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Consumption of a mediterranean lean fish enriched with Platelet-Activating Factor inhibitors extracted from olive pomace favorably modulates hemostasis and thrombosis in healthy adults with overweight

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Abstract

Blood coagulation prevents excessive bleeding but also contributes to thrombosis. Studies suggest that consuming more than two fish meals weekly reduces platelet aggregation induced by various agonists. Among them, Platelet-Activating Factor (PAF), a potent inflammatory lipid, plays a key role in atherogenesis. Previous studies demonstrated that a polar lipid extract from olive pomace (OOPLE), containing PAF inhibitors, reduced atherogenesis in animals. This double-blind, randomized, crossover trial investigated the effects of consuming gilthead sea bream fed with OOPLE-enriched feed (EF) versus conventional feed (CF) on platelet aggregation and hemostatic markers in adults with overweight. Participants (35–70 years, BMI 25–35 kg/m²) consumed two servings of EF or CF per week for one month, separated by a one-month washout. EF intake significantly reduced platelet aggregation in response to PAF (44%) and ADP (67%) compared to CF (p=0.002), and slightly decreased activated partial thromboplastin time by 0.82 seconds (p=0.025). No significant differences were found in glucose and lipid metabolism, other hemostatic biomarkers, or red blood cell membrane fatty acid profiles between the two interventions. These findings suggest that incorporating olive pomace-derived polar lipids into aquaculture feed may enhance the cardioprotective effects of fish by reducing platelet activation—supporting a novel strategy to improve the health benefits of farmed fish.

Keywords

PAF; human platelet aggregation; gilthead sea bream; aquaculture feed; olive pomace; polar lipids; fibrinogen.

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1. Introduction

Cardiovascular diseases (CVDs) still remain the leading cause of mortality worldwide among noncommunicable diseases with unhealthy diet identified as a modifiable behavioral risk factor and overweight/obesity as a metabolic risk factor [1]. The consumption of at least two servings of fatty fish per week (150g/portion) that can provide up to 2g of eicosapentanoic (EPA) and docosahexanoic (DHA) acids, is recommended by the European Society of Cardiology (ESC) and American Heart Association (AHA) for protection against heart diseases [2, 3]. Intervention trials among healthy participants revealed that fish intake mostly affects lipidemic profile and in particular an average daily intake of EPA and DHA over 0.8g resulted in decreased triglyceride (TAG) levels while an amelioration for high-density lipoprotein (HDL) levels was observed when more than 5 servings of fatty fish per week were consumed [4]. A recent comprehensive and detailed systematic review on the impact of ω-3 fatty acids on heart health found that increasing EPA and DHA levels—primarily through supplements—modestly lowers the likelihood of death and incidents related to coronary heart disease while also decreasing blood TAG levels [5].

Blood coagulation is a double-edged sword in cardiovascular diseases—necessary for preventing excessive bleeding but also a key factor in thrombosis, which can lead to heart attacks and strokes. This process entails the activation of both coagulation factors and platelets, ultimately resulting in the production of thrombin and fibrinogen [6]. Beyond coagulation factors, endothelial cells lining the blood vessels play also a crucial role in maintaining vascular stability and modulating coagulation processes. Overall, platelets and coagulation factors are fundamental to blood clotting, highlighting their essential roles in hemostasis but also having a significant impact on atherogenesis and atherothrombosis [7]. Activated platelets release various soluble compounds such as adenosine diphosphate (ADP), thrombin, platelet factor 4 (PF4) and platelet-activating factor (PAF) that are key drivers of platelet activation and clot formation. Among them, PAF chemically identified as 1-O-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine [8], is the most potent lipid inflammatory mediator, implicated in almost every pathological condition involving inflammation, cellular injury, and apoptosis such as atherogenesis and atherosclerosis [9]. PAF exerts its effects by binding to the PAF receptor (PAFR), a G-protein coupled receptor located on the plasma and nuclear membrane that is also expressed in immune and coagulation system cells and triggers a series of intracellular signaling events, including platelet aggregation, leukocyte adhesion, enhanced vascular permeability and the secretion of inflammatory mediators [10].

Modifying dietary habits, either alone or alongside established antiplatelet treatments, may help control platelet activity and lower CVDs risk. The groundbreaking studies by Bang and Dyerberg on Greenland Eskimos demonstrated prolonged bleeding times, attributed to reduced platelet aggregation from a diet rich in ω-3 polyunsaturated fatty acids (PUFAs) [11]. Based on these findings, recent research suggests that fish consumption—likely due to its content of ω-3 fatty acids and bioactive polar lipids—may positively influence platelet count and activity [12, 13]. A recent review of intervention studies [4] concluded that consuming more than two fish meals per week—supplying 0.40-3.40 g of EPA and DHA per day—was linked to reduced platelet aggregation induced by collagen, ADP, PAF, and arachidonic acid (AA). Conversely, none of the interventions significantly impacted fibrinogen levels, and in most cases, tissue plasminogen activator (tPA) or plasminogen activator inhibitor-1 (PAI-1) levels or activity remained unchanged.

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The global rise in fish demand over recent decades has led to increased reliance on farmed fish and the expansion of aquaculture. This sector is expected to play a vital role in building equitable food systems that support both human health and environmental sustainability [14]. However, the future of aquaculture depends on reducing dependence on marine-based raw materials by incorporating in the feeds plant-based ingredients and animal by-products. Although significant amounts of fishmeal (FM) and fish oil (FO) have been successfully replaced with agricultural alternatives and low-trophic organisms, research has primarily focused on enhancing fish growth and health, with the impact of these dietary shifts on the nutritional value of fish and human health remaining largely unexplored [15]. A limited number of dietary intervention studies have explored the impact of farmed fish with different feeding regimens on cardiovascular disease-related biomarkers in adults [16-20]. These studies primarily assessed serum lipid profiles, along with few circulating markers of vascular and systemic inflammation. Findings indicate that consuming farmed fish raised on alternative diets results in metabolic profiles that are either unchanged or less favorable compared to those associated with conventional fed farmed fish.

Our previous studies have demonstrated that a specific polar lipid extract from either olive oil or olive pomace (OOPLE) contain PAF inhibitors acting also as PAFR antagonists, which not only inhibit atherogenesis in experimental models but also reduce the thickness of existing atheromatous plaques and preserve vessel wall elasticity [21-24]. The most potent PAF inhibitor from the aforementioned polar lipid extract has been already identified as a glyceryl-ether-*sn*-2-acetyl glycolipid, based on chemical determinations and mass spectrometry analysis [22, 25]. The protective effects of both dietary interventions were linked to decreased *ex vivo* platelet aggregation and reduced plasma oxidation. Among all biochemical parameters analyzed, only the reduction in PAF-induced platelet aggregation was directly correlated with the attenuation of atherosclerotic lesions. Additionally, olive pomace has already been tested as a fish oil substitute in aquafeeds, demonstrating positive effects on fish growth [26]. These by-products have the potential to support the production of value-added foods including fish with functional properties while contributing to sustainable waste management and efficient resource utilization, particularly in the primary production sector [27]. Indeed, daily intake of a yogurt enriched with OOPLE has been shown to reduce platelet sensitivity to PAF, lower inflammatory markers, regulate PAF metabolism beneficially, and positively influence gut microbiota and fecal metabolites in healthy, predominantly overweight individuals [28-30].

This study aimed to explore the effects of the consumption of a fish raised on a diet where fish meal and fish oil were partially replaced with OOPLE compared to the consumption of fish raised on a conventional fish-oil diet, on platelet aggregation and key hemostatic markers in predominantly overweight but otherwise healthy adults. Based on previous studies with OOPLE in both animals and humans, we hypothesized that the intake of a fish raised on a diet enriched with OOPLE would have a stronger effect on these health biomarkers compared to fish raised on a conventional diet.

2. Materials and Methods

2.1. Production of Gilthead seabream (*Sparus aurata*) fillets and analysis

The extraction method for the OOPLE used in fish feed enrichment has been previously described [29]. In summary, OOPLE was obtained from wet olive pomace, a by-product of Koroneiki olives from Crete, processed using a two-phase centrifugal system. The pomace was extracted with absolute ethanol, filtered to remove solids, and the resulting

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filtrate—rich in polar lipids—was concentrated. To remove neutral lipids, it was washed twice with n-hexane, then further concentrated under vacuum to eliminate solvent residues. Previous analyses of this specific extract, obtained from various olive oil production seasons and supplemented with 30% w/w maltodextrin, revealed the following chemical composition per 100 g of the final OOPLE extract: approximately 57.0 ± 2.0 g carbohydrates, 2.5 ± 0.5 g proteins, 8.0 ± 1.5 g polar lipids, 9.5 ± 0.5 g dietary fiber, 6.0 ± 1.0 mg phenolic compounds (expressed as gallic acid), 17.0 ± 0.5 g ash, and 6.2 ± 0.6 g moisture [29]. To obtain the final material for fish feed, OOPLE was incorporated into maltodextrin—a plant-derived polysaccharide with high adsorption capacity—and into soy protein -a raw material widely used in aquafeeds-. The incorporation process was conducted under vacuum coating to ensure complete lipid entrapment within the final matrix. The approximate composition of the mixture (OOPLE-mix) was 40% OOPLE, 34% maltodextrin, and 26% concentrated soy protein and was stored at –80°C until use in fish feed.

Gilthead sea bream (Sparus aurata) fillets—both conventional and enriched—were supplied by AVRAMAR Aquaculture SA, with details of aquaculture practices provided elsewhere [31]. A pilot feeding trial was undertaken to evaluate the effects of dietary supplementation with OOPLE in gilthead sea bream. The OOPLE was incorporated into feed at inclusion levels ranging from 1% to 4% (w/w). Fish were monitored throughout the trial to assess growth performance and health status. Growth and health outcomes were satisfactory across all inclusion levels. For the subsequent human dietary intervention study, a 2% (w/w) inclusion rate of OOPLE was selected based on technical feasibility and cost-effectiveness. Briefly, gilthead sea bream with an initial average body weight of 294 g were randomly assigned to two groups following a 15-day acclimatization period. The control group received a standard commercial diet, while the experimental group was fed an identical base diet in which 13.3% w/w of the formulation was replaced with OOPLE-mix. Final chemical analysis of the conventional and experimental feeds showed minimal differences. Per 100 g of feed, both formulations contained approximately 46 g protein, 16.5 g fat, 2.8 g fiber, and 20 g carbohydrates, with a moisture content of 7.5% and ash content of approximately 7%. At the conclusion of the trial, fish had reached an average weight of 494 g. They were filleted, packed in polystyrene boxes with ice, and transported to the Department of Nutrition and Dietetics at Harokopio University. Fillets were stored at –20°C until distribution to study participants.

2.2. Fatty acid analysis and biological activity of Gilthead seabream fillets

Conventional and enriched fillets were lyophilized and total lipids (TL) were extracted using the Bligh–Dyer method [32]. Half of the extracted lipids were further separated into polar lipids (PL) and neutral lipids (NL) by counter-current distribution [33]. Fatty acid analysis was then conducted on the TL and PL fractions and was based on ISO-12966 2. Briefly, fatty acid methyl esters (FAME) were generated through the methanolysis of extracts and subsequently extracted using hexane containing an internal standard, methyl nonanoate, along with BHT. Fatty acid methyl esters were analyzed with gas chromatography using a Shimadzu Nexis GC-2030 (Kyoto, Japan) gas chromatograph equipped with flame ionization detector, split-splitless injector, and an autosampler by injecting an aliquot (1µL) of liquid sample into the gas chromatograph at a split ratio 1:50. Separation of FAME was achieved on an Agilent (Santa Clara, California, USA) DB-23 column (60.0 m × 0.25 mm, 0.15 µm). Helium (He) 32 mL/min and Hydrogen (H_2) 24 mL/min, were used as the carrier gas at a flow rate of 200 mL/min, injector and detector were held at 250 and 250 ^{o}C, respectively. A standard FAME mixture including 37 components was used for the identification of the peaks (Sigma L9405,

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St Louis, MO, USA). Identification of fatty acids was based on the retention times of the reference standard. The quantitative determination of fatty acid methyl esters was carried out based on the responses of the standard, normalized to the response of C16:0.

PL and TL fractions were also assessed for their ability to inhibit human platelet-rich plasma (PRP) aggregation induced by PAF, ADP, and thrombin receptor-activating peptide (TRAP) using a Chrono-Log aggregometer (model 440VS, Havertown, PA, USA). Blood was obtained from healthy volunteers who did not participate in the intervention study, following an overnight fast (minimum of 8 hours), at least 24 hours after their last alcohol intake, and after a minimum two-week period without antibiotics, aspirin, or anti-inflammatory drugs. The procedure followed has been previously described [28]. PRP (0.250 mL) was preincubated for 1 minute at 37 °C, with the extracts (dissolved in bovine serum albumin at 2.5 mg/mL), under continuous stirring at 1000 rpm, followed by the addition of the agonist—PAF, ADP, or TRAP. Reversible aggregation was induced using PAF (10–20 μM, dissolved in bovine serum albumin at 2.5 mg/mL), ADP (1–4 mM, dissolved in saline), and TRAP (2–5 μM, dissolved in saline). Aggregation was measured both before (considered as 0% inhibition) and after the addition of various sample concentrations. The percentage of inhibition (ranging from 20% to 80%) was plotted against sample concentration, producing a linear relationship. From this curve, the sample concentration required to achieve 50% inhibition of agonist-induced aggregation (IC_{50}) was determined, representing the inhibitory concentration for fifty percent inhibition.

2.3. Ethical approval

The study was conducted at the Department of Nutrition and Dietetics at Harokopio University of Athens, Greece, and was registered in ClinicalTrials.gov (NCT04440202). Before participation, all subjects received detailed information about the study's objectives and procedures and provided written informed consent. Adhering to the principles of the Declaration of Helsinki, the study protocol received approval from the Ethics Committee of Harokopio University of Athens on March 2nd, 2018.

2.4. Trial design

The study was a double-blind randomized controlled clinical trial with a 1:1 allocation ratio. It compared two dietary treatments, i.e. farmed fish fed an OOPLE -enriched diet (enriched fish, EF) versus farmed fish fed a fish oil diet (conventional fish, CF), using a cross-over design. The study was conducted from October 2021 to July 2022 and included three phases: treatment period one (1 month), a wash-out period (at least 1 month), and treatment period two (1 month). Participants were evenly assigned to the two dietary treatments across the two study periods. The choice of a one-month intervention period for each fish was made since a previous study in which participants consumed the same extract (OOPLE) showed that a 4-week period is sufficient to detect changes in platelet sensitivity [28]. Moreover, given that during the period of the intervention, there was both a surge in COVID-19 cases as well as the administration of booster vaccine doses that have been both associated with platelet activation, the duration was set to be as short as possible to minimize the likelihood of either occurring [34].

2.5. Participants and settings

Eligible participants were adults aged 35-70 years with a body mass index (BMI) of 25.0-35.0 kg/m² and a low habitual fish consumption, i.e. ≤1 portion per week (one portion

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was defined as 150 g of cooked fish). Exclusion criteria included pregnancy, presence of diabetes mellitus, active cancer, cardiovascular disease, chronic inflammatory or psychiatric disorders, use of antidiabetic, anxiolytic, antidepressant, or cortisol medications, use of n-3 fatty acid (FA) supplements, excessive habitual alcohol intake (>210 g and >140 g ethanol/week for men and women, respectively), adherence to a weight-loss diet, or recent lifestyle changes (within the past 6 months). Participants were recruited from the community through word of mouth and informational posters. All participants resided in the Attica region of Greece. The study was conducted at the Laboratory of Nutrition and Clinical Dietetics in collaboration with the Laboratory of Biology, Biochemistry, Physiology, and Microbiology of Harokopio University of Athens, Greece.

2.6. Intervention

Participants were randomly assigned to one of two fish treatment sequences (CF/EF or EF/CF), receiving each treatment for one month with a minimum one-month wash-out period in between. The fish species studied was gilthead seabream (*Sparus aurata*) and two different types were compared: farmed fish fed an OOPLE-enriched diet (EF) and farmed fish fed a fish oil-based diet (CF). Fish fillets were provided to participants with instructions to consume 2 servings per week on days of their choice (a total of 8 servings, each being approximately 200 g cooked). At the start of each treatment period, participants received oral and written information on fish preparation, including instructions to bake or grill the fish and the option to add seasonings and spices as desired. Participants were asked to maintain their usual dietary and physical activity habits throughout the study and to avoid intentional weight loss. During the wash-out period, no fish portions were provided, and participants were advised to follow their regular dietary habits. Before and after each fish consumption period, participants visited the premises of Harokopio University of Athens early in the morning for blood sampling and the collection of anthropometric and lifestyle data. All visits were conducted after an overnight fast (at least 8 hours), at least 24 hours after the last episode of alcohol intake, at least 2 weeks without reception of antibiotics, aspirin, or anti-inflammatory drugs, and 1-2 days after consuming the last fish serving. Additionally, participants were enrolled in the study at least two months after either a COVID-19 diagnosis or receiving a booster vaccine dose.

2.7. Primary outcomes and other outcome measures

Participants were evaluated in terms of anthropometric indices, lifestyle habits, blood pressure and various laboratory parameters at the beginning and at the end of each treatment period. The primary outcome was the *ex vivo* assessment of human platelet aggregation in platelet rich plasma (PRP) along with the evaluation of coagulation parameters (i.e. prothrombin time, international normalized ratio and activated partial thromboplastin clotting time) and circulating concentrations/activities of hemostatic markers (i.e. tPA, PAI-1, fibrinogen and sP-selectin).

Anthropometric indices: Height was measured with a stadiometer to the nearest 0.5 cm, with participants' occipital prominence, upper back, buttocks, calves and heels being in touch with the vertical line of this stadiometer, and their head placed in the Frankfort horizontal plane. Body weight was measured with a digital scale (TANITA BC-418MA, Tokyo, Japan) to the nearest 100 g, with participants wearing light clothing and being barefoot. The BMI was computed as [body weight (kg) ÷ (height (m)^2]. Waist circumference (WC) was

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measured with a non-elastic tape to the nearest 0.1 cm, at the midpoint between the lowest rib and the upper border of the iliac crest, at the end of a normal exhalation.

Dietary habits: Habitual dietary intake over the last month was assessed through a validated 76-item semi-quantitative food frequency questionnaire (FFQ) [35]. Participants were asked to report how often, on average, they consumed a defined portion of each food item according to a 6-grade scale (never/rarely, 1-3 times/month, 1-2 times/week, 3-6 times/week, 1 times/day, or ≥2 times/day). Dietary intake of individual foods/beverages (e.g. potatoes, olive oil, coffee) and food groups (e.g. dairy products, fruits, vegetables) was estimated and expressed as servings/day based on the serving sizes provided in the dietary guidelines for Greek adults [36]. Participants' daily energy and macronutrient intake were also estimated based on this FFQ. Also, participants' level of adherence to the Mediterranean diet was evaluated through the Mediterranean Diet Score (MedDietScore) [37]. This a-priori dietary index takes into account the habitual consumption of 11 food items, namely whole grains, potatoes, fruits, vegetables, legumes, full-fat dairy products, fish, poultry, red meat, olive oil and alcohol, each being scored using a 0-5-point scale. In specific, items representative of the Mediterranean diet (whole grains, potatoes, fruits, vegetables, legumes, fish, and olive oil) are given a score from 0 to 5 for a very rare to a very frequent consumption, respectively, the opposite scale is used for items not typical of the Mediterranean diet (full-fat dairy products, poultry and red meat), while alcohol gets a score of 0-5 for consumption of 0/>7, 6-7, 5-6, 4-5, 3-4 and <3 standardized servings per day, respectively (one standardized serving corresponds to 12 grams of ethanol). The total MedDietScore ranges from 0 to 55, with higher values indicating a greater level of adherence to the Mediterranean diet.

Physical activity habits: Physical activity level was assessed through the Athens Physical Activity Questionnaire (APAQ) [38]. The APAQ collects data on physical activities performed during the past week at work, at home and for recreation, as well as time spent in sedentary activities and sleep. Each activity is assigned a value of metabolic equivalent of tasks (MET) based on the Compendium of Physical Activities [39], and its daily energy expenditure is calculated based on the following equation: [MET value x body weight (kg) x activity duration (hours)]. For each participant, total energy expenditure (TEE) (kcal/day) was calculated by summing the energy expenditure of each activity, while physical activity level (PAL) was calculated as the ratio of total daily energy expenditure to basal metabolic rate (BMR) (1 kcal/kg/hour).

Smoking habits: Participants were assessed with respect to smoking habits and categorized as never-smokers, former smokers and current smokers. Former smokers and current smokers were further grouped as ever smokers.

Blood pressure: Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured with an automatic sphygmomanometer operating on the oscillometric principle (OMRON HEM-7130, Kyoto, Japan). Measurements were performed in the left arm with the subject being in a seated position, after an at least 8-hour fasting period and at least 30 minutes without engaging in any kind of physical activity or smoking [40]. Two blood pressure measurements were taken from each participant separated by a 2-minute interval, and their average was calculated and used for analyses.

Blood sampling: Venous blood samples were collected after a 10-hour overnight fast. Serum was separated using silicone-coated tubes (BD Vacutainer® Plus Plastic Serum Tubes), allowing the blood to clot at room temperature for 60 minutes before centrifugation (1500 x

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g for 10 minutes at 20°C). Plasma was obtained from EDTA tubes (BD Vacutainer® spray-coated K2EDTA Tubes). A whole blood count in EDTA anticoagulated whole blood was performed on a BC-3000 Plus Auto Hematology Analyzer (Shenzhen Mindray Bio-Medical Electronics C., Ltd) at room temperature. All serum and plasma samples were stored at -80°C until further biochemical analysis.

Blood analyses: Standard biochemical marker measurements in serum were conducted using the ADVIA® Clinical Chemistry System (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) employing enzymatic methods. Specifically, glucose (mg/dL) was measured using glucose oxidase, triacylglycerols (TAG, mg/dL) with phospho-glycerol oxidase, and total cholesterol (mg/dL) with cholesterol esterase/cholesterol oxidase. High-density lipoprotein cholesterol (HDL-cholesterol, mg/dL) and low-density lipoprotein cholesterol (LDL-cholesterol, mg/dL) were measured using the same method as for total cholesterol, after first removing non-HDL and non-LDL lipoproteins, respectively. In the second step, specific surfactants were used to selectively identify HDL- and LDL-particles. Insulin (µIU/mL) was measured with Atellica® IM Analyzer (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) employing a 2-site sandwich immunoassay using direct chemiluminescent technology.

Citrated plasma was used for the determination of the activity of t-PA (IU/mL) and PAI-1 (AU/mL) with human chromogenic activity kits (intra-assay CV%: <10% and inter-assay CV%: <10%, Assaypro LLC, St. Charles, Missouri) as well as for the evaluation of fibrinogen (mg/dL) by the Clauss method using Dade® Fibrinogen Determination Reagents, activated partial thromboplastin time (aPTT, sec) with Pathromtin® SL reagent, prothrombin time (PT, sec) with Thromborel® S reagent and International Normalized Ratio (I.N.R.)

The concentrations of serum sP-selectin (ng/mL) and PF4, also known as CXCL4 (ng/mL) were determined with ELISA kits (Quantikine, R&D Systems, intra-assay CV%: <8.9%, inter-assay CV%: <12%) and (kit Quantikine, R&D Systems, intra-assay CV%: <8.1%, and inter-assay CV%: <12%), respectively.

All the above measurements were performed in duplicate.

Ex vivo assessment of human platelet aggregation: The procedure followed has been previously described [28]. Briefly, blood was collected using trisodium citrate as an anticoagulant, and PRP was obtained through centrifugation. Platelet-poor plasma (PPP) was obtained by further centrifugation of the remaining residue. Light transmission aggregometry in a Chrono-Log aggregometer (model 440VS, Havertown, PA, USA) was used to assess platelet aggregation in human PRP following stimulation with various concentrations of PAF, ADP and collagen (COL). The minimum-irreversible platelet aggregation induced by an agonist was considered 100% aggregation. Within the 20%–80% range of reversible platelet aggregation, a linear relationship exists between the agonist concentration and platelet aggregation percentage. The concentration of an agonist required to induce 50% platelet aggregation (EC_{50} or half-maximal effective concentration) was calculated from this linear portion of the curve. As expected, a lower EC_{50} value indicates greater potency in platelet aggregation. Bovine serum albumin (BSA), PAF, ADP, and COL were obtained from Sigma-Aldrich (St. Louis, MO, USA). EC50 values were expressed as nM for PAF, µM for ADP, and µg/mL for COL.

Red blood cell isolation and fatty acids (FA) analysis: The procedure followed has been previously described [41]. Briefly, red blood cell pellet was prepared from EDTA tubes (BD

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Vacutainer® spray-coated K2EDTA Tubes) after recently plasma collection. The determination of fatty acids profile was performed by gas chromatography as it is described in session 2.2.

2.8. Sample size, randomization, allocation and implementation

A priori power analysis determined that a sample size of 25 participants was sufficient to achieve an 83% statistical power at a 5% significance level for two-sided hypotheses, assessing a 1 standard deviation (SD) difference between groups based on the EC_{50} values of platelet aggregation induced by PAF. An additional 20% was accounted for potential dropouts. Ultimately, 34 individuals were included in the study, of whom 32 completed it, while 2 dropped out—one participant was unable to consume the required amount of fish, while blood sampling was not possible for another one.

Participants were randomly assigned to each treatment group using a computerized random number generator. The allocation process was managed exclusively by one research dietitian, ensuring that all other investigators remained blind to group assignments. Additionally, participants were unaware of the specific fish treatments they received, as the two types of raw fish appeared identical, and their cooked forms exhibited similar sensory characteristics, such as odor, taste, and aftertaste.

2.9. Compliance

Throughout the study (fish consumption and wash-out periods), participants documented the days they consumed fish, any medications taken, vaccination for COVID and any short-term illnesses (such as cold of flu), in print self-monitoring forms. The forms were collected during the scheduled appointments with the research dietitian. To further monitor adherence, a research investigator contacted participants midway through each intervention period to assess compliance and identify any unexpected issues, such as illness, difficulties with fish deliveries, or accidental loss of servings during cooking. Additionally, compliance with the study protocol was evaluated through FA analysis of red blood cell (RBC) membranes, which were collected at the start and at the end of each treatment period.

2.10. Statistical methods

Data analysis followed the per-protocol (PP) approach. All statistical analyses were conducted using Stata Statistical Software, Release 12 (College Station, TX: StataCorp LP). P-values are presented for two-sided tests; the significance (α) level was set to 0.05 for all two-sided tests.

Primary and secondary outcomes were analyzed for sequence, carry-over, and period effects using analysis of variance (ANOVA) for a 2×2 crossover design (pkcross). To assess the normality of the data distribution, the Shapiro-Wilk test and normal Q-Q plots were utilized. Data are summarized as absolute frequencies for categorical variables (i.e., sex), and as mean ± standard deviation for continuous variables if normally distributed or as median (25th, 75th percentiles) if non-normally distributed. Fisher's exact test was used to compare the intervention groups for sex distribution. Two-independent-samples t tests or Mann–Whitney tests were used to compare the intervention groups for continuous variables with normal and non-normal distribution, respectively.

The FA composition of lipid extracts from the CF and EF fillets is expressed as % percentage of total FA and is based on the analysis of three samples of CF and EF fillets, respectively; only data for FA ≥ 0.2% of total FA are presented. The biological activity of the

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TL and PL extracts from the CF and EF fillets against platelet aggregation is expressed as IC_{50} (mg sample/mL cuvette aggregometer) and is based on the analysis of three samples of CF and EF fillets, respectively; two-independent-samples t test was used to compare the two fish species. Demographic characteristics, anthropometric measurements, hematological and coagulation parameters, biochemical and hemostatic markers, and the FA composition (%) of RBC membranes of the participants are presented for the two intervention groups (CF/EF vs EF/CF) at baseline and at the beginning of the second intervention period.

Anthropometric measurements, hematological parameters, classical biochemical markers, coagulation parameters, markers of hemostasis and *ex vivo* platelet aggregation of the participants are presented for each fish treatment at the end of each intervention period. Relative change (%) was calculated as the change between the values at the end and the values at the beginning of each intervention period. Two-paired samples t-tests or Wilcoxon matched-pairs signed-rank tests were used to compare the values at the end of treatment with the values at the beginning of treatment per fish treatment and per intervention period, as well as for the combined results from the two periods for variables with normal distribution and non-normal distribution, respectively.

To evaluate compliance to the study protocol, baseline and follow-up data for the participant's dietary habits (i.e., energy and macronutrient intake, food and food group consumption, adherence to the Mediterranean diet) were compared with paired-samples t-tests or Wilcoxon signed-rank tests for continuous variables with normal and non-normal distribution, respectively. In addition, the FA composition (%) of RBC membranes before treatment and the pre-post treatment change by period and type of treatment were evaluated; data are presented as mean before treatment ± standard deviation, and as mean change ± standard deviation and 95% confidence interval (CI) of the change, respectively. Change was calculated as the FA content (%) after treatment minus the FA content (%) before treatment. Two-paired-samples t tests were used to compare the values after treatment with the values before treatment.

Finally, the effect size of the enriched fish (EF) treatment compared to the conventional fish (CF) treatment (treatment effect) regarding the FA composition of the RBC membranes, platelet aggregation markers and aPTT was estimated using the mixed-effects linear regression with random effects at the level identified by the variable ID, that is, the participant level; the dependent variable was the outcome variable evaluated post-treatment, the exposure was the fish type consumed (EF versus CF), the covariates were the outcome variable evaluated prior to fish treatment, the sequence (i.e., the between-subjects factor representing the two intervention groups of the study), and sex of the participants. Values for PAF (EC_{50}), ADP (EC_{50}) and collagen (EC_{50}) were ln-transformed values so that residuals are approximately normally distributed. Results are presented as the mean difference between the EF and CF treatments for the untransformed variables or as the exponentiated mean difference (or the ratio of the geometric mean of the outcome variable at the end of the EF treatment over the geometric mean of the outcome variable at the end of the CF treatment) for the ln-transformed variables; the values of standard error (SE), the 95% confidence interval (95% CI) are also reported.

3. Results

3.1. FA analysis and biological activity of fish fillets

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The total lipid content (TL) of the fillet from CF was 3.51±0.71 % (w/w lyophilized fish mass), specifically 0.80±0.08 % (w/w) in PL and 2.71±0.63 % (w/w) in NL. In the EF, the corresponding values were 4.58±0.69 % (w/w) for TL, 1.02±0.16 % (w/w) for PL and 3.56±0.54 % (w/w) for NL. Although the diet enriched with OOPLE led to higher levels of lipids in EF, the differences in the lipid content between the CF and EF fillets were not significant. In both fish fillets, the proportion of PL in relation to TL was comparable between the CF and EF fillets, specifically 22.81% in the CF and 22.26% in the EF. The FA composition of the fillets was analyzed from both CF and EF, and is expressed as a percentage of total FA content in Table 1. No significant differences were observed between the two fish species in the relative composition of major FA classes, including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), as well as ω-3 and ω-6 fatty acids.

Table 2 summarizes the results of the biological activity of TL and PL extracts from CF and EF in relation to platelet aggregation. The PL extract from EF demonstrated markedly stronger inhibitory activity against the two aggregation agonists namely PAF and ADP (p=0.018 for PAF and p=0.03 for ADP), and an inhibitory trend was also observed for TRAP-induced aggregation (p=0.06). This is evidenced by significantly lower IC_{50} values— representing the concentration required to inhibit agonist-induced aggregation by 50%—compared to the corresponding extract from CF. A similar trend was observed for the TL extract, although the significant inhibition was limited to PAF-induced aggregation (p=0.032).

3.2. Description of intervention groups at the beginning of treatment periods

The participants in the study were middle-aged adults, with overweight or obesity and normoglycemia. At baseline, there were no notable differences between the CF/EF and EF/CF groups in all the evaluated parameters with the exception of glucose levels that were higher in the EF/CF group compared to the CF/EF group (Table 3). Furthermore, the two groups did not differ with respect to FA composition (%) of RBC membranes regarding EPA, DHA as well as SFA, MUFA, PUFA, ω-3 and ω-6 FA. (Table S1). At the onset of the second period, no significant differences were observed in any of the assessed parameters (Table 3), including the FA composition of RBC membranes (Table S1).

3.3. Endpoints and changes in outcomes after fish treatments

Within group changes and between-group differences in participant's dietary habits are presented in Table S2. Energy intake decreased in both groups (CF: from 2,105 ± 512 to 1,934 ± 468 kcal/day, p=0.009; EF: from 2,094 ± 456 to 1,921 ± 429 kcal/day, p=0.008), but was similar between groups at follow-up (p=0.911). Moreover, protein intake increased in the CF group (from 15.0 ± 2.5 to 16.0 ± 2.5 %E, p=0.003), but was similar between groups at follow-up (p=0.936). At the end of the study, no significant between-group differences were evident in the consumption of the food groups (all p>0.050). MedDietScore values were also similar between groups at follow-up (p=0.928). With regard to physical activity habits, no significant within-group changes were observed in participants' TEE (CF: from 2827 ± 490 to 2850 ± 567 kcal/d, p=0.686; EF: from 2843 ± 537 to 2890 ± 649 kcal/d, p=0.424) and PAL (CF: from 1.36 ± 0.14 to 1.37 ± 0.16, p=0.762; EF: from 1.35 ± 0.13 to 1.37 ± 0.21, p=0.441); between-group differences at follow-up were also non-significant (p=0.794 and p=0.980, respectively).

Tables 4 and 5 present the data concerning anthropometric measurements, blood pressure levels, hematological and coagulation parameters, as well as biochemical and hemostatic markers at the end of the first and second intervention periods involving

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consumption of either the CF or the EF, respectively. Both fish interventions reduced triglyceride levels (Table 4, CF: p=0.031; Table 5, EF: p=0.043). LDL-cholesterol levels were lower following the first intervention period of both fishes (Table S3, CF: p=0.034 and EF: p=0.018). Notably, only CF consumption sustained a reduction in LDL-cholesterol through the second period (Table S3, p=0.039), resulting in an overall decrease (Table 4, p=0.003). EF intake significantly lowered total cholesterol after the first period (Table S3, p=0.006), whereas CF consumption was associated with consistently lower total cholesterol levels overall (Table 4, p=0.020). In contrast, CF intake was linked to an increase in glucose concentrations at the end of the first period (Table S3, p=0.028) and a reduction in WBC count (Table 4, p=0.047), primarily observed at the conclusion of the second period (Table S3, p=0.046).

EF intake resulted in reduced platelet sensitivity to PAF during both periods (p=0.030 and p=0.006 for the first and second periods, respectively), as well as to ADP during the first period (p=0.039), as evidenced by significantly elevated EC_{50} values, representing the concentration required to achieve 50% agonist-induced aggregation (Table S4). Conversely, CF consumption led to increased platelet sensitivity to ADP in both periods (p=0.049 and p=0.007 for the first and second periods, respectively in Table S4). Collagen-induced PRP aggregation remained unaffected by either intervention. Similarly, serum PF4 and sP-selectin levels did not exhibit significant changes across all interventions (Tables 4, 5 and S4).

Regarding the evaluation of coagulation markers, PT and I.N.R. remained unaffected by either intervention while aPTT was significantly reduced following EF consumption during both intervention periods (p=0.029 and p=0.004 for the first and second periods, respectively in Table S4), although values remained within the normal range. Fibrinogen levels presented a significant increase after EF intake during the first period (p=0.021, Table S4). Among the two fibrinolysis markers, tPA activity increased significantly following EF consumption at the end of the first intervention period (p=0.021), as well as after CF consumption at the end of the second period (p=0.021). PAI activity, however, did not exhibit significant changes across any of the interventions (Tables 4, 5 and S4).

3.4. Compliance to the study protocol

Participants demonstrated full adherence to the study protocol, as indicated by self-reported data and corroborated by the increased percentages of total ω-3 FA and DHA in RBC membranes at the end of each intervention period compared to baseline levels even though no significant difference was recorded (Table S5). On the nutrient level, ω-3 FA intake increased in both groups (CF: from 0.60 ± 0.13 to 0.75 ± 0.13 %E, p<0.001; EF: from 0.63 ± 0.23 to 0.74 ± 0.09 %E, p=0.010), but was similar between groups at follow-up (p=0.654) (Table S2). On the food/food group level, fish consumption increased in both groups [CF: from 0.18 (0.04, 0.35) to 0.70 (0.53, 0.70) servings/day, p<0.001; EF: from 0.25 (0.18, 0.35) to 0.70 (0.53, 0.70) servings/day, p<0.001), but was similar between groups at follow-up (p=0.819) (Table S2). The assessment of the effect size of the intervention with enriched fish (EF) compared to conventional fish (CF), in terms of the FA composition of RBC membranes, revealed no significant differences (Table S6).

3.5. Effect of fish type consumed

No significant sequence, carry-over or period effects were observed for changes in any primary or secondary outcome measures (data not shown). No significant differences

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were observed between the two fish treatments in terms of hematological parameters, lipid profile or markers of glucose homeostasis (data not shown). Similarly, PF4, sP-selectin and fibrinogen levels as well as PAI-1 and tPA activities, did not differ between the two fish treatments (data not shown).

Based on the *ex vivo* PRP aggregation results, consumption of the EF fish significantly altered platelet responsiveness compared to CF fish. Specifically, EF intake led to a 44% increase in the EC_{50} value for PAF (p=0.002) and a 67% increase in the EC_{50} value for ADP (p=0.002), indicating a markedly reduced platelet sensitivity to both agonists. Furthermore, aPTT values were, on average, 0.82 seconds shorter following EF intake compared to CF (p=0.025), although they remained within the normal range (Table 6). These findings highlight distinct effects of the two fish types on platelet function and coagulation parameters.

4. Discussion

In the present study, the effects of consuming gilthead sea bream reared on a diet containing 13.3% w/w OOPLE-mix (2% w/w OOPLE) were evaluated in comparison to those of gilthead sea bream raised on a conventional fish oil-based diet. The investigation focused on markers of hemostasis and thrombosis in adult participants with overweight or obesity, yet otherwise healthy. This double-blind, randomized crossover trial demonstrated that the consumption of two enriched fish (EF) meals per week (approximately 200 g cooked per meal) over a one-month period significantly attenuated platelet aggregation in response to PAF and ADP by 44% and 67%, respectively, and led to a reduction in activated partial thromboplastin time (aPTT) by 0.82 seconds, suggesting a modest yet measurable influence on intrinsic and common coagulation pathways, compared to the control fish (CF) group. These findings suggest distinct modulatory effects of the two fish diets on platelet function and coagulation parameters. Conversely, no significant differences were observed between the two dietary treatments regarding the other hemostatic and thrombotic biomarkers.

Although the substitution of FM and FO with agricultural alternatives in aquaculture feeds has been the subject of extensive investigation, yielding promising outcomes pertaining to the preservation or even enhancement of growth performance and the health of fish, the effects of these dietary modifications on the nutritional quality of fish and subsequent implications for human health remain significantly under-researched [15]. The gilthead sea bream is recognized as one of the predominant species in Mediterranean aquaculture. Previous experiments have demonstrated that substituting 8% w/w of FO with olive pomace (OP) in the diet of gilthead sea bream, resulted in satisfactory growth performance compared to those fish that were sustained on traditional feed formulations. Furthermore, the TL content of gilthead sea bream subjected to an OP-enriched diet displayed significantly enhanced inhibitory effects against platelet aggregation induced by PAF [26]. In the current investigation, the fish feed was supplemented with a specific polar lipid extract derived from OP (OOPLE), which has been previously characterized by its PAF-inhibitory properties and has exhibited anti-atherogenic effects in experimental models, alongside beneficial modulation of both platelet aggregation and inflammatory responses in healthy subjects [21-23, 28]. In the present study, fishes that were nurtured on the enriched diet demonstrated an elevated lipid content compared to that observed in fishes receiving the conventional feed; however, no significant differences were identified between the two groups in terms of TL content and FA composition within the fillets, expressed as a percentage of total FA content. The lipid profile

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and FA composition of the EF are in accordance with findings documented in prior research [42, 43]. Additionally, the PL extract from the EF exhibited markedly enhanced anti-platelet aggregation activity when stimulated by either PAF or ADP, findings that corroborate those reported in earlier studies [26].

Regarding *ex vivo* PRP aggregation, the consumption of EF was associated with a diminished responsiveness of platelets to PAF across both intervention periods and a similar trend was observed for ADP during the first period, as indicated by the significantly increased EC_{50} values, which denote the concentration necessary to elicit 50% platelet aggregation induced by the agonist. In contrast, the intake of CF resulted in an enhanced platelet sensitivity to ADP throughout both periods while PAF-induced aggregation was unaffected. Notably, collagen-induced aggregation of PRP exhibited no significant changes as a result of either dietary intervention. The above results support the conclusion that the reduced platelet sensitivity to PAF and ADP is due to the enrichment of the fish feed with OOPLE. The literature presents conflicting evidence regarding the effect of fish consumption on ADP- and collagen-induced platelet aggregation in healthy individuals, with some studies reporting a decrease in platelet aggregation —typically in interventions where participants consumed more than three fish meals per week—, while others observe no significant effect [4]. The present findings are not in agreement with those reported by Mori et al. [44], who observed a reduction in platelet aggregation in response to collagen and PAF after fish intake. However, in their study, participants exhibited higher blood pressure, cholesterol, and triglyceride levels, and the intervention involved a substantially higher fish intake (one fish meal per day) sustained over a much longer period (12 weeks). In contrast, the findings of the present study align with our previous research, which demonstrated that an eight-week intake of farmed gilthead sea bream from apparently healthy volunteers, resulted in an enhanced platelet response to ADP while a modest reduction of PAF-induced aggregation was also recorded [45]. With respect to the influence of fish consumption, it seems that platelet aggregation responses are modulated by several factors, including the background diet, the frequency of fish meal consumption per week and the duration of the dietary intervention, as well as the metabolic status of the participants and the specific agonist employed to induce aggregation. Furthermore, a meta-analysis of randomized controlled trials in adults found that supplementing with ω-3 polyunsaturated fatty acids (α-linolenic acid, EPA, DHA) did not significantly influence ADP- and collagen-induced platelet aggregation in healthy individuals [46]. Although the beneficial impact of ω-3 PUFAs on platelet function may be partly due to increased ω-3 and reduced ω-6—particularly arachidonic acid—levels in platelet phospholipids, research showed no significant differences in collagen- or thrombin-induced platelet aggregation when comparing equal amounts of fish and plant oils with varying ω-6 to ω-3 ratios [47]. The marked reduction in platelet aggregation in response to PAF and ADP following consumption of the EF cannot be attributed to its ω-3 and ω-6 FA content, as both fishes exhibited comparable levels of these FA. Furthermore, the ω-3 and ω-6 FA composition of RBC membranes in participants did not differ significantly between the two dietary interventions. Conversely, the observed effects are in agreement with the *in vitro* inhibitory activity of lipid extracts derived from the EF on PAF- and ADP-induced aggregation, as well as with prior dietary intervention studies indicating that the intake of PAF antagonists reduces platelet responsiveness to PAF and ADP [28, 48, 49].

Collectively, the findings indicate that the observed biological effects cannot be ascribed to individual fatty acids, including ω-3 PUFAs, but are more likely a result of enrichment with OOPLE. Olive pomace (OP) is a complex matrix containing carbohydrates—

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primarily in the form of polysaccharides—lipids, phenolic compounds, and inorganic elements. Its phenolic profile closely mirrors that of olive oil (OO), with predominant constituents such as tyrosol (T), hydroxytyrosol (HT), and p-coumaric acid, as previously reported by Ribeiro et al. [50]. Ethanolic extracts derived from OP have demonstrated the ability to inhibit platelet aggregation *in vitro*. This anti-platelet activity is largely attributed to the presence of polyphenols, particularly oleuropein and HT, both of which are known to interfere with platelet activation pathways and the biosynthesis of eicosanoids [51, 52]. Nevertheless, it is important to recognize that the concentrations of phenolics employed in these *in vitro* studies often exceed those achievable through a typical diet, raising questions about their direct physiological relevance. In contrast, human intervention trials have focused on extra virgin olive oil (EVOO), especially formulations rich in oleocanthal or HT. These studies have shown reductions in collagen-induced platelet aggregation and decreases in serum thromboxane B2 levels within hours of consumption, in both healthy individuals and patients with diabetes [53, 54]. However, the polar lipid extract used in the present trial contained only trace amounts of phenolic compounds—approximately 1,000 times less than the lipid fraction. This stark discrepancy suggests that the reduced platelet sensitivity to PAF and ADP observed in the trial is primarily attributable to the polar lipids themselves. The possibility of a synergistic interaction between polar lipids and phenolic microconstituents cannot be excluded. Both T and oleuropein have been shown to inhibit PAF-induced platelet aggregation [24]. Further supporting the role of synergy, recent evidence has demonstrated that HT, in combination with 3',4'-dihydroxyphenylglycol in proportions similar to those found in EVOO, can attenuate collagen-induced platelet aggregation in diabetic rat models [55]. These findings point toward a complex interplay between lipid and phenolic constituents in modulating platelet function, highlighting the potential of OOPLE as a multi-component therapeutic agent.

In the assessment of coagulation biomarkers, PT and I.N.R. exhibited no alterations as a result of either intervention. Conversely, aPTT demonstrated a significant decrease subsequent to the consumption of EF, while an elevation in fibrinogen concentration was noted after EF intake during the initial time frame, which may have played a role in the observed reduction in aPTT. The marginal—yet within the normal range—decrease in aPTT alongside the increase in fibrinogen levels, indicative of a reduced clot formation duration, may signify a compensatory response to the markedly diminished primary hemostatic function via platelet aggregability, as neither platelet count nor mean platelet volume displayed any significant variation. The potential for alterations in vitamin K biosynthesis by the gut microbiota remains a plausible consideration, given that it has been documented that the OOPLE may affect its microbial composition [29, 56]. ω-3 PUFA are postulated to exert influence over blood coagulation factors, although findings from various studies have been inconsistent. An extensive review encompassing eight RCTs with a total of 822 participants—of which 408 received at least 1.5 g/day of EPA and DHA—revealed no significant alterations in PT, PTT, or aPTT [57]. In a similar vein, multiple investigations utilizing fish oil reported no noticeable impact on PT or aPTT; however, one study indicated a modest reduction in the activity of intrinsic and common pathway factors following the administration of 0.9 g/day of ω-3 over a period of 30 days [58]. In contrast, an increase in PTT was observed among subjects with hypercholesterolemia following the intake of 2–4 g/day of ω-3 PUFAs for durations ranging from 9 to 12 weeks [59, 60]. With respect to fish consumption, the majority of the interventions failed to demonstrate a significant impact on fibrinogen levels in participants with normal weight or overweight, whereas an upregulation of fibrinogen was recorded in

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individuals who consumed a daily meal of trout raised on a marine diet for a duration of 8 weeks [4, 19].

No significant differences were observed between the two fish treatments regarding several other thrombosis-related biomarkers. Levels of PF4 and sP-selectin remained stable across both intervention periods, indicating that these markers of platelet activation were not notably influenced by the dietary intervention. These findings align with the majority of existing studies, which similarly report no effect of fish consumption on plasma markers of platelet activation, including sP-selectin and PF4 [61]. An exception is a single study involving a small cohort of healthy Japanese individuals, in which daily intake of 200–400 g of fish over 17 days led to a significant reduction in PF4 levels, accompanied by decreases in platelet count and mean platelet volume [62]. With respect to fibrinolytic activity, tPA activity showed an increase following EF consumption at the end of the first intervention period, and a similar increase was observed after CF consumption at the end of the second period; however, no significant differences in tPA activity were detected between the EF and CF treatments. The activity of PAI-1 remained unchanged throughout the study. The impact of fish intake on tPA and PAI-1 levels or activities has been explored in various intervention studies, but the results have been largely inconsistent and inconclusive. Most studies using fish or fish oil report no significant effect on tPA or PAI-1 activity or antigen levels in healthy individuals [63]. However, some earlier studies suggested a potential enhancement of fibrinolytic capacity—reflected by increased tPA activity and reduced PAI-1 levels—following diets rich in lean fish or fish oil, while others reported a significant increase in plasma PAI-1 activity [64, 65, 66].

In addition to effects on thrombosis-related parameters, lipid profile assessments revealed that both fish consumption led to reductions in serum concentrations of triglycerides, while CF consumption also resulted in decreased levels of LDL-cholesterol, and total cholesterol. Nonetheless, the magnitude of lipid reduction did not differ significantly between the EF and CF interventions, suggesting that while both fish types may support cardiovascular health, the observed lipid-lowering effects cannot be attributed exclusively to the modified diet of the EF. Our findings are consistent with the majority of studies reporting beneficial effects on triglyceride and cholesterol levels when daily intake of EPA and DHA exceeds approximately 1 g per day [4].

5. Limitations and Strengths

A number of methodological strengths and limitations should be considered to accurately interpret the findings of the present study. A primary limitation pertains to sample size estimation, which was based solely on the EC_{50} value of PAF post- versus pre-intervention. Additionally, the study cohort predominantly comprised middle-aged individuals of Greek origin without a history of cardiovascular disease, thus limiting the external validity and generalizability of the results to more diverse populations. Furthermore, other studies are warranted to explore the long-term implications of such dietary interventions. Conversely, the study possesses several methodological strengths. In the absence of prior research on the effects of differently fed farmed gilthead sea bream on platelet aggregation and circulating haemostatic biomarkers in healthy adults, the cross-over design represents a significant advantage, as it allows each participant to serve as their own control, thereby reducing inter-individual variability. Moreover, the absence of significant baseline differences between the intervention sequences and the lack of carry-over effects for primary outcomes enhance the internal validity of the results. Compliance with the dietary intervention was further

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substantiated through fatty acid profiling of erythrocyte membranes, providing objective biochemical confirmation of adherence.

6. Conclusion

In conclusion, this study provides evidence that the inclusion of polar lipid-rich olive pomace extracts in aquaculture feed can enhance the cardioprotective profile of gilthead sea bream, primarily through attenuation of platelet aggregation and modulation of coagulation parameters. These findings underscore the potential of sustainable aquafeed innovations to produce functional seafood products that may contribute to the prevention of thrombotic cardiovascular events, particularly in populations at elevated cardiometabolic risk. Further studies are warranted to explore the long-term implications of such dietary interventions and to elucidate the underlying mechanisms driving these effects.

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Table 1. Fatty acid profile of total (TL) and polar (PL) lipids extracts from CF and EF fillets.

Fatty acids	CF (TL)	EF (TL)	CF (PL)	EF (PL)
C14:0	4.05±0.25	4.06±0.24	1.32±0.04	1.30±0.05
C15:0	0.33±0.04	0.37±0.03	0.24±0.01	0.26±0.01
C16:0	18.82 ± 1.01	19.37±1.15	23.84±1.17	24.42±1.46
C16:1	6.21 ± 0.24	6.38±0.32	2.01±0.07	2.28±0.09
C17:0	0.81±0.03	0.86±0.04	0.49±0.01	0.51±0.01
C17:1	0.77±0.02	0.74±0.03	0.23±0.01	0.21±0.01
C18:0	4.01±0.26	3.68±0.18	5.22±0.24	5.38±0.22
C18:1ω9c	25.41±1.26	26.25±1.83	15.83±0.65	16.47±0.82
C18:2ω6c	12.09±0.43	11.61±0.70	7.06±0.21	7.85±0.39
C18:3ω6	0.25±0.01	0.23±0.01	0.15±0.01	0.14±0.01
C18:3ω3	1.84±0.04	1.94±0.06	0.78±0.04	0.82±0.03
C20:0	0.37±0.03	0.31±0.01	0.19±0.01	0.18±0.01
C20:1(ω9)	2.43±0.07	2.08±0.06	1.01±0.04	0.94±0.03
C20:2(ω6)	0.42±0.02	0.43±0.01	0.40±0.01	0.40±0.02
C20:3ω6	0.27±0.01	0.25±0.01	0.39±0.01	0.38±0.01
C20:4ω6	0.95±0.08	0.77±0.06	2.43±0.08	2.03±0.10
C20:5ω3	7.91±0.32	7.84±0.39	13.01±0.39	12.74±0.76
C22:1ω9	0.29±0.01	0.33±0.01	0.05±0.00	0.05±0.00
C22:6(ω3)	12.23±0.37	11.72±0.59	24.77±1.79	22.80±1.59
SFA	28.39±1.13	28.85±1.73	31.30±1.27	32.55±2.27
MUFA	35.11±1.78	35.97±2.52	19.13±0.96	20.04±1.20
PUFA	35.96±1.89	35.18±2.29	48.99±2.47	47.41±3.32
ω-6	13.98±0.56	13.50±0.68	10.43±0.37	10.93±0.55
ω-3	21.98±1.02	21.68±1.30	38.56±2.04	36.49±2.19
ω-3/ ω-6	1.57±0.05	1.61±0.06	3.69±0.13	3.34±0.11

Data are presented as mean (±standard deviation) of % percentage of total FA and are based on the analysis of three samples of CF and EF, respectively. Only fatty acids that their percent was ≥ 0.2% of total fatty acids are presented. CF (PL): polar lipids extract from conventional fish; CF (TL): total lipids extract from conventional fish; EF (PL): polar lipids extract from enriched fish; EF (TL): total lipids extract from enriched fish; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids.

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Table 2. Biological activity of the total and polar lipid extracts from CF and EF against platelet aggregation.

	IC_{50} TL (mg sample/mL cuvette aggregometer).			IC_{50} PL (mg sample/mL cuvette aggregometer).		
Fish species	**PAF**	**ADP**	**TRAP**	**PAF**	**ADP**	**TRAP**
CF	0.946±0.101	0.844±0.135	0.866±0.121	0.027±0.005	0.782±0.116	0.716±0.136
EF	0.693±0.097*	0.845±0.144	0.931±0.150	0.014±0.003*	0.522±0.061*	0.485±0.090

Data are presented as mean (±standard deviation) and are based on the analysis of three samples of CF and EF, respectively. Two-independent-samples t test was used to compare fish species. * indicates significant difference of EF extract compared to CF corresponding extract ($p<0.05$). ADP: adenosine diphosphate; CF: conventional fish; EF: enriched fish; IC_{50}: inhibitory concentration for fifty percent inhibition; PAF: platelet-activating factor; PL: polar lipids extract; TL: total lipids extract; TRAP: thrombin receptor-activating peptide.

Table 3. Demographic characteristics, smoking habits, anthropometric measurements, blood pressure levels, hematological and coagulation parameters, and biochemical and hemostatic markers of the intervention groups at the beginning of each period

Variable	**First period** at baseline (time= 0).			**Second period** at baseline (time= 2).		
	CF/EF n=16	**EF/CF n= 16**	***p***	**CF/EF n=16**	**EF/CF n= 16**	***p***
Sex (F/M), n	7/9	9/7	0.724	7/9	9/7	0.724
Age (years)	47.4 ± 7.2	52.9 ± 8.3	0.057			
Current smoking (yes/no), n	5/11	5/11	>0.999			
Ever smoking (yes/no), n	7/9	9/7	0.724			
SBP, mmHg	127.5 ± 17.6	135.2 ± 12.5	0.194	135.4 ± 16.7	129.4 ± 15.8	0.307
DBP, mmHg	76.0 ± 9.1	77.8 ± 6.7	0.535	75.5 ± 6.6	72.8 ± 10.4	0.381
BMI (kg/m^2)	29.7 ± 2.7	30.2 ± 3.7	0.665	30.5 ± 3.0	30.4 ± 3.6	0.930
WBC count (10^3/µL)	5.9 (5.0, 6.9)	5.8 (5.3, 6.9)	0.806	5.7 (5.2, 7.4)	6.0 (5.3, 7.3)	0.792
Lymphocyte count (10^3/µL)	1.9 ± 0.6	1.9 ± 0.5	0.683	1.9 (1.8, 2.0)	2.0 (1.6, 2.3)	0.879
Platelet count (10^3/µL)	233.0 (194.0, 278.5)	246.0 (197.5, 290.0)	0.836	253.5 ± 76.5	267.4 ± 80.2	0.620
Mean platelet volume (fL)	7.97 ± 0.97	8.22 ± 0.78	0.429	7.85 (7.40, 8.65)	8.20 (7.85, 8.45)	0.227
Glucose (mg/dL)	87.81 ± 7.53	95.13 ± 11.06	**0.037**	93.0 (91.50, 106.50)	100.50 (92.50, 105.50)	0.865
Insulin (µIU/mL)	8.20 (6.40, 11.85)	11.1 (6.4, 14.0)	0.283	9.45 (8.55, 11.05)	12.45 (7.30, 17.65)	0.175

Triglycerides (mg/dL)	110.0 (87.0, 169.0)	110.0 (66.5, 138.0)	0.440	107.0 (85.0, 139.5)	107.0 (76.0, 141.0)	0.806
Total cholesterol (mg/dL)	199.63 ± 22.15	202.13 ± 25.35	0.769	199.25 ± 28.17	203.50 ± 22.70	0.642
HDL-cholesterol (mg/dL)	53.0 (48.5, 60.0)	53.5 (49.0, 71.0)	0.865	53.38 ± 11.96	54.88 ± 15.78	0.764
LDL-cholesterol (mg/dL)	140.50 ± 21.06	145.19 ± 27.97	0.596	138.94 ± 32.47	146.94 ± 30.12	0.476
tPA (IU/mL)	0.53 (0.34, 0.62)	0.48 (0.35, 0.80)	0.880	0.85 (0.75, 1.42)	0.95 (0.78, 1.28)	0.806
PAI-1 (AU/mL)	70.66 (56.80, 80.70)	67.66 (54.21, 74.73)	0.720	70.03 (53.72, 78.56)	68.09 (57.74, 79.60)	0.678
Prothrombin time (sec)	11.73 ± 0.81	11.76 ± 0.83	0.932	11.30 ± 0.67	11.45 ± 0.83	0.579
INR	0.95 ± 0.07	0.95 ± 0.07	0.942	1.0 ± 0.06	1.01 ± 0.08	0.524
aPTT (sec)	30.78 ± 3.33	32.23 ± 3.79	0.257	32.08 ± 3.03	32.69 ± 4.23	0.642
Fibrinogen (mg/dL)	314.5 (288.0, 352.0)	343.0 (306.5, 380.0)	0.193	322.5 (283.0, 343.0)	352.5 (322.5, 414.5)	0.082
sP-selectin (ng/mL)	83.66 ± 25.53	95.21 ± 29.16	0.243	80.10 ± 28.89	91.05 ± 32.15	0.319
PF4 (ng/mL)	8909.05 (8481.45, 9744.8)	9398.35 (8214.60, 9872.85)	0.792	8142.35 (7564.0, 9475.75)	8958.75 (8067.35, 9827.55)	0.309
PAF (EC_{50}) (nM)	89.04 (29.76, 148.53)	57.54 (29.88, 101.17)	0.486	39.28 (27.30, 58.11)	36.22 (27.23, 46.20)	0.498
ADP (EC_{50}) (μM)	4.72 (2.58, 6.72)	2.67 (1.82, 4.51)	0.062	0.74 (0.46, 1.33)	0.62 (0.45, 1.06)	0.462

Collagen (EC_{50}) (μg/mL)	0.59 (0.39, 0.84)	0.61 (0.41, 0.72)	0.910	0.44 (0.32, 0.60)	0.34 (0.28, 0.58)	0.597

Categorical variables (i.e., sex and smoking) are presented as absolute frequencies, n. Continuous variables are presented as mean ± standard deviation if normally distributed or as median (25^{th}, 75^{th} percentiles) if non-normally distributed. Fisher's exact test was used to compare groups for sex distribution. Two-independent-samples t tests or Mann–Whitney tests were used to compare the intervention groups for continuous variables with normal and non-normal distribution, respectively; p-values are presented for two-sided tests. The significance (α) level was set to 0.05 for all two-sided tests. ADP: adenosine diphosphate; aPTT: activated partial thromboplastin time; BMI: body mass index; DBP, diastolic blood pressure; HDL: high density lipoprotein; INR: International Normalized Ratio; LDL: low density lipoprotein; PAF: platelet-activating factor; PAI-1: plasminogen activator inhibitor-1; PF4: platelet factor 4; SBP, systolic blood pressure; tPA: tissue plasminogen activator; WBC: white blood cells.

Table 4. Anthropometric measurements, blood pressure levels, hematological parameters, classical biochemical markers, coagulation parameters, markers of hemostasis and ex vivo platelet aggregation of the participants on the conventional fish (CF) diet.

	End of 1st Period	% Change	End of 2nd Period	% Change	*p**
BMI (kg/m^2)	29.9 ± 2.9	0.55 (-0.08, 1.03)	30.4 ± 3.7	-0.40 (-0.70, 0.44)	0.780
SBP (mmHg)	129.3 ± 13.9	0.29 (-4.30, 7.14)	129.7 ± 16.8	-0.82 (-10.12, 1.58)	0.245
DBP (mmHg)	77.7 ± 8.4	0.34 (-2.67, 6.63)	76.8 ± 7.9	3.21 (-1.07, 6.03)	0.219
WBC count (10^3/μL)	5.7 (5.0, 7.6)	-0.93 (-10.03, 3.25)	5.4 (4.9, 7.0)	-8.47 (-15.49, 0.39)	**0.047**
Lymphocyte count (10^3/μL)	1.9 ± 0.5	2.50 (-7.89, 12.92)	1.9 (1.6, 2.2)	0.0 (-12.13, 16.25)	1.000
Platelet count (10^3/μL)	231.0 (189.5, 277.0)	-4.05 (-8.80, 7.04)	260.9 ± 74.0	-1.48 (-11.90, 8.57)	0.308
Mean platelet volume (fL)	7.96 ± 0.81	0.57 (-1.75, 2.08)	8.10 (7.60, 9.0)	1.25 (-1.81, 3.37)	0.482
Glucose (mg/dL)	91.19 ± 8.07	2.76 (-1.0, 8.95)	102.50 (92.50, 107.50)	0.0 (-3.23, 3.40)	0.156
Insulin (μIU/mL)	6.90 (6.25, 11.60)	-11.95 (-27.31, 28.97)	11.20 (8.20, 13.80)	-5.36 (-21.59, 10.84)	0.121
Triglycerides (mg/dL)	104.5 (82.5, 146.5)	-16.29 (-36.20, 8.16)	91.5 (71.0, 131.0)	-13.30 (-18.78, 8.62)	**0.031**
Total cholesterol (mg/dL)	192.06 ± 18.66	-4.42 (-8.81, 1.73)	198.19 ± 21.03	-3.87 (-7.04, 2.56)	**0.020**
HDL-cholesterol (mg/dL)	55.5 (45.0, 63.5)	2.38 (-9.22, 10.27)	54.63 ± 14.80	-1.47 (-6.87, 4.81)	0.940
LDL-cholesterol (mg/dL)	132.94 ± 20.67	-6.23 (-9.96, 0.64)	138.38 ± 23.64	-6.47 (-11.69, 1.17)	**0.003**
PAF (EC50) (nM)	56.60 (34.04, 82.48)	-17.35 (-53.75, 43.35)	31.66 (17.47, 52.28)	-9.02 (-38.49, 44.91)	0.411
ADP (EC50) (μM)	3.32 (2.81, 4.65)	-12.91 (-46.91, 10.88)	0.45 (0.35, 0.63)	-21.21 (-37.79, -8.36)	**0.006**
Collagen (EC50) (μg/mL)	0.43 (0.40, 0.62)	-5.32 (-42.26, 34.82)	0.35 (0.30, 0.52)	2.06 (-37.30, 51.56)	0.239
Prothrombin time (sec)	11.73 ± 0.76	0.43 (-0.92, 1.57)	11.49 ± 0.73	0.89 (-1.25, 2.31)	0.779
INR	0.95 ± 0.07	0.54 (-1.14, 1.94)	1.02 ± 0.07	1.01 (-1.40, 2.16)	0.743
aPTT (sec)	30.81 ± 2.68	0.63 (-1.07, 2.47)	32.25 ± 4.49	-2.57 (-4.59, 2.05)	0.431
Fibrinogen (mg/dL)	322.5 (309.0, 337.0)	3.15 (-3.43, 10.38)	332.0 (317.5, 404.0)	-3.53 (-10.33, 2.25)	0.794
sP-selectin (ng/mL)	84.62 ± 26.52	0.01 (-4.47, 6.24)	93.71 ± 28.11	0.68 (-2.08, 13.84)	0.190
PF4 (ng/mL)	8880.35 (7874.5, 10886.45)	1.13 (-5.82, 5.59)	9065.4 (8290.25, 9861.25)	0.56 (-5.81, 5.84)	0.955
tPA (IU/mL)	0.65 (0.44, 0.89)	26.50 (-17.59, 91.28)	1.17 (0.92, 1.52)	20.44 (3.23, 27.36)	**0.006**
PAI-1 (AU/mL)	63.12 (53.31, 80.33)	0.74 (-13.47, 8.58)	69.39 (58.89, 75.0)	-3.90 (-9.34, 9.15)	0.455

Data are summarized as mean ± standard deviation if normally distributed or as median (25th, 75th percentiles) if non-normally distributed. Relative change (%) was calculated as the change between the values at the end and the values at the beginning of each treatment period. Two-paired samples t-tests or Wilcoxon matched-pairs signed-rank tests were used to compare the values at the end of treatment with the values at the beginning of treatment for the combined results from the two periods for variables with normal distribution and variables with non-normal distribution, respectively; p-values are presented for two-sided tests; the significance (α) level was set to 0.05 for all two-sided tests. ADP: adenosine diphosphate; aPTT: activated partial thromboplastin time; BMI: body mass index; DBP, diastolic blood pressure; CF: conventional fish; EF: enriched fish; HDL: high density lipoprotein; INR: International Normalized Ratio; LDL: low density lipoprotein; PAF: platelet-activating factor; PAI-1: plasminogen activator inhibitor-1; PF4: platelet factor 4; SBP, systolic blood pressure; tPA: tissue plasminogen activator; WBC: white blood cells.

Table 5. Anthropometric measurements, blood pressure levels, hematological parameters, classical biochemical markers, coagulation parameters, markers of hemostasis and ex vivo platelet aggregation of the participants on the enriched fish (EF) diet.

	End of 1st Period	% Change	End of 2nd Period	% Change	*p**
BMI (kg/m^2)	30.3 ± 3.6	0.26 (-0.68, 1.36)	30.6 ± 3.2	-0.55 (-0.97, 1.18)	0.466
SBP (mmHg)	131.4 ± 14.2	-3.09 (-4.32, 1.56)	127.4 ± 15.1	-2.25 (-6.10, 4.81)	0.084
DBP (mmHg)	77.0 ± 9.1	-1.62 (-7.00, 7.90)	73.8 ± 9.7	3.31 (-4.64, 7.98)	0.666
WBC count (10^3/μL)	6.1 (5.3, 6.7)	1.72 (-12.04, 15.93)	5.1 (4.8, 7.6)	-3.39 (-11.82, 5.68)	0.633
Lymphocyte count (10^3/μL)	1.9 ± 0.4	-2.08 (-6.20, 13.33)	1.9 (1.6, 2.2)	0.0 (-10.73, 5.90)	0.637
Platelet count (10^3/μL)	236.0 (203.5, 265.5)	-2.19 (-9.35, 2.84)	258.4 ± 73.4	2.97 (-4.02, 9.08)	0.985
Mean platelet volume (fL)	8.28 ± 0.89	0.03 (-1.23, 2.88)	8.05 (7.40, 8.80)	1.14 (-2.89, 5.06)	0.238
Glucose (mg/dL)	93.44 ± 8.03	-3.87 (-5.22, 1.67)	97.0 (92.0, 105.5)	-0.44 (-6.72, 3.76)	0.160
Insulin (μIU/mL)	9.4 (6.7, 12.9)	-10.18 (-41.46, 26.28)	11.75 (8.35, 16.40)	10.73 (-7.82, 53.30)	0.911
Triglycerides (mg/dL)	81.0 (59.5, 121.5)	-16.81 (-20.81, 1.0)	99.0 (80.5, 154.0)	-5.22 (-22.55, 17.79)	**0.043**
Total cholesterol (mg/dL)	192.38 ± 24.57	-5.83 (-8.37, -1.20)	202.31 ± 24.73	2.15 (-6.19, 12.47)	0.334
HDL-cholesterol (mg/dL)	55.0 (47.0, 75.0)	0.0 (-8.01, 9.05)	52.81 ± 13.94	-0.10 (-6.34, 5.02)	0.903
LDL-cholesterol (mg/dL)	137.25 ± 27.60	-6.32 (-11.51, -0.88)	143.31 ± 26.98	4.45 (-7.91, 18.27)	0.583
PAF (EC50) (nM)	61.23 (47.77, 112.48)	26.57 (5.09, 94.99)	58.10 (24.95, 88.40)	22.0 (3.56, 57.09)	**<0.001**
ADP (EC50) (μM)	3.50 (2.69, 4.77)	17.20 (2.90, 61,89)	0.78 (0.49, 1.25)	7.53 (-8.69, 18.15)	**0.026**
Collagen (EC50) (μg/mL)	0.53 (0.42, 0.66)	-12.75 (-45.87, 12.38)	0.52 (0.34, 0.57)	10.94 (-15.12, 52.35)	0.562
Prothrombin time (sec)	11.64 ± 0.66	0.0 (-1.67, 1.78)	11.37 ± 0.72	0.87 (-1.36, 1.34)	0.723
INR	0.94 ± 0.06	0.0 (-2.07, 2.10)	1.0 ± 0.07	0.99 (-0.98, 1.52)	0.806
aPTT (sec)	31.67 ± 3.77	-1.55 (-3.38, -0.47)	30.50 ± 3.25	-4.08 (-7.13, -1.92)	**<0.001**
Fibrinogen (mg/dL)	358.5 (331.0, 393.5)	7.69 (0.99, 15.58)	332.0 (280.0, 358.5)	2.29 (-4.64, 9.30)	**0.042**
sP-selectin (ng/mL)	95.19 ± 31.58	0.64 (-7.32, 8.60)	78.56 ± 25.62	-1.61 (-6.97, 10.08)	0.676
PF4 (ng/mL)	8993.45 (8324.1, 9502.8)	0.22 (-7.36, 5.01)	8493.0 (8245.4, 9610.2)	6.86 (-4.20, 11.11)	0.331
tPA (IU/mL)	0.75 (0.52, 0.86)	36.19 (13.0, 90.34)	0.96 (0.76, 1.54)	5.44 (-6.15, 13.69)	**0.010**
PAI-1 (AU/mL)	65.86 (56.13, 78.89)	1.97 (-7.66, 13.15)	70.05 (56.63, 80.81)	0.84 (-9.81, 4.70)	0.837

Data are summarized as mean ± standard deviation if normally distributed or as median (25th, 75th percentiles) if non-normally distributed. Relative change (%) was calculated as the change between the values at the end and the values at the beginning of each treatment period. Two-paired samples t-tests or Wilcoxon matched-pairs signed-rank tests were used to compare the values at the end of treatment with the values at the beginning of treatment for the combined results from the two periods for variables with normal distribution and variables with non-normal distribution, respectively; p-values are presented for two-sided tests; the significance (α) level was set to 0.05 for all two-sided tests. ADP: adenosine diphosphate; aPTT: activated partial thromboplastin time; BMI: body mass index; DBP, diastolic blood pressure; CF: conventional fish; EF: enriched fish; HDL: high density lipoprotein; INR: International Normalized Ratio; LDL: low density lipoprotein; PAF: platelet-activating factor; PAI-1: plasminogen activator inhibitor-1; PF4: platelet factor 4; SBP, systolic blood pressure; tPA: tissue plasminogen activator; WBC: white blood cells.

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Table 6. Effect size of the enriched fish (EF) treatment compared to the conventional fish (CF) treatment (treatment effect) regarding platelet aggregation markers and activated partial thromboplastin time.

	Effect size (SE)	95% CI	*p*
aPTT (sec)	-0.82 (0.37)	-1.54, -0.11	**0.025**
PAF (EC_{50})* (nM)	1.44 (1.13)	1.14, 1.82	**0.002**
ADP (EC_{50})* (μM)	1.67 (1.18)	1.20, 2.33	**0.002**
Collagen (EC_{50})* (μg/mL)	1.11 (1.08)	0.95, 1.29	0.204

*ln-transformed values so that residuals are approximately normally distributed; Effect size of treatment was estimated using the mixed-effects linear regression with random effects at the level identified by the variable ID, that is, the participant level; the dependent variable was the outcome variable evaluated post-treatment, the exposure was the fish type consumed (EF versus CF), the covariates were the outcome variable evaluated prior to fish treatment, the sequence (i.e., the between-subjects factor representing the two intervention groups of the study), and sex of the participants. Results are presented as the mean difference between the EF and CF treatments for the untransformed variables or as the exponentiated mean difference (or the ratio of the geometric mean of the outcome variable at the end of the EF treatment over the geometric mean of the outcome variable at the end of the CF treatment) for the ln-transformed variables; the values of standard error (SE), the 95% confidence interval (95% CI) and the p-values are also reported. ADP: adenosine diphosphate; aPTT: activated partial thromboplastin time; PAF: platelet-activating factor.

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The data supporting this article have been included as part of the Supplementary Information.