Fish Oil-derived N-3 Polyunsaturated Fatty Acids and Extracellular Vesicles

(NCT03203512)

Study Protocol

Feb 2017

Study objectives

The main objectives of the study were divided into two strands (**Figure 1**).

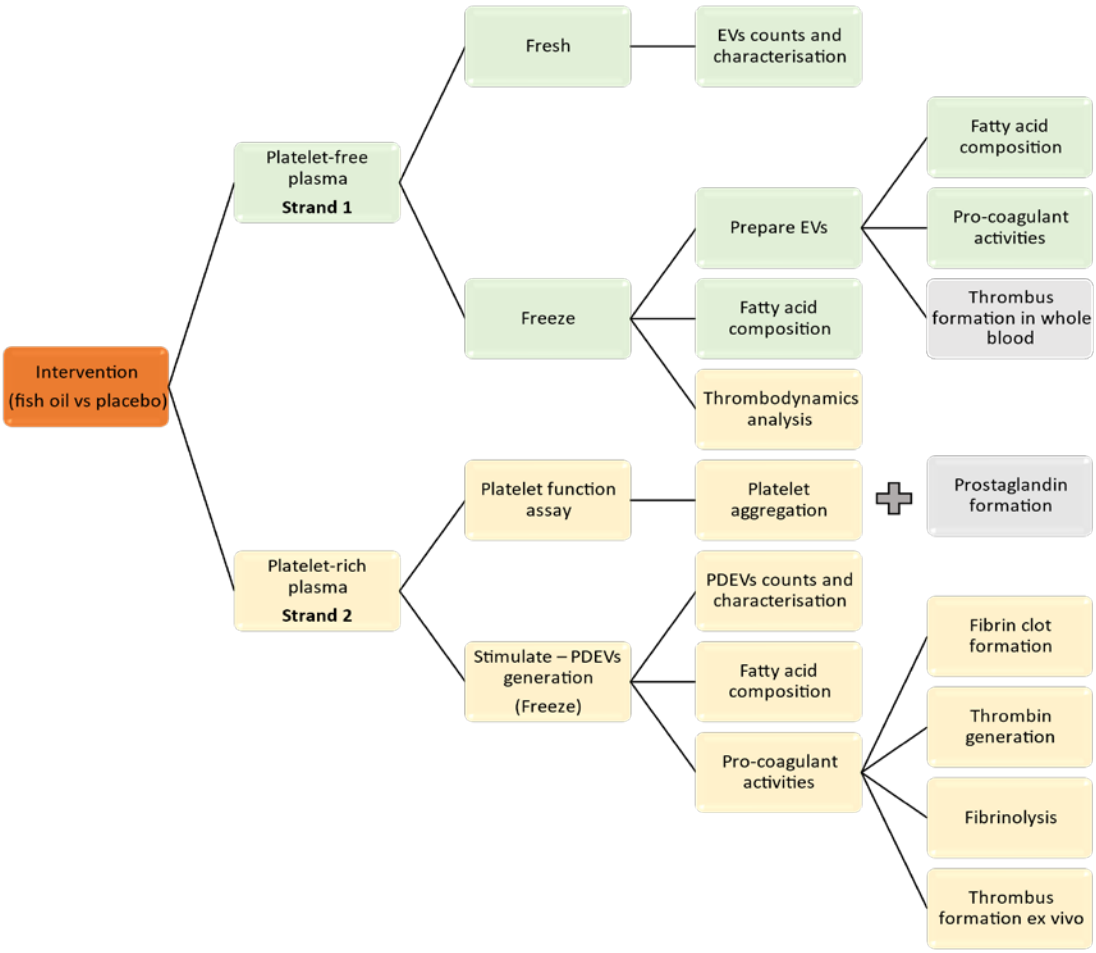

Figure 1. Overview of the experimental work. Strand 1 focused on the numeration and functional analysis of circulating EVs; strand 2 focused on the numeration and functional analysis of platelet-derived EVs. EVs, extracellular vesicles; PDEVs, platelet-derived EVs.

Strand 1: the influence of n-3 PUFA supplementation on the number and phenotype of total circulating EVs, and their fatty acid composition and coagulatory activities.

Strand 2: the influence of n-3 PUFA on the production of EVs by platelets taken from subjects and generated *in vitro* as well as their fatty acid composition and coagulatory activities.

Study design

The study was a randomised, double-blind, controlled crossover intervention. A total of 40 subjects aged between 40 and 70 years with moderate CVD risk (see criteria below) were recruited and randomly allocated to consume either fish oil or control (high-oleic safflower oil) in the first 12-week intervention period. After a 12-week washout, they then crossed over to the other intervention for a further 12 weeks. Random assignment of subjects for intervention order ("1" and "2") was performed using online software (https://www.randomizer.org/). Fish oil and control capsules were of identical appearance and blinded by an individual not involved in the study. All investigators involved in the study remained blind until all sample and statistical analysis had been completed.

The length of the intervention and washout periods was based on evidence that incorporation of n-3 PUFA in all cell types reaches a plateau after 12 weeks' intervention and that a 12- week washout period is sufficient to avoid carryover effects (Walker et al., 2015).

During the study, there were four intervention visits, which took place at the beginning and end of each 12-week intervention period (weeks 0, 12, 24 and 36). Before each study visit, subjects were asked to abstain from alcohol and strenuous exercise during the 24 hours prior to the study day. On each visit day, subjects were asked to come to the nutrition unit in an unfed state (fasted, not eating or drinking anything but water from 8 pm the night before). After taking height, weight and blood pressure measurements, a blood sample of approximately 100 ml was collected into vacutainer tubes containing 3.2% sodium citrate (Greiner Bio-One, Gloucestershire, UK) for EV-related analysis (details see below). After the first visit, subjects were asked to start to consume 6 capsules per day of either fish oil (Wiley's Finest Easy Swallow Minis, Canada), providing a total daily intake of 1.8 g n-3 PUFA, or high-oleic safflower oil (Wiley's Finest, Canada), providing 740mg oleic acid + 120mg linoleic acid per day. Subjects were advised to take capsules with breakfast, lunch and dinner (2 at each meal). Weeks 12-24 represented a washout period and then subjects crossed over to the other intervention product until week 36 (**Figure 2**).

Subjects were asked to complete food frequency questionnaires at home before their first visit and again during each arm of the intervention study (weeks 0, 12, 24 and 36). These were used to assess their habitual diet and to confirm that they had low consumption of oily fish (less than one portion per month).

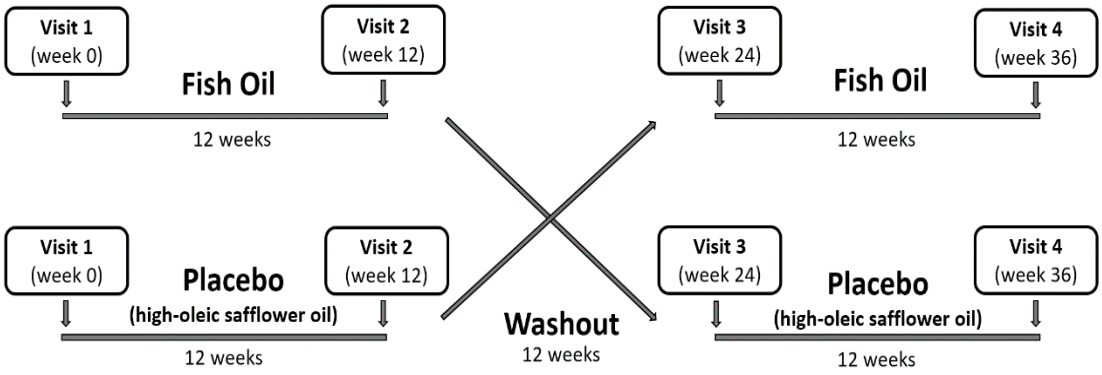

Figure 2. Study timeline

Study methods

Blood collection and processing

On the visit day, subjects were asked to come to the Hugh Sinclair Unit of Human Nutrition in an unfed state (fasted, not eating or drinking anything but water from 8 pm the night before). Venous blood samples were collected from fasted subjects into vacutainer tubes containing 3.2% sodium citrate (Greiner Bio-One, Gloucestershire, United Kingdom), and were kept at room temperature and processed within 30 minutes after transfer into polypropylene tubes (Fisher Scientific, Loughborough, United Kingdom). Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 175 x g for 15 minutes, with no brake at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 13,000 x g at room temperature for 2 minutes and removal of the supernatant from the platelet pellet. To obtain platelet-free plasma (PFP), blood was centrifuged at 1500 x g for 15 minutes at room temperature and the upper two-thirds were collected and centrifuged once again at 13,000 x g for 2 minutes at room temperature, before collecting the upper three quarters from each tube as PFP. Freshly prepared PRP and PPP were used in the platelet aggregation assay. Some of the PFP was used for the enumeration and characterization of circulating total EVs, while the remainder was stored at − 80°C for

analysis of thrombogenic activity of PFP (thrombin generation and fibrin clot properties) and of EVs (thrombin generation).

Isolation of PDEVs generated *in vitro* from unstimulated and stimulated platelets

The isolation process for PDEVs generated in vitro from unstimulated and stimulated (thrombin receptor activator peptide 6, TRAP-6) platelets consisted of i) isolation of platelets from whole blood, ii) stimulation or not of isolated platelets and iii) isolation of EVs from the supernatants of stimulated and unstimulated platelets.

To isolate platelets, PRP was centrifuged at 1,000 x g, for 10 min, with low brake, at room temperature in the presence of prostacyclin (PGI2, 1 µg/ml; Sigma-Aldrich, Dorser, UK), as described by Armstrong et al (Armstrong et al., 2017). The pellet was washed twice to obtain washed platelets in modified Tyrode's (MTH) buffer prepared by the supplementation of Tyrode`s HEPES stock (containing 134 mmol/l NaCl, 2.9 mmol/l KCl, 0.34 mmol/l Na2HPO4, 12 mmol/l NaHCO3, 1 mmol/l MgCl2 and 20 mmol/l HEPES; Ph 7.4; filtered on 0.22 µm) with 0.1% of glucose (Sigma-Aldrich, Dorset, UK). Platelet concentration in the suspension was counted (Sysmex, USA) and adjusted to 3x108 platelets/ml with MTH buffer. Washed platelets at 3 x 108 platelets/ml were supplemented with 2 µM of CaCl2 (Sigma-Aldrich, Dorset, UK) then immediately stimulated with either 30 □ M of TRAP-6 (stimulated platelets) or PBS (un-stimulated platelets). Stimulated and unstimulated platelets were incubated at 37°C for 2 hours in non-stirring conditions. Platelets were then removed by two sequential centrifugations at 1,200 x g for 10 minutes. The upper 90% of the supernatant, which contains the EVs, was collected and pelleted by centrifugation at 15,000 x g for 30 minutes at 4 °C. The supernatant was discarded and the pellet rich in PDEVs was suspended. Isolated PDEVs were pooled in a vial to ensure their homogeneity and then divided into aliquots of 30 µl each and stored at -80 °C until use.

Preparation of pooled vesicle free plasma (VFP)

Venous blood samples were collected from three healthy, fasted subjects as described above to prepare pooled VFP. Whole blood was first centrifuged twice at 2,500 x g for 15 min to remove blood cells and obtain a pooled plasma. Vesicles were removed from pooled plasma by ultracentrifugation at 20,000g for 1 hour at 4 °C, then the supernatant was collected, and the remaining pellets (EVs) was discarded. The vesicle poor plasma was ultracentrifuged at 100,000g for 1 hour at 4 °C, followed by filtration on 0.1 µm (filter info) four times. This filtered supernatant was considered VFP and stored in 1 ml aliquots at -80 °C until use.

Isolation, enumeration and characterization of circulating EVs using nanoparticle tracking analysis (NTA)

PFP was subjected to size exclusion chromatography (SEC) using Izon qEV columns (Izon Science Ltd, Oxford, United Kingdom) to isolate EVs prior to NTA. PBS was used to elute 10 sequential fractions of 0.5ml and fractions 7-9 were diluted with PBS to achieve the recommended concentration range of particles (1-10 x 10^8 vesicles/ml) (Gardiner et al., 2013, Vestad et al., 2017; Dragovic et al., 2011) for analysis on the NanoSight 300 (Malvern, Amesbury, United Kingdom). Data from 5 separate videos, each of 60 seconds' duration, were analyzed using the NTA 3.20 instrument software, which identifies individual particles and estimates size based on the Stokes-Einstein Equation (van der Pol et al., 2010). A threshold of 70nm was set to ensure minimal interference by small lipoproteins. Total circulating EV numbers enumerated by NTA were defined as total EVs (TEVs).

Enumeration and characterization of circulating EVs using flow cytometry

For enumeration and phenotyping of circulating EVs by flow cytometry (BD FACSCanto II flow cytometer, BD Biosciences, Wokingham, United Kingdom), an initial EV size gate was set using non-fluorescent, silica ApogeeMix beads with diameters of 180nm, 240 nm, 300nm, 590nm, 880nm, and 1300nm (Apogee flow systems, Hemel Hempstead, United Kingdom) and additionally, fluorescent latex beads with diameters of 110nm

and 500nm. The 240nm silica beads corresponded to the lowest reliable detection limit of the flow cytometer and any particles below this size (on SSC) were excluded to minimize background noise. In order to exclude platelets and cellular material, the upper detection gate would ideally be set at 1µm, but the closest available diameter of silica beads was 880 nm, so the gate was set just above this on scatter mode.

The following fluorochrome-coupled antibodies and their corresponding isotypes were used for EV phenotyping: Annexin V conjugated to Allophycocyannin (APC) (Thermo Fisher Scientific, Basingstoke, United Kingdom) for phosphatidylserine (PS) positive EVs, anti-CD41 conjugated to Phycoerythrin (PE) (Diagnostica Stago LTD, Theale, United Kingdom) for platelet-derived EVs (PDEVs) and anti-CD105 conjugated to eFluor450 (Life Technologies LTD (Invitrogen division), Paisley, United Kingdom) for endothelial-derived EVs (EDEVs). PFP (5 µl) was incubated with FcR blocking reagent (5µl) (Miltenyi Biotec Ltd, Surrey, United Kingdom) and Annexin V buffer (10mM HEPES, 140mM NaCl, 2.5mM $CaCl^2$, pH 7.40; Cambridge Bioscience, Cambridge, United Kingdom) for 15 minutes in the dark at room temperature, before incubation with antibodies and isotype-matched controls for a further 15 minutes in the dark at room temperature. Samples were then diluted with 200µl Annexin V buffer and analyzed by flow cytometry, as illustrated in **Figure 3**. EVs staining positive for Annexin V were classified as PS+EVs, those staining positive for Annexin V and CD41 were classified as PS+ platelet-derived EVs (PDEVs) and those staining positive for Annexin V and CD105 were classified as PS+ endothelium-derived EVs (EDEVs). Matching isotype controls (IgG1-PE and IgG1-eFlour450) were used as appropriate. Absolute numbers of vesicles were calculated using BD Trucount tubes (BD Biosciences, Wokingham, United Kingdom).

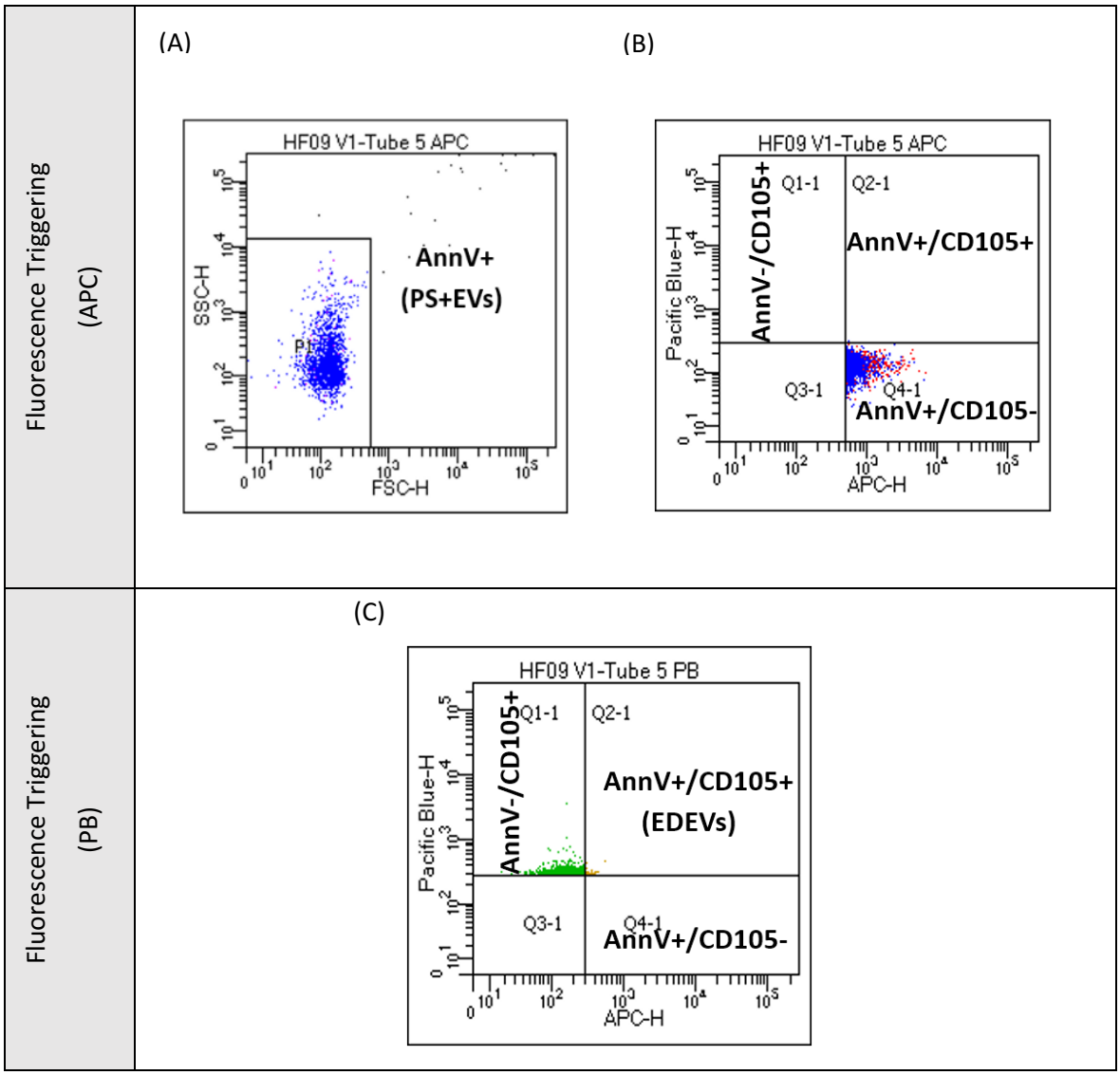

Figure 3. Flow cytometric analysis of a typical sample of PFP labelled with Annexin V-APC, anti-CD41-PE and anti-CD105-eFluor450. EVs were characterized as follows: (**A**) All Annexin-APC fluorescently labelled particles were identified as PS+EVs (in blue); (**B**) APC vs PE quadrant plot were set to identify both Annexin-APC and CD41-PE labelled particles as PDEVs (in red); (**C**) APC vs PB quadrant plot to identify both Annexin-APC and CD105-eFluor450 labelled particles as EDEVs (in yellow). *AnnV, Annexin V; APC, Allophycocyanin; EDEVs, endothelial-derived extracellular vesicles; PB, Pacific Blue; PDEVs, platelet-derived extracellular vesicles; PE, phycoerythrin; PS, phosphatidylserine.*

For characterisation of PDEVs generated in vitro using flow cytometry, all antibodies and reagents were filtered using 100 nm syringe filters (Sartorius, UK) and 100 nm centrifugal devices (Sartorius, UK) and analysed for 1 minute to evaluate the level of background particles that fell in the EV gate before each analysis day. To measure the PS expression of PDEVs, samples were assessed for their ability to bind cell marker-

specific monoclonal antibody anti CD41 conjugated to PE (Biocytex, Marseille, France) to detect platelet-derived EVs and Annexin V conjugated to APC (ThermoFisher, UK), which binds externalized PS residues. For this double staining, frozen PDEVs were thawed, then 2 µl of each PDEV sample was incubated with both anti CD41 and Annexin V (3µl each) for at least 30 minutes in the dark at room temperature. The incubation was followed by dilution with 70 µl of Annexin V-binding buffer (10mM HEPES, 140mM NaCl, 2.5mM CaCl2, pH 7.4) and samples were analysed by FCM. Events were collected with low flow rate for 1 minute on the threshold corresponding to the labelled fluorophore avoiding an event rate higher than 5,000 events/second. For anti CD41, sample incubation with its isotype control had lower fluorescence than the antibody on its own; therefore the background was set on the fluorescence produced by the antibody only. EV positive events were calculated, by subtracting the number of events obtained from the antibody mix on each experimental day from the absolute count of positive events of the labelled sample (**Figure 4**). Data was captured using FACSDiva Software version 6.1.3 and analysed using FlowJo version 10.

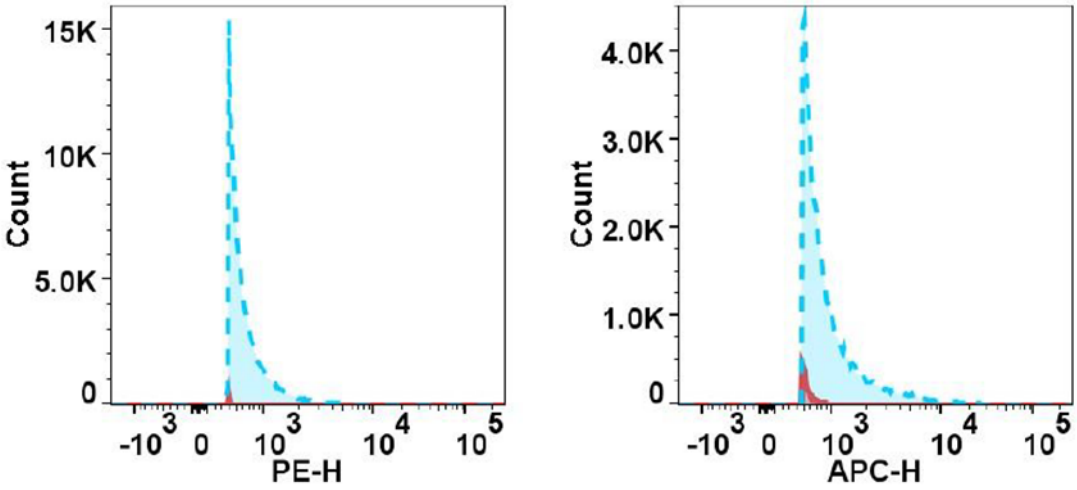

Figure 4. Representative flow cytometry histograms with signal triggered in fluorescence mode showing mean fluorescence intensity (MFI) of the antibody background (red) and PDEVs positive events (blue). PE = CD41, APC = Annexin V. The number of positive events was calculated subtracting the events collected in blue minus events collected in red.

Functional assays of PDEVs generated *in vitro*

Measurement of clot formation induced by PDEVs

The impact of PDEVs (before and after intervention) on fibrin clot formation in VFP was assessed in 96-well flat-bottom microplates (Greiner Bio-One, Stonehouse, Gloucestershire, UK) by a change in turbidity of the VFP at 405 nm (A405) every 30 seconds, for 1 hour at 37°C using a Flex Station 3 plate reader (Molecular Devices, United State). Therefore, pooled PFP (positive control) and VFP (negative control), served as benchmarking in this assay. The assay was then set up by diluting 30% of VFP in clot buffer (10 mM Tris [pH 7.4] and 0.01% Tween 20) and 5 µg/ml protein concentration of PDEVs (final concentration) was then added. Fibrin clot formation was initiated by addition of 5 mM CaCl2 in a final volume of 100 µl of clotting mixture. Plates were read at 37 °C for 1 hour at 30 second intervals using a fluorescence plate reader (FlexStation 3, United State) at wavelength of 405 nm. Data were then analysed by an online application (https://drclongstaff.shinyapps.io/Clot_or_HaloCL/) to obtain relative parameters: time to 50% change, absorbance at 50% change, absorbance at peak, area under curve.

Measurement of thrombin generation

Thrombin formation was assessed using a commercially available, plate-based thrombin generation assay (Technothrombin TGA kit, Austria), which assesses a change in fluorescence as a result of cleavage of a fluorogenic substrate by thrombin over time upon activation of the clotting cascade by tissue factor. Two separate analyses were conducted: (i) determination of the effect of n-3 PUFA supplementation on thrombin generation in PFP from study samples relative to VFP and (ii) determination of the effects of in vitro-generated PDEVs or circulating EVs derived from study samples on thrombin generation in VFP. Prior to analysis, a thrombin calibration curve was constructed from dilutions of lyophilised Hepes-NaCl-buffer containing 0.5 % bovine serum albumin and ~ 1000 nM thrombin in buffer with BSA. A kinetic reading of a plate was initiated by additional of 50 µl of fluorogenic substrate solution containing fluorogenic substrate 1 mM Z-G-G-R-AMC and 15 mM CaCl2.

Calibration curves were recorded at 37 °C for 10 minutes with 30 seconds intervals using the plate reader (FlexStation 3, United State) at 360 nm for excitation and at 460 nm for emission.

The methods for both approaches were based on the use of pooled VFP as a negative control to allow assessment of thrombin generation specifically resulting from the presence of EVs. For the first approach (i), 40 µl aliquots of either pre-thawed study sample PFP or pooled VFP or pooled PFP were added into the plate. For the second approach, PDEVs or circulating EVs (10 µl of EV suspensions at 5 µg protein/ml, final concentration) produced from either unstimulated or stimulated platelets (UP-EVs and SP-EVs, respectively) from intervention samples, or PBS (negative control) were added to 30 µl VFP. This was followed by addition of a 10 µl suspension of phospholipid micelles containing recombinant human tissue factor (TF) in Tris-Hepes-NaCl buffer (RCL), which was provided in the kit. Formation of thrombin was initiated by additional of 50 µl of fluorogenic substrate solution containing fluorogenic substrate 1 mM Z-G-G-R-AMC and 15 mM CaCl2. Plates were immediately read at 37 °C for 1 hour at 1 min intervals using a fluorescence plate reader (FlexStation 3, United State) at excitation and emission wavelengths of 360 and 460 nm, respectively. All samples were measured in duplicate. Fluorescent intensity was detected by TGA Evaluation Software to calculate thrombin generation in samples. Data were then analysed by the TGA Evaluation Software manually to convert the unit of thrombin generation from RFU to nM and presented as three variables: lag time, time to the peak, the concentration of thrombin (nM), velocity-index and area under curve.

Measurement of fibrinolysis by PDEVs

The assessment of fibrinolysis induced by PDEVs added to VFP was performed in 96-well flat-bottom microplates (Greiner Bio-One, Stonehouse, Gloucestershire, UK) by a change in turbidity of VFP at 405 nm (A405) every 30 seconds, for 8 hours at 37°C using a Flex Station 3 plate reader (Molecular Devices, United State. In this assay, pooled PFP (positive control) and VFP (negative control), therefore, served as benchmarking.

The assay for fibrinolysis of PDEVs generated in vitro was set up by diluting 30% of VFP in clot buffer (10 mM Tris [pH 7.4] and 0.01% Tween 20) in the presence of tPA (100 pM; Sigma-Aldrich, Dorset, UK) and 5 µg/ml protein concentration of PDEVs (final concentration) was then added. Fibrin clot formation to lysis was initiated by addition of 5 mM CaCl2 in a final volume of 100 µl of clotting mixture. Plates were read at 37 °C for 8 hours at 30 second intervals using a fluorescence plate reader (FlexStation 3, United State) at wavelength of 405 nm. Data were then analysed by an online application (https://drclongstaff.shinyapps.io/Clot_or_HaloCL/) to obtain relative parameters: area under curve, time to 50% lysis from zero, absorbance at %50 lysis, time clotting to 50% lysis (time between 50% clotting and lysis), time peak to 50% lysis (time between the peak and 50% lysis).

Measurement of *in vitro* thrombus formation under flow

PDEVs prepared from the study samples were subjected to analysis of their effect on thrombus formation in whole blood under flow, a specialist technique conducted by Dr Joanne Mitchell from the School of Biological Sciences, University of Reading. The assay was performed by adding either buffer (control) or 30 µl of PDEVs generated from TRAP-6 stimulated platelets (final protein concentration of EVs: 5 µl/ml) into whole blood in modified Tyrode's-HEPES buffer (134 mM NaCl, 2.9 mM KCL, 0.34 mM Na2HPO4, 20 mM HEPES, 1 mM MgCl2, pH 7.4). Cellix Vena8 Fluoro+ biochips with capillaries 0.01 cm high and 0.04 cm wide were coated with collagen (100 mg/ml) prior to thrombus formation studies. Whole citrated blood labelled with 3,3'-Dihexyloxacarbocyanine Iodide (DiOC6) was flowed for 10 minutes over the collagen coated capillary chambers to form platelet thrombi at an arterial shear rate of 1000s-1. Images were taken every 2-4 seconds using a Nikon A1R fluorescence confocal microscope at 20X magnification. Data were analysed using Fiji (Image J) by measuring the fluorescence intensity of the DiOC6 over time which corresponded to the increase in thrombus size.

Assessment of platelet aggregation

Freshly prepared PRP (40 µl) was added to appropriate wells of microplate containing six separate agonists (ADP, CRP-XL, EPI, TRAP-6, U46619 and AA) and the plate was tapped gently to ensure that the PRP reached the bottom. The plate was shaken at 1,200 rpm at 37°C for 5 minutes using a plate shaker (Thermo-shaker, Grant Instruments, UK).PRP or PPP were then added to the relevant control wells (agonist free) in plate which was tapped sharply to remove any aggregates from the center of the wells. A plate-reader (Tecan Microplate Reader Spark, Switzerland) was used to determine the absorbance at 405nm. Absorbance data were converted to a percent aggregation by reference to the absorbance of PRP and PPP as 0% and 100% aggregation controls, respectively. Dose-response curves in response to each agonist were obtained and curves were fitted by a four-parameter logistic non-linear regression using Prism (Version 8.2, GraphPad Software, Inc., San Diego, CA).

Clot growth and fibrinolysis

Clot growth and fibrinolysis in samples of PFP was assessed using a thrombodynamics analyzer and thrombodynamics kit (HemaCore, Moscow, Russia). PFP (120µl) of PFP was thawed in a 37 °C water bath for 5 min. Plasma placed into a special plastic vial contained a lyophilized solution of protein-inhibitor and incubated for 15 minutes at 37°C within the thermostat of the analyzer, following which 4nM of tissue-type plasminogen activator (tPA, final concentration) (Sigma-Aldrich, Dorset, UK) was added. This step was followed by the addition of a lyophilized solution of calcium salt. The sample was immediately transferred into an optically transparent cuvette with two thin channels, which were placed into the 37°C temperature-controlled chambers of the instrument. Finally, an activating insert, the end edges of which were covered with immobilized tissue factor to activate clotting, was gently placed fully into the cuvette. Growth and lysis of the fibrin clot was quantified using video microscopy software over a 60 minutes period (see **Figure 5**).

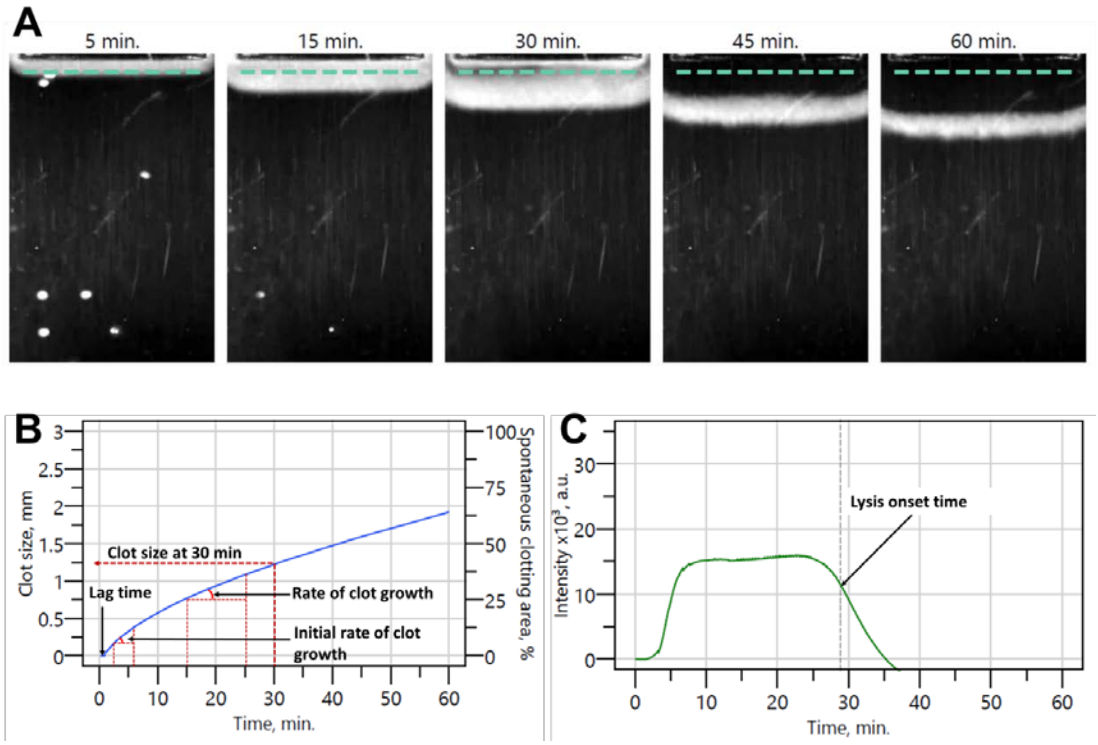

Figure 5. Clot formation is activated by introducing the TF bearing surface to PFP and fibrinolysis was assessed by addition of 4 nM tPA. (**A**) Representative images of coagulation process and fibrinolysis were recorded over 60 min and all parameters were calculated by the thrombodynamics software; (**B**) Plot of fibrin clot growth versus time was constructed to calculate coagulation-related parameters. Lag time was calculated as the time from the beginning of the measurement until the beginning of the clot growth when the first significant levels of fibrin can be detected. The rate of clot growth and the initial rate of clot growth, which indicate the propagation stage of blood coagulation were calculated on the interval 15-25 minutes and 2-6 minutes after the beginning of clot growth. The clot size was measured at the 30th minute of clot growth. Clot density was measured as amount of light scattering from a fibrin clot; (**C**) Plot of fibrin clot intensity versus time was constructed to calculate fibrinolysis-related parameters. Lysis onset time was calculated as the time, when the light scattering intensity (green line) in the clot reach to 30% reduction from the beginning and the lysis progression was calculated as the linear rate of the light scattering intensity decrease as the percentage of the initial value in the following 5 min.

Conventional cardiovascular risk markers assessment

Anthropometric data included height, weight and body mass index (BMI), the latter recorded using the Tanita MC-780MA P (Tanita Europe BV, Netherlands). SBP and diastolic BP (DBP) were recorded as the average of three using an Omron M2 Upper Arm Blood Pressure Monitor (OMRON Healthcare Europe BV, United Kingdom). An overnight fasting blood sample of approximately 9ml was taken into a serum-separating tube (Greiner Bio-One, Gloucestershire, United Kingdom) for the analysis

of blood lipid and glucose concentrations. Serum-separating tubes were kept upright at room temperature for 30 minutes (and no more than 60 minutes) before centrifuging at 1700 x g for 15 minutes at room temperature. The plasma (160µl) was collected and analyzed by iLab (iLab 600 Clinical Chemistry System, Diamond Diagnostics, United States) for triacylglycerol (TAG), TC, HDL-C, and glucose concentrations using standard reagent kits (Werfen Limited, Warrington, United Kingdom).

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(NCT03203512)

Statistical Analysis Plan

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Statistical analysis

Data are expressed as mean ± SEM if normally distributed and as median with inter-quartile range when not. The Kolmogorov-Smirnov test was applied to assess the normal distribution of continuous variables and all variables with non-normal distribution were log-transformed to achieve normal distribution before analysis. A general linear model with fixed factors of treatment and period was conducted to determine the differences in the effect of two treatments and two periods on biological characteristics and EVs parameters. A paired t-test comparing the baseline data (before fish oil and before control oil) was performed to check potential carry-over effects. All statistical analyses were performed with SPSS Statistics version 25 and a p-value < 0.05 was considered statistically significant.