High dose fish oil supplements are more effective than oily fish in altering the number and function of extracellular vesicles in healthy human subjects: A randomized, double-blind, placebo-controlled, parallel trial

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Abstract

N-3 polyunsaturated fatty acids (n-3 PUFAs) delivered by fish oil supplements alter the number and functions of circulating extracellular vesicles (EVs), but consumption of oily fish does not reproduce this effect. In order to assess the effects of fish oil supplements and oily fish, at a level achievable in the diet, on EV numbers, composition and procoagulant activity in healthy human volunteers, forty-two healthy subjects were assigned to one of three treatment groups: (i) fish oil supplements plus white fish meals, (ii) control supplements plus oily fish meals or (iii) control supplements plus white fish meals for 12 weeks in a randomized, double-blind, placebo-controlled, parallel trial; circulating EVs were enumerated and their procoagulant activity assessed using thrombin generation and fibrinolysis assays. Our results showed that fish oil supplements decreased circulating EV numbers and reduced EV-stimulated thrombin generation, but the consumption of oily fish at half the dose of EPA had no effect on either EV number or thrombogenic capacity. Consumption of both oily fish and fish oil supplements increased the EPA and DHA contents of EVs and the proportion of EPA in circulating EVs was strongly associated with EV-stimulated thrombin generation. This study revealed that the additional 1 g/d EPA delivered in the fish oil supplements is required to decrease the numbers and thrombogenic capacity of EVs, since oily fish at a level achievable in the diet had no effect. Increasing EPA intake beyond current guidelines for oily fish consumption may therefore be required for cardiovascular benefits relating to EVs.

Abbreviations:

AA: arachidonic acid
ALA: α-linolenic acid
BMI: body mass index
CVD: cardiovascular disease
DBP: diastolic blood pressure
DGLA: dihomo-γ-linolenic acid
DHA: docosahexaenoic acid
DPA: docosapentaenoic acid

EPA: eicosapentaenoic acid

ETA, eicosatetraenoic acid

EV: extracellular vesicle

GLA: gamma linolenic acid

HDL-C: high-density lipoprotein cholesterol

HPA: heneicosapentaenoic

LDL-C: low-density lipoprotein cholesterol

MUFAs: monounsaturated fatty acids

NTA: nanoparticle tracking analysis

PDEVs: platelet-derived extracellular vesicles

PS: phosphatidylserine

PUFA: polyunsaturated fatty acid

SA: stearidonic acid

SBP: systolic blood pressure

SFAs: saturated fatty acids

T2D: type 2 diabetes

VDP: vesicle-depleted plasma

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Keywords: cardiovascular disease; extracellular vesicles; fish oil; oily fish; thrombosis; coagulation.

Introduction

Extracellular vesicles (EVs) are lipid bilayer-enclosed vesicles naturally present in the bodily fluids of healthy individuals and derived from almost all cells under both physiological and pathological conditions. The properties and functions of EVs are diverse and are primarily determined by their cellular origin, the stimulus triggering their release, and their specific cargo, which includes nucleic acids, proteins and lipids (1). EVs contribute to the regulation of normal physiological processes, such as blood coagulation, intercellular communication and tissue repair (2-3), and may serve as potential biomarkers for diseases, such as cardiovascular diseases (CVDs), type 2 diabetes (T2D), and cancer (4-7).There is particular interest in the association of EV numbers with endothelial dysfunction (7), platelet activation (8), vascular inflammation (9), and the aspects of blood coagulation pathways (10), as well as being correlated with CVDs and metabolic syndrome (4,7,11), all of which suggest that EVs have potential as a novel biomarker of cardiometabolic diseases.

Dietary n-3 polyunsaturated fatty acids (n-3 PUFAs), which are abundant in oily fish and fish oils, have long been associated with protection from CVDs and although the strength of evidence has at times been questioned, the most recent meta-analyses and systematic reviews broadly support the view that n-3 PUFA supplementation lowers risk of either CVD-related death or all-cause mortality or both (12-13). Furthermore, recent analysis of 17 prospective studies demonstrated that risk of death from all causes and death from CVDs was significantly lower in the highest vs the lowest quintile for circulating n-3 PUFAs (14). A range of potential mechanisms of this cardiovascular benefit have been considered (15), but to date, very few studies have explored the effects of n-3 PUFAs on EV numbers and/or function. A small number of studies have demonstrated a decrease in the numbers of EVs after the intervention with n-3 PUFA supplements (16-20). We have previously demonstrated that supplementation with n-3 PUFAs decreased the numbers of circulating total EVs, EV subtypes from platelet and endothelial cells as well as their coagulatory behavior in individuals with moderate risk of CVD (21). However, there is no information about whether oily fish, consumed at a level that is achievable through the diet, affects either EV numbers or function. Furthermore, there is a lack of insight as to whether EPA and DHA perform differently in relation to effects on EVs. The

current study therefore examines, for the first time, whether n-3 PUFAs delivered in the form of oily fish are able to modify the profile and coagulatory behavior of EVs in the circulation in the same way as fish oil supplements, consumed at a dose sufficient to lower numbers of EVs, and whether the effect is driven by EPA or DHA.

Methods

This study was conducted at the School of Sport and Exercise Sciences, Liverpool John Moores University, from October 2016 to January 2017 and carried out according to the guidelines in the Declaration of Helsinki, with ethical approval from the National Research Ethics Service (S16SPS041). Written informed consent was obtained from participants.

Trial Design

The trial was a randomized, double-blind, placebo-controlled, parallel trial. Eligible participants were allocated randomly (block randomization was performed using Excel by a member of staff unrelated to the trial) to one of the three groups as follows: (i) fish oil capsules provided as 2.2 g/d of n-3 PUFA ethyl esters plus two white fish meals per week, (ii) control capsules containing refined olive oil plus two oily fish meals providing the equivalent of 2.2 g/d of n-3 PUFAs (one meal containing salmon and the other mackerel) and (iii) control capsules plus two white fish meals (**Table 1**). Although the doses of EPA were not matched, they represented optimal levels of intake which could be achieved through the diet or supplementation, and in the case of the supplements, a dose which previous evidence has shown would be sufficient to lower the number of EVs (20). The fish meals were provided as ready meals supplied by Soulmate Food (Liverpool, UK) and the capsules were supplied by Wiley's Finest (Granville, Ohio, USA). Servings of mackerel, salmon and white fish contained within the ready meals were 229 g, 240 g and 110 g respectively. The fatty acid compositions of the fish oil capsules, control capsules and fish meals were analyzed by the West Yorkshire analytical services (Leeds, UK) and are shown in **Table 1**. Capsules were coded by an independent researcher and the code was broken after all data analysis had been completed. All meals were delivered chilled and distributed to participants twice a week, with storage and heating instructions provided by the supplier**.** Participants attended three visits during the intervention: at screening, baseline, and the end of intervention (12 weeks). Compliance to the intervention was confirmed verbally during each week of the trial. A three-day food diary was administered three days prior to the start of the intervention and

during the last three days of the intervention to confirm that participants maintained consistent habitual dietary intakes during the study. Researchers involved in the measurement and assessment of study outcomes generated the random allocation sequence, enrolled the participants and assigned the interventions, but were blinded to the allocation of treatment; all participants were blinded to the interventions as well.

Participants

Participants older than 40 years were recruited through the media (BBC local and national radio, online forums, social media, societies, and flyers in the street) and a total of 42 participants all completed the study (54.97± 1.45 years; 28 females), as illustrated in the participant flow diagram (**Supplemental Figure 1**). The Framingham Risk Score was used to identify participants who were at above average risk of developing CVD (22), defined as a relative risk (RR) of 1.5 based on scoring a minimum of 2 points against one or more of the criteria listed in **Supplemental Table 1**, which includes family history of myocardial infarction or type 2 diabetes (23). Exclusion criteria included the following: smoking; infection; fever of unknown origin; immune disorders, including HIV; autoimmune diseases; medical conditions requiring immediate intervention; unstable or rapidly progressive neurological diseases; a history of hemorrhagic or ischemic stroke within the last 3 months; consuming oily fish more than once per week on average and/or n-3 index > 6%; taking any medication and/or dietary supplements; allergy, hypersensitivity, or intolerance to fish, fish oils or n-3 fats; any known food allergies; alcohol misuse; pregnant or breastfeeding.

Blood collection and processing

Venous blood samples were collected into 3.2% sodium citrate and processed to platelet-free plasma (PFP) as previously described (24). PFP was aliquoted and stored at −80 °C for further analysis.

EV isolation

EVs were isolated using size exclusion chromatography (SEC) (Izon, Oxford, UK). PFP (0.5 ml) was thawed at room temperature on a sample roller and loaded onto a qEV original column which had been pre-flushed with 30 ml phosphate-buffered saline (PBS). A further 5 ml PBS was passed through the column to elute the EVs based on their size and nine 0.5 ml fractions were collected. Fractions 7 to 9 were pooled for EV quantification and analysis.

Enumeration and characterization of EVs

Nanoparticle tracking analysis (NTA)

The concentration and size distribution of circulating EVs were determined by NTA using a NanoSight 300 (NS300; Malvern, Amesbury, UK) (24). The size of circulating EVs detected by NTA ranged from 70 nm to approximately 350 nm, but the majority (>80%) were 100-200 nm.

Flow cytometry (FCM)

The concentrations of circulating EV subpopulations including phosphatidylserine (PS) positive EVs, platelet-derived EVs (PDEVs) and endothelial-derived EVs (EDEVs) were determined by flow cytometry (FCM; Canto II Flow Cytometer, BD Biosciences, UK), using blue (488 nm), a red (633 nm) and violet (405 nm) lasers (24).

Fatty acid compositions of red blood cells (RBCs) and EVs

Lipid extracts were prepared from RBCs and EVs (isolated by SEC, as above) and separated by solid phase extraction. Briefly, pooled fractions of EVs (800 µl) and RBCs (50 µl) were mixed with 5 ml of chloroform/methanol (2/1) containing 50 mg/l butylated hydroxytoluene as antioxidant and then centrifuged at 1,000 x g for 10 min. The lower phase was collected and dried under nitrogen at 40 °C. Dry toluene (0.5 ml) was added to the total lipid extract, followed by methanol (1 ml) containing 2% (v/v) sulphuric acid. The tubes were capped and incubated at 50°C for 2 h. After cooling, samples were neutralised with 0.25M $KHCO_3$/0.5M K_2CO_3 (1 ml) and lipid was extracted by adding dry hexane (1 ml) and centrifuged at 250 x g for 2 min at room temperature. The upper phase containing the fatty acid methyl esters (FAMEs) was collected and analysed using gas chromatography on a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard, California, United States) equipped with flame ionisation detection (Agilent Technologies, Cheadle, United Kingdom). FAMEs were separated in a BPX-70 fused silica capillary column (30mx0.25mmx25µm; SGE Analytical Science, United Kingdom) at a split ratio of 30:1 and an injection volume of 5 µl. The temperature of both injector and detector were kept at 300 °C and the program was set at an initial temperature of 115 °C for 2 min, increased at 10 °C/min to 200 °C, held at this temperature for 16 min, and then finally increased at 60 °C/min to 240 °C for 2 min (total run time 29.2 minutes). Helium was used as carrier gas (velocity: 29 cm/sec; pressure: 21:96psi and flow rate: 1.0 ml/min) and make up gas, flow rate: 45ml/min. Hydrogen was used as detector gas were hydrogen flow rate: 40ml/min; air flow: 120 ml/min.

Samples were analysed by using ChemStation software (Agilent Technologies, Cheadle, United Kingdom) and Microsoft Excel (Microsoft Corporation, United States).

Coagulatory function of EVs

Measurement of thrombin generation

Thrombin formation was assessed using the Technothrombin MP kit (Technoclone, Vienna), which is based on the thrombin-dependent cleavage of a fluorogenic substrate over time, as previously described (21). Two separate analyses were conducted: (i) determination of the effects of intervention on thrombin generation in PFP from study samples compared with that in pooled vesicle-depleted plasma (VDP) alone and (ii) determination of the effects of intervention on thrombin generation in VDP plus EVs isolated by SEC from study samples compared with that in VFP alone. The methods for both approaches were based on the use of pooled VDP as a negative control to allow assessment of thrombin generation specifically resulting from the presence of EVs and VDP was prepared from three healthy volunteers as previously described (21). For the first approach, 40 µl aliquots of either pre-thawed study sample PFP or pooled VDP were added into the plate. For the second approach, EV fractions were first eluted by SEC were concentrated using Vivaspin™ 6 Sample Concentrators with 100,000 MWCO (Fisher Scientific, Loughborough) at 1500 x g for 40 min and EV aliquot (10 µl) with final protein concentration of 5 µg/ml was then added to 30 µl of VDP. Data were then analyzed by the TGA Evaluation Software manually to convert the unit of thrombin generation from RFU to nM and presented as lag time, peak concentration of thrombin (nM), velocity-index and area under the curve (AUC).

Clot formation and fibrinolysis

The clot-forming capacity of EVs was assessed by isolating EVs using SEC, adjusting the concentration to 5 µg/ml and applying clot formation and lysis assays adapted from previous studies (25-26) to compare clot formation and fibrinolytic activity in VDP with VDP plus added EVs. The clot formation assay was performed in duplicate in 96 well plates by incubating EVs (10 µl, final concentration 5 µg/ml) and VFP (30 µl) with Tween Tris buffered saline (40 µl, containing 10 mM Tris pH 7.4; 0.01% Tween 20 (T/T)) and 20 µl of 5.3 mM $CaCl_2$. The clot was measured at 405 nm every 30 s for 1 h at 37 °C using a FlexStation 3 microplate reader (Molecular Devices, San Jose, USA). The fibrinolytic activity of EVs was assessed by isolating

EVs and measuring their ability to initiate plasmin generation (enzyme important for degrading the blood clot) using a chromogenic assay. In brief, EVs (10 µl, final concentration 5 µg/ml) were incubated with VFP (30 µl), Tween Tris buffered saline (30 µl, containing 10 mM Tris pH 7.4; 0.01% Tween 20 (T/T)), tissue plasminogen activator (tPA) to stimulate clot breakdown (10 µl, final concentration 100 pM) and 20 µl of 5.3 mM $CaCl_2$. The kinetics measurement was started immediately after adding the calcium and readings were taken every 30 s at 405 nm for 4 h using a FlexStation 3 microplate reader (Molecular Devices, San Jose, USA). All data were analyzed using an online tool for analysis of clot and lysis using the Shiny App developed by Longstaff (27) and presented as area under the curve (AUC) and time to full lysis, respectively.

Plasma lipid analysis

The plasma lipid profile before and after the treatment was assessed using a Daytona Plus clinical chemistry analyzer (Randox). Plasma total cholesterol and triacylglycerol were analyzed using enzyme-based assays and high-density lipoprotein cholesterol (HDL-C) was analyzed using a clearance assay, while low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald formula.

Power calculation

The sample size calculation was performed according to Julious *et al.* (28) for the primary endpoints of the proportions of both EPA and DHA in RBC, in which 10 subjects would provide 80% power in detecting a difference of 0.9 wt% in EPA of RBC with α=0.05 and a within-subject standard deviation of 1.0, and 15 subjects would provide 80% power in detecting a difference of 1.6 wt% in DHA of RBC with α=0.05 and a within-subject standard deviation of 0.8.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Differences between three groups were determined using a general linear model with fixed factors of treatment and period followed by post-hoc analysis using Bonferroni tests where applicable. The strengths of correlations among EV numbers and EV coagulatory functions with fatty acid compositions of both EVs and RBCs were assessed by Pearson's correlation coefficient or Spearman's correlation coefficient where appropriate. Significantly associated variables were entered into a multivariate regression model, and all variables with *P*-values < 0.05 were subsequently incorporated into a

stepwise multivariate regression model, in which parameters of $F \leq 0.05$ were entered and $F \geq 0.10$ were removed, to identify independent predictors of EV related parameters. *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed using IBM SPSS statistics 27.

Results

A total of 42 participants aged 55 ± 2 years completed the 12-week intervention. The three groups did not differ significantly with regard to their physical characteristics, BP, lipid and glucose profile (**Table 2**).

Consumption of fish oil supplements and oily fish altered the fatty acid profiles of circulating EVs and RBCs, but had no effects on blood lipid profile

The proportions of both EPA and DHA in both circulating EVs and RBCs were significantly increased following intervention with fish oil supplements and oily fish, with a substantial overall increase in total n-3 PUFAs (**Table 3** and **Table 4**) The degree of incorporation of n-3 PUFAs in the two groups was comparable, despite the fact that the fish oil supplements had a higher content of EPA+DHA (2 g vs 1 g) and a higher ratio of EPA to DHA (3:1 vs 1:1) than the oily fish meals (**Table 1**). There was a decrease in the proportion of total n-6 PUFAs in RBCs after consumption of the consumption of fish oil supplements or oily fish compared to the control group, but there was no difference between the fish oil supplements and control groups (**Table 4**). There were no accompanying changes in the proportions of any other fatty acids in either circulating EVs or RBCs following the consumption of fish oil supplements or oily fish (**Table 3** and **Table 4**). There was no significant effect of either oily fish or fish oil supplements on the blood lipid profile, although there were trends for a reduction in plasma triacylglycerol concentration and an increase in LDL-C concentration following intervention with both oily fish and fish oil supplements (**Supplemental Table 2**).

Fish oil supplements, but not oily fish, decreased numbers of circulating EVs

Supplementation with fish oil significantly decreased numbers of circulating EVs, whereas the consumption of oily fish had no effect (**Figure 1A**). There was no effect of either oily fish or fish oil supplements on the mean size of the EV population (**Figure 1B**) or on numbers of PS-positive circulating EVs, PDEVs and EDEVs (**Supplemental Table 2**).

Fish oil supplements, but not oily fish, reduce the ability of EVs to support thrombin generation

The thrombogenicity of EVs following intervention was analyzed using two approaches. In the first approach, the difference in thrombin generation between PFP from study subjects and pooled VFP from healthy subjects indicated the degree to which EVs influenced TF-stimulated thrombin generation in plasma before and after the intervention. PFP from subjects supplemented with fish oil had significantly lower thrombogenic capacity than that from subjects in the oily fish or control groups, as determined by peak thrombin concentration, AUC (**Figure 2A and 2B**) and velocity index (treatment: P=0.015; time: P=0.276; time*treatment interaction: P=0.016). The second approach, SEC-isolated EVs from study subjects were added to pooled VFP from healthy subjects, demonstrated that EVs from subjects supplemented with fish oil had significantly lower thrombogenic capacity than those from subjects in the oily fish or control groups, as determined by peak thrombin concentration and AUC (**Figure 2C and 2D**).

Fish oil supplements and oily fish have no effect on the ability of EVs to induce clot formation and lysis

There was no significant effect of either oily fish or fish oil supplements on clot formation or lysis supported by isolated EVs from subjects, as determined by AUC and time to full lysis (**Supplemental Table 2**).

Fatty acid profiles of circulating EVs and RBC are associated with numbers and coagulatory activity of circulating EVs

The proportions of EPA, DHA, ETA and total n-3 PUFAs in circulating EVs were significantly associated with the numbers of circulating EVs (**Supplemental Table 3**) and the proportions of EPA, DHA, DPA, stearic acid, linoleic acid, total n-3 PUFAs, total MUFAs and total n-6 PUFAs in RBCs were significantly correlated with circulating EV numbers (**Supplemental Table 4**). Stepwise regression analysis suggested that the proportions of EPA in circulating EVs and RBCs explained 28.0% and 31.5% of the variance for circulating EV numbers, respectively (**Figures 3A and 3B**).

The proportions of EPA, ALA, stearic acid, linoleic acid and total n-3 PUFAs in circulating EVs were significantly associated with EV-supported thrombin generation parameters. EPA and total n-3 PUFAs in circulating EVs were also significantly associated with EV-supported clot

formation parameters (**Supplemental Table 3**). The proportion of EPA in circulating EVs independently predicted 11.5% of the variance for EV-supported thrombin generation (AUC) and 10.6% of the variance in EV-supported clot formation (AUC) respectively (**Figures 3C and 3D**). Furthermore, the proportions of EPA, DHA, DPA, oleic acid, total n-3 PUFAs, total MUFAs and total n-6 PUFAs in RBCs were associated with EV-supported thrombin generation and clot formation parameters (**Supplemental Table 4**). The proportion of EPA in RBCs explained 14.3% of the variance in EV-supported thrombin generation (AUC) and 13.6% of the variance in EV-supported clot formation (AUC) respectively (data not shown).

Discussion

This study demonstrated that fish oil supplements are more effective than oily fish in decreasing numbers of circulating EVs and reducing their procoagulant activity, and that these effects appear to be attributable to the additional 1 g/d EPA delivered in the fish oil supplements. Consumption of both oily fish and fish oil supplements increased the proportions of EPA and DHA in circulating EVs and RBCs to a similar degree, despite the dose of n-3 PUFAs in the oily fish meals being half that provided by the supplements. The proportion of EPA in circulating EVs and RBCs was strongly associated with circulating EV numbers and EV-stimulated thrombin generation, which also suggests that EPA, rather than DHA, is driving the effects.

The dramatic reduction in numbers of circulating EVs after fish oil supplements confirms some previous observations (16-20) and the reduction in thrombin generation supported by EVs is consistent with a small number of studies in healthy individuals (21, 29), or post-myocardial infarction patients (19). However, not all studies agree; healthy subjects consuming 1.2 g/d EPA+DHA for 4 weeks did not experience an alteration in the number of circulating PS-positive EVs (30), and PDEV numbers were not significantly altered after an 8-week intervention with 1.5 g EPA+DHA per day (20). Furthermore, to our knowledge, this is the first study comparing the effects of n-3 PUFAs in the form of oily fish, at a level achievable in the diet, with fish oil supplements on EV numbers and functional activity. Ideally, the two treatments would have delivered equivalent doses and ratios of EPA and DHA, but the design of the study was dependent on the limited options and availability of oily fish meals and supplements. As a result,

the doses of EPA and DHA delivered in the supplements were approximately 1.5 g/d and 0.5 g/d respectively, compared with approximately 0.6 g/d and 0.4 g/d respectively in the oily fish meals.

A small number of studies have compared the intervention with n-3 PUFAs in the form of oily fish and fish oil capsules on the incorporations of EPA and DHA into plasma and cellular lipids. Harris *et al.* demonstrated the equivalent incorporations of EPA and DHA into RBCs and plasma phospholipids following consumption of oily fish or fish oil capsules providing equal amounts of EPA and DHA (31). Another study demonstrated that EPA content was higher in RBCs and platelets following supplementation, but DHA content was higher in RBCs and platelets following intervention with salmon, and this was most likely due to a ratio of EPA:DHA of 1.6:1 in the supplement group compared with 1:2.4 in the salmon group, explaining the greater accumulation of DHA in the salmon group (32). The current study reports a similar degree of incorporation of EPA, DHA and total n-3 PUFAs into both RBCs and EVs in the fish oil and oily fish groups, even though the supplements provided a higher EPA: DHA ratio compared to oily fish meals. It should also be noted that n-3 PUFAs are present in the ethyl ester form in supplements, whereas they are present as triglycerides and phospholipids in oily fish, where they are considered to have superior bioavailability, so comparisons between the two should take this into account (33-34).

The fact that fish oil supplements, but not oily fish, decreased EV numbers and their thrombogenic capacity in the current study suggests that the additional 1g/d EPA delivered in the fish oil supplements against an equivalent DHA background is required for the effects. There is limited insight into differential effects of EPA and DHA on EV properties, but one study reported that thrombin generation stimulated by PDEVs was decreased after a 24h intervention with an EPA-rich oil (1 g EPA with an EPA/DHA ratio of 5:1), but not a DHA-rich oil (1 g DHA with an EPA/DHA ratio of 1:5) or a placebo treatment (29). The current study demonstrated that the proportion of EPA in circulating EVs and RBCs independently predicted circulating EV numbers and EV-supported thrombin generation, supporting the argument that EPA is the main driver of the effects of n-3 PUFAs on EV function. Nomura *et al.* conducted three trials, which provided patients with hyperlipidemia and type 2 diabetes with pure EPA at a dose of 1.8 g/d for

four weeks (16) or six months (17), or combined with 2 mg/d pitavastatin for six months (18). They demonstrated significant decreases in numbers of PDEVs, monocyte-derived EVs (16, 18) and EDEVs (17) after EPA supplementation.

The exact mechanisms by which n-3 PUFAs decrease numbers of circulating EVs and inhibit their coagulatory function are not fully understood, but it is well appreciated that the incorporation of n-3 PUFAs into cell membrane phospholipids may play a fundamental role in modulating the lipid composition and function of cells, thereby altering the generation and behavior of circulating EVs (1-2, 35-37). Externalization of PS in the outer leaflet during cell activation is a key step of EV generation and a key contributor to the thrombin-generating capacity of EVs, and it has been demonstrated to be inhibited by flaxseed oil-derived n-3 PUFA (38). Larson and colleagues also reported that PS exposure by platelets was reduced by approximately 50% after 28 days of n-3 PUFA supplementation in healthy subjects (39). Underlying mechanisms may also involve lipid rafts, which are involved in EV formation, cargo loading and fusion with target cells, and have been reported to be altered by n-3 PUFAs, giving rise to the suggestion that this alteration may disrupt EV shedding and behavior (40-41). The decrease in total n-6 PUFAs in RBCs reported in the current study may provide an additional explanation. N-3 and n-6 PUFAs are metabolized in a competitive manner, and n-6 PUFA-derived lipid mediators enhance platelet aggregation, thrombin generation and inflammation. Thus, the incorporation of n-3 PUFAs into cell membranes may decrease EV generation and coagulatory function through modification of the lipid mediator profile (42-43).

The favorable effects of EPA on EVs have previously been attributed to more rapid incorporation of EPA into the phospholipids of cell membranes compared to DHA (35-37,44). For example, the consumption of 1 g/d EPA for 3 days significantly increased concentrations of EPA in plasma and RBC phospholipids, while a similar increase in DHA required 6 days of consumption at a dose of 1 g/d (36). Similarly, enrichment of EPA in plasma phospholipids occurred earlier and was more marked and more dose-dependent than that of DHA when supplemented at doses ranging from 1-4 g/d (37,44). Although there was a trend towards a higher EPA content in RBCs in the fish oil group compared to the oily fish group in the current

study, this was not statistically significant and it may be that the unique effects of EPA are not limited to its incorporation into plasma membranes and that similar incorporation in the fish oil and oily fish groups does not therefore imply that the effects will be the same. They could, for example, be related to effects of EPA on cardiometabolic factors, such as platelet activation and endothelial dysfunction, and these in turn affect EV generation and function (45-50). The behavior of EVs is dependent on its cargo, which may also be modified by fatty acid supplementation, and this may influence not only procoagulatory activity, but also fibrinolysis (51).

The strengths of this study include the fact that it explores the effects of consumption of oily fish at a level achievable in the diet on EV numbers and thrombogenic capacity, and suggests that a higher dose of EPA than is possible through dietary intervention may be required to achieve significant effects. The main limitations of the study relate to it being a parallel trial, which potentially introduces confounding variables, such as the influence of biological sex on outcomes (52), and differences in baseline circulating EV numbers between the fish oil and control groups, which could influence the interpretation of the findings. Future studies may benefit from a cross-over design and further exploration of sex-specific effects to validate and expand upon these conclusions. It is also notable that there were no effects of n-3 PUFAs on EV subtypes, despite the dramatic effects on total EV numbers. EV subtypes were analyzed by flow cytometry, which has a detection limit of 200 nm, which means that they only represent EV subtypes larger than 200nm, whereas total EVs were analyzed by NTA. Thus, flow cytometry, and therefore subtype analysis, only represents a small proportion of total EVs. The interpretation is that fish oil affected smaller EVs, but it is not possible to identify which subtypes are altered using currently available techniques.

In conclusion, this study demonstrates that fish oil supplements delivering a high dose of EPA were more effective than oily fish in altering the number and thrombogenicity of EVs. EPA therefore appears to be a key factor driving alterations in EV number and function, an observation supported by a strong association between the EPA content of EVs and their biological activity. The study suggests that increasing EPA intake beyond the current dietary

guidelines for oily fish consumption may offer additional benefits, particularly in relation to EV numbers and functions.

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Author contribution

GC, JP, PY and PC designed the study together with the funders. GC and JP ran the intervention study, HF conducted the fatty acid composition analysis under the supervision of PC, and AS conducted the remaining experimental work, under the supervision of PY. AS and RZ analyzed data and performed statistical analyses; AS, RZ and PY wrote the manuscript; PY had primary responsibility for final content, and all authors read and approved the final manuscript.

Data Availability: Data described in the manuscript will be made publicly and freely available without restriction from the University of Reading Research Data Archive at https://doi.org/10.17864/1947.001350

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Table 1. Fatty acid compositions of fish oil, control oil capsules and the whole homogenized fish meals

	Fish oil capsules		**Control capsules (olive oil)**			**Piri Piri Fish (Salmon)**	**Katsu Fish Curry (Mackerel)**	**Piri Piri Fish (White Fish)**
Fatty acids	g/capsule	(wt%)	g/capsule	(wt%)	**Parameters**	g/portion	g/portion	g/portion
Myristic Acid (14:0)	0.0003	0.0375	0.0001	0.0198	Meal weight	534	586	378
Tetracosanoic (24:0)	0.0038	0.3875	0.0007	0.0761	Fish weight	226	229	110
Oleic acid (18:1n-9)	0.0042	0.425	0.6694	66.945	Total calories	837 kcal	845 kcal	861 kcal
Linoleic acid (18:2n-6)	0.0016	0.1625	0.0871	8.7133	Protein	69	70	72
DGLA (20:3n-6)	0.0018	0.1875	0	0	Carbohydrates	56	57	58
AA (20:4n-6)	0.0195	1.95	0	0	Fat	36	36	37
ALA (18:3n-3)	0.002	0.2	0.0065	0.6567	ALA (18:3n-3)	0.7	0.9	0.6
ETA (20:4n-3)	0.0206	2.0625	0	0	SA (18:4n-3)	1.6	4.5	0.2
EPA (20:5n-3)	0.7543	75.437	0.0003	0.03208	EPA (20:5n-3)	0.7	2.2	0.04
HPA (21:5n-3)	0.0197	1.975	0	0	DPA (22:5n-3)	0.3	0.4	<0.04
DPA (22:5n-3)	0.0166	1.6625	0.0005	0.0506	DHA (22:6n-3)	0.7	3.2	0.1
DHA (22:6n-3)	0.2671	26.712	0.0006	0.0604	Total n-3 PUFAs	4.0	11.2	0.94
Total SFAs	0.0042	0.425	0.1401	14.011				
Total MUFAs	0.0108	1.0875	0.6771	67.718				
Total n-3 PUFAs	1.1038	110.38	0.0079	0.7998				
	g/day (2 capsules)					**g/day (oily fish)**		

Total n-3 PUFA	2.2	Total n-3 PUFA	2.2
EPA	1.5	EPA	0.4
DHA	0.53	DHA	0.56
EPA+DHA	2	EPA+DHA	1

Data are expressed as the gram per capsules (g/capsules) and the percentages of the weight of each individual fatty acid relative to the total weight of all fatty acid (wt%) in either fish oil or control oil capsules, and gram per portion (g/portion) in fish meals. *AA, arachidonic acid; ALA, alpha-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; GLA, gamma linolenic acid; HPA, heneicosapentaenoic; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SA, stearidonic acid; SFAs, saturated fatty acids.*

Table 2. Baseline subject characteristics

	Fish oil supplement (n=15)	Oily fish (n=14)	Control (n=13)	P value
Subject characteristics				
Age (years)	57.23±2.76	56.14±2.50	51.16±2.00	0.209
Male: Female ratio	3:10[†]	5:9	2:9[†]	0.603
Height (m)	1.69±0.02	1.65±0.02	1.68±0.02	0.561
Weight (kg)	75.86±3.40	68.85±2.83	73.63±4.64	0.370
BMI (kg/m^2)	27.10±1.45	25.16±0.98	25.83±1.50	0.675
SBP (mm Hg)	127.91±4.67	128.15±3.96	125.18±6.13	0.855
DBP (mm Hg)	69.66±2.25	72.30±2.88	72.81±2.35	0.586
Biochemical values (mmol/L)				
Total cholesterol	4.90±0.28	4.30±0.17	4.57±0.30	0.079
HDL-C	1.41±0.05	1.15±0.08	1.48±0.08	0.062
LDL-C	2.94±0.23	2.55±0.18	2.66±0.23	0.106
Triacylglycerol	1.21±0.15	1.31±0.29	0.95±0.15	0.769

Data are mean ± SEM. Differences in baseline characteristics between the three groups were determined using a general linear model. [†] Data of gender in three subjects were missing. *AUC, area under the curve;BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure*.

Table 3. Effect of fish oil supplements and oily fish on the fatty acid composition of circulating EVs

Fatty Acids	**Fish oil supplement**		**Oily fish**		**Control**		**P value (treatment)**
	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	
Palmitic acid (16:0)	24.83±0.64	26.05±0.61	25.86±0.62	25.15±0.55	24.50±0.38	24.75±0.52	0.372
Stearic acid (18:0)	9.66±0.91	8.71±0.55	8.93±0.75	8.56±0.57	9.83±0.74	9.60±0.80	0.570
Oleic acid (18:1, n-9)	30.28±1.03	28.43±0.66	30.34±1.08	29.21±1.07	30.52±1.08	29.89±0.93	0.805
Linoleic acid (18:2, n-6)	17.15±0.44	17.48±0.44	17.56±0.77	18.58±0.96	17.44±1.05	17.97±1.08	0.740
AA (20:4, n-6)	3.41±0.21	2.90±0.13	3.68±0.20	3.05±0.23	3.69±0.16	3.44±0.23	0.247
ALA (18:3, n-3)	0.25±0.02	0.25±0.01	0.25±0.02	0.20±0.02	0.23±0.01	0.23±0.02	0.386
EPA (20:5, n-3)	**0.43±0.03**	**1.07±0.06**	**0.45±0.03**	**1.03±0.06**	**0.44±0.03**	**0.48±0.04**	**<0.001**
DPA (22:5, n-3)	0.36±0.02	0.39±0.02	0.44±0.02	0.42±0.02	0.36±0.02	0.31±0.01	0.249
DHA (22:6, n-3)	**1.06±0.06**	**1.45±0.07**	**1.10±0.08**	**1.53±0.06**	**0.91±0.07**	**0.94±0.10**	**<0.001**

Total SFA	35.25±1.06	35.54±0.87	35.44±1.03	34.32±0.93	35.28±0.70	35.37±1.07	0.883
Total MUFA	33.34±1.11	31.84±0.76	33.72±1.08	32.47±1.03	33.93±1.11	33.33±0.95	0.735
Total n-3 PUFA	**2.76±0.13**	**4.11±0.17**	**2.98±0.13**	**4.17±0.14**	**2.98±0.19**	**2.88±0.17**	**0.003**
Total n-6 PUFA	22.79±0.52	22.60±0.50	23.54±0.86	24.14±1.04	23.59±1.07	24.02±1.20	0.465

Data are mean ± SEM. Differences in the fatty acid composition of circulating EVs after intervention between the three groups were determined using a general linear model, including post-hoc analysis with Bonferroni tests for treatment, period and treatment*time interaction with differences shown at $P < 0.05$. **EPA**: There was a significant effect of the treatment on the proportion of EPA in the circulating EVs ($P<0.001$) and significant time*treatment interaction ($P<0.001$) with a significant effect of time ($P<0.001$); the proportion of EPA has increased after fish oil and oily fish groups compared to the control group (both $P<0.001$), but there was no difference between fish oil and oily fish groups. **DHA**: There was a significant effect of the treatment on the proportion of DHA in the circulating EVs ($P<0.001$) and significant time*treatment interaction ($P=0.004$) with a significant effect of time ($P<0.001$); the proportion of DHA has increased after fish oil and oily fish groups compared to the control group ($P=0.004$ and $P<0.001$, respectively), but there was no difference between fish oil and oily fish groups. **Total n-3 PUFA**: There was a significant effect of the treatment on the proportion of total n-3 PUFA in the circulating EVs ($P=0.003$) and significant time*treatment interaction ($P<0.001$)with a significant effect of time ($P<0.001$); the proportion of total n-3 PUFA has increased after fish oil and oily fish groups compared to the control group ($P=0.004$ and $P=0.024$, respectively), but there was no difference between fish oil and oily fish groups. *AA, arachidonic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids.*

Table 4. Effect of fish oil supplements and oily fish on the fatty acid composition of RBCs

Fatty Acids	Fish oil supplement		Oily fish		Control		P value (treatment)
	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	
Palmitic acid (16:0)	24.42±0.17	24.72±0.24	24.78±0.32	24.81±0.32	24.39±0.26	24.26±0.19	0.403
Stearic acid (18:0)	12.59±0.22	13.15±0.21	12.51±0.17	13.21±0.29	12.93±0.24	13.62±0.32	0.265
Oleic acid (18:1, n-9)	18.60±0.36	17.69±0.29	18.97±0.43	17.86±0.40	18.70±0.29	18.08±0.27	0.812
Linoleic acid (18:2, n-6)	17.17±0.40	15.00±0.41	16.77±0.79	14.66±0.58	17.33±0.50	15.88±0.61	0.456
AA (20:4, n-6)	12.84±0.41	12.48±0.31	12.73±0.41	12.97±0.36	13.16±0.37	14.13±0.45	0.122
ALA (18:3, n-3)	0.45±0.04	0.36±0.02	0.46±0.04	0.38±0.02	0.39±0.03	0.30±0.03	0.174
EPA (20:5, n-3)	**1.07±0.07**	**2.77±0.16**	**1.00±0.07**	**2.22±0.11**	**0.93±0.08**	**1.20±0.07**	**<0.001**
DPA (22:5, n-3)	2.07±0.06	2.98±0.10	2.08±0.09	2.63±0.10	2.21±0.12	2.44±0.08	0.179
DHA (22:6, n-3)	**3.92±0.17**	**5.31±0.16**	**3.72±0.15**	**5.69±0.19**	**3.51±0.15**	**4.51±0.18**	**0.005**
Total SFA	37.15±0.20	38.03±0.23	37.45±0.28	38.17±0.26	37.47±0.40	38.05±0.32	0.781
Total MUFA	20.13±0.41	18.66±0.32	20.66±0.50	18.83±0.46	20.17±0.37	19.12±0.35	0.796
Total n-3 PUFA	**7.74±0.22**	**11.59±0.33**	**7.46±0.22**	**11.09±0.33**	**7.20±0.27**	**8.60±0.26**	**<0.001**
Total n-6 PUFA	**32.36±0.41**	**29.35±0.47**	**31.71±0.60**	**29.50±0.57**	**32.48±0.50**	**31.91±0.48**	**0.037**

Data are mean ± SEM. Differences in the fatty acid composition of RBCs after intervention between the three groups were determined using a general linear model, including post-hoc analysis with Bonferroni tests for treatment, period and treatment*time interaction with differences shown at $P < 0.05$. **EPA**: There was a significant effect of the treatment on the proportion of EPA in the circulating EVs ($P<0.001$) and significant time*treatment interaction ($P<0.001$) with a significant effect of time ($P<0.001$); the proportion of EPA has increased after fish oil and oily fish groups compared to the control group (both $P<0.001$), but there was no difference between fish oil and oily fish groups. **DHA**: There was a significant effect of the treatment on the proportion of EPA in the circulating EVs ($P=0.005$) and significant time*treatment interaction ($P=0.001$) with a significant effect of time ($P<0.001$); the proportion of EPA has increased after fish oil and oily fish groups compared to the control group ($P=0.022$ and $P=0.007$, respectively), but there was no difference between fish oil and oily fish groups. **Total n-3 PUFA**: There was a significant effect of the treatment on the proportion of total n-3 PUFA in the circulating EVs ($P<0.001$) and significant time*treatment interaction ($P<0.001$)with a significant effect of time ($P<0.001$); the proportion of total n-3 PUFA has increased after fish oil and oily fish groups compared to the control group ($P<0.001$and $P=0.001$, respectively), but there was no difference between fish oil and oily fish groups. **Total n-6 PUFA**: There was a significant effect of the treatment on the proportion of total n-6 PUFA in the circulating EVs ($P=0.037$) and significant time*treatment interaction ($P=0.008$)with a significant effect of time ($P<0.001$); the proportion of total n-6 PUFA has increased after oily fish group compared to the control group ($P=0.043$), but there was no difference between fish oil control groups. *AA, arachidonic acid; ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids.*

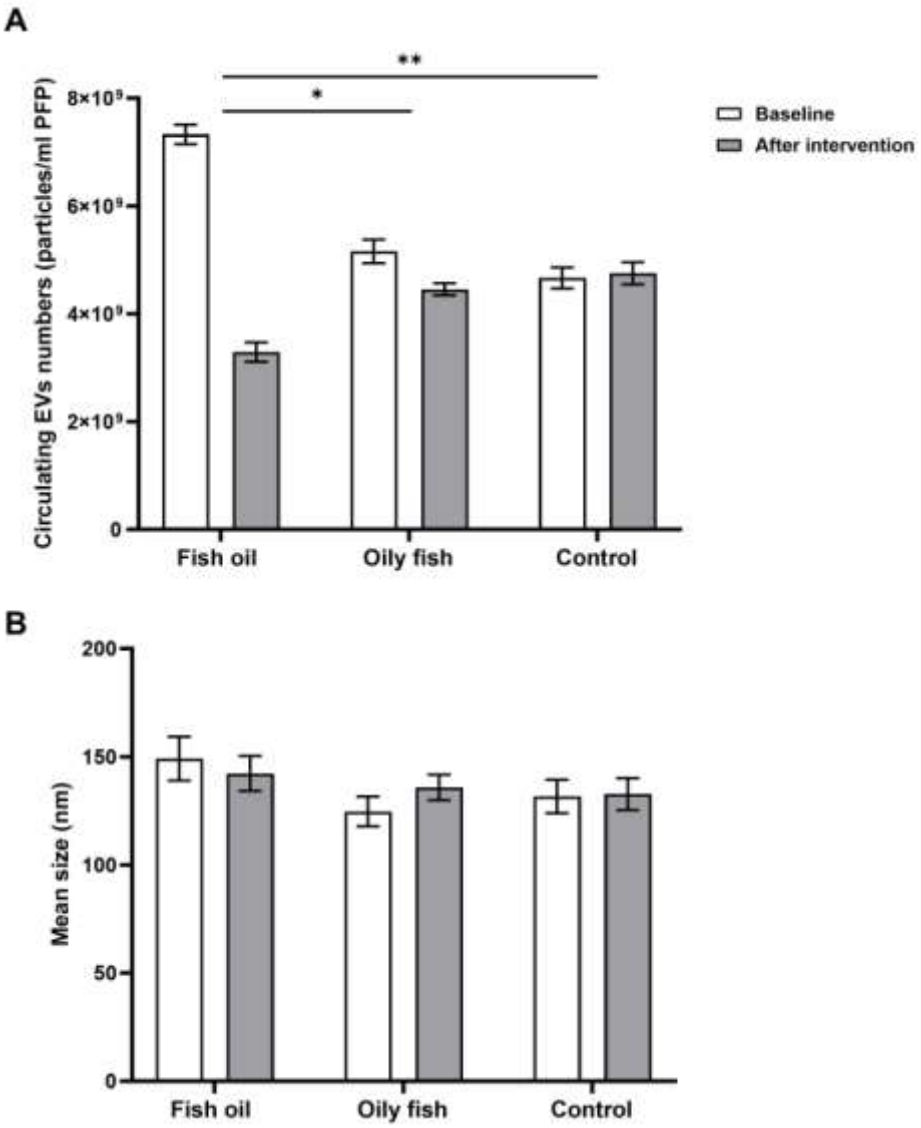

Figure 1: Effects of oily fish and fish oil supplements on the numbers and mean size of circulating EVs determined by NTA. Data were analyzed by using a general linear model, including post-hoc analysis with Bonferroni tests for treatment, period and treatment*time interaction with differences shown at $P<0.05$. **A:** There was a significant effect of treatment on EV number ($P=0.004$) and significant time*treatment interaction ($P<0.001$), with a significant effect of time ($P<0.001$); EV numbers were significantly decreased after fish oil supplement compared to oily fish ($P=0.023$) and control ($P=0.007$). **B:** There were no statistically significant effects of treatment and time interaction on EV size ($P=0.299$ and $P=0.389$, respectively). *$P < 0.05$ and **$P < 0.01$. *EV, extracellular vesicles; PFP, platelet-free plasma.*

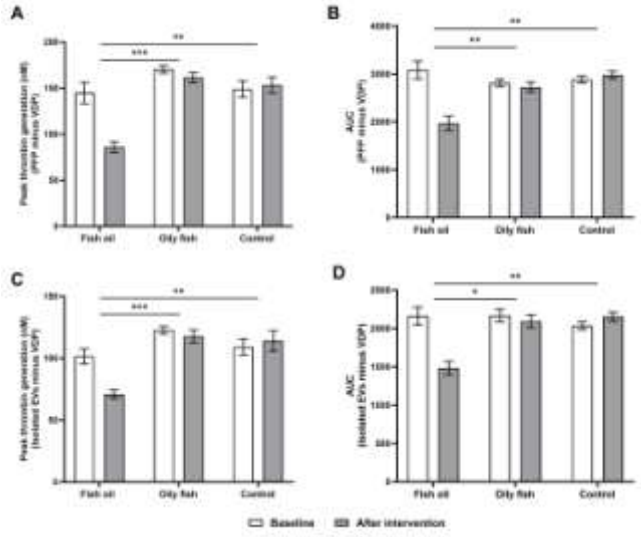

Figure 2: Effects of oily fish and fish oil supplements on circulating EV-supported thrombin generation. Data were analyzed by using a general linear model, including post-hoc analysis with Bonferroni tests for treatment, period and treatment*time interaction with differences shown at $P<0.05$. **A**: There was a significant effect of treatment on EV-supported (PFP minus VDP) thrombin peak concentration ($P<0.001$) and significant time*treatment interaction ($P=0.001$), with a significant effect of time ($P=0.010$), in which fish oil supplement significantly decreased thrombin peak concentration compared to oily fish ($P<0.001$) and control ($P=0.001$). **B**: There was a significant effect of the treatment on EV-supported (PFP minus VDP) thrombin AUC ($P<0.001$) and significant time*treatment interaction ($P<0.001$), with a significant effect of time ($P=0.007$), in which fish oil supplement significantly decreased thrombin AUC compared to oily fish ($P=0.001$) and control ($P=0.004$). **C**: There was a significant effect of the treatment on EV-supported (Isolated EVs minus VDP) thrombin peak concentration ($P<0.001$) and significant time*treatment interaction ($P<0.001$), with a significant effect of time ($P<0.001$), in which fish oil supplement significantly decreased thrombin peak concentration compared to oily fish ($P<0.001$) and control ($P=0.001$). **D**: There was a significant effect of the treatment on EV-supported (Isolated EVs minus VDP) thrombin AUC ($P=0.001$) and significant time*treatment interaction ($P<0.001$), with a significant effect of time ($P<0.001$), in which fish oil supplement significantly decreased thrombin AUC compared to oily fish ($P=0.033$) and control ($P=0.001$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. *AUC, area under curve; EVs, extracellular vesicles; PFP, platelet-free plasma; VDP, vesicles-depleted plasma.*

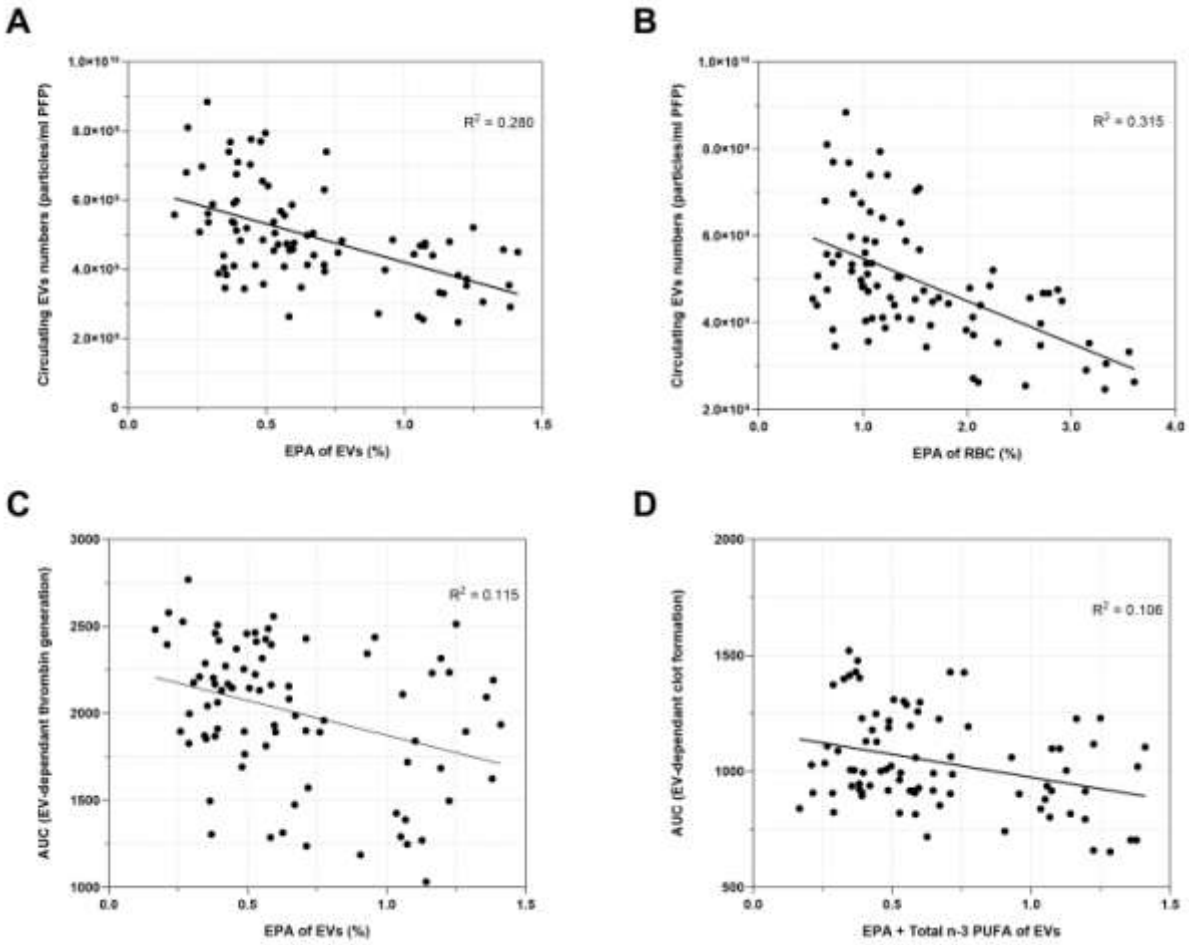

Figure 3: Association between fatty acid profiles of circulating EVs and RBCs with numbers and coagulatory activity of circulating EVs. Data were analyzed by using a stepwise multivariate regression model to identify independent predictors of EV parameters. **A**: The proportion of EPA in circulating EVs independently explained 28.0% of the variance for circulating EV numbers. **B**: The proportion of EPA in RBCs independently explained 31.5% of the variance for circulating EV numbers. **C:** The proportion of EPA in circulating EVs independently explained 11.5% of the variance for AUC in EV-dependent thrombin generation. **D:** The proportion of EPA in circulating EVs independently explained 10.6% of the variance for AUC in EV-dependent clot formation. *AUC, area under curve; EPA, eicosapentaenoic acid; EV, extracellular vesicles; RBC, red blood cells; PUFAs, polyunsaturated fatty acids.*