

Study Protocol

Subjects

Physically active individuals who are amateur endurance athletes (runners, cyclists) with a minimum of 3 years of training experience, training 3–7 times per week for a total of 60–240 minutes per week, will participate in the study. Participants must not have consumed any form of omega-3 supplement for 4 weeks prior to the start of the study and must not have consumed fatty marine fish ≥ 3 times per week during that period.

Study design

The study will be a randomized, crossover trial. The study will be conducted in 2 stages. At the beginning of the first stage, all participants will undergo an assessment of basic anthropometric parameters using the InBody Data Management System. Subsequently, the participants will be randomly assigned to 4 groups: a group supplemented with EPA+DHA fatty acids (animal source), a group supplemented with ALA fatty acids (plant source), a placebo-supplemented control group, and a control group without supplementation. Then, for the next 4 weeks, participants assigned to the first group will take 3630 g of EPA + DHA (1980 g EPA + 1650 g DHA), and participants assigned to the second group will take 3500 g of ALA fatty acids. The third group will take a placebo containing MCT fatty acids at a dose of 3500 g. The fourth group will not take any supplement. The supplements will be taken in capsule form – 3 capsules at breakfast and 3 capsules at dinner. After 4 weeks of supplementation, there will be a 3-month washout period. After the 3-month break, the second stage will begin, which will also last 4 weeks, during which the groups will be crossed over. During this time, the group that previously received EPA+DHA will receive the ALA supplement, and the group previously supplemented with ALA will receive the EPA+DHA supplement. The group previously receiving placebo will not take anything, and the previous control group will receive placebo.

At the beginning and at the end of both the first and the second stage (two periods of 4-week supplementation), 10 ml of blood will be collected from the antecubital vein at rest for biochemical and genetic analyses. A buccal swab from the inner cheek will be collected once, at the beginning of the study, for genetic analyses. Also before and after both 4-week supplementation periods, the following assessments will be performed: electromyography (EMG), tensiomyography (TMG), cognitive function tests, psychological assessments, and measurements of body mass and body composition.

Planned blood analyses

- Complete blood count (hemoglobin, hematocrit, erythrocytes, leukocytes, platelets).
- Blood lipid profile: total cholesterol (TC), LDL cholesterol, HDL cholesterol, triglycerides.
- Parameters characterizing immune system function (concentrations of C-reactive protein, IL-1, IL-6, IL-10, TNF α , IL-4, IL-1ra).
- Activity of antioxidant enzymes (SOD, CAT, GPx, GR), concentrations of non-enzymatic antioxidants (GSH, sirtuin 1 and 3, uric acid), total antioxidant status (TAS) and total oxidative status (PerOX).
- Biomarkers of oxidative stress: concentration of malondialdehyde (MDA), concentration of advanced oxidation protein products (AOPP), cyclooxygenase-2.
- Omega-3 fatty acid index (HS-Omega-3 Index®), including the ratio of saturated to monounsaturated fatty acids (SFA/MUFA), the TRANS fat index, and the ratio of

arachidonic acid to eicosapentaenoic acid (AA/EPA), will be determined using gas chromatography with flame-ionization detection (GC-FID). The omega-3 index is defined as the sum of EPA and DHA expressed as a percentage of the total fatty acid content in erythrocyte membranes. It is measured in dried blood spots (DBS) and is a qualitative (%) rather than a quantitative ($\mu\text{g/ml}$) expression of the total fatty acid content. DBS analysis is a repeatable and relatively non-invasive measurement. The analysis will be performed in a certified laboratory (Centro Diagnostico Delta, Apollosa, Italy; certificate no. 8449/2010).

Planned genetic analyses

- Analysis of polymorphisms in genes of enzymes responsible for omega-3 fatty acid metabolism—elongation (ELOVL2) and desaturation (FADS1/2)—using DNA isolated from buccal epithelial cells collected from the inner cheek. Six polymorphisms in the ELOVL2 and FADS1/2 genes will be analyzed to identify genetic variants that influence the variability of omega-6/omega-3 fatty acid concentrations in plasma and cell membranes.
- Transcriptomic analyses will be conducted in peripheral blood mononuclear cells (PBMC) using microarrays enabling simultaneous assessment of approximately 60,000 transcripts, covering the entire human transcriptome. These will also allow evaluation of selected long non-coding RNA (lncRNA) molecules. PBMC represent an attractive material for studying the effects of supplementation on gene expression in humans due to observed changes that correlate well with alterations in other tissues (e.g., adipocytes), and their collection is minimally invasive. High-throughput transcriptomic studies will provide significantly expanded knowledge on the mechanisms of observed supplementation effects and guide further research.
- Cell metabolism analyses (ATP-rate assay, Cell Mito Stress, Glycolytic Rate) will be performed on previously cryopreserved PBMC using the Seahorse analyzer (Agilent). After thawing, viable cell counts will be determined, cells will be seeded onto poly-D-lysine coated measurement plates, and analyses will be conducted according to the manufacturer's protocol.
- Epigenetic analysis through quantitative DNA methylation assessment will enable evaluation of epigenetic markers associated with biological aging processes (epigenetic clock analysis), lifestyle- and environment-sensitive epigenetic markers (e.g., smoking, physical activity, diet, supplementation, sleep duration), epigenetic markers linked to disease risk (e.g., type 2 diabetes, cancers, cardiovascular diseases), and epigenetic BMI markers. The analysis will be performed on blood samples and buccal swabs using Illumina iScan microarray technology for genome-wide DNA methylation analysis (>900,000 CpG markers) and/or high-throughput Ion AmpliSeq DNA sequencing for targeted analysis of precisely selected markers, following manufacturers' protocols and leveraging prior experience. In recent years, DNA methylation has gained significant importance as a marker of overall health status, biological aging pace, and disease risk. Epigenetic aging and potential epigenetic rejuvenation through health-promoting interventions have become particularly attractive topics. The discovery of DNA methylation's link to aging processes marked a breakthrough in longevity research. Notably, only about 30% of aging pace depends on genetic variability, while the remaining 70% is influenced by lifestyle, which modifies DNA methylation. DNA methylation, in turn, affects gene expression, leading to functional changes in the body. A key recent finding in epigenetics is that DNA methylation is reversible, and reversing methylation of specific genes not only

leads to rejuvenation but also reduces various risk factors. Literature shows positive effects of physical activity, anti-inflammatory diets, and supplements on DNA methylation and aging processes, but longitudinal studies assessing the impact of specific factors on methylation markers over time are still lacking and highly desirable.

Other planned analyses

- Measurement of body composition and mass using the 8-point bioelectrical impedance analyzer InBody.
- Electromyography (EMG) will be performed using surface electrodes attached to the biceps brachii muscle (long head) of the right and left upper limbs and the lateral head of the gastrocnemius muscle of the right and left lower limbs, which will be disinfected beforehand. The test is conducted in a supine or seated position during muscle contraction. EMG is a technique for obtaining, recording, and analyzing muscle bioelectrical signals, which arise from physiological changes in muscle fibers. Participants will refrain from intense exercise for at least 48 hours prior to testing. The EMG will be performed at rest without warm-up. The test is non-invasive, harmless, and can be repeated multiple times.
- Tensiomyography (TMG) assessment will be performed using electrodes attached to the biceps brachii muscle (long head) of the right and left upper limbs and the lateral head of the gastrocnemius muscle of the right and left lower limbs, which will be disinfected beforehand. The test uses a very low voltage and current, completely non-invasive to the body. The electrodes deliver an electrical impulse generated by the electrostimulator to induce muscle contraction and precisely measure muscle contraction velocity. Participants will refrain from intense exercise for at least 48 hours prior to testing. The TMG will be performed at rest without warm-up. The test is harmless and can be repeated multiple times.
- Cognitive and Visual Function **Tests** Assessment of cognitive, visual, and visuo-motor functions will be conducted using the Synaptec Sensory Station platform. The test involves a series of assessments evaluating human visual functions. The participant stands 3 meters from the screen, where the software guides the entire procedure. Each test is preceded by instructions and a practice trial. Besides cognitive functions, the system does not assess other motor parameters, so the test does not burden systems other than the visual and nervous systems.

Participants / Group Size, Age, Sex, Health Status

The study will be conducted in a group of 80 healthy men aged 40–60 years who train endurance disciplines (runners, cyclists).

Inclusion and Exclusion Criteria

The selection of participants will be based on meeting the following inclusion criteria:

- Voluntary consent to participate in the study,
- Minimum 3 years of endurance training experience,
- Weekly training time: 60–240 minutes,
- Number of training sessions per week: 3–7,
- Good health status,

- No intake of omega-3 supplements, anti-inflammatory or antioxidant supplements, and/or anti-inflammatory drugs for at least 1 month prior to the study and during its duration.

The primary exclusion criteria will be:

- Failure to meet all inclusion criteria and health contraindications (hemophilia, blood clotting disorders),
- Unavailability on the day of testing (e.g., injury, diagnosed inflammation).

Expected Therapeutic/Cognitive Benefits

Improvement in neuromuscular conduction and cognitive functions in participants.
Improvement in the omega-3 index, antioxidant status, cellular metabolism, and other measured biochemical parameters.