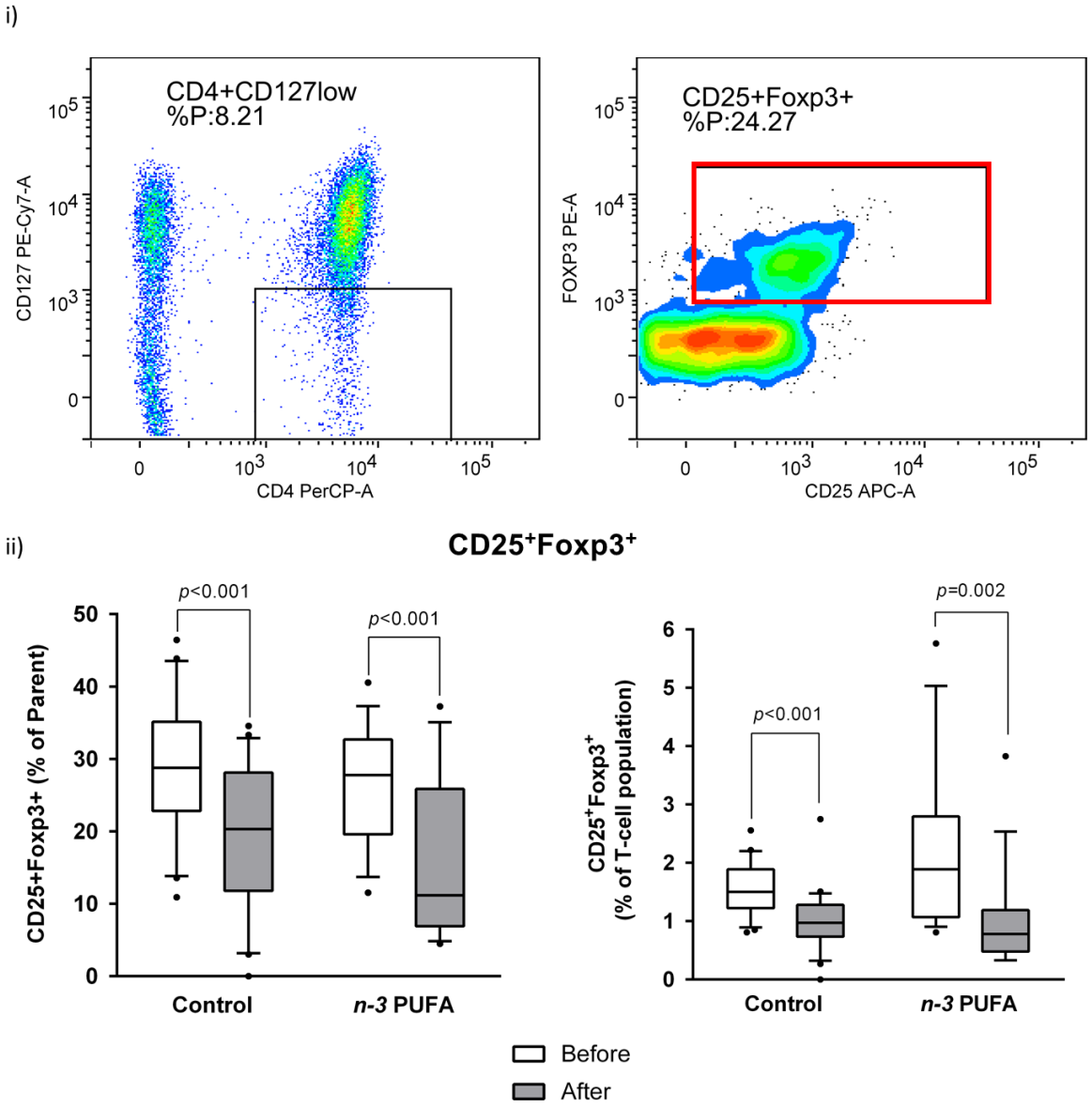


Figure 5.3. Effects of regular (Control group) and *n-3* polyunsaturated acid (PUFA)-enriched hen egg (*n-3* PUFAs group) consumption on the frequency of peripheral regulatory T cells (Treg) in healthy young individuals. 5.3. i) shows the representative gating strategy, while the relative frequencies are presented as box-and-whisker plots at 5.3. ii). PUFA—polyunsaturated fatty acid; Paired t-test, $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

The observed decrease was 1.5-fold in the Control group and 1.6-fold in the *n-3* PUFAs group. Additional analysis of Foxp3 expression in T helper cells (representative gating dot plots shown in Figure 5.4. i-iv) showed that the observed differences were due to the significant reduction of $CD4^{+}CD25^{+}Foxp3^{high}$ subpopulation corresponding to 'real' regulatory T cells (nTreg) in both groups (Figure 5.5. i, ii), while the observed increase in recently activated

$CD4^+CD25^+Foxp3^{-/low}$ T cell frequencies represents a relative increase since their frequencies within the total T cell pool remained unchanged (Figure 5.5. i).

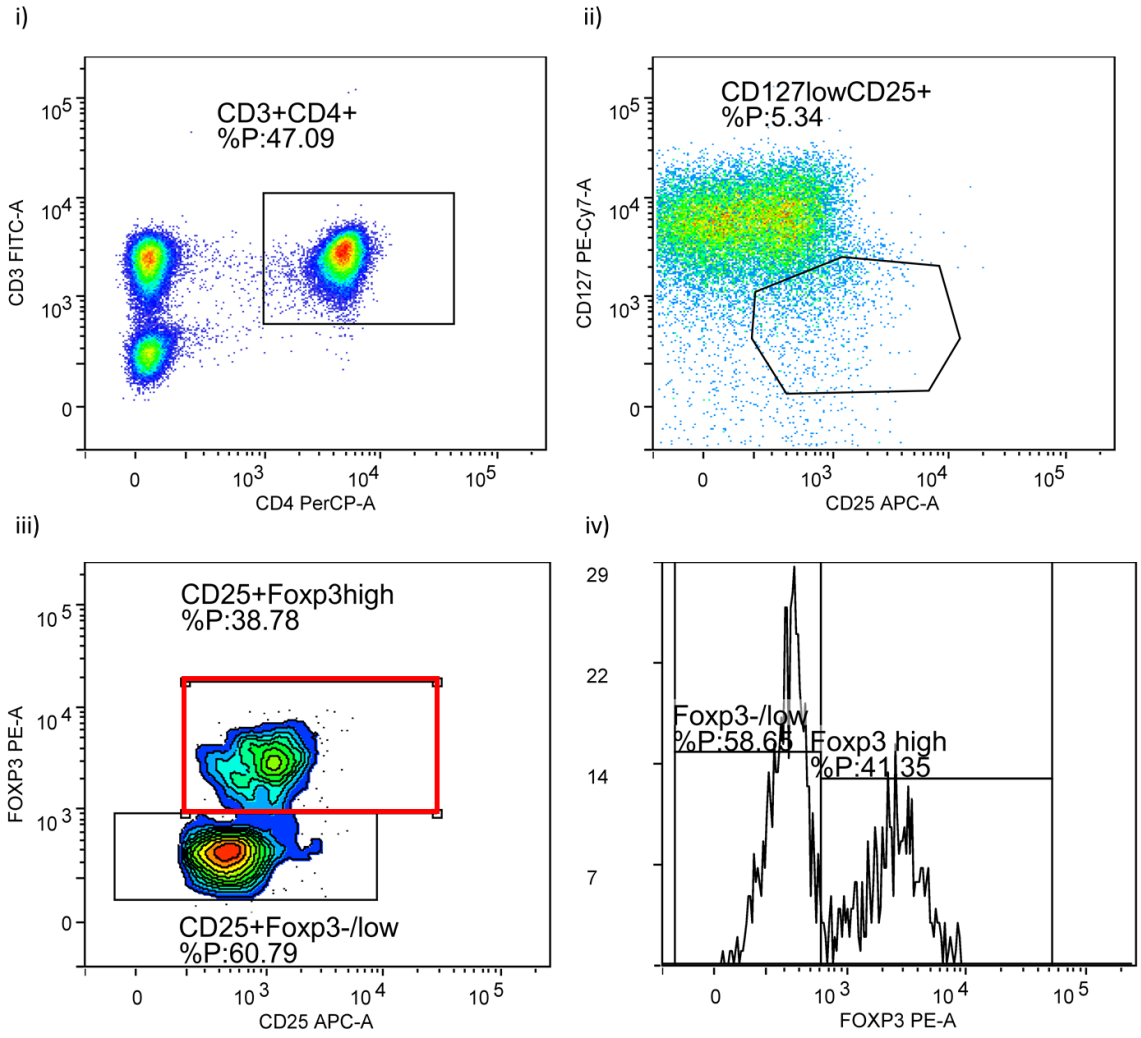


Figure 5.4. Representative dot plots illustrating gating strategy for $CD25^+CD127^{low}$ subpopulation (ii) of T helper lymphocytes ($CD3^+CD4^+$) (i) for the expression of Foxp3 transcription factor (iii-iv). two T cell subpopulations were identified within the $CD4^+CD127^-CD25^+$ T cell pool: $CD4^+CD127^-CD25^+Foxp3^{-/low}$ recently activated T helper cells, and $CD4^+CD127^-CD25^+Foxp3^{high}$ subpopulation corresponding to regulatory T cells.

There were no significant differences in these subpopulations of cells between the groups, neither prior to entering the dietary protocols nor at the end of the protocols. An additional finding of this study was that the rates of recently activated T cells were positively associated (Figure 5.6. i, ii), while the % of nTreg was inversely related to serum IL-22 levels in the *n-3* PUFAs group (Figure 5.6. iii, iv).

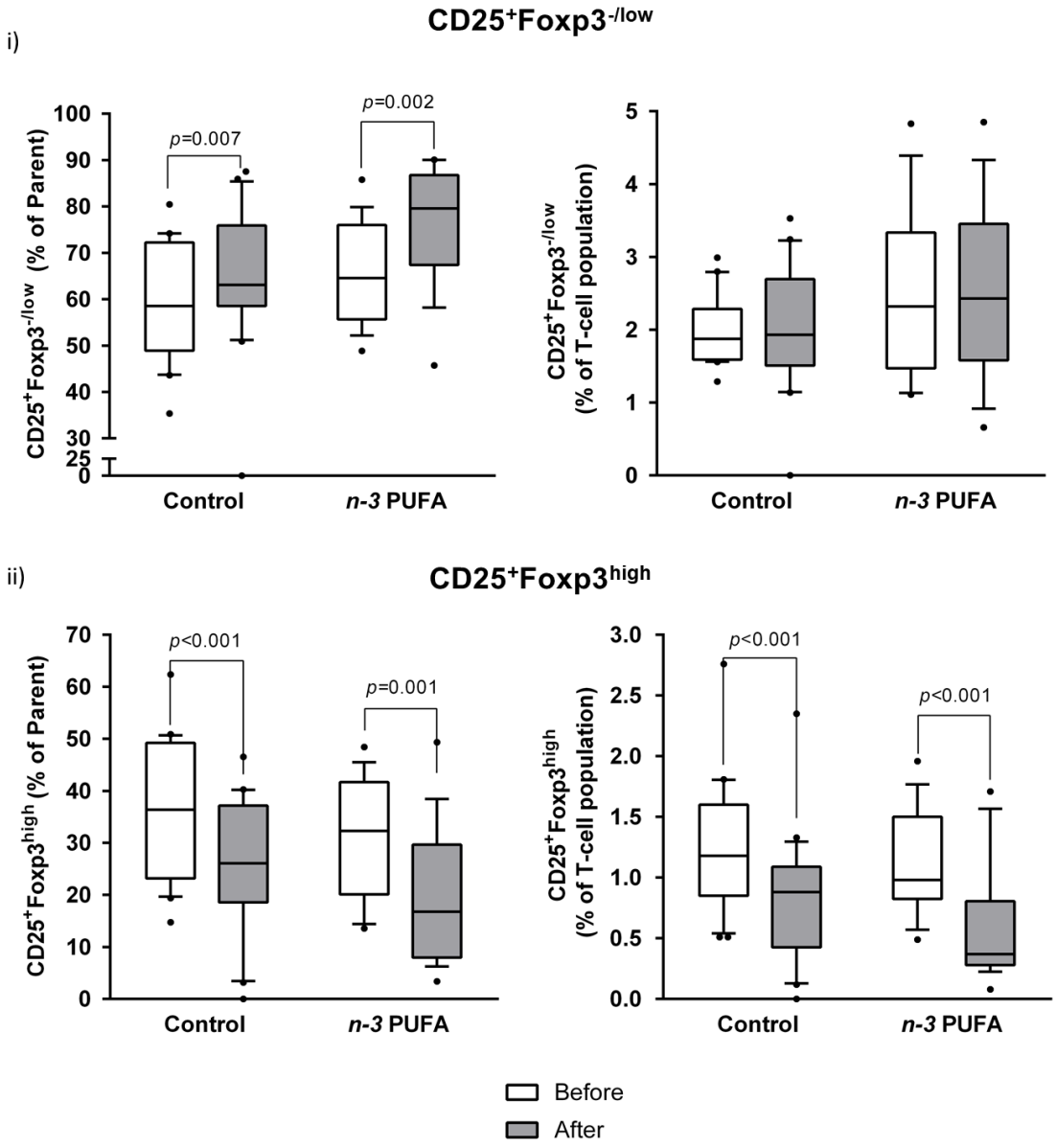


Figure 5.5. Effects of regular (Control group) and *n-3* polyunsaturated acid (PUFA)-enriched hen egg (*n-3* PUFAs group) consumption on the frequency of peripheral regulatory T cells (Treg) in healthy young individuals. Relative frequencies of $CD25^{+}Foxp3^{-/low}$ and $CD25^{+}Foxp3^{high}$ are presented as box-and-whisker plots at i) and ii), respectively. PUFA—polyunsaturated fatty acid; Paired t-test, $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

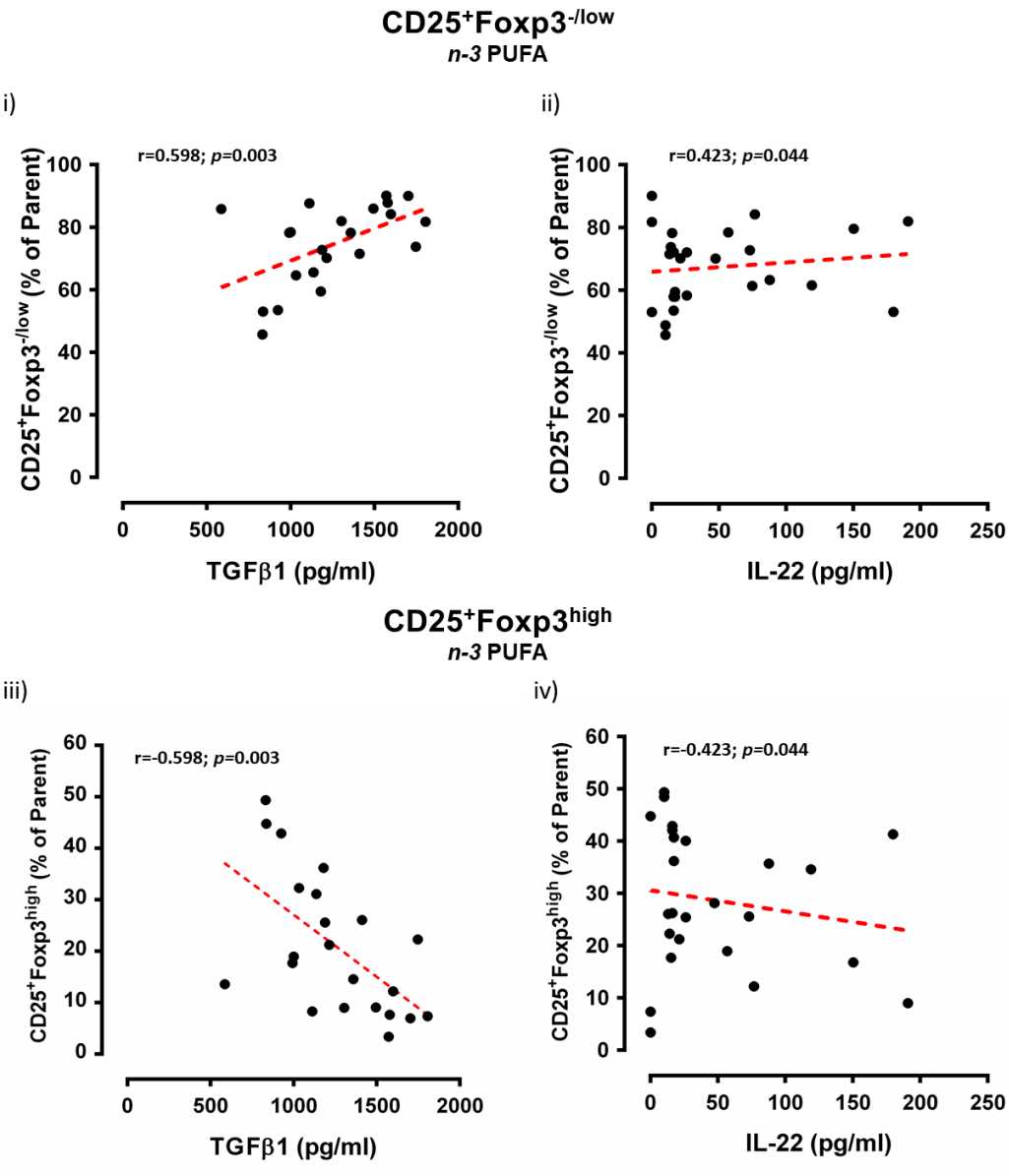


Figure 5.6. Differential correlation of Foxp3-expressing subpopulations with TGF-β1 (secreted upon PBMC stimulation) and serum levels of IL-22. PUFA—polyunsaturated fatty acid; TGF-β1—Transforming Growth Factor Beta-1; PBMC—peripheral blood mononuclear cells; IL-22 - Interleukin 22. r—Spearman correlation coefficient; significance level $p < 0.05$.

Represenatative dot plots illustrating gating strategy, exclusion of doublets and dead cells, gating of lymphocytes, $CD3^+$ T cells and $CD3^+CD4^+$ T cells, prior to Th17 lymphocyte analysis, are shown in Figure 5.7. (i-v).

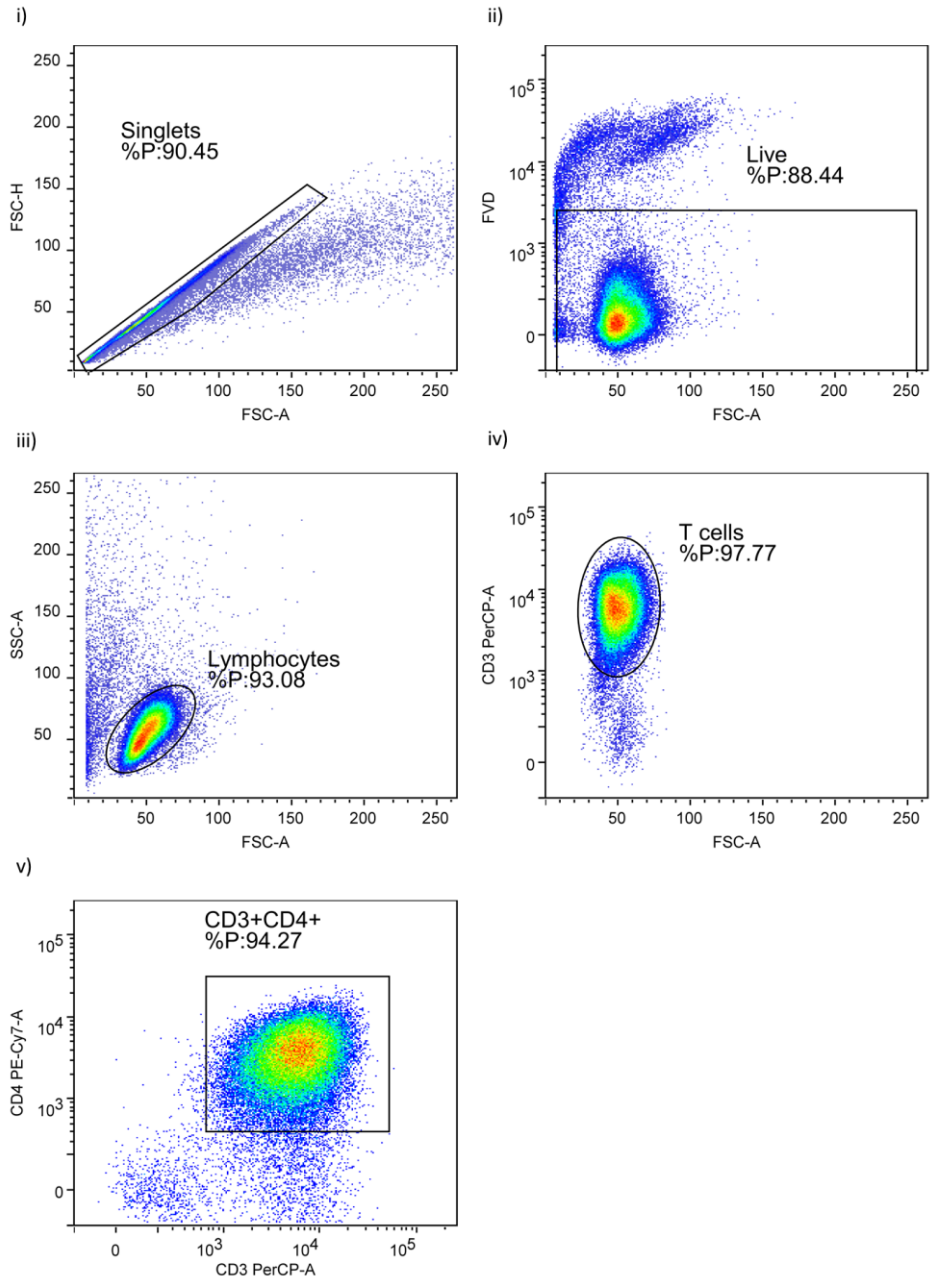


Figure 5.7. Representative dot plots illustrating gating strategy, including the exclusion of doublets using forward scatter area (FSC-A) versus forward scatter width (FSC-W) analysis (i), gating on live cells negative for amine-reactive fixable viability dye (ii), lymphocytes (iii), $CD3^+$ T cells (iv) and $CD3^+CD4^+$ T cells (v).

In the present study, a significant reduction in the frequency of total IL-17 secreting peripheral T helper cells was observed at the end of the protocol in the Control group (p = 0.008; Figure 5.8. i, ii).

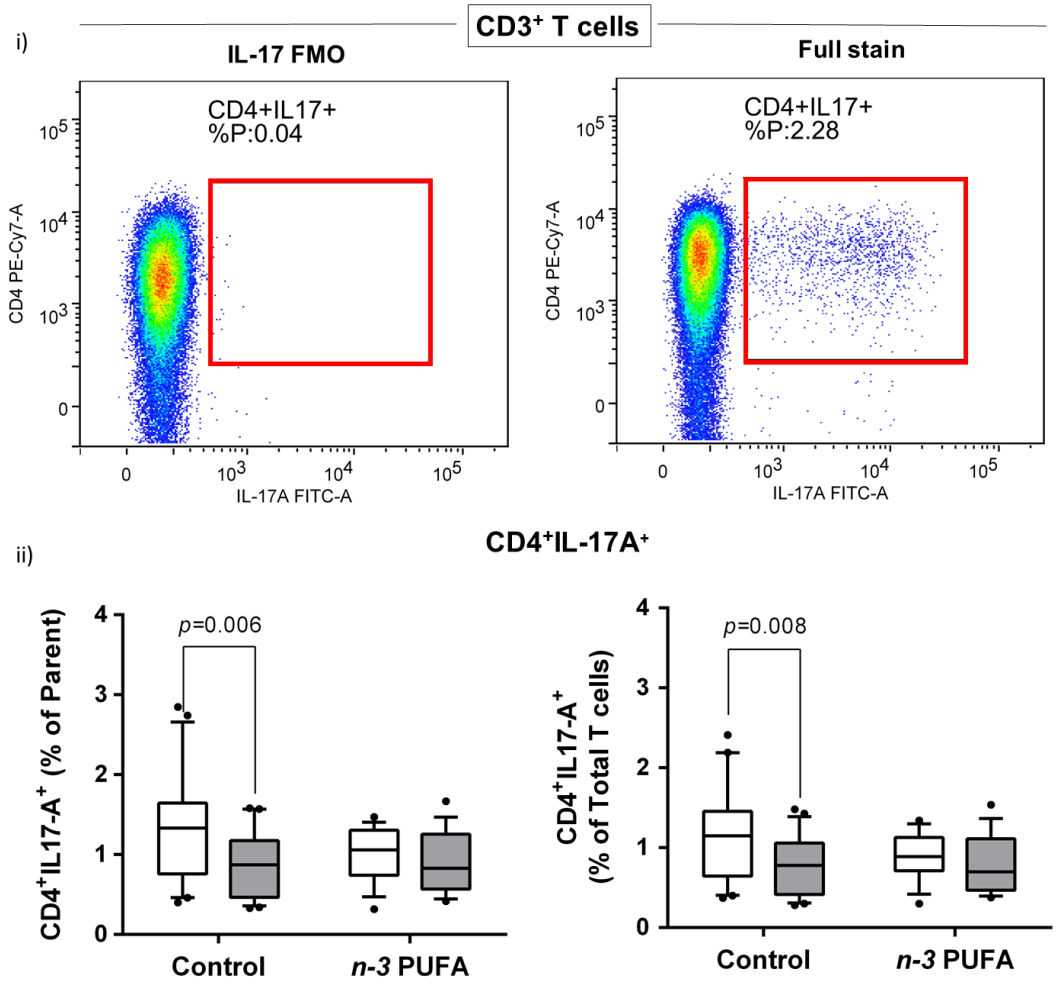


Figure 5.8. Effects of regular (Control group) and *n-3* polyunsaturated acid (PUFAs) enriched hen egg (*n-3* PUFAs group) consumption on the frequency of peripheral T helper cells in healthy young individuals. $CD3^+$ T cells were analysed for IL-17A expression (i). There was a significant decrease in Th17 cell frequency in Control group following dietary protocol (ii). Paired t-test, $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

These cells were further immunephenotyped and, based on their CCR6 expression, subdivided to Th17 ($CCR6^+IL\text{-}17^+$) and non-Th17 ($CCR6^-IL\text{-}17^+$) T helper cells, with the latter corresponding mostly to IL-17-secreting Th1 and Th2 T helper cells. This further revealed that the frequencies of Th17 cells were significantly reduced at the end of both dietary protocols ($p = 0.009$ and $p = 0.008$ in the case of the Control group and *n-3* PUFAs group, respectively; Figure 5.9. i, ii). Interestingly, in the case of $CCR6^-IL\text{-}17^+$ T cells (non-Th17 cells), the Control group had significantly reduced frequency of these cells ($p = 0.033$, Figure 5.9. iii), while the participants from the *n-3* PUFAs group had significantly increased frequency of the same T cell subpopulation ($p < 0.001$, Figure 5.9. i).

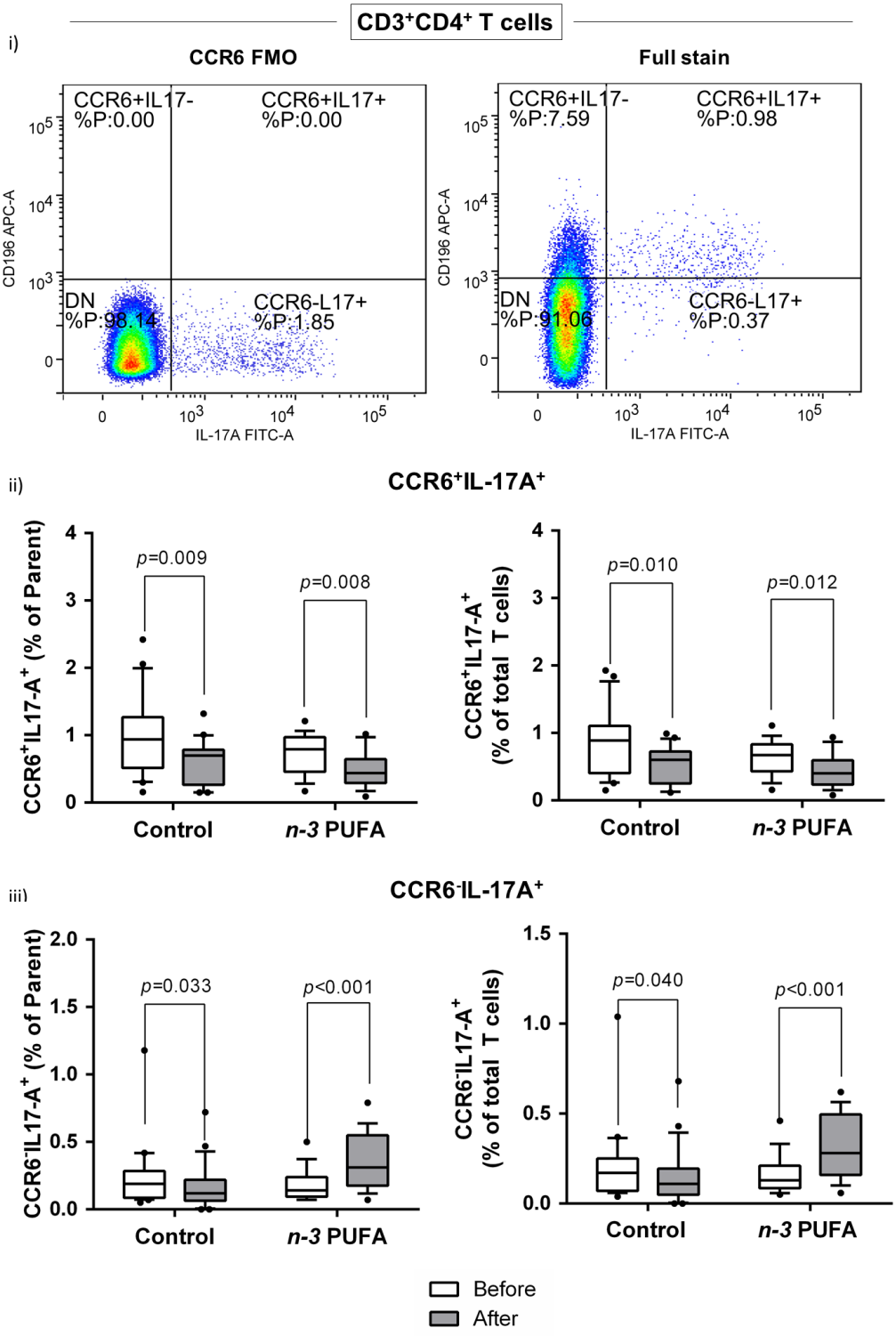


Figure 5.9. Effects of regular (Control group) and *n-3* polyunsaturated acid (PUFAs) enriched hen egg (*n-3* PUFAs group) consumption on the frequency of peripheral T helper cells in healthy young individuals. T cells were analysed for CCR6 expression; hence two subpopulations were identified: 1) $CD4^+CD196^+IL17^+$, Th17 cells and 2) $CD4^+CD196^-IL17^+$, non-Th17 cells (i). There was a significant decrease in Th17 cell frequency in both groups following dietary protocol (ii). The frequency of non-Th17 cells was significantly reduced in the Control group, while the same T cell subpopulation was

increased in *n-3* PUFAs group after dietary protocol (iii). Paired t-test, $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

5.3. Content of fatty acids in chicken feed mixture and edible part of eggs

Fatty acid profiles of chicken feed mixture (g/100 g of total fatty acids) and edible part of eggs (mg/100 g edible part) used in the present study are described in Table 5.2. The average egg weight of both types was 60 g. Each *n-3* PUFAs egg contained on average 351 mg of *n-3* PUFAs (ALA 230.5 mg/egg, DHA 105.5 mg/egg, EPA 15.1 mg/egg). Each control egg, produced on the same farm, contained on average 83 mg of *n-3* PUFAs (ALA 36 mg/egg, DHA 47 mg/egg, EPA 0 mg/egg).

5.4. Hemodynamic parameters and lipid profile

BMI, BP, HR, hsCRP, and serum lipid profile values in Control and *n-3* PUFAs group before and after respective protocol are shown in Table 5.3. There was no significant difference in BMI after consumption of *n-3* PUFAs or regular hen eggs compared to baseline (initial) measurements within the *n-3* PUFAs or Control group. SBP and MAP significantly decreased, while DBP and HR values were similar after consumption of regular hen eggs compared to the baseline measurement in the Control group. There was no significant change in SBP, DBP, MAP, and HR values before and after dietary protocol in *n-3* PUFAs group. The values of SBP, DBP, MAP, and HR did not significantly differ between Control and *n-3* PUFAs group before or after corresponding dietary protocols. The values of hsCRP were not significantly changed by consumption of regular or *n-3* PUFAs enriched eggs compared to baseline measurements within the groups, or there was no difference in hsCRP between the groups.

There were no significant differences in serum LDL cholesterol, HDL cholesterol, triglycerides and total cholesterol concentrations after regular or *n-3* PUFAs enriched eggs consumption compared to baseline conditions within the groups. Serum cholesterol and HDL cholesterol concentrations were higher in Control group than in *n-3* PUFAs group after completion of the respective diet protocol (but still within the normal reference level).

Table 5.2. Fatty acids profile of hens feeding mixture and edible parts of eggs

Fatty Acid	Feeding Mixture (*N* = 3) (g/100 g Total Fatty Acids)		Eggs (*N* = 10) (mg/100 g Egg [1])	
	Control	*n-3* PUFAs	Control	*n-3* PUFAs
∑SFA	16.6 (0.2)	16.8 (0.2)	2082.6 (83.1)	2162.1 (52.6)
∑MUFA	26.5 (0.1)	25.9 (0.1)	2669.6 (84.8)	2917.5 (137.9) *
∑*n-6* PUFA	51.9 (0.1) *	23.4 (0.2)	1417.7 (119.3) *	1182.6 (111.9)
LA	51.9 (0.1) *	23.2 (0.2)	1274.4 (127.3)	1106.6 (108.6)
AA	N/F	0.17 (0.01)	125.4 (6.1) *	67.1 (3.6)
∑*n-3* PUFA	5.05 (0.04)	33.9 (0.3) *	138.2 (28.4)	585.2 (77.1) *
ALA	5.05 (0.04)	28.5 (0.2) *	59.9 (16.1)	384.2 (64.0) *
EPA	N/F	1.87 (0.03)	N/F	25.2 (3.2)
DHA	N/F	3.54 (0.07)	78.3 (13.8)	175.8 (25.9) *
∑*n-6*/*n-3* PUFA	10.3 *	0.69	10.3 *	2.02

Data are presented as arithmetic mean (standard deviation, SD). *n-3* PUFA—*n-3* polyunsaturated fatty acids; *N*—number of analysis; ∑SFA—saturated fatty acids (C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C23:0); ∑MUFA—monounsaturated fatty acids (C14:1, C16:1, C18:1n9t, C18:1n9c, C20:1n9, C22:1n9); ∑*n-6* PUFA—polyunsaturated fatty acids (C18:2n6c, C18:3n6, C20:3n6, C20:4n6, C22:2n6); LA—linoleic acid (C18:2n6c); AA—arachidonic acid (C20:4*n*6); ∑*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); N/F—not found. 1 edible part; * $p < 0.05$ Control vs. *n-3* PUFAs.

Table 5.3. The effect of regular (Control group) and *n-3* PUFAs enriched hen eggs (*n-3* PUFAs group) consumption on anthropometric, hemodynamic, and biochemical parameters

Parameter	Control		*n-3* PUFAs	
	Before	After	Before	After
BMI (kg/m2)	24.3 (3.1)	23.0 (6.1)	23.3 (3.6)	21.9 (6.8)
SBP (mmHg)	117 (10) †	112 (11)	115 (11)	112 (11)
DBP (mmHg)	77 (8)	73 (8)	77 (8)	74 (8)
MAP (mmHg)	90 (7) †	86 (8)	90 (7)	87 (8)
HR (beats per min)	77 (11)	73 (11)	78 (13)	76 (11)
hsCRP (mg/L)	1.6 (2.2)	1.8 (2.6)	1.3 (1.2)	1.8 (2.1)
cholesterol (mmol/L)	5.2 (0.9)	5.3 (1.0) *	4.6 (0.9)	4.7 (0.7)
triglycerides (mmol/L)	1.1 (0.5)	1.1 (0.4)	1.3 (1.0)	1.2 (0.6)
HDL cholesterol (mmol/L)	1.5 (0.4)	1.5 (0.4) *	1.3 (0.2)	1.3 (0.2)
LDL cholesterol (mmol/L)	3.1 (0.7)	3.1 (0.8)	2.7 (0.6)	2.8 (0.4)

Data are presented as arithmetic mean (standard deviation (SD). *n-3* PUFA—*n-3* polyunsaturated fatty acids; BMI—body mass index; SBP—systolic blood pressure; DBP—diastolic blood pressure; MAP—mean arterial pressure; HR—heart rate; HDL–high-density lipoprotein; LDL—low-density lipoprotein. * $p < 0.05$ difference between Control and *n-3* PUFA group; † $p < 0.05$ difference within the each group.

5.5. Body composition and body fluid status

Body composition and body fluid status responses to dietary protocols are presented in Table 5.4. Fat% significantly increased, and Fat Free Mass% (FFM%) and Total Body Water% (TBW%) significantly decreased after consumption of three regular hen eggs per day during three weeks compared to baseline measurement in the Control group. There was no significant difference in FFM%, Fat%, TBW%, Extracellular Water% (ECW%), Intracellular Water% (ICW%), Plasma Fluid (PF), Interstitial Fluid (IF), or Body Density before and after dietary protocol within the *n-3* PUFAs group. There was no difference in FFM%, Fat%, TBW%, ECW%, ICW%, PF, IF, or Body Density between the groups neither before nor after respective study protocols.

Table 5.4. Body composition and body fluid status responses to regular (Control group) and *n-3* PUFAs enriched hen eggs (*n-3* PUFAs group) consumption

Parameter	Control		*n-3* PUFAs	
	Before	After	Before	After
Fat Free Mass (%)	79.1 (8.6) *	75.7 (8.4)	77.7 (6.9)	77.8 (6.7)
Fat (%)	20.9 (8.6) *	24.3 (8.4)	22.3 (6.9)	22.2 (6.7)
Total Body Water (%)	56.7 (5.4) *	54.2 (5.8)	56.6 (6.2)	55.5 (4.8)
Extracellular Water (%)	43.0 (4.1)	42.8 (4.4)	41.4 (1.2)	41.3 (1.4)
Intracellular Water (%)	57.0 (4.1)	57.2 (4.4)	58.6 (1.2)	58.7 (1.4)
Plasma Fluid (L)	3.64 (0.68)	3.56 (0.72)	3.46 (0.96)	3.34 (0.89)
Interstitial Fluid (L)	12.73 (2.36)	12.44 (2.51)	12.11 (3.38)	11.68 (3.12)
Body Density (kg/L)	1.051 (0.020)	1.044 (0.018)	1.048 (0.015)	1.048 (0.014)

Data are presented as arithmetic mean (standard deviation (SD). *n-3* PUFA- *n-3* polyunsaturated fatty acids. * $p < 0.05$ difference within the each group.

5.6. Serum fatty acids profile

Serum fatty acids profile (a total of 37 fatty acids) in respect to dietary protocols is presented in Table 5.5. The serum concentration of ALA significantly increased, while EPA and DHA tended to increase following consumption of *n-3* PUFAs enriched eggs compared to baseline measurement within the *n-3* PUFAs group. The serum concentration of other measured fatty acids was similar before and after dietary protocol with *n-3* PUFAs enriched eggs. As a result of *n-3* PUFAs enriched hen eggs consumption, serum *n-6*/*n-3* ratio decreased by approximately 30% in *n-3* PUFAs group. Serum fatty acids profile was not significantly changed, and *n-6*/*n-3* ratio decreased by approximately 16% following dietary protocol with regular eggs compared to baseline measurement in the Control group. At baseline measurement prior to entering the study, serum concentration of C16:0 palmitic acid, C18:0 stearic acid, C18:1[cis-9] oleic acid, C20:3[cis-8,11,14] dihomo-gamma-linolenic acid, and C20:4[cis-5,8,11,14] arachidonic acid was significantly higher in the Control compared to the *n-3* PUFAs group. Serum DHA concentration was significantly increased, and C20:4[cis-5,8,11,14] arachidonic acid was significantly decreased in the *n-3* PUFAs group compared to the Controls after completion of the respective dietary protocol.

Table 5.5. The effect of regular (Control group) and *n-3* PUFAs enriched hen eggs (*n-3* PUFAs group) consumption on serum fatty acids profile

Parameter	Control		*n-3* PUFAs	
	Before	After	Before	After
SFA (µmol/L)				
C4:0 Butyric acid	N/F	N/F	N/F	N/F
C6:0 Caproic acid	N/F	N/F	N/F	N/F
C8:0 Caprylic acid	N/F	N/F	N/F	N/F
C10:0 Capric acid	N/F	N/F	N/F	N/F
C11:0 Undecylic acid	N/F	N/F	N/F	N/F
C12:0 Lauric acid	<LOQ	<LOQ	<LOQ	<LOQ
C13:0 Tridecylic acid	N/F	N/F	<LOQ	<LOQ
C14:0 Myristic acid	35.0 (14.5)	25.5 (5.6)	42.0 (12.3)	32.7 (5.2)
C15:0 Pentadecylic acid	12,70	<LOQ	<LOQ	10,90
C16:0 Palmitic Acid	825.4 (127.6) *	767.1 (185.1)	515.2 (170.5)	552.7 (253.8)
C17:0 Margaric acid	9,91	<LOQ	10,80	10.3 (0.8)
C18:0 Stearic acid	224.9 (40.2) *	213.8 (36.4)	153.1 (61.0)	161.1 (78.6)
C20:0 Arachidic acid	<LOQ	<LOQ	<LOQ	<LOQ
C21:0 Heneicosanoic acid	N/F	N/F	N/F	N/F
C22:0 Behenic acid	<LOQ	<LOQ	<LOQ	<LOQ
C23:0 Tricosanoic acid	<LOQ	<LOQ	<LOQ	<LOQ
C24:0 Lignoceric acid	<LOQ	<LOQ	<LOQ	<LOQ
PUFA (µmol/L)				
n-5				
C14:1[cis-9] Myristoleic acid	<LOQ	<LOQ	13,7	<LOQ
C15:1[cis-10] Cis-10-Pentadecenoic acid	<LOQ	<LOQ	<LOQ	<LOQ
***n*-7**				
C16:1[cis-9] Palmitoleic acid	61.9 (26.5)	53.7 (22.2)	47.9 (19.3)	45.5 (19.1)
C17:1[cis-10] cis-10-Heptadecenoic acid	8,90	<LOQ	5,50	<LOQ

(Continuation of the table on the next page)

Parameter	Control		*n-3* PUFAs	
	Before	After	Before	After
***n*-9**				
C18:1[trans-9] Elaidic acid	N/F	N/F	N/F	N/F
C18:1[cis-9] Oleic acid	562.0 (108.2) *	507.1 (151.2)	372.4 (152.7)	383.6 (235.4)
C20:1[cis-11] 11-Eicosenoic acid	<LOQ	<LOQ	<LOQ	<LOQ
C22:1[cis-13] Erucic acid	N/F	N/F	<LOQ	<LOQ
C24:1[cis-15] Nervonic acid	N/F	N/F	<LOQ	<LOQ
n-6				
C18:2[trans-9,12] Linoelaidic acid	N/F	N/F	N/F	N/F
C18:2[cis-9,12] Linoleic acid	1065.4 (136.8)	961.0 (95.1)	849.1 (291.6)	889.3 (363.8)
C18:3[cis-6,9,12] gamma-Linolenic acid	21.0 (5.2)	16.9 (4.1)	18.2 (5.0)	16.4 (6.6)
C21:2[cis-11,14] Eicosadienoic acid	7.1 (0.5	7,40	8,10	7,8
C20:3[cis-8,11,14] Dihomo-gamma-linolenic acid	55.4 (15.9) *	45.5 (10.8)	29.8 (8.3)	32.1 (11.1)
C20:4[cis-5,8,11,14] Arachidonic acid	355.2 (91.4) *	363.8 (74.6) *	231.9 (92.1)	236.8 (106.2)
C22:2[cis-13,16] 13,16-Docosadienoic acid	N/F	N/F	N/F	N/F
n-3				
C18:3[cis-9,12,15] alpha-Linolenic acid	10.3 (2.2)	10.5 (3.4)	11.9 (1.6)	19.3 (6.2) *
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	9.2 (2.4)	10.7 (4.5)	10.4 (1.7)	16.0 (6.5)
C22:6[cis-4,7,10,13,16,19] cis-4,7,10,13,16,19-Docosahexaenoic acid	39.1 (11.1)	50.8 (14.1)	33.7 (11.4)	52.8 (28.0) †
***n-6*/*n-3* PUFAs**	14.9	12.5	13.1	9.3

Results are expressed as arithmetic mean (standard deviation, SD). *n-3* PUFAs—*n-3* polyunsaturated fatty acids; SFA—saturated fatty acids. <LOQ—below limit of quantification; N/F—not found. * $p < 0.05$ difference between Control and *n-3* PUFA group; † $p < 0.05$ before vs. after within the group (Control or *n-3* PUFA).

5.7. Serum pro- and anti-inflammatory cytokines, chemokines, growth factors and soluble cell adhesion molecules protein concentration

Serum pro- (INFγ, TNF-α, IL-17A, IL-6, IL-23, and IL-9) and anti-inflammatory cytokines (IL-21, IL-22, and IL-10), chemokines (SDF-1α), LAP, vascular growth factors (VEGF-A, VEGF-D) and soluble cell adhesion molecules (sICAM-1, sVCAM-1), protein concentration values in the Control and *n-3* PUFAs group before and after respective protocol are described in Table 5.6. INFγ protein concentration significantly decreased, and IL-10 serum concentration significantly increased following consumption of *n-3* PUFAs enriched eggs compared to baseline measurement within the *n-3* PUFAs group.

Serum protein concentration of other measured cytokines, chemokines, growth factors, or soluble cell adhesion molecules (TNF-α, IL-17A, IL-6, IL-21, IL-22, IL-23, IL-9, SDF-1α, LAP, VEGF-A, VEGF-D, sICAM-1, and s-VCAM-1) were similar before and after dietary protocol with *n-3* PUFAs enriched eggs. In Controls, IL-17A, IL-10, and VEGF-A significantly increased following consumption of regular hen eggs compared to the baseline measurement. Serum protein concentration of other measured cytokines, chemokines, growth factors or soluble cell adhesion molecules (INFγ, TNF-α, IL-6, IL-21, IL-22, IL-23, IL-9, SDF-1α, LAP, VEGF-D, sICAM-1, and s-VCAM-1) were similar before and after dietary protocol with regular eggs. Only serum protein concentration of IL-10 was significantly increased in *n-3* PUFAs group compared to the Controls after completion of the respective dietary protocol.

Table 5.6. The effect of regular (Control group) and *n-3* PUFAs enriched hen eggs (*n-3* PUFAs group) consumption on serum pro- and anti-inflammatory cytokines, chemokines, vascular growth factors, and soluble cell adhesion molecules protein concentration

Parameter	Control		*n-3* PUFAs	
	Before	After	Before	After
IFNγ (pg/mL)	14.3 (8.7)	16.6 (9.9)	31.9 (31.9) †	12.2 (5.5)
TNF-α (pg/mL)	11.1 (4.4)	13.8 (6.1)	9.1 (3.0)	17.9 (16.4)
IL-17A (pg/mL)	7.3 (4.8)	9.7 (4.7) †	6.63 (2.57)	9.2 (8.9)
IL-6 (pg/mL)	32.4 (12.9)	38.8 (17.1)	32.6 (13.3)	45.8 (28.5)
IL-21 (pg/mL)	42.3 (30.1)	46.8 (30.5)	51.6 (48.2)	80.7 (47.4)
IL-22 (pg/mL)	31.6 (21.8)	41.2 (34.3)	36.5 (47.3)	76.2 (59.2)
IL-23 (pg/mL)	3.6 (0.5)	3.7 (0.5)	3.50 (0.31)	4.0 (1.1)
IL-9 (pg/mL)	4.9 (4.7)	5.9 (5.2)	4.0 (2.1)	7.6 (11.7)
IL-10 (pg/mL)	2.6 (1.1)	3.2 (1.3) †	2.7 (1.5)	6.7 (4.6) *,†
SDF-1α (ng/mL)	1.0 (0.2)	0.8 (0.3)	1.3 (0.6)	0.8 (0.4)
LAP (ng/mL)	32.5 (16.8)	27.4 (11.1)	30.3 (11.8)	29.4 (15.9)
VEGF-A (pg/mL)	611.5 (480.9)	684.3 (465.9) †	527.8 (331.9)	705.1 (602.6)
VEGF-D (pg/mL)	0.4 (0.1)	0.4 (0.1)	0.5 (0.1) *	0.5 (0.3)
sICAM-1 (ng/mL)	91.5 (51.2)	95.2 (50.2)	103.2 (46.5)	107.0 (49.6)
sVCAM-1 (ng/mL)	50.7 (17.7)	48.2 (18.7)	53.0 (23.1)	55.1 (21.9)

Data are presented as arithmetic mean (standard deviation, SD). *n-3* PUFAs—*n-3* polyunsaturated fatty acids; IFNγ—interferon gamma; TNF-α—tumor necrosis factor alpha; IL-17A—interleukin 17A; IL-6—interleukin 6; IL-21—interleukin 21; IL-22—interleukin 22; IL-23—interleukin 23; IL-9—interleukin 9; IL-10—interleukin 10; SDF-1α—stromal cell-derived factor 1 alpha; LAP—latency associated peptide; VEGF-A—vascular endothelial growth factor A ; VEGF-B—vascular endothelial growth factor D; sICAM-1—soluble intercellular adhesion molecule 1; sVCAM-1—soluble vascular cell adhesion molecule 1. * $p < 0.05$ difference between Control and *n-3* PUFA group; † $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

5.8. *n-3* PUFA supplementation changes the ratio of pro- and anti-inflammatory lipid mediators originating from *n-6* (AA) and *n-3* (EPA) fatty acids

Serum concentrations of pro-inflammatory eicosanoids (LTB4 and PGE2) from AA, and inflammation resolving oxylipins (LTB5 and PGE3) and resolvins (RvE1) from EPA and DHA before and after the dietary protocols are shown in Figures 5.10., 5.11. and 5.12. LTB4 and PGE3 serum levels were significantly increased in the Control group after three-week consumption of regular hen eggs ($p = 0.021$ and $p = 0.014$, respectively, Figure 5.10. B; Figure 5.11. A), while their levels remained unchanged in the *n-3* PUFAs group. Average serum

concentrations of LTB5 at the end of the dietary protocol were significantly increased in both groups, compared to their respective baseline levels ($p < 0.0001$ and $p = 0.012$, respectively; Figure 5.10. A).

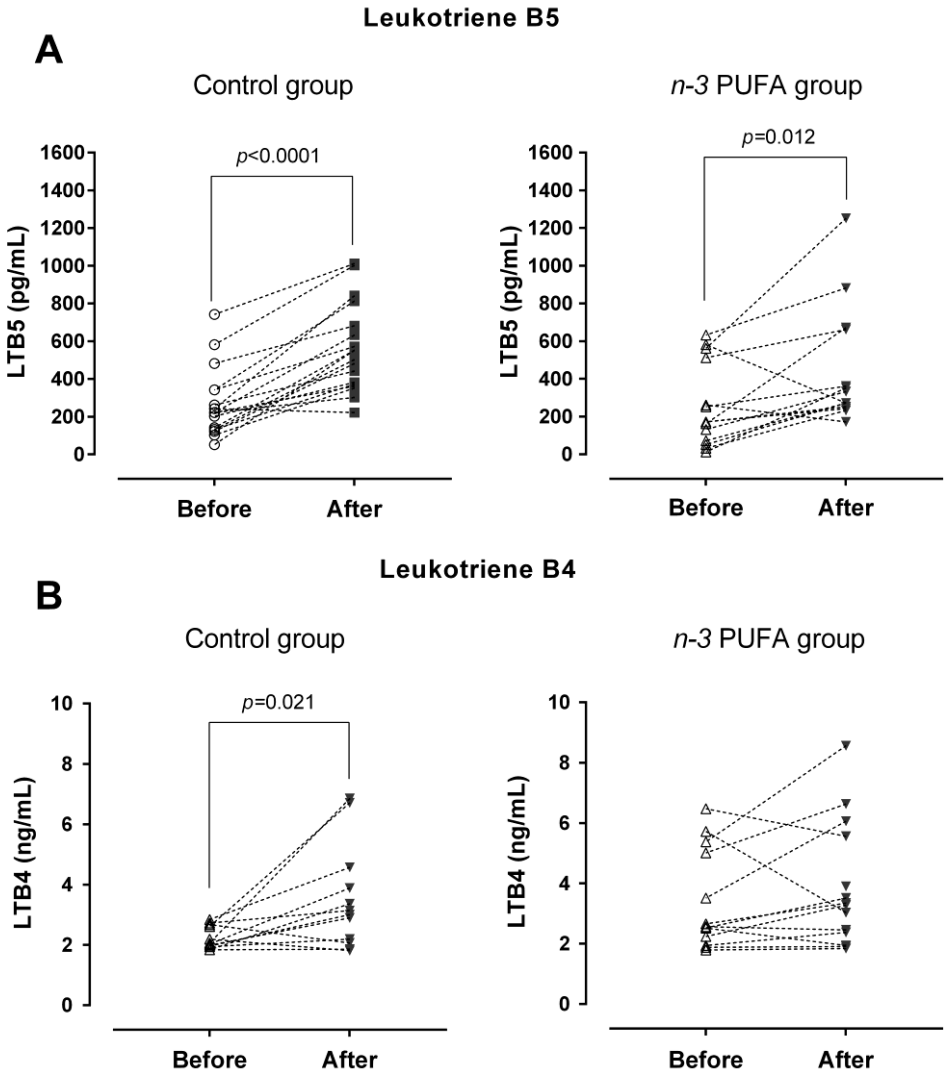


Figure 5.10. Effects of regular (Control group) and *n-3* polyunsaturated acid (PUFA)-enriched hen egg (*n-3* PUFA group) consumption on the serum concentrations of inflammation resolving leukotriene B5 (A) and pro-inflammatory leukotriene B4 (B) lipid mediators originating from *n-3* (eicosapentaenoic acid; EPA) and *n-6* (arachidonic acid; AA) fatty acids. PUFA—polyunsaturated fatty acid; LTB4 – leukotriene B4; LTB5—leukotriene B5. Paired t-test; $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

Serum concentrations of PGE2 were not significantly affected by any of the dietary protocols (Figure 5.11. B). To address the proportions of pro-inflammatory and anti-inflammatory lipid mediators, prostaglandin E2/E3 and leukotrienes B4/B5 ratios were calculated and compared

across the measurements. These results showed a significant decrease in prostaglandin E2/E3 ratio following *n-3* PUFA dietary protocol (p = 0.014; Figure 5.11. C), while the leukotrienes B4/B5 ratio remained unchanged in both groups.

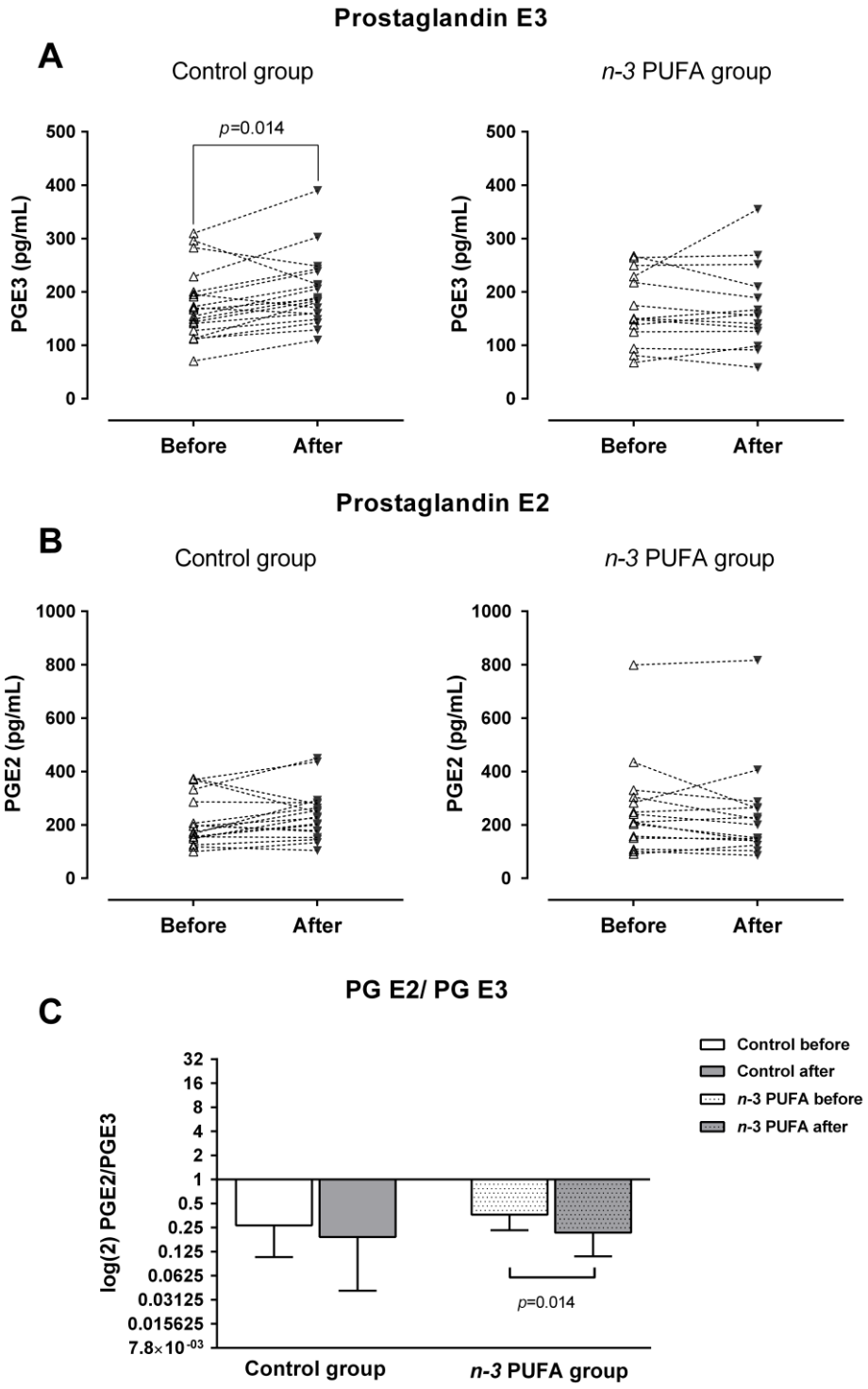


Figure 5.11. Effects of regular (Control group) and *n-3* polyunsaturated acid (PUFA)-enriched hen egg (*n-3* PUFA group) consumption on the serum concentrations of inflammation resolving prostaglandin E3 (A) and pro-inflammatory prostaglandin E2 (B); lipid mediators originating from *n-3*

(eicosapentaenoic acid; EPA) and *n-6* (arachidonic acid; AA) fatty acids. The ratio between prostaglandin E2 and prostaglandin E3 is shown at Panel (C). PUFA—polyunsaturated fatty acid; PGE2 - prostaglandin E2; PGE3—prostaglandin E3. Paired t-test; $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

Serum level of RvE1 was significantly increased in the *n-3* PUFAs group after the three-week consumption of *n-3* PUFA-enriched hen eggs ($p = 0.013$; Figure 5.12.).

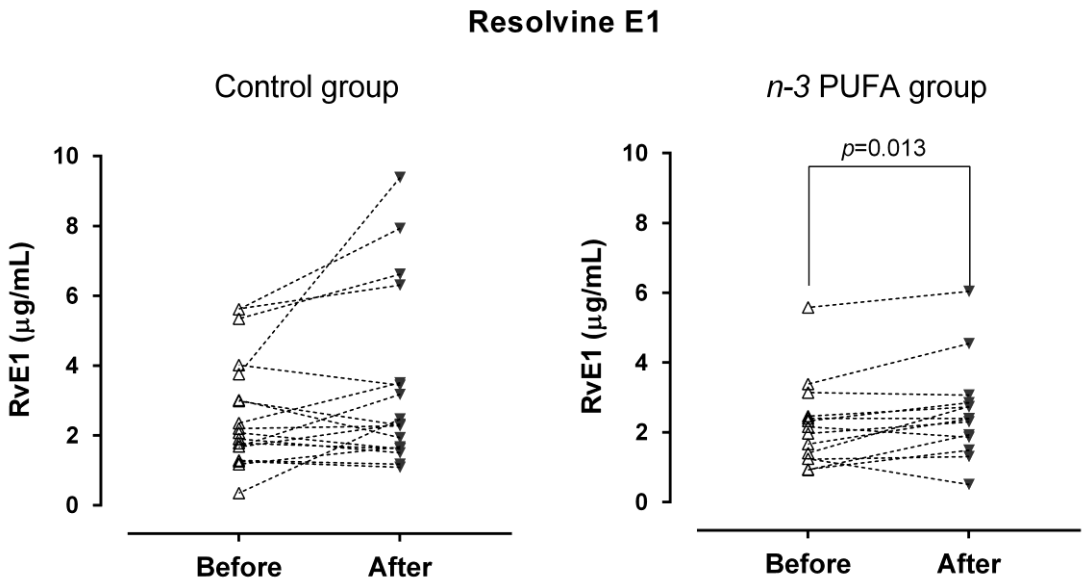


Figure 5.12. Effects of regular (Control group) and *n-3* polyunsaturated acid (PUFA)-enriched hen egg (*n-3* PUFA group) consumption on the serum concentrations inflammation resolving resolvin E1, lipid mediator originating from *n-3* (eicosapentaenoic acid; EPA) fatty acids. PUFA—polyunsaturated fatty acid; RvE1—resolvin E1. Paired t-test; $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

5.9. PBMC-derived cytokines following PMA–ionomycin activation

Concentrations of anti-inflammatory (IL-10, TGF-β1) (Figure 5.13.), pro-inflammatory cytokines (IL-6, IL-23, IL-17A) and MCP-1 (Figure 5.14.) chemokine in both Control and experimental group, before and after dietary protocols were determined in supernatants from PBMC cell cultures upon PMA/ionomycin stimulation. TGF-β1 production by peripheral blood mononuclear cells following *n-3* PUFA dietary protocol was significantly increased ($p = 0.026$; Figure 5.13. A), while IL-6 production was significantly decreased ($p = 0.05$; Figure 5.14. B), compared to the respective baseline levels. In addition, end-point TGF-β1 levels were

significantly lower in the Control group, compared with the end-point levels measured in the *n-3* PUFAs group ($p = 0.004$; Figure 5.13. A).

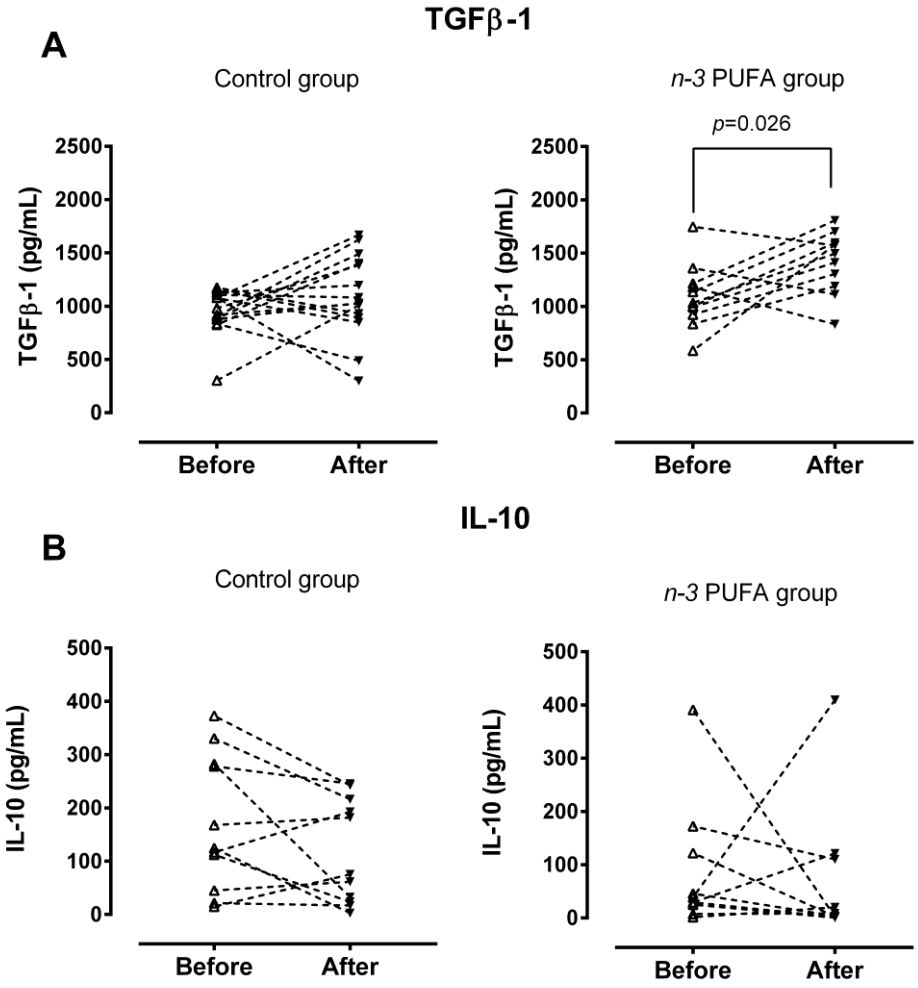


Figure 5.13. Levels of anti-inflammatory cytokines secreted by PBMCs upon PMA/ionomycin stimulation in young individuals following regular (Control group) or *n-3* polyunsaturated acid (PUFA)-enriched hen egg (*n-3* PUFA group) consumption. There was a significant increase in TGF-β1 level in *n-3* PUFA group (A), while no significant changes were found for IL-10 (B). PBMC—peripheral blood mononuclear cells; TGFβ-1—Transforming Growth Factor Beta-1; IL-10—Interleukin 10. Paired t-test; $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

Target cytokine and chemokine production by PBMC was unaffected by the consumption of regular hen eggs (Control group, Figures 5.13. and 5.14). Furthermore, the intergroup analysis revealed significant differences in baseline and end-point IL-17A production, namely, in the Control group, levels of IL-17 secreted by PBMC upon PMA/ionomycin stimulation were significantly higher both prior and after consumption of regular hen eggs, compared to *n-3* PUFAs group ($p < 0.001$ and $p = 0.001$, respectively; Figure 5.14. A).

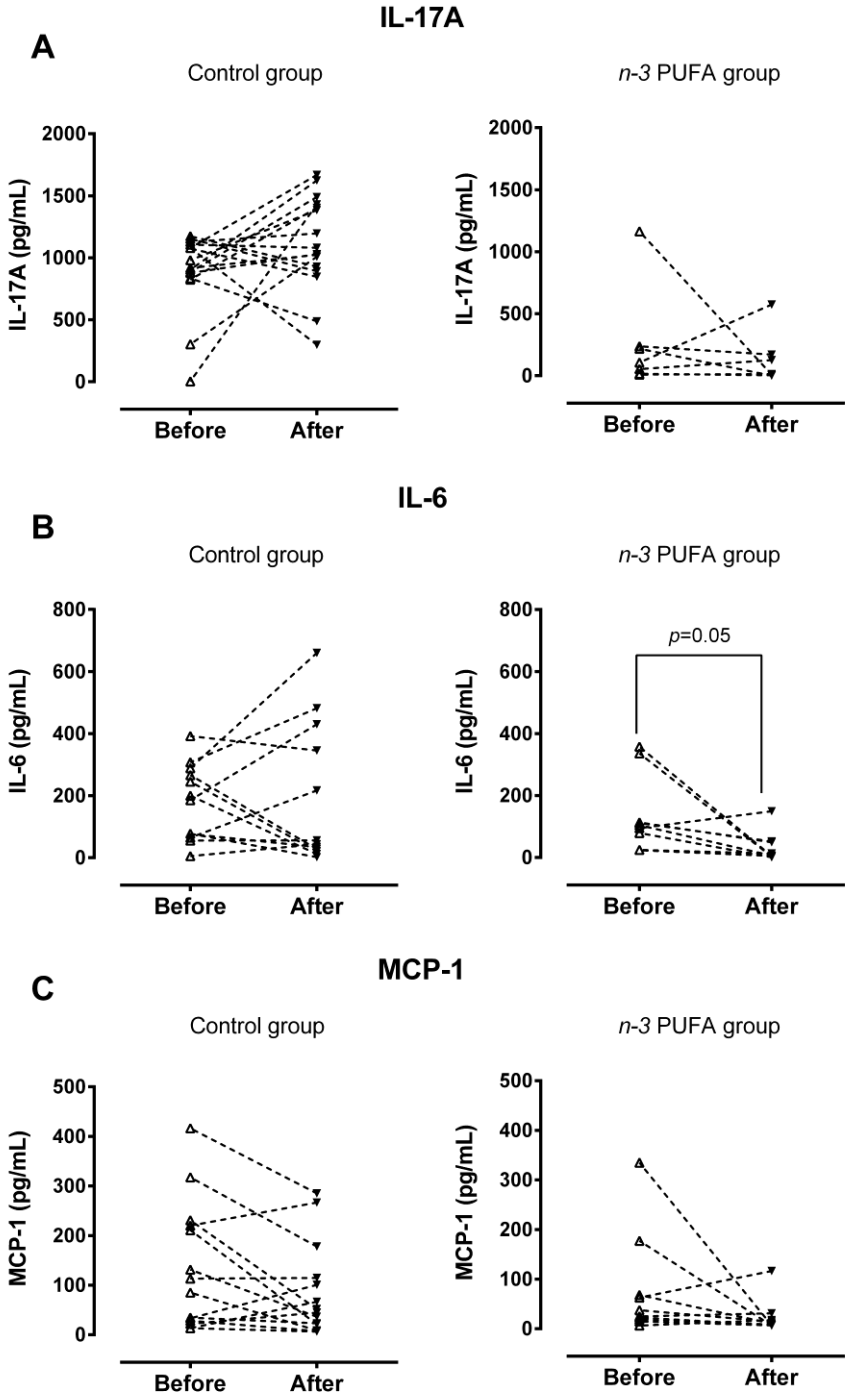


Figure 5.14. Levels of pro-inflammatory cytokines and chemokines secreted by PBMCs upon PMA/ionomycin stimulation in young individuals following regular (Control group) or *n-3* polyunsaturated acid (PUFA)-enriched hen egg (*n-3* PUFA group) consumption. No significant changes were found for IL-17A (A) or MCP-1 (C), while IL-6 (B) production was significantly decreased in *n-3* PUFA group. PBMC—peripheral blood mononuclear cells; IL-17A—Interleukin 17A; IL-6—Interleukin 6; MCP-1—Monocyte Chemoattractant Protein-1. Paired t-test; $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

5.10. Correlation analysis based on Tregs and Th17

Correlation analysis was performed to assess relationships between biochemical parameters (hsCRP, fasting lipid profile), serum concentrations of eicosanoids and resolvins, PBMC-derived cytokines and chemokines, and peripheral Treg and Th17 lymphocyte frequencies.

In the Control group, rates of peripheral blood CD25/Foxp3-expressing lymphocytes were positively associated with the rates of peripheral blood IL-17 producing CD4 T cell subset ($r = 0.326$; $p = 0.035$) and inversely associated with the serum fasting cholesterol levels ($r = -0.332$; $p = 0.036$) and BMI ($r = -0.441$; $p = 0.0036$). However, after further identification of nTreg ($CD25^{+}Foxp3^{high}$) and recently activated T cells ($CD25^{+}Foxp3^{neg/low}$) among CD25/Foxp3-expresing T helper cells, associations with $CD4^{+}IL\text{-}17^{+}$ T cell pool were lost. Interestingly, the BMI status was still inversely related to nTreg ($r = -0.535$; $p < 0.0001$) but positively associated to the rates of recently activated T helper cells ($r = 0.399$; $p = 0.009$). There was a positive correlation between prostaglandin E2 and E3 serum concentrations ($r = 0.819$; $p = 0.0002$), while there was a significant negative correlation found between hsCRP level and prostaglandin E2/E3 ratio ($r = -0.438$; $p = 0.006$).

In the *n-3* PUFAs group, peripheral nTreg lymphocytes were negatively associated with non-Th17 IL-17A secreting T helper cells ($CCR6^{-}IL\text{-}17^{+}$; $r = -0.613$, $p < 0.0001$) and PBMC-derived TGF-β1 ($r = -0.616$, $p = 0.002$), while recently activated T helper cells were positively associated with $CCR6^{-}IL\text{-}17^{+}$ ($r = -0.591$, $p = 0.0003$) and PBMC-derived TGF-β1 ($r = -0.522$, $p = 0.012$). Interestingly, rates of peripheral non-Th17 lymphocytes were positively associated with PBMC-derived IL-17A levels ($r = 0.456$, $p = 0.032$), while there was no association to 'real' Th17 cells. Leukotriene B5 serum concentrations negatively correlated with concentrations of PBMC-derived IL-17A ($r = -0.743$, $p = 0.029$). HDL-cholesterol levels negatively correlated with hsCRP ($r = -0.408$, $p = 0.042$).

5.11. Forearm skin microvascular endothelium-dependent and endothelium-independent vasodilation

Consumption of *n-3* PUFAs enriched hen eggs significantly improved ACh-induced dilation (AChID) (an increase of 14.4%) (Figure 5.15. A) of forearm skin microcirculation compared to baseline measurement within the *n-3* PUFAs group. Contrarily, consumption of regular hen eggs did not induce any significant change in AChID of forearm skin microcirculation compared to baseline in the Control group. There was no significant difference in AChID at baseline or following respective hen eggs consumption between the groups. SNP-induced

dilation (SNPID) (Figure 5.15. B) was similar between baseline and regular or *n-3* PUFAs eggs consumption within the groups, and similar between Control and *n-3* PUFAs groups.

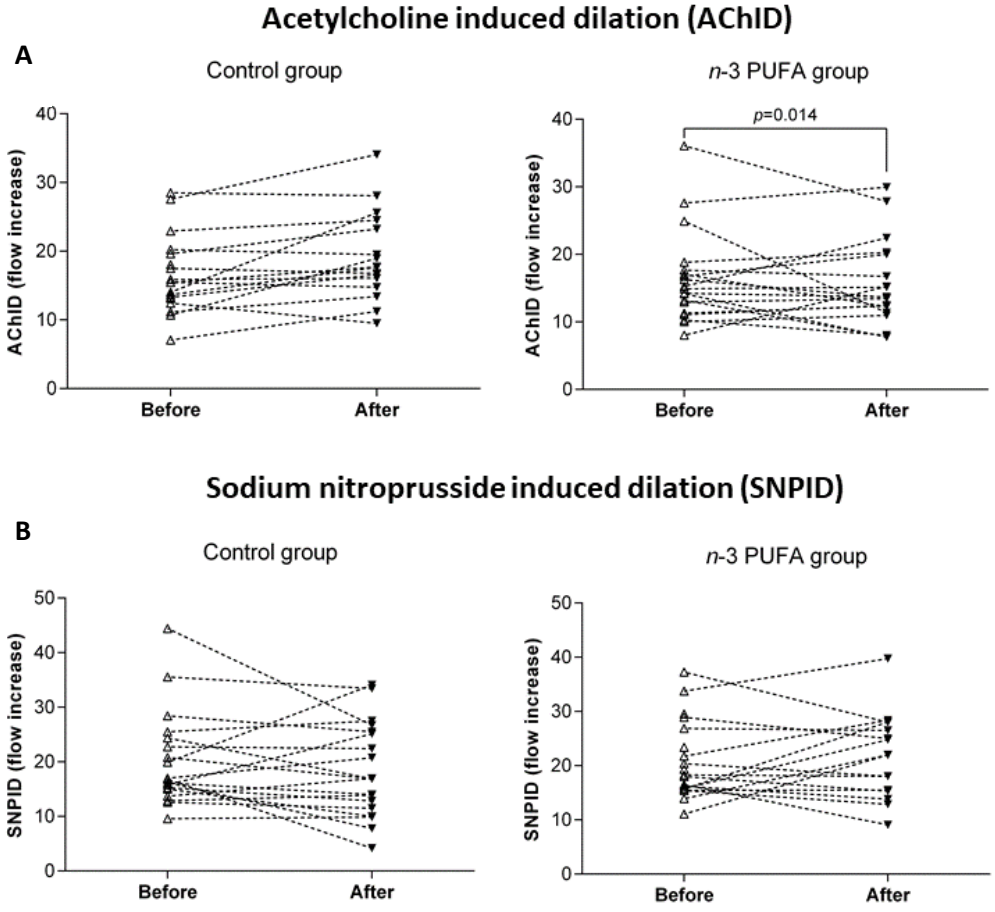


Figure 5.15. The effect of the three-week regular (Control group) and *n-3* PUFAs (*n-3* PUFAs group) enriched hen eggs consumption on skin microvascular endothelium-dependent and endothelium-independent vasodilation in young healthy individuals. (**A**) Acetylcholine-induced dilation (AChID), and (**B**) Sodium nitroprusside-induced dilation (SNPID). AChID and SNPID are expressed as flow increase following ACh or SNP administration compared to baseline flow. Data are presented as arithmetic mean (standard deviation, SD). *n-3* PUFA- *n-3* polyunsaturated fatty acids. Paired t-test; $p < 0.05$ difference between before and after within each group (Control or *n-3* PUFA).

5.12. Dietary habits of the study population

Adherence of participants to the guidelines recommended by WHO in dietary habits of study cohort is shown in Table 5.7. (Food Based Dietary Guidelines, FBDG) [26,27]. Majority of study population consumed less than five recommended meals per day with no major differences between sexes or residence. Based on entire study population, 72% of participants

failed to adhere to the guidelines for fruits and 76% for fish daily intake, while 64% of participants respected recommendations for protein, 86% for carbohydrate and 81% for vegetable intake. Daily intake of fluids, dairy products, whole grains, and nuts was equally distributed between participants who followed guidelines and those that failed to do so. The sstudy population consumed more than recommended sweets and sodas, therefore they failed to adhere to the guidelines. More than half of the study population failed to drink enough fluids to meet the guidelines.

Large percentage of women consumed recommended amount of vegetables and carbohydrates, while men mainly adhered to the guidelines on protein intake. Men also followed guidelines on carbohydrates and vegetable intake. Only 15% women and 33% men consumed satisfactory quantities of fish, while 21% women and 33% men included recommended amount of fruits in their diet. Overall, men had significantly higher protein intake ($p = 0.02$) compared to women. According to residence, both groups mainly adhered to the guidelines for vegetable, carbohydrate and protein intake. Only 27% native and 26% non-native students included recommended amount of fruits in their diet. Fish was also among foodstuffs that was under-consumed in both groups. Native students consumed sweets more than recommended daily intake.

Three or less meals per day was consumed by 56% of study population, with major part of that being women ($p = 0.033$) while men consumed at least four meals per day ($p = 0.027$). Native students mainly consumed three or less meals per day, while non-natives students consumed five or more meals or at least four meals with prevailing three or less meals per day. More than half of non-native students consumed at least four meals per day which is not the case in native group. When comparing dietary habits between Control and *n-3* PUFA groups, we found no significant differences. Both groups mainly adhered to the recommended guidelines for protein, carbohydrate and vegetable intake, while they failed to adhere with the guidelines regarding intake of fish.

5.13. Correlation analysis based on dietary habits

Correlation analysis was performed between biochemical parameters, anthropometric parameters, and dietary habits of the study population. Based on study population, a significant positive correlation between haematocrit values and frequency of vegetable consumption (r =

0.594, p = 0.0004) was found. A significant positive correlation was also found between the number of meals consumed and the frequency of fruit consumption (r = 0.534, p = 0.0008).

According to residence, in non-native group a significant negative correlation was found between triglyceride levels and frequency of fruit (r = −0.556, p = 0.04) or vegetable (r = −0.685, p = 0.009) consumption, as well as between age of participants and frequency of sweets consumption (r = −0.569, p = 0.03). In native group, age of participants correlated positively to cholesterol levels (r = 0.544, p = 0.01).

Women's BMI correlated positively to cholesterol (r = 0.819, p = 0.0001) and LDL-cholesterol (r = 0.856, p < 0.0001) levels, as well as age of female participants and their body weight (r = 0.643, p = 0.003) did. Men's triglyceride levels correlated negatively to frequency of vegetable consumption (r = −0.753, p = 0.0005) which was also found in high-cholesterol subgroup (r = −0.713, p = 0.003). Additionally, in high-cholesterol group, positive correlation was found between number of meals and frequency of carbohydrates (r = 0.616, p = 0.01), dairy (r = 0.546, p = 0.03), sweets (r = 0.673, p = 0.006), and coffee (r = 0.552, p = 0.03) consumption.

According to our experimental groups, in both Control and *n-3* PUFAs group, age of participants is positively correlated to cholesterol levels (Control: r = 0.549, p = 0.003; *n-3* PUFAs: r = 0.571, p = 0.041). There was also a significant positive correlation between number of meals and the amount of fruits consumed (Control: r = 0.698, p = 0.012; *n-3* PUFAs: r = 0.531, p = 0.034). In *n-3* PUFAs group, cholesterol and LDL-cholesterol serum levels correlated negatively with vegetable intake (r = -0.665, p = 0.013 and r = -0.613, p = 0.026, respectively), while the age of participants positively correlated with fish intake (r = 0.619, p = 0.01). In Control group, erythrocytes, haemoglobin and haematocrit levels were positively correlated with nuts intake (r = 0.515, p = 0.032; r = 0.549, p = 0.01; r = 0.595, p = 0.005). Sweets intake positively correlated with juice intake in *n-3* PUFAs group (r = 0.566, p = 0.022) and with number of consumed meals in Control group (r = 0.733, p = 0.001).

5.14. Analysis of covariance (ANCOVA)

To test the possibility of confounding effects of age, BMI status, serum lipids, hsCRP and sex on primary (frequencies of peripheral Tregs/Th17 lymphocytes) and secondary study outcomes (lipid mediators' concentration, cytokine profile and microvascular reactivity) (dependable variable) a series of ANCOVA (Analysis of covariance) was performed. However, no statistically significant cofounding effect was detected.

5.15. Harms

There were no adverse effects of interventions on participants in clinical terms. Although, there were difficulties concerning eating three hard-boiled eggs every day for three weeks, as some participants found it difficult. Participants that were lost from the follow-up due to personal reasons didn't show up for the second appointment despite numerous notifications from the researcher. Since the missed the follow-up date, they were entirely excluded from final analysis.

Table 5.7. General dietary habits of the study population

Parameter	Study Population	Women	Men	*p*	Native	Non-Native	*p*	Control	*n-3* PUFAs	*p*
Average meals per day (SD)	3.49 (0.77)	3.32 (0.75)	3.66 (0.77)	-	3.36 (0.66)	3.67 (0.89)	-	3.43 (0.75)	3.69 (0.87)	-
Five or more meals per day	10.81	10.53	11.11	0.954	4.55	20	0.137	4.76	25	0.074
At least four meals per day	32.43	15.79	50	0.027 *	31.82	33.33	0.923	42.86	18.75	0.104
Three or less meals per day	56.76	73.68	38.89	0.033 *	63.64	46.67	0.306	52.38	56.25	0.815
Adherence to the recommended guidelines for daily intake of fruit units	27.03	21.05	33.33	0.400	27.27	26.67	0.967	23.81	37.5	0.367
Adherence to the recommended guidelines for daily intake of protein units	64.86	42.11	77.78	0.027 *	59.09	66.67	0.641	61.9	68.75	0.666
Adherence to the recommended guidelines for daily intake of carbohydrate units	86.49	78.95	83.33	0.733	86.36	86.67	0.979	80.95	81.25	0.982
Adherence to the recommended daily fluid intake guidelines	48.65	42.11	55.56	0.413	50	46.67	0.842	57.14	43.75	0.419
Adherence to the recommended guidelines for daily intake of dairy products	51.35	52.63	52.94	0.872	45.45	60	0.385	52.38	56.25	0.815
Adherence to the recommended guidelines for daily intake of vegetable units	81.08	94.74	66.67	0.063	90.91	66.67	0.065	85.71	75	0.294
Adherence to the recommended guidelines for daily intake of whole grains	43.24	42.11	44.44	0.886	31.82	60	0.089	47.62	43.75	0.815
Adherence to the recommended guidelines for daily intake of fish	24	15.8	33.3	0.214	22.73	26.67	0.784	23.81	18.75	0.711
Adherence to the recommended guidelines for daily intake of nuts	45.95	36.84	55.56	0.254	18.18	26.67	0.538	42.86	43.75	0.957
Failure to adhere to the recommended guidelines for daily intake of sweets	75.68	73.68	77.78	0.772	86.36	60	0.066	19.05	31.25	0.391
Failure to adhere to the recommended guidelines for daily intake of sodas	51.35	42.11	61.11	0.248	59.09	40	0.254	42.86	62.5	0.236

Results are expressed as percentage (%) of participants regarding consumption of food/beverages in terms of recommended guidelines. SD – standard deviation; PUFAs – polyunsaturated fatty acids. Chi-square test. Significance level $p < 0.05$ *. Women vs. Men; Native vs. Non-native; Control vs. *n-3* PUFAs.

6. DISCUSSION

Beneficial effects of *n-3* PUFA supplementation are well documented in the literature, but these studies usually involved *n-3* or *n-6* PUFAs addition to various cell cultures (79,143,144) or, in the case of human studies, oil capsules were mostly given to patients with certain comorbidities (145–147). The salient findings of the present study are differential effects of the regular hen egg and *n-3* PUFA-enriched hen egg consumption on the serum levels of lipid mediators, representation of peripheral T helper cell subsets (recently activated T helper cells, nTreg, Th17, and non-Th17 IL-17A secreting T helper lymphocytes), and their functional capacity for cytokine secretion.

Both diets significantly altered systemic levels of pro-inflammatory, and inflammation resolving lipid mediators; however, only the *n-3* PUFAs group showed a significant shift towards anti-inflammatory prostanoids and increased levels of pro-resolvins. Both study groups showed reduced frequencies of peripheral nTreg lymphocytes and decreased rates of peripheral Th17 cells. Their functional capacity for cytokine secretion was significantly altered only in the *n-3* PUFAs group in terms of increased anti-inflammatory TGF-β1and IL-10 secretion, and reduced pro-inflammatory IL-6 and INFγ secretion.

We established that this particular study population normally consumed extremely low amounts of *n-3* PUFA-rich food such as fish and nuts (148). Also, through diet analysis, we found no significant differences regarding food or beverage intake between the Control and *n-3* PUFAs group. Therefore, we can assume that the main effect of *n-3* PUFAs in the present study came from the consumption of *n-3* PUFAs enriched hen eggs. Assessment of serum fatty acid profile before and after finishing respective dietary protocols confirmed excellent compliance of participants and significant changes to the fatty acid composition and *n-6*/*n-3* PUFAs ratio (113).

6.1. Effects of *n-3* PUFAs enriched hen eggs on lipid mediator biosynthesis, cytokine secretion, and T cell differentiation

In healthy individuals, nTreg cells represent around 2–10% of the total T helper cells pool, with slightly lower frequencies found in peripheral blood (149,150). Treg cells have shown noticeable therapeutic potential in terms of their expansion in order to control autoimmune and inflammatory disorders or, at the other side of the therapeutic spectra, their depletion to promote T effector cell function and eliminate cancer (150,151). However, most recent studies reported

their substantial phenotypic and functional variability beyond sole immunosuppression (152–156). Decreased rates of peripheral Treg cells following *n-3* PUFA-enriched functional food consumption in our study can be explained by the previously reported inhibitory effect of dietary DHA on both migratory and suppressive Treg cells functions which was proven as dose-dependent in vitro and in vivo (100 μmol/L) (151,157). Interestingly, a diet rich in *n-3* PUFAs upregulates expression of Treg cell markers, TGF-β1, and Foxp3 (151,157), which is opposite to our results, where we found negative correlation between TGF-β1 supernatant concentration and the frequencies of peripheral Tregs.

Lipid mediators and cytokines examined in the present study (secreted predominantly by T cells) act synergistically to constrain inflammation and to promote resolution after pathogen elimination, predominantly through refining T cell functions (61,69,70,158). Physiological role of cytokines is to act as signalling molecules and humoral regulators of cell activation, differentiation, proliferation, and cytokine production, including chemokines, interleukins (IL), growth factors, and interferons (IF) (69–71). T lymphocytes manage cell immunity through activation of phagocytes and by promoting the destruction of pathogens and infected cells while also producing cytokines with specific effects on the other immune cells (61,66). Here we report significantly altered cytokine secretion by T lymphocytes following *n-3* PUFAs supplementation, characterized by cytokine profile towards reduced inflammation.

TGF-β1 and IL-10 cytokines have a central role in maintaining the immune balance by limiting immune reactions and promoting additional inducible regulatory T cell differentiation (iTreg) (159), thus preventing uncontrolled inflammation and/or autoimmunity (160–163). Rosa et al. (2012) (164) demonstrated that tissue levels of TGF-β1 were increased in rats following EPA and DHA administration through fish oil. Similarly, increased TGF-β1 mRNA and protein secretion in colonic cell lines in response to commensal bacteria *Lactobacillus gasseri* was enhanced by pre-treatment with EPA (165). This is in the line with present finding of enhanced capacity of activated PBMCs for TGF-β1 secretion following consumption of *n-3* PUFA-enriched eggs. Interestingly, levels of secreted TGF-β1 negatively correlated with the frequency of peripheral nTreg cell population in the *n-3* PUFAs group. Even though TGF-β1 exhibits both anti- and pro-inflammatory properties (166,167), the latter is manifested only in combination with IL-6 (promotion of Th17 differentiation) (168) which was decreased in the *n-3* PUFAs group.

Pro-inflammatory cytokines have a detrimental role in the differentiation and survival of Th17 cells as well as in mediating Th17 effector functions. In addition, previous research suggests that they can be downregulated by increased RvE1 production (158), which we have partly confirmed by the observation that IL-6 production by peripheral lymphocytes was reduced, in parallel with increased systemic levels of RvE1 in the *n-3* PUFAs group. In addition, there was decreased prevalence of peripheral Th17 lymphocytes following hen eggs consumption, independently of their *n-3* PUFA enrichment.

Inhibitory effects of supplemental ALA and DHA on the COX pathway were previously documented in human umbilical vein endothelial cells (HUVECs) (79) and bovine aortic endothelial cells (BAECs) (143,144). In addition, Araujo et al. (2019) (79) failed to find a significant association between increased EPA administration and production of PGE3 in HUVECs, suggesting ALA/DHA-specific effects on the COX pathway. A possible explanation was given by Malkowski et al. (2001) (169), who described decreased flexibility of EPA when bound to COX activity site due to an additional double bond which results in low oxygenation and enzymatic conversion to PGE3. This could also explain the results of the present study in which we were not able to prove significant effects of *n-3* PUFAs supplementation through functional food on systemic levels of both pro- and anti-inflammatory (PGE2 and PGE3, respectively) lipid metabolites derived via the COX pathway. Even though there were no significant changes observed in PGE2 and PGE3 serum concentrations individually, there was a significant decrease in endpoint PGE2/E3 serum concentration ratio in the *n-3* PUFAs group, compared to baseline ratio, which indicates a slight shift in favour of anti-inflammatory metabolites.

Further finding of this study were significantly increased serum concentrations of inflammation resolving five-series of leukotrienes/LTB5 in both groups at the end of dietary protocols and increased levels of pro-inflammatory four-series of leukotrienes/LTB4 in the Control group which indicates that the activity of the LOX pathway is preferred over the production of COX pathway metabolites from EPA. It has been previously shown that supplemental DHA increases the production of LOX pathway metabolites and resolvins in HUVECs (79). This information and the fact that DHA can be retro-converted to EPA following supplementation (170,171) support our finding of increased production of RvE1 in the *n-3* PUFAs group following diet protocol. Both E- and D-series resolvins have a crucial role in response to acute inflammation

(172), especially in allergic response and asthma (158,173), and neuroinflammation (80,174), and exhibit therapeutic potential for the treatment of inflammatory bowel disease (175).

Haworth et al. (2008) (158) reported that increased RvE1 decreases IL-17A concentration by ~70% in an animal model. This effect, however, could not be observed in our study population. As elaborated in the Results Section, in our cohort of healthy young adults, a significant difference was observed regarding IL-17A levels in the Control group, both prior and after the protocol, when compared to the *n-3* PUFAs group. Therefore, we performed series of correlation analyses between the IL-17A levels and anthropometric/biochemical parameters which could not explain the baseline differences in IL-17A production between the groups. Ongoing acute infection or underlying immune-mediated inflammatory disorders were eliminated based on the medical records and blood tests. However, a significant positive association was observed between RvE1 and systemic levels of IL-10, IL-22, IL-6, and IL-9 only in the *n-3* PUFAs group. In addition, the functional capacity of lymphocytes to produce IL-17A was inversely related to systemic levels of pro-resolving LTB5 in the *n-3* PUFAs group, which is, alongside RvE1, also an EPA-derived mediator. Furthermore, the inhibitory effect of RvE1 on IL-6 production was previously reported in human neutrophil cell lines (176), and IL-6 was another cytokine measured in our study, namely, IL-6 secretion by lymphocytes upon PMA/ionomycin activation was significantly decreased in *n-3* PUFAs group at the end of the dietary protocol.

6.2. Effects of *n-3* PUFAs enriched hen eggs on microvascular endothelial function

This is the first randomized, double-blind, placebo-controlled interventional study that investigated the effect of *n-3* PUFAs enriched hen eggs consumption on endothelial microvascular function, and their anti-inflammatory potential in the young healthy individuals. The novel finding of this study regarding microcirculation is that consumption of *n-3* PUFAs enriched hen eggs improved endothelium-dependent vasodilation of skin microcirculation independently of BP or body composition and fluid status changes in healthy individuals. Observed enhancement in endothelial function was accompanied by a previously mentioned significant increase in IL-10 (anti-inflammatory cytokine), and a decrease in INFγ (pro-inflammatory cytokine) serum protein concentration.

On the other hand, consumption of regular hen eggs did not significantly affect microvascular endothelial function but significantly increased IL-17A (pro-inflammatory cytokine) and

VEGF-A (vascular endothelial growth factor), as well as IL-10 serum protein concentration. Thus, our study suggests that changes in the balance between pro-and anti-inflammatory cytokines could be a potential moderator of enhanced endothelial function following increased consumption of *n-3* PUFAs in the form of functional food in a healthy population. Furthermore, consumption of hen eggs per se, either regular or *n-3* PUFAs, even three pieces a day for three weeks, did not have any harmful effects on metabolic (e.g., serum lipid profile; liver function not assessed), inflammatory or functional vascular parameters (LDF measurement of forearm skin microvascular reactivity) in participants of the study and did not increase CV risk in healthy subjects.

n-3 PUFAs have the potential to modify vascular endothelial function by being incorporated into endothelial cell membrane phospholipids (at the expense of *n-6* PUFAs, such as arachidonic acid), resulting in modulation of different factors which determine endothelial function itself (e.g., synthesis of vasoactive mediators derived from the endothelium, oxidative stress level, endothelial activation and inflammation, etc.) (177). The present study demonstrated a significant decrease in serum arachidonic acid at the expense of increased *n-3* PUFAs, which may be a source for anti-inflammatory and vasoprotective eicosanoids, contributing to enhanced microvascular response, as observed.

A number of multiple epidemiological, experimental, and clinical studies in patients with increased CV risks or already existing CV diseases, such as hypertriglyceridemia, peripheral artery disease, diabetes mellitus type 2, healthy smokers, etc. reported that *n-3* PUFAs may reduce the risk of CV diseases, at least in part, by improving vascular function (94,178,179). A detailed overview of these studies in CV patients was recently presented in two reviews by Zehr and Walker (94), and Du et al. (180), respectively. Importantly, referenced studies utilized pharmacological supplementation of *n-3* PUFAs in the form of capsules, but not functional food.

The present study demonstrated, for the first time, that peripheral microvascular endothelial-dependent vasodilation, but not endothelial-independent is improved in response to *n-3* PUFAs enriched hen eggs consumption in the young healthy individuals. Our results are in agreement with improved endothelium-dependent vasodilation following iontophoretic applications of ACh and SNP in healthy individuals who took fish oil supplementation (EPA + DHA) for 8 months (181). In contrast, a randomized cross-over designed study of three diets, each lasting for 4 weeks reported that postprandial forearm skin post-occlusive reactive hyperaemia was

significantly increased only after monounsaturated fatty acids-rich Mediterranean diet, but not low-fat diet enriched in ALA in 20 healthy men (182). On the other hand, previously we reported that the young healthy participants who consumed 777 mg of *n-3* PUFAs/day in enriched eggs for 3 weeks had improved skin microvascular reactivity in response to PORH (183). Altogether, these results suggest that a threshold dose of *n-3* PUFAs in food/serum needs to be achieved to observe effects in microcirculatory vasodilator responses.

Still, mechanisms mediating endothelium-dependent vasodilation in both micro- and macro-vasculature, both in patients and healthy individuals, are not known. It has been suggested that *n-3* PUFAs may improve endothelial function by increasing the bioavailability of main endothelial vasodilator NO (increasing NO production and/or decreasing oxidative stress level) (Figure 1.4. a), and/or by changing the expression/activity of other endothelial-derived vasoactive mediators, in particular, metabolites of arachidonic acid (e.g., COX metabolites PGI2, PGH2, TXA2; CYP450 metabolites epoxyeicosatrienoic acids, EETs; and 20-hydroxyeicosatetraenoic acid, 20-HETE) (Figure 1e) (2,103,106,108,184,185).

Additionally, inflammatory stimuli, such as cytokines and chemokines, can activate the endothelium, as an early event in the initiation of adhesion of monocytes and other leukocytes to the endothelium, preceding their infiltration and extravasation to the site of injury (109). Activated endothelium interacts with blood leukocytes, which is characterized by increased expression of cell adhesion molecules (e.g., ICAM-1, and VCAM-1), and increased secretion of chemokines (e.g., LAP, SDF-1α) and pro-inflammatory cytokines (e.g., IL-6 and TNF-α), and with the progression of inflammation with a release of a variety of vascular growth factors (VEGF-A and VEGF-D), which under certain circumstances may act as angiogenic mediators that promote atherogenic plaque progression and instability (Figure 1c,d). On the other hand, VEGF is important for endothelial integrity and vascular function, since it is a potent growth factor for endothelial cells and inducer of angiogenesis (110). A previous study by Wu and colleagues showed that fish-oil supplementation enhanced mobilization of bone-marrow-derived EPCs independently of eNOS genotype in patients with moderate risk for CV diseases (105). In the present study, SDF-1 serum levels were unaffected by diets, suggesting there were no alterations in EPCs numbers in the blood, possibly due to non-existent or low risk for CV diseases in our cohort.

There is solid proof that *n-3* PUFAs can decrease (or prevent) endothelium–leukocyte interactions by decreasing the expression of cell adhesion molecules (ICAM, VCAM, and/or

E-selectin) (109,111). Few animal feeding studies that included rats and mice, all reported reduced VCAM-1 expression after feeding with DHA and/or EPA. In healthy individuals, *n-3* PUFAs may reduce levels of soluble adhesion molecules (e.g., reduced sICAM-1 after supplementation of 2–6.6 g of EPA + DHA daily for 8–12 weeks; reduced sVCAM-1 after supplementation of 1 g EPA + DHA for 12 weeks) (112,186,187). However, there are also reports on no change (e.g., no change in sICAM-1 and sVCAM-1 after 1.35, 2.7, or 4.05 g EPA daily for 12 weeks in healthy young and older men) (188), or even increase in sCAMs following *n-3* PUFAs intake (e.g., increased sVCAM-1 after 1.3 g DHA and 700 mg EPA daily for 8 weeks in healthy men and women (189); increase in sICAM-1 after 1.37 g EPA and 240 mg DHA daily for 8 weeks in healthy individuals) (190). The present study demonstrated that consumption of *n-3* PUFAs enriched hen eggs (but also of regular eggs) did not induce a significant change in sICAM-1 and sVCAM-1 levels in healthy individuals. Thus, we may exclude endothelial activation as a mechanism of action.

As mentioned earlier, *n-3* PUFAs may be able to influence the levels of circulatory pro- and anti-inflammatory molecules (cytokines, chemokines, and growth factors) directly. *n-3* PUFAs intake is associated with reduced concentrations of acute-phase protein reactants (CRP), pro-inflammatory eicosanoids, cytokines, chemokines, and other inflammation biomarkers (111). Moreover, in addition to inhibiting pro-inflammatory mediators, some animal studies also report that *n-3* PUFAs may reciprocally increase the concentration of the anti-inflammatory cytokine IL-10 (191,192). While the results on the effect of *n-3* PUFAs on inflammatory molecules in population with increased CV risk remain inconclusive, the majority of studies in healthy individuals demonstrated that *n-3* PUFAs did not significantly affect the serum concentration of any of the cytokines (e.g., IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α, and INFγ) and chemokines (e.g., CCL2, CCL3, CCL5, and CCl11) measured in these studies (111).

On the other hand, fish oil supplements to healthy individuals decreased the production of TNF-α, IL-1β, and IL-6 by endotoxin-stimulated monocytes or mononuclear cells (193). The potential reason may be an insufficient dose of provided *n-3* PUFAs (< 2 g EPA + DHA), together with basally low levels of cytokines and chemokines in serum of healthy participants. The latter is supported by the fact that the anti-inflammatory effects of *n-3* PUFAs have been repeatedly confirmed in studies employing individuals suffering from autoimmune or inflammatory disorders (194). To our knowledge, the present study is the first study to report

that functional food rich in *n-3* PUFAs decreased INFγ and increased IL-10 serum concentration in healthy individuals. Furthermore, consumption of regular hen eggs for three weeks increased IL-10 (less than observed in the *n-3* PUFA group) but also increased IL-17A and VEGF-A serum concentration in healthy individuals. These results may indicate that unlike the uptake of *n-6* PUFAs, which leads to an increase in the concentration of pro-inflammatory cytokines (such as IL-17A) and angiogenic growth factors (VEGF-A), uptake of *n-3* PUFAs may contribute to maintaining a favorable anti-inflammatory milieu in healthy individuals.

6.2.1. Effects of n-3 PUFAs enriched hen eggs on serum lipid profile

Supplementation with EPA and DHA can reduce serum lipids, especially triglycerides in hyperlipidemic individuals (195,196). In the present study, consumption of *n-3* PUFAs enriched hen eggs did not induce a significant change in serum lipid profile compared to baseline measurement; triglycerides level decreased by approximately 8% ($p > 0.05$). At the end of the protocol, total cholesterol and HDL cholesterol levels were slightly higher in the Control group than in *n-3* PUFAs group. However, this difference is statistically rather than clinically relevant, since both total and HDL cholesterol levels were within the normal reference range. Thus, we conclude that the consumption of 3 eggs per day does not pose a risk for unfavorable changes in serum lipid profile for healthy individuals.

Results of our study are consistent with the results of a meta-analysis by Leslie et al., who reported that only very high doses of EPA and/or DHA-enriched food sources (≥4 g/day) have the ability to reduce serum triglycerides (by 9–26%) in normolipidemic to borderline hyperlipidemic and otherwise healthy individuals (197). Several studies demonstrated that consumption of *n-3* PUFAs enriched hen eggs has the potential to decrease serum triglycerides (but not total, HDL and LDL cholesterol levels) in healthy individuals (183,198–200), but the lack of consistent study design for elevating *n-3* PUFAs through dietary modifications, particularly functional food, continues to be a limitation in the field. Interestingly, it has been suggested that higher doses of EPA and DHA may be required to decrease serum triglycerides then are needed to improve endothelial dysfunction (94), and furthermore, the endothelial function may be improved in the absence of a decrease in triglycerides (94), which is in accordance with the present results.

6.2.2. Effect of n-3 PUFA enriched hen eggs on blood pressure level, body composition, and body fluid status

n-3 PUFAs supplementation may induce clinically relevant BP decrease in patients with untreated hypertension (201), in patients with essential hypertension (202,203), and in mildly hypercholesterolemic but normotensive individuals (204). However, such a significant effect of *n-3* PUFAs in lowering BP level was not observed in normotensive individuals (205,206), and was not observed in the present study. For example, supplementation of *n-3* PUFAs 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) in normotensive individuals (207–209) did not significantly change BP. Still, our earlier study demonstrated that consumption of *n-3* PUFAs enriched hen eggs (777 mg/day) for three weeks significantly reduces BP in healthy normotensive individuals, similar to a study of Oh et al. (198). Surprisingly, BP level significantly decreased in healthy individuals who consumed three regular hen eggs for three weeks. Thus, a decrease in BP could not be attributed to *n-3* PUFAs in the present study.

The influence of *n-3* PUFAs intake on body composition is unclear. One systematic review of clinical trials did not find any important effect of dietary *n-3* PUFAs intake on body weight in mostly overweight and obese adults (210). Another meta-analysis of only randomized clinical trials that explicitly examined body composition-related measures and used only *n-3* PUFAs of fish provenience reported minimal non-significant change in body weight (590 g) and other body composition-related outcomes (such as BMI, body fat percentage, and waist circumference) between intervention and control groups (211). Even consumption of seven *n-3* PUFAs enriched hen eggs per week for 24 weeks did not cause any significant change in body weight compared to baseline in healthy volunteers (212). Consistently, in the present study, we did not observe any significant change in measured body composition parameters and body fluid status following consumption of *n-3* PUFAs enriched hen eggs in healthy lean individuals. Interestingly, body fat mass significantly increased, and fat-free-mass, as well as total body water, significantly decreased following consumption of regular hen eggs in the Controls, an observation for which the mechanism is still not clear.

Since in the present study *n-3* PUFAs consumption was not accompanied by changes in serum lipid profile, BP level, body composition, and body fluid, it seems plausible that improved microvascular endothelial function due to *n-3* PUFAs intake is a consequence of the unique effect of increased serum *n-3* PUFAs concentration, decreased *n-6*/*n-3* PUFAs ratio, and

favorable anti-inflammatory milieu rather than systemic hemodynamic and/or obesity-related changes in healthy individuals.

6.3. Dietary habits of the study population

A dietary assessment questionnaire in the present study was designed to examine participants' dietary habits but also to provide insights into differences between sexes and differences in habits of students in regard of residence status in the city of studying. Dietary habits in Croatian students were addressed earlier (213,214), but those were mainly large observational studies focusing on nutritional status rather than its relation to anthropometric and biochemical parameters of a small, isolated population. Thus, in the present study, we have performed additional analyses on these parameters that reflect cardiovascular status in relation to dietary habits.

The main findings of the present study were that our participants (mainly students) skipped meals and had quite an irregular intake of carbohydrates, sweets, and sodas. Furthermore, a major part of men followed recommendations regarding protein intake, which may characterize men as "protein type". On the other hand, contrary to popular opinion that women are "carb type" and usually find a resort in refined sugars, almost all women in our study adhered to guidelines on carbohydrate and vegetable intake. Natives who live in family homes consumed vegetables regularly, while non-natives relied on whole grains and proteins.

Results of the present study suggest that our study population typically consumes extremely low amounts of *n-3* PUFAs rich foods (fish and nuts), which is rather discouraging, considering the known beneficial effect of polyunsaturated fatty acids on microvascular reactivity, oxidative stress reduction, and lipid profile repairmen (14,183,215,216).

According to WHO, the basics of good dietary habits are at least five portions of fruit and vegetables per day (excluding starchy roots), total fat intake reduced to less than 30 % (preferably saturated and trans-fats replaced with unsaturated), reduced salt intake to less than 5 g per day and intake of free sugars reduced to < 5 % through five or more meals per day (three main meals and two snacks) (217,218). In the present study, students' average intake of three meals per day is below recommended intake. Only a small percentage of medical students consumed five or more meals daily and those were prevalently non-native students.

Young adults, specifically the university population, have been shown to be prone to poor dietary choices (219,220). The quality of lifestyle/food consumed varied greatly between those studying away from home and those studying in home residence; nevertheless, often both groups tend to reach for junk food in a stressful environment (121). Therefore, native students in our study expectedly consumed more vegetables in the comfort of a family home, while non-native students had more discipline in taking regular daily meals and consumed fewer sweets as they got older.

Unhealthy dietary choices and nutrition, physical inactivity, cigarette smoking, and heavy alcohol consumption are harmful habits contributing to the increasing prevalence of obesity, cardiovascular disease, type 2 diabetes, hypertension, and several types of cancer in all industrialized countries (116,221–223). The most common unhealthy dietary habits among students are irregular/skipped meals paired with fried food consumption, low fruit and vegetable intake, and frequent snacks in between meals (224), which is in accordance with our results. Male students are usually shown more prone to being overweight and obese when compared to female students (225,226). Results of our present study point in the same direction since there was a significant negative correlation found between triglyceride levels and vegetable/fruit consumption of our study participants.

Although in our research elevated cholesterol/LDL-cholesterol levels and weight problems were correlated positively to the age of participants, problems like obesity accompanied by disorders such as insulin resistance and hyperlipidemia usually affect individuals since childhood; therefore, it is never too early to take preventive measures (227).

Hyperlipidemia is one of the main causes of increased risk for cardiovascular diseases. Hypercholesterolemia and hypertriglyceridemia (can occur individually or in combination) are the most common disorders globally and can be prevented by certain lifestyle changes such as regular exercise, a low-fat diet, and consumption of food rich in fiber (116,228–230). As suggested by Tsai et al. (2020) (231), there is a decreased incidence of CVD later in life when leading a healthy lifestyle in young adulthood. A shift to a healthier lifestyle is certainly beneficial for all age groups, but in this particular age group, it serves as a prevention measure above all.

Average biochemical parameters and lipid profile analysis of our study population were in the reference range; however, when evaluating individual results, it was indicated that almost half

of participants had borderline cholesterol and LDL-cholesterol levels. According to Pletcher et al. (2010) (232), even the modest rises in LDL-cholesterol are associated with a significantly higher risk of atherosclerosis. LDL-cholesterol levels during young adulthood are highly correlated with lipid levels later in life. As a result of non-optimal lipid levels, atherosclerotic changes begin to occur which is associated with coronary artery diseases later in life. A study by Zhang et al. (2019) (233) with a total sample size of more than 36,000 individuals confirms that higher cholesterol levels (especially LDL-cholesterol) and exposure to high blood pressure during young adulthood are highly associated with increased CVD risks later in life.

Participants in the study that had higher levels of cholesterol also had a high intake of foods of animal origin, such as proteins and dairy products. With the higher number of meals, this group also simultaneously consumes more carbohydrates, sweets, and coffee. This is in accordance with studies reporting that excessive consumption of meat and high-fat dairy products alter lipid levels in a negative context (234–236). A positive correlation between serum cholesterol levels and BMI was recently reported by Nwaiwu and Ibe (2015) (237) in children and Laclaustra et al. (2018) (238) in adults. Approximately 23% of women and 13% of men gain more than 20 kg between the ages of 18 and 55 years (239).

Unhealthy diet and physical inactivity are more common among those who gain more weight through the years, and so is the incidence of type 2 diabetes, hypertension, and other CDV diseases. Hypertension is five times more frequent among obese and overweight people than normal weighted people; therefore, overweight/obesity is contributing to a global increase in cardiovascular disease (240). Weight gain occurs more frequently during early to middle adulthood with greater weight gain among women being explained by weight retention after childbirth (241).

To grasp the specificity of local cuisine that may influence our results, we have assembled a dietary assessment questionnaire in the form of a table with offered answers regarding food items, portions, and preparation methods in accordance with these specificities and assumed dietary habits of students in the research area. It is based on what food items do students consume and do they follow recommended guidelines rather than the exact amount in grams, millilitres, etc. Instructions on filling out the forms were in accordance with standard three-day food records, which note everything consumed during a time period of three days; two questionnaires for weekdays and one for a weekend day (avoid celebrations and similar occasions (117,138,242).

Its adjustment compared to FFQ and 24 h is a simplification of the standardized form with offered options for foods, portions and preparation, in order to avoid handwriting and reduce the longevity of filling the forms. The questionnaire used in the present study is different from standard FFQ, which is usually displayed as a list of items (91,207); in a way, it takes into account the method of preparation and thus provides more information. Our questionnaire is closest to the standard 24 h method in terms of gathering information on the type of food and its characteristics, preparation method, and quantity consumed (136), although we mainly emphasized adherence of participants with general guidelines on intake for our area of research.

6.4. Limitations and future research

Limitations of the present study are short duration of the protocol. Further, the fact that the study population was composed of young, healthy individuals was a limitation itself since there were no inflammation or impairments present that required mitigation in such sense. There are a lot of confounding factors present in this study, and although there was no significant effect detected during analysis, this is not a confirmation that it would still be the case if sample size was larger.

Future research should include longer interventions (e.g. six months) with more rigorous checks throughout duration and several follow-ups (e.g. monthly appointments) to determine long-term intake of *n-3* PUFAs through enriched food in healthy population. This study had surrogate endpoints and hard endpoints are needed to prove possible preventive health effects.

7. CONCLUSIONS

Based on the conducted research and the obtained results, the following can be concluded:

- Both dietary protocols (regular and enriched hen eggs) resulted in decreased rates of peripheral Th17 cells and reduced frequencies of peripheral nTreg lymphocytes. Their functional capacity for cytokine secretion was significantly altered only in the *n-3* PUFAs group through increased anti-inflammatory TGF-β1 and IL-10, and reduced pro-inflammatory IL-6 and IFNγ secretion.

- Diet enriched with *n-3* PUFAs (functional food in the form of enriched hen eggs) changes immune response towards inflammation resolving conditions through modification of key mediators of inflammation and altered frequency of particular lymphocyte subpopulations resulting in favourable cytokine milieu in human model without underlying comorbidities.

- *n-3* PUFAs enriched diet resulted in a significant shift towards anti-inflammatory prostanoids and increased levels of pro-resolvins.

- We present evidence that *n-3* PUFAs from functional food (enriched hen eggs) can increase serum-free *n-3* PUFAs concentration and significantly change the fatty acid composition and *n-6*/*n-3* PUFAs ratio.

- Improved microvascular endothelium-dependent vasodilation in *n-3* PUFAs group provides the first potential mechanism for enhanced microvascular function.

8. SUMMARY

INFLUENCE OF OMEGA-3 ENRICHED HEN EGGS CONSUMMATION ON MICROVASCULAR REACTIVITY AND SYSTEMIC INFLAMMATION IN HEALTHY YOUNG PEOPLE – RANDOMIZED CONTROLLED STUDY

Objectives: To determine the effects of *n-3* polyunsaturated fatty acid (PUFA) supplementation, i.e., α-linolenic (~230 mg), eicosapentaenoic (~15 mg), and docosahexaenoic acid (~105 mg), on the prevalence of T helper (Th17) and regulatory (Treg) lymphocytes, cytokine profile, other inflammatory parameters and lipid metabolites, microvascular reactivity and endothelium-dependent/-independent mechanisms of vasodilation in healthy individuals.

Study Design and Setting: This was a randomized, double-blind, placebo-controlled, prospective, intervention study. Research was carried out at Department of Physiology and Immunology, Faculty of Medicine Osijek, Josip Juraj Strossmayer University of Osijek in Osijek, Croatia

Participants and Methods: 44 healthy adults of both sexes were recruited according to inclusion/exclusion criteria for the participation in the study. 42 participants were randomized and divided into: 1) Control group (*N*=21) that consumed regular hen eggs (*n-3* PUFAs: ~249 mg/per day), 2) *n-3* PUFAs group (*N*=21) that consumed enriched hen eggs (*n-3* PUFAs: ~1053 mg/per day). Two participants from *n-3* PUFA were lost to follow-up due to personal reasons, with total number of participants included in final analysis being *N*=19. The study protocol included two appointments and participants were instructed to eat three hard-boiled hen eggs/day for three weeks. Venous blood samples were taken before and after the dietary protocol for serum and peripheral blood mononuclear cells collection/isolation. Anthropometric and biochemical measurements, blood pressure, body composition and fluid status, serum lipid and free fatty acids profile were measured before and after respective dietary protocol. Concentrations of lipid mediators, inflammatory and endothelial activation markers were measured via ELISA and Luminex assays. Frequencies of Tregs and Th17 lymphocytes were determined by flow cytometry. Skin microvascular blood flow in response to iontophoresis of acetylcholine and sodium nitroprusside was assessed by laser Doppler

flowmetry. An adjusted dietary assessment questionnaire was used to determine the dietary habits of the study population.

Results: Both study groups showed reduced frequencies of peripheral Treg and Th17 lymphocytes. Functional capacity for cytokine secretion was significantly altered in the *n-3* PUFAs group through increased anti- (transforming growth factor β1, interleukin 10) and reduced pro-inflammatory (interleukin 6, interferon gamma) cytokine secretion. Significantly increased levels of resolvins were observed only in the *n-3* PUFAs group as well as significantly improved acetylcholine-induced dilatation.

Conclusion: Diet supplemented with *n-3* PUFAs alters immune response towards inflammation resolving conditions through effects on lipid mediators and cytokine secretion by T lymphocytes, as well as it provides potential mechanisms for enhanced microvascular function since it improved microvascular endothelium-dependent vasodilation in human model without underlying comorbidities.

Trial registration: Clinical Trials ID NCT02720250

Keywords: cytokines, diet habits, functional foods, inflammation, microcirculation, omega-3 fatty acids

9. SAŽETAK

UTJECAJ KONZUMACIJE KOKOŠJIH JAJA OBOGAĆENIH OMEGA-3 MASNIM KISELINAMA NA MIKROVASKULARNU REAKTIVNOST I SUSTAVNU UPALU KOD ZDRAVIH MLADIH LJUDI – RANDOMIZIRANA KONTROLIRANA STUDIJA

Ciljevi: Odrediti učinke suplementacije s *n-3* polinezasićenim masnim kiselinama (PUFA), tj. α-linolenske (~230 mg), eikosapentanoična (~15 mg) i dokosaheksanoična kiseline (~105 mg), na zastupljenost T pomoćničkih (Th17) i regulatornih (Treg) limfocita, profil citokina, druge upalne parametre i lipidne metabolite, mikrovaskularnu reaktivnost i endotel-ovisne/-neovisne mehanizme vazodilatacije u zdravih osoba.

Dizajn studije i mjesto provođenja: Ovo je bila randomizirana, dvostruko slijepa, placebom kontrolirana, prospektivna, intervencijska studija. Istraživanje je ostvareno na Katedri za fiziologiju i imunologiju Medicinskog fakulteta u Osijeku.

Sudionici i metode: 44 zdrave osobe oba spola regrutirano je prema uključnim/isključnim kriterijima za sudjelovanje u studiji. 42 ispitanika je randomizirano i podijeljeno u: 1) kontrolnu skupinu (*N*=21) koja je konzumirala obična kokošja jaja (*n-3* PUFA: ~249 mg/dan), 2) *n-3* PUFA skupinu (*N*=21) koja je konzumirala obogaćena kokošja jaja (*n-3* PUFA: ~1053 mg/dnevno). Dva ispitanika iz *n-3* PUFA skupine su se povukla iz studije iz privatnih razloga, te je konačan broj analiziranih *N*=19. Protokol studije uključivao je dva termina, a ispitanicima je dana uputa da jedu tri tvrdo kuhana kokošja jaja dnevno tijekom tri tjedna. Uzorci venske krvi uzeti su prije i nakon dijetalnog protokola za prikupljanje/izolaciju seruma i mononuklearnih stanica periferne krvi. Antropometrijska i biokemijska mjerenja, krvni tlak, tjelesni sastav i status tekućine, profil lipida i slobodnih masnih kiselina u serumu mjereni su prije i nakon dijetalnog protokola. Koncentracije lipidnih medijatora, upalnih i endotelnih aktivacijskih markera mjerene su ELISA i Luminex testovima. Učestalost Treg i Th17 limfocita određena je protočnom citometrijom. Mikrovaskularni protok krvi u koži kao odgovor na iontoforezu acetilkolina i natrijevog nitroprusida procijenjen je laser Doppler flowmetrijom. Ispitanici su ispunjavali prilagođen upitnik u svrhu utvrđivanja prehrambenih navika.

Rezultati: Obje skupine se pokazale smanjenu frekvenciju perifernih Treg i Th17 limfocita. Funkcionalni kapacitet za izlučivanje citokina značajno je promijenjen u *n-3* PUFA skupini kroz povećano lučenje anti- (transformirajući faktor rasta β1, interleukin 10) i smanjeno lučenje pro-upalnih (interleukin 6, interferon gama) citokina. Značajno povećane razine resolvina opažene su samo u *n-3* PUFA skupini, kao i značajno poboljšana dilatacija izazvana acetilkolinom.

Zaključak: Prehrana obogaćena s *n-3* PUFA mijenja imunološki odgovor u smjeru smanjenja upale kroz učinke na lipidne medijatore i izlučivanje citokina od strane T limfocita, te također pruža potencijalne mehanizme za poboljšanu mikrovaskularnu funkciju budući da poboljšava endotel-ovisnu vazodilataciju u ljudskom modelu bez komorbiditeta u podlozi.

Registracija studije: Clinical Trials ID NCT02720250

Ključne riječi: citokini, prehrambene navike, funkcionalna hrana, upala, mikrocirkulacija, omega-3 masne kiseline

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11. BIOGRAPHY

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Work experience

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Scientific Laboratory Technician – Faculty of Medicine Osijek, Josip Juraj Strossmayer University of Osijek

- 2017. – CURRENT– Osijek, Croatia

Assistant - Faculty of Medicine Osijek, Josip Juraj Strossmayer University of Osijek

Education

- 2017 – CURRENT – Osijek, Croatia

Postgraduate doctoral study Biomedicine and Healthcare, Faculty of Medicine Osijek

- 2014. – 2017. – Osijek, Croatia

Master of Nature and Environmental Protection (mag. prot. nat. et amb.) - Graduate University Study Programme in Nature and Environmental Protection - Department of Biology, Josip Juraj Strossmayer University of Osijek

- 01.10.2015 – 29.01.2016. – Vienna, Austria

ERASMUS+ student exchange program – BOKU – University of Natural Resources and Life Sciences in Vienna, Austria

- 2010.-2014. - Osijek, Croatia

Bachelor's Degree of Biology (univ. bacc. biol.) - Bachelor of Science Undergraduate Programme - Department of biology, Josip Juraj Strossmayer University of Osijek

Training

- 23.10.2017. – 27.10.2017. – Zagreb, Croatia

Annual Flow Cytometry Course 2017 – Srebrnjak Children's hospital

- 28.06.2017. – 08.07.2017. – Zagreb, Croatia

LABANIM - a training course for people working with experimental animals (B category) – Faculty of Science, University of Zagreb

Language skills

- Mother tongue: Croatian
- Other: English (C2)

Honours and awards

- 2017 - Best poster award – Croatian Physiological Society

4th Congress of Croatian Physiological Society and 2nd Regional Congress on Physiological Societies // Abstract title: Positive effect of omega-3 enriched diet on reducing oxidative stress level in young healthy women

- 2020 – Dean’s award – Faculty of Medicine Osijek

Dies Doctorandorum 2020 // Abstract title: Positive effect of *n-3* PUFAs consumption on serum levels of anti-inflammatory cytokines in young healthy participants

Projects

- “Production and Medical Testing of Functional Food” (MedResFF); Funded by: European Structural and Investment Funds, to Scientific Centre of Excellence for Personalized Health Care, J.J. Strossmayer University of Osijek, #KK.01.1.1.01.0010.

- “Impaired Vasorelaxation and Endothelial Leukocyte Interaction (ELI) in Development of Atherosclerotic Lesions “(V-ELI Athero, HRZZ-IP-2014-09-6380), Croatian Science Foundation.
- "Interaction of renin - angiotensin and adrenergic systems in oxidative stress-induced endothelial activation" (RAS-AdrenOX, IP-2016-06-8744), Croatian Science Foundation.

Journal articles and review articles in CC journals:

1. Stupin, Ana; Cvetko, Ana; Kralik, Gordana; Mihalj, Martina; Šušnjara, Petar; **Kolobarić, Nikolina**; Breškić Ćurić, Željka; Lukinac, Ana Marija; Kibel, Aleksandar; Selthofer-Relatić, Kristina et al. The effect of *n-3* polyunsaturated fatty acids enriched hen eggs consumption on IgG and total plasma protein N-glycosylation in healthy individuals and cardiovascular patients. // Glycobiology, 2021 (2021) doi:10.1093/glycob/cwab051

2. Breškić Ćurić, Željka; Masle, Ana Marija; Kibel, Aleksandar; Selthofer-Relatić. Kristina; Stupin, Ana; Mihaljević, Zrinka; Jukić, Ivana; Stupin, Marko; Matić, Anita; Kozina, Nataša **et al.** Effects of *n-3* Polyunsaturated Fatty Acid-Enriched Hen Egg Consumption on the Inflammatory Biomarkers and Microvascular Function in Patients with Acute and Chronic Coronary Syndrome—A Randomized Study. // Biology, 10 (2021), 8; 774, 20 doi:10.3390/biology10080774

3. Stupin, Ana; Mihalj, Martina; **Kolobarić, Nikolina**; Šušnjara, Petar; Kolar, Luka; Mihaljević, Zrinka; Matić, Anita; Stupin, Marko; Jukić, Ivana; Kralik, Zlata et al. Anti-inflammatory potential of *n-3* polyunsaturated fatty acids enriched hen eggs consumption in improving microvascular endothelial function of healthy individuals - clinical trial. // International journal of molecular sciences, 21 (2020), 11; 4149, 20 doi:.org/10.3390/ijms21114149

4. Mihaljević, Zrinka; Matić, Anita; Stupin, Ana; Frkanec, Ruža; Kelava, Vanja; Tartaro Bujak, Ivana; **Kolobarić, Nikolina**; Kibel, Aleksandar; Drenjančević, Ines. Arachidonic Acid Metabolites of CYP450 Enzymes and HIF-1α Modulate Endothelium-Dependent Vasorelaxation in Sprague-Dawley Rats under Acute and Intermittent Hyperbaric Oxygenation. // International journal of molecular sciences, 21 (2020), 17; 6353, 21 doi:.org/10.3390/ijms21176353

5. **Kolobarić, Nikolina**; Gradinjan Centner, Maja; Šušnjara, Petar; Matić, Anita; Drenjančević, Ines. Anthropometric and Biochemical Parameters in Relation to Dietary Habits as Early Indicator of Cardiovascular Impairment in Young Adult Cohort. // International journal of environmental research and public health, 17 (2020), 24; 1-14 doi:10.3390/ijerph17249208

Journal articles and review articles in other journals:

1. Stupin, Ana; Drenjančević, Ines; Šušnjara, Petar; Debeljak, Željko; **Kolobarić, Nikolina**; Jukić, Ivana; Mihaljević, Zrinka; Martinović, Goran; Selthofer-Relatić, Kristina. Is There Association between Altered Adrenergic System Activity and Microvascular Endothelial Dysfunction Induced by a 7-Day High Salt Intake in Young Healthy Individuals. // Nutrients, 13 (2021), 5; 1731, 16 doi:.org/10.3390/nu13051731

2. **Kolobarić, Nikolina**; Drenjančević, Ines; Matić, Anita; Šušnjara, Petar; Mihaljević, Zrinka; Mihalj, Martina. Dietary Intake of *n-3* PUFA-Enriched Hen Eggs Changes Inflammatory Markers' Concentration and Treg/Th17 Cells Distribution in Blood of Young Healthy Adults—A Randomised Study. // Nutrients, 13 (2021), 6; 1851, 20 doi:10.3390/nu13061851

3. Jukić, Ivana; **Kolobarić, Nikolina**; Stupin, Ana; Matić, Anita; Kozina, Nataša; Mihaljevič, Zrinka; Mihalj, Martina; Šušnjara, Petar; Stupin, Marko; Breškić Ćurić, Željka et al. Carnosine, small but mighty - prospect of use as functional ingredient for functional food formulation. // Antioxidants, 10 (2021), 7; 1037, 31 doi:10.3390/antiox10071037

4. Mihalj, Martina; Stupin, Ana; **Kolobarić, Nikolina**; Tartaro Bujak, Ivana; Matić, Anita; Kralik, Zlata; Jukić, Ivana; Stupin, Marko; Drenjančević, Ines. Leukocyte activation and antioxidative defense are interrelated and moderately modified by *n-3* polyunsaturated fatty acid-enriched eggs consumption—double-blind controlled randomized clinical study. // Nutrients, 12 (2020), 10; 3122, 21 doi:10.3390/nu12103122

5. **Kolobarić, Nikolina**; Stupin, Ana; Šušnjara, Petar; Barić, Lidija; Matić, Anita. Effect of low selenium diet on glutathione peroxidase 3 concentration in male Sprague-Dawley rats serum. // Southeastern European Medical Journal, 3 (2019), 1; 50-59

Conference papers in CC journals

1. Šušnjara, Petar; Stupin, Ana; **Kolobarić, Nikolina**; Mihaljević, Zrinka; Drenjančević, Ines. Four Nutrients - Enriched Hen Eggs Consumption Improves Skin Microvascular Reactivity in Healthy Individuals. // XII ISIN Conference on Immunonutrition July 14– 16, 2021 (Online): Abstracts in Annals of Nutrition and Metabolism / Marcos, Ascension; Castell, Margarida; Pérez Cano, Francisco J. (ur.). online, 2021. str. 12-13

2. Drenjančević, Ines; Stupin, Ana; Barić, Lidija; Stupin, Marko; Mihalj, Martina; Mihaljević, Zrinka; Matić, Anita; Jukić, Ivana; Šušnjara, Petar; Kozina, Nataša; **Kolobarić, Nikolina**. Increased Oxidative Stress Underlies Impaired Endothelial Function and Vascular Reactivity with High Salt Dietary Intake, Independently of Blood Pressure Changes – Translational Studies. // Journal of Vascular Research 56(suppl 1):1–134, Maastricht, Netherlands, 2019. pg. 29-30 doi:10.1159/000499516.

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Membership in scientific societies and associations:

Croatian Physiological Society

12. APPENDICES

Annex 1. *24 Hour Food Recall* form

Annex 2. *CONSORT 2010 checklist* of information for randomized trial

Annex 1. *24 Hour Food Recall* form **DATE: _____**

24 Hour Food Recall
- FORM-

FIRST NAME/ LAST NAME_____
DATE OF BIRTH: _____
SEX: F M
HEIGHT: _____
WEIGHT: _____

At the first appointment participants were each given three identical forms for tracking food and beverage consumption for the past 24 hours. The first two forms refer to weekdays and the third one to weekend days. All participants were informed by the researcher via text message/e-mail about filling in the form for the day before so they don't subconsciously alter their usual diet habits. The form itself is constructed as a table for the sake of simplicity and transparency. Table is divided into four main parts: **BREAKFAST**, **LUNCH**, **DINNER** and **SNACK**. Each meal involves food and beverages. Meals are divided into: **FRUIT** (fresh / dry); **VEGETABLES** (fresh / frozen / cooked / baked); **DAIRY PODUCTS** (milk / cheese / yogurt); **MEAT** (white meat /red meat /fish / processed meat products); **CARBS** (bread / pasta / bagels / potatoes/ rice); **CEREALS** (oats /corn cereal / sweetened cereal); **BEVERAGES** (water / mineral water / coffee / coffee with milk/ tea / natural juice / soda / alcohol); **SWEETS** (chocolate / chips / cookies / cake); **NUTS** (almonds / hazelnuts / walnuts). Method of preparation and portion sizes were taken into consideration depending on the type of food. The participant fills the table in such way as he/she places a plus/tick symbol in the places provided for it in accordance with what he/she consumed that day and how it was prepared. After each meal there is a section called **COMMENT** where it is necessary to emphasize whether the participant was taking any dietary supplement or anything that could significantly affect or alter nutrient intake that day. If not present in the table, what was consumed can also be written in this section. If any of the meals are skipped also indicate.

*Portion sizes are described as: small (1/2 of a cup / slice / piece); medium/normal (cup / slice / piece); large (2 cups / slices / pieces).

**Way of consumption (dairy products / nuts): separately – a cup of milk, a piece of cheese, a handful of almonds; as part – cereals with milk, sandwich with ham and cheese, cereals with nuts.

BREAKFAST	**DAIRY PRODUCTS**		**WAY OF CONSUMPTION**		**PORTION SIZE**					
		DIVISION	**Separately**	**As part**	**Small**	**Medium**	**Large**			
		Cheese								
		Milk								
		Yoghurt								
		_____								
	MEAT		**PREPARATION**				**PORTION SIZE**			
		DIVISION	**Raw**	**Cooked**	**Baked**	**Fried**	**Small**	**Medium**	**Large**	
		White meat								
		Red meat								
		Fish								
		Meat products								
	CARBOHYDRATES		**PREPARATION**				**PORTION SIZE**			
		DIVISION	**Raw**	**Cooked**	**Baked**	**Fried**	**Small**	**Medium**	**Large**	
		Bread								
		Bagels								
		Pasta								
		Potatoes								
		Rice								
	FRUIT		**PREPARATION**		**PORTION SIZE**					
		DIVISION	**Fresh**	**Dried**	**Small**	**Medium**	**Large**			
		Apple/Pear								
		Banana								
		Berries								
		_____								
	VEGETABLES		**PREPARATION**				**PORTION SIZE**			
		DIVISION	**Fresh**	**Frozen**	**Cooked**	**baked**	**Small**	**Medium**	**Large**	
		Legumes								
		Tuberous/Roots								
		Cabbage								
		Bulbs								
		Pumpkins								
	CEREALS		**PORTION SIZE**							
		DIVISION	**Small**	**Medium**	**Large**					
		Oats								
		Corn								
		Sweetened								
		_____								
	NUTS		**WAY OF CONSUMPTION**		**PORTION SIZE**					
		DIVISION	**Separately**	**As part**	**Small**	**Medium**	**Large**			
		Almonds								
		Hazelnuts								
		Walnuts								
	BEVERAGES		**AMOUNT**							
		DIVISION	**Glass/cup**	**2x**	**3x**	**5x**	**More than 5x**			
		Water								
		Mineral water								
		Coffee								
		Coffee with milk								
		Tea								
		Fresh natural juice								
		Soda								
		Alcohol								

COMMENT:

_____

_____

LUNCH	**MEAT**		**PREPARATION**				**PORTION SIZE**		
		DIVISION	**Raw**	**Cooked**	**Baked**	**Fried**	**Small**	**Medium**	**Large**
		White meat							
		Red meat							
		Fish							
	CARBOHYDRATES		**PREPARATION**				**PORTION SIZE**		
		DIVISION	**Raw**	**Cooked**	**Baked**	**Fried**	**Small**	**Medium**	**Large**
		Bread							
		Bagels							
		Pasta							
		Potatoes							
		Rice							
	VEGETABLES		**PREPARATION**				**PORTION SIZE**		
		DIVISION	**Fresh**	**Frozen**	**Cooked**	**baked**	**Small**	**Medium**	**Large**
		Legumes							
		Tuberous/Roots							
		Cabbage							
		Bulbs							
		Pumpkins							
		_____							
	BEVERAGES		**AMOUNT**						
		DIVISION	**Glass/cup**	**2x**	**3x**	**5x**	**More than 5x**		
		Water							
		Mineral water							
		Coffee							
		Coffee with milk							
		Tea							
		Fresh natural juice							
		Soda							
		Alcohol							
	SOUPS		**PORTION SIZE**						
		DIVISION	**1x**	**2x**	**3x**				
		Chicken							
		Beef							
		Veggie							
		Cream							

COMMENT:

_____

_____

DINNER	**DAIRY PRODUCTS**		**WAY OF CONSUMPTION**		**PORTION SIZE**					
		DIVISION	**Separately**	**As part**	**Small**	**Medium**	**Large**			
		Cheese								
		Milk								
		Yoghurt								
		_____								
	MEAT		**PREPARATION**				**PORTION SIZE**			
		DIVISION	**Raw**	**Cooked**	**Baked**	**Fried**	**Small**	**Medium**	**Large**	
		White meat								
		Red meat								
		Fish								
		Meat products								
		_____								
	CARBOHYDRATES		**PREPARATION**				**PORTION SIZE**			
		DIVISION	**Raw**	**Cooked**	**Baked**	**Fried**	**Small**	**Medium**	**Large**	
		Bread								
		Bagels								
		Pasta								
		Potatoes								
		Rice								
	FRUIT		**PREPARATION**		**PORTION SIZE**					
		DIVISION	**Fresh**	**Dried**	**Small**	**Medium**	**Large**			
		Apple/Pear								
		Banana								
		Berries								
		_____								
	VEGETABLES		**PREPARATION**				**PORTION SIZE**			
		DIVISION	**Fresh**	**Frozen**	**Cooked**	**Baked**	**Small**	**Medium**	**Large**	
		Legumes								
		Tuberous/Roots								
		Cabbage								
		Bulbs								
		Pumpkins								
		_____								
	CEREALS		**PORTION SIZE**							
		DIVISION	**Small**	**Medium**	**Large**					
		Oats								
		Corn								
		Sweetened								
		_____								
	BEVERAGES		**AMOUNT**							
		DIVISION	**Glass/cup**	**2x**	**3x**	**5x**	**More than 5x**			
		Water								
		Mineral water								
		Coffee								
		Coffee with milk								
		Tea								
		Fresh natural juice								
		Soda								
		Alcohol								

COMMENT:

_____

_____

SNACK	NUTS		WAY OF CONSUMPTION		PORTION SIZE		
		DIVISION	Separately	As part	Small	Medium	Large
		Almonds					
		Hazelnuts					
		Walnuts					
		_____					
	FRUIT		WAY OF CONSUMPTION		PORTION SIZE		
		DIVISION	Fresh	Dried	Small	Medium	Large
		Apple/Pear					
		Banana					
		Berries					
		Citrus					
		_____					
	SWEETS		PORTION SIZE				
		DIVISION	Small	Medium	Large		
		Chocolate					
		Chips					
		Cookies					
		Cake					
		_____					
	BEVERAGES		AMOUNT				
		DIVISION	Glass/cup	2x	3x	5x	More than 5x
		Water					
		Mineral water					
		Coffee					
		Coffee with milk					
		Tea					
		Fresh natural juice					
		Soda					
		Alcohol					

COMMENT:

_____

_____

Annex 2. *CONSORT 2010 checklist* of information for randomized trial

CONSORT 2010 checklist of information

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	Title page
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	70, 72
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	1-11
	2b	Specific objectives or hypotheses	12-13
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	14
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	None
Participants	4a	Eligibility criteria for participants	14
	4b	Settings and locations where the data were collected	14
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	14-15
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	15, 17-26
	6b	Any changes to trial outcomes after the trial commenced, with reasons	None
Sample size	7a	How sample size was determined	15-16
	7b	When applicable, explanation of any interim analyses and stopping guidelines	None
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	16

	8b	Type of randomisation; details of any restriction (such as blocking and block size)	16
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	16
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	16
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	16
	11b	If relevant, description of the similarity of interventions	None
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	26
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	26
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	27 Figure 5.1.
	13b	For each group, losses and exclusions after randomisation, together with reasons	27
Recruitment	14a	Dates defining the periods of recruitment and follow-up	27
	14b	Why the trial ended or was stopped	None
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 5.1.
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	27
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	28-53
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	28-53

Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	None
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	None
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	68
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	56
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	56-68
Other information			
Registration	23	Registration number and name of trial registry	14
Protocol	24	Where the full trial protocol can be accessed, if available	Not available
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	After title page