Dietary fat modulation of extracellular vesicles

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Declaration

I confirm that this is my own work and the use of all materials from other sources has been acknowledged.

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List of commonly used abbreviations:

ALA - Alpha linolenic acid

AUC - area under the curve

BP - blood pressure

BMI - body mass index

CHD - coronary heart disease

CVD - cardiovascular disease

DHA - docosahexaenoic acid

DBP - diastolic blood pressure

EPA - eicosapentaenoic acid

EVs - extracellular vesicles

FCM - flow cytometry

CM - chylomicrons

GC - gas chromatography

HDL - high-density lipoprotein

IE - interesterified fats

ISEV- International Society for Extracellular Vesicles

IHD - ischaemic heart disease

LDL -low density lipoprotein

MUFAs - monounsaturated fatty acids

n-3 PUFAs - n-3 polyunsaturated fatty acids

NTA - nanoparticle Tracking Analysis

PFP - platelet-free plasma

PBS - phosphate-buffered saline

PS – phosphatidylserine

RBC - red blood cells

RCTs - randomised controlled trials

RR - relative risk

SFA - saturated fatty acids

SBP - systolic blood pressure

SEC - size exclusion chromatography

SSC - side scatter,

TAG – Triacylglycerol

TC - total cholesterol

TFs - tissue factors

tPA - tissue plasminogen activator

TFA - trans fatty acids

VFP - vesicle-free plasma

VLDL - very low-density lipoprotein

W-H ratio - waist to hip ratio

Abstract

Cardiovascular diseases (CVDs) are considered as the major cause of death worldwide. Dietary fats have been reported to be involved in the causation and prevention of CVD. It is advised to cut down the consumption of SFA (saturated fatty acids) and TFA (trans fatty acids) due to their negative impact on CVD risk, particularly with regard to levels of blood cholesterol. On the other hand, n-3 PUFAs (n-3 polyunsaturated fatty acids) are reported to protect against CVD due to their role in reducing blood TAG (triacylglycerol). Extracellular vesicles (EVs) are small membrane-bound vesicles released from all cell types and their levels are reported to be increased in CVD, suggesting their potential use as a disease biomarker. Studying EVs as potential biomarkers involves their purification from contaminating particles; this is of particular importance in postprandial studies after a high fat meal, where the number of lipoproteins particles (whose size overlaps with that of EVs) increases substantially.

The first study in this thesis examined whether there was co-isolation and/or interference of lipoproteins during EV isolation and analysis using size-dependent isolation methods. Following consumption of a high fat meal by healthy volunteers, EVs were isolated from platelet-free plasma by size exclusion chromatography (SEC) and the EV fractions were shown to have very little contamination with the apolipoproteins, apoB48 or apoB100. Lipoprotein fractions prepared from platelet-free plasma by density gradient centrifugation and analysed by flow cytometry (FCM) to visualise annexin V-positive, platelet-derived and endothelial-derived EVs were virtually devoid of EVs. Furthermore, when purified lipoproteins were applied to size exclusion columns, they eluted later than EVs, with little cross-contamination. These findings together suggested that even after consuming high fat meal, EVs could be isolated and analysed without significant contamination from lipoproteins

The second study described in this thesis explored the effects of high fat meals containing different commercially available fats on the number and thrombogenic activity of EVs during the postprandial period. Numbers and thrombogenic activity of EVs increased during the postprandial period, reaching a peak at 4h, but there was no difference in response to the type of fat in the meal, and in particular, to interesterified (IE) fats compared to traditional commercial fats.

The final study was based on published data showing that n-3 PUFA supplements decrease numbers of circulating EVs, advancing on this by comparing the effects of fish oil supplements with two oily fish meals per week on EV number, composition and procoagulant activity. Supplementation with fish oil significantly decreased both number and thrombogenic capacity of EVs, while consumption of oily fish at a level achievable in the diet had no effect on either EV number or thrombogenic capacity.

In summary, numbers of EVs are increased during the postprandial period after a high fat meal and the EVs are more thrombogenic, but the type of fat in the meal does not appear to influence either the number or thrombogenicity, and the analysis of EVs during the postprandial period is not hampered by the presence of lipoproteins. In a chronic intervention study, n-3 PUFA from fish oil supplements reduced EV number and procoagulant activity, but consumption of two oily fish meals per week had no effect.

Table of contents:

Chapter 1: Literature Review

The objective of this chapter is to review the evidence on EV and CVD and the impact of dietary fat modulation on EV and CVD.

1.1 Introduction to extracellular vesicles

Almost all types of cells release very small membrane-derived vesicles enclosed by a lipid bilayer and carry several types of cargo, such as nucleic acids, proteins and lipids, reflecting the cells of their origin [1]. EVs can be isolated from all body fluids, including plasma, saliva, urine, serum and amniotic fluid [2]. The discovery of cell-derived extracellular vesicles dates back to 1946, when it was shown that prolonged centrifugation (150 min at 130,000g) increases the clotting time of the supernatant. When the pellet was added to plasma, the clotting time was shortened [3], which suggests that subcellular factors must stimulate blood clotting. In 1967, the subcellular fraction of plasma was investigated by electron microscopy and found to contain small EVs released from platelets and termed as "platelet dust"; this was thought to be a cellular debris with no biological value [4]. However, this view changed when the particles were suggested to have a role in antigen presentation [5]. Since then, EVs have been reported to be involved in the regulation of normal physiological processes, such as blood coagulation, intracellular communication, vascular repair and could be used as vehicle to deliver drugs [6]. Their levels are reported to be increased under pathological conditions such as in CVD, type 2 diabetes, cancer and pulmonary hypertension, suggesting their potential use as a disease biomarker [7-10]. According to the World Health Organization, any substance or mechanism that influences or predicts a disease and can be measured in the body is termed as a biomarker [11]. Consequently, the concentration and content of EVs can be measured and could provide useful information when making comparisons between health and disease status [12].

1.2 Classification and biogenesis of EVs

Due to their small size, heterogeneity and the lack of specific methods to discriminate between the different types of EVs, their classification is challenging, and the nomenclature is not fully standardized [6]. Cell derived vesicles used to be named after the cells or tissues from which they were released, e.g., prostasomes; vesicles derived from prostate, dexasomes; dendritic derived vesicles and matrix vesicles; vesicles derived from cartilage and synaptic vesicles; neurons derived vesicles [6]. However, more recently, the nomenclature of EVs refers to their size and the mechanism of production into the extracellular space, where they classified into three main categories [13]. Exosomes (30-150 nm) are released through the fusion of multivascular endosomes, microvesicles (100-1000 nm) produced by the direct blebbing of vesicles from the plasma membrane and apoptotic bodies (50-2000 nm) generated through programmed cell death [14].

1.2.1 Exosomes

Exosomes are the smallest cell-derived vesicles released from the endolysosomal pathway by the inward budding of cell membrane to create early endosomes (Figure 1.1) [14]. Another inward budding occurs to the endosomal membrane to produce intraluminal vesicles (late endosomes), which are also termed multivascular endosomes [15]. The complexes of endosomal sorting complex required for transport (ESCRT) are involved in multivesicular endosome formation. The complex ESCRT-0 produces clustered cargo on the surface of membrane. These are recruited by ESCRT -I and II to form buds and therefore the formation of multivascular endosomes [16]. Multivesicular endosomes either fuse with the plasma membrane and release their internal vesicles as exosomes, or fuse with lysosomes for cargo degradation [14].

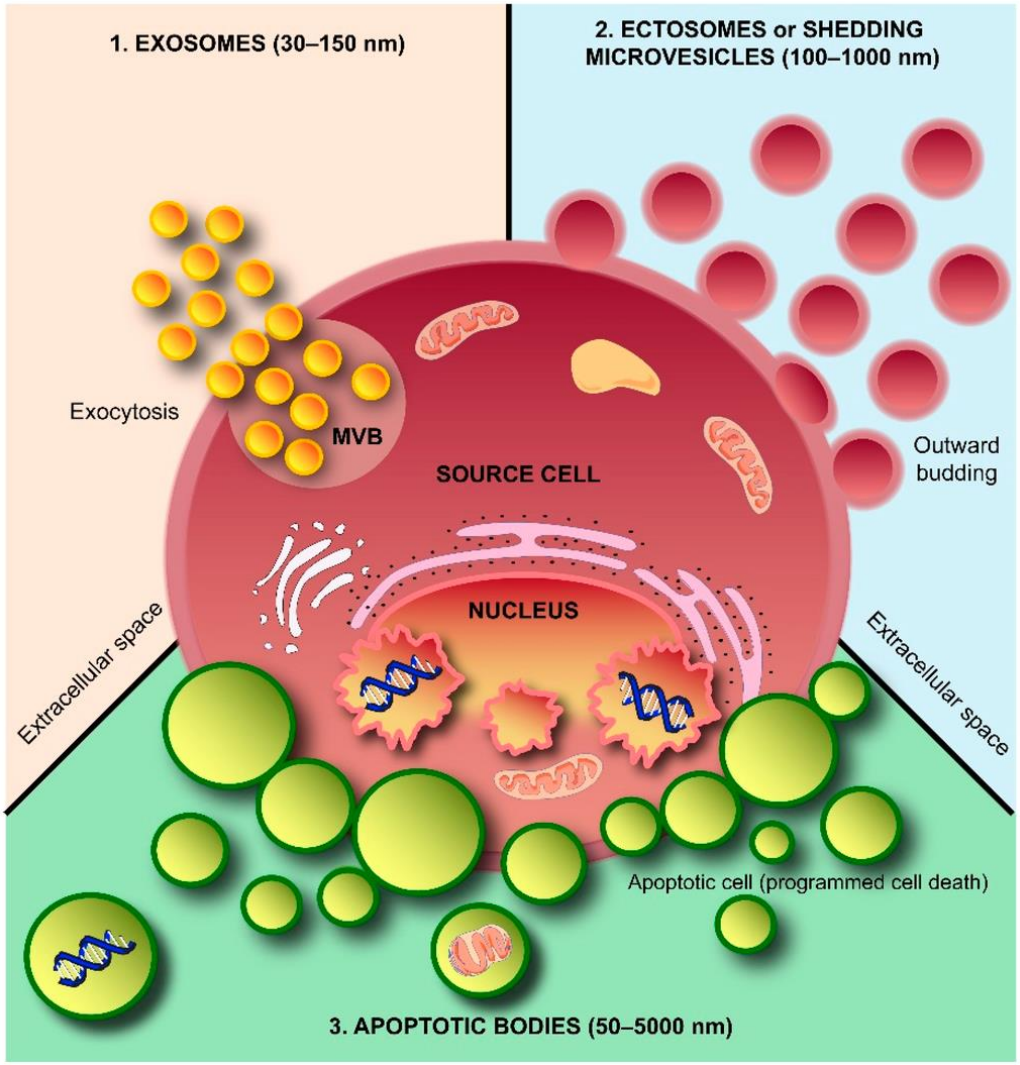

Figure 1.1 Mechanisms of EV release. 1) Exosomes are derived from the endolysosomal pathway by the inward budding of cell membrane to create multivascular endosomes which fuse with the plasma membrane and release their internal vesicles as exosomes, **2)** Microvesicles are formed through the budding and pinching of the surface of plasma membrane, **3)** Apoptotic bodies are released through the process of membrane blebbing from dying cells during apoptosis [14].

1.2.2 Microvesicles

Microvesicles are larger than exosomes and are primarily distinguished from exosomes by the mechanism of their formation through the budding and pinching of the plasma membrane surface (Figure 1.1) [14]. This occurs by disruption of the asymmetric distribution of protein and phospholipid of the plasma membrane (Figure 1.2) [17]. The asymmetry of a cell is regulated and maintained by three types of proteins located on the cell membrane: translocase,

which transfers phosphatidylserine (PS) from the outer to the inner leaflet of the cell; floppase, which transfers lipids from the inner to outer leaflet and scramblase, which permits lipids to move randomly between both leaflets [17]. When cells are activated, the calcium concentration increases to a point that dysregulates these proteins and therefore disrupts the asymmetric structure of the cell and stimulates the exposure of PS [14, 17]. The increased concentration of calcium also stimulates the protein calpain, which in turn induces the release and budding of PS expressing microvesicles [14].

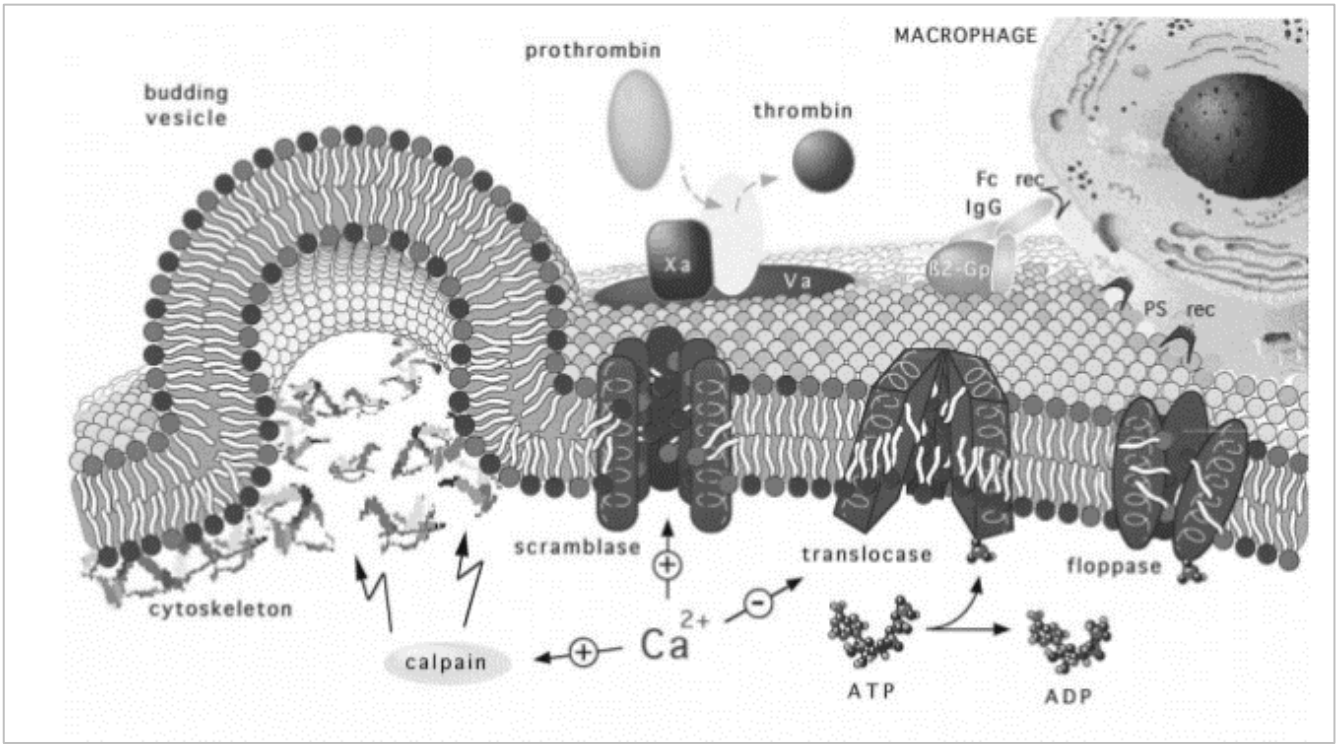

Figure 1.2 The mechanism of the dysregulation of the asymmetric structure of protein and phospholipid contents of plasma membrane. The asymmetry of the cell membrane is maintained by proteins, including translocase, floppase, and scramblase. When cells are activated, the calcium concentration increases to dysregulate these proteins and therefore disrupt the asymmetric structure of the cell and stimulate the exposure of PS [17].

1.2.3 Apoptotic bodies

Apoptotic bodies are the largest EVs produced through the process of membrane blebbing from dying cells during apoptosis (Figure 1.1) [14, 18]. The mechanism of apoptotic body formation is regulated via several stages, which include condensation of both nucleus and cytoplasm,

nuclear rupture, separation of protuberance, cell shrinkage, mitochondrial swelling and PS exposure [14, 19]. Production of apoptotic bodies occurs when cells are weakened by the cleavage of the cell's main structure, which includes the cytoskeleton. This is achieved by cysteine proteases, which are responsible for protein cleavage [14]. Increased calcium concentration also stimulates the release of apoptotic bodies and exposes PS [20]. The formed apoptotic bodies are packed with enzymatically active and toxic components, which are phagocytosed by macrophages to avoid the leakage of these components and prevent tissue damage and inflammation [14]. The current review focuses only on exosomes and microvesicles since apoptotic bodies are produced through programmed cell death and eliminated by phagocytosis, and therefore not directly relevant to serving as potential biomarkers for cardiovascular disease.

1.3 Protein and lipid composition of EVs

Apart from their physical properties and biogenesis, EVs can be identified on the basis of their unique protein and lipid composition which can be characterised by western blotting, enzyme-linked immunosorbent assay and FCM; the latter is the most widely used method to phenotype and determine the cellular origin of EVs [21]. However, EV characterization is challenging because of the diversity of cellular origin of EVs and the absence of a standard method to identify EV surface markers [21]. Additionally, the overlap in surface markers make it difficult to distinguish between the different types of EVs, for instance, Wubbolts et al. reported CD63 as a membrane protein associated with lysosomes, which is an organelle involved in the formation of exosomes [22]. However, CD63 was also found to be expressed in microvesicles [23].

Exosomes and microvesicles contain diverse types of proteins and lipids molecules. According to the statistics from the ExoCarta, an online database to provide an update on the exosomal

molecular composition, exosomes have 9,769 proteins and 1,116 lipids demonstrating the complexity of their composition [24]. The protein content of exosomes predominantly reflects the cells from which they originate, and because they originate from the fusion of multivesicular endosomes with plasma membrane, the proteins that are involved in the production of multivesicular endosomes such as Alix, tumor susceptibility gene 101 protein (TSG101), ESCRT and major histocompatibility complex class II (MHC class II), are commonly expressed on the exosome surface [25]. In addition, the membrane of exosomes is highly enriched with tetraspanin proteins including CD9, CD63, CD37, CD81 and CD82 [2]. Moreover, heat shock proteins (Hsp70, Hsp90) are reported to express on the surface of exosomes [26]. Similar to exosomes, microvesicles also express tetraspanins, such as CD9, CD63 and CD81, which can be detected by FCM [23]. Since different EV subtypes express several common proteins, this limits the use of these markers to general EV detection rather than specific subclasses of EVs.

Lipidomics studies have shown that EVs are enriched with different types of lipids. Exosomes are reported to contain mainly PS, cholesterol, phosphatidylcholine and sphingomyelin, whereas the lipid component of microvesicles comprises PS, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol [22, 27].

1.4 EV analysis

1.4.1 EV isolation methods

Analysis of circulating EVs requires purification from contaminants to improve the accuracy of downstream analysis, such as EV quantification, characterization, activity, and functions. However, isolating a pure EV sample is challenging because of the complexity of biological fluids and the overlap in the physiochemical and biochemical properties of EVs [28]. There are several methods to isolate EV based on size, mass, density, antigen exposure, and the lack of

a standardized isolation method makes it difficult to compare results between laboratories [29]. The International Society for Extracellular Vesicles (ISEV) has provided considerations and recommendations applicable to all isolation methods to overcome the pitfalls in EV isolation. These recommendations include, selecting isolation methods suitable for the downstream analysis, isolate EV before concentrating them, confirm and compute EVs and non-EV components to assess the efficacy of the isolation method, adequate reporting of the isolation method and its effectiveness [29].

The commonly used method for EV isolation include differential centrifugation, density gradient centrifugation, size exclusion chromatography, ultrafiltration, immuno-capture and precipitation [29]. The principles of these methods, their advantages, and disadvantages are presented in Figure 1.3 and Table 1.1. Differential centrifugation isolates EVs according to their size and density by increasing the centrifugal force to pellet EV at the bottom of the tube [29]. Despite the wide use of this method, it has major limitations, where it co-isolate lipoproteins and produce EV aggregates [29, 30]. The density gradient centrifugation isolates EV based on their density, and maintain the physical properties of the isolated particles, but it is complex, expensive, and time consuming [29-31]. In the ultrafiltration method, EVs are isolated and concentrated through a filter by pressure to remove contaminants. This results in high yield of EVs (80%), however, the use of pressure results in structurally deformed EVs [29]. EVs can also be isolated by precipitation, where a solvent used to precipitate soluble proteins, leaving EVs in the supernatant [32]. Although, it is quick, simple technique and produces high yield of EVs (90 %), the use of solvent affects EV composition and structure [29, 32, 33]. The immune capture method uses magnetic beads coated with antibody that bind to antigen on EV surface. This produces functionally active EVs, but expensive and not suitable for large sample size [34, 35]. In this project, size exclusion chromatography (SEC) has been

used to isolate EVs based on the reported advantages in terms of removing most of contaminating particles, time efficiency, cost, and enrichment of EVs [29, 36].

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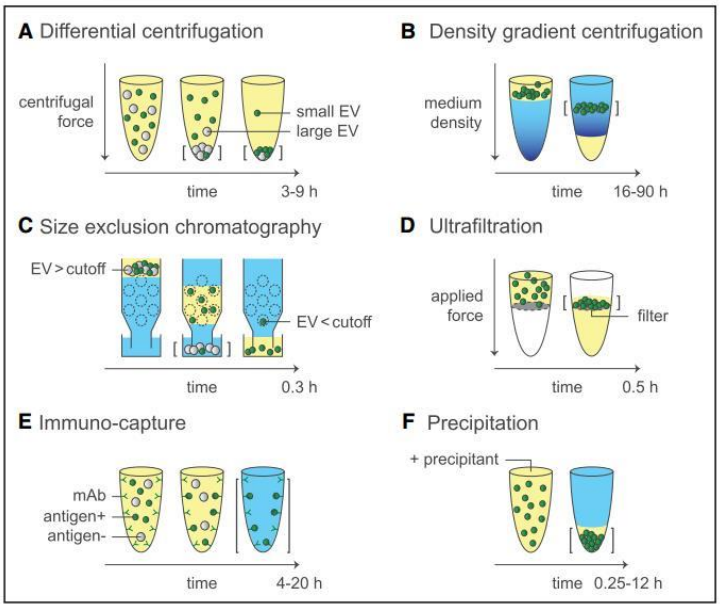

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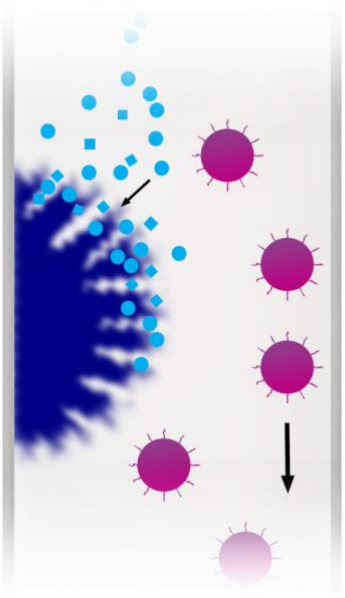

Figure 1.3 Biophysical and biochemical methodologies for EV isolation. (1.A) Differential centrifugation separates particles according to their size, but protein aggregates may co-pellet with EVs. **(1.B)** Density gradient centrifugation uses solutions of fixed density to enable isolation of particles according to their density. **(1.C)** and **(2)** Size exclusion chromatography contains a porous matrix which separates particles according to their size. Particles and soluble components smaller than the size cut off enter the matrix pores temporarily and elute in the earlier fractions, while EVs and particles larger than the size cut off are not trapped by the pore and elute earlier. The size cut-off is determined by the choice of the exclusion matrix (qEV original column separate particles in the range 70-1000 nm). **(1.D)** In ultrafiltration, EVs are separated through a filter in which soluble proteins and particles smaller than the filter pores are pushed through the filter and EVs are retained on the filter (Vivaspin™ 6 ultrafiltration device utilizes a 100,000 molecular weight cut-off). **(1.E)** In immune capture isolation, EVs are isolated based on their immune phenotype by using coated beads with antibody to capture antigens on the EV surface. **(1.F)** In precipitation, EVs are separated by a precipitating agent and then pelleted by centrifugation [29].

Table 1.1 Summary table of the principles, advantages, and disadvantages of the current methods for EVs isolation.

Isolation method	**Isolation principle**	**Advantages**	**Disadvantages**
Differential centrifugation [29, 30]	Separates particles based on size and density by gradually increasing the centrifugal force to pellet the particles at the bottom of the centrifuge tube	• Removes platelets, bulky debris • Precipitates EVs in the pellet. • Concentrates samples 8-fold	• Co-isolation of lipoproteins • EV clumping • Low purity • Low recovery • Time consuming
Density gradient centrifugation [29, 31]	Isolates EVs using density gradient solutions in which particles are isolated solely based on their density	• Prevents mixing of the density gradient • Maintains the physical characteristics of the isolated particles	• Co-isolation of lipoproteins • Time consuming • Expensive
SEC [29, 33, 36]	Enables EV isolation solely according to size.	• High yield of EVs (40-90%) • Removes most of soluble proteins, lipids, and cell debris • Maintains the biological properties of EVs. • Time efficient	• Co-isolation of contaminants above the size cut-off • Ignore exosomes of size <70 nm, separate particles in the range 70-1000 nm
Ultrafiltration [29]	EVs are separated and concentrated through a filter by pressure or centrifugation to remove contaminants.	• Rapid and simple • High yield of EVs (80%)	• Pressure could deform the structure of large EVs • Produces EV particle aggregates
Immune capture [34, 35]	EVs isolated by binding to magnetic beads coated with antibody, which bind to antigen on the surface of EVs	• Easy to perform • Produces functionally active EVs • High sensitivity and specificity	• Expensive • Not suitable for plasma containing platelets
Precipitation [29, 32, 33]	A solvent (acetone) added to plasma to precipitate soluble proteins, leaving EVs in the supernatant.	• High yield of EVs (90 %) • Uses only a small volume of plasma • Time efficient and simple • Isolates highly pure EVs • Inexpensive	• Partially removes soluble proteins • The solvent affects EV composition and structure

1.4.2 Enumeration and phenotyping of EVs

Several techniques have been used for EV enumeration including FCM, resistive pulse sensing (RPS), nanoparticle tracking analysis (NTA) and Transmission electron microscopy [30]. These methods can quantify the total concentration of EVs, and some can determine the physical and biological characteristics of EVs [30]. One of the challenges that hinder EV detection and identification is their small size; where the detection of small particles is based on their light scattering signal, small EVs scatter less light and have a smaller surface for antigen exposure [6, 37]. The concentration of EVs in plasma is reported to range from 10^4 to 10^{12} EVs/ml and this variation is attributed to the lack of sensitivity, differences in resolution and variability associated with the detection techniques [37]. For instance, the detected vesicle concentrations vary according to the technique's minimum detectable vesicle size, where the minimum detectable vesicle size for NTA (NS500) is 70-90 nm, 70-100 nm for resistive pulse sensing, but it is 150–190 nm for dedicated flow cytometry (FCM) and 270-600 nm for conventional FCM [37]. In this project, the upper detection limit for the FCM was set by approximately 1 μm size silica beads (ApogeeMix), while the lower detection limit was set by 240 nm size beads to exclude background noise. This includes all vesicles of size between 240nm-1μm.

This variability in detection threshold hinders the reproducibility and comparability of the results across the laboratories. The ISEV has also published guidelines to optimize EV detection [29] which were followed in this thesis for the flow cytometric analysis of EVs. These guidelines include equipment maintenance, optimizing the instrument to measure EV, determine detection limit and calibrate detectors, use controls to confirm the presence of EV and specificity of labelling and measure sufficient EV to determine distribution.

Flow cytometry is a powerful tool to analyse EV particles where they pass in a single file through a laser beam. EV particles, therefore, scatters light and emits fluorescence signals that

captured by multiple measurement channels [29]. This technique enables the use of fluorescent antibodies that can detect EV subtypes [29]. However, the use of FMC to analyse EV has a drawback where it cannot visualise small EVs due to the setting of detection threshold [38]. The nanoparticles tracking analysis employs both light scattering and Brownian motion to obtain information on size distribution and concentration of the analysed particles, however, NTA cannot determine EV subtypes or composition [39, 40]. In addition, EVs can be detected using transmission electron microscopy which utilises electron beams to illuminate through the samples and transmitted to bright field images that can be used for structure verification. This method can employ the use of immunogold labelling and provide information on EV protein content [39, 41]. However, it is not suitable for the quantitative analysis of EV. The Resistive pulse sensing can also be used for EV detection where it used the biological nanopore as a coulter counter for EV analysis which allows size and concentration measurement but cannot determine the particles type and composition [39, 40]. In this project, two detection methods were combined to analyse EVs, FCM was used to determine the cellular origin of EVs and NTA was used to assess the concentration of EVs and their size distribution including small EVs where it was set to detect particles in the size range 70-1000 nm. Table 1.2 summarise the advantages and disadvantages of EV detection methods.

Table 1.2 Summary table of the principles, advantages, and disadvantages of the current methods for EVs detection.

EV Detection Technique	Principle	Advantages	Disadvantages
Nanoparticle Tracking Analysis (NTA) [39, 40]	Uses dynamic light scattering and Brownian motion to determine the size and concentration of particles	• Easy to use • Provide information on size variation and concentration	• Sensitive to vibration • Require large sample size (> 250 µL) • Cannot determine particle type or composition
Transmission electron microscopy [39, 41]	Utilises electron beams to illuminate through samples	• Enables the use of immunogold-labelling • Can reveal information on EV proteins, disease markers and mechanism associated with plasma EVs	• Not appropriate for quantitative analysis • EV morphology may be damaged by the sample preparing steps (dehydration)
Flow Cytometry [29, 38]	Detect particles through light scattering or fluorescent excitation	• Enables the use of fluorescent ligands, anti-bodies for EV sub-type detection.	• Excluding smaller EVs from the analysis due to lower detection limit
Resistive pulse sensing [39, 40]	Uses the biological nanopore as a Coulter counter for EV analysis	• Enables size and concentration measurement	• Cannot determine the particle type or chemical makeup

1.5 The role of EVs in CVD

According to the World Health Organization, CVD is responsible for the death of 17.9 million people worldwide [42]. The main risk factors include high blood pressure, smoking, high cholesterol, physical inactivity and unhealthy diet [43]. EVs are reported to have pathophysiological effects in CVD and have been suggested to serve as potential novel biomarkers for CVD, which makes cardiovascular health one of the most studied areas in relation to EVs [44]. EVs are reported to be produced from the cells of the cardiovascular system, such as cardiomyocytes, endotheliocytes, fibroblasts, platelets, smooth muscle cells, leucocytes, monocytes and macrophages [45]. Elevated levels of circulating endothelial EVs have been found to be strongly associated with higher levels of TAG [46]. High concentrations of TAG have been shown to be associated with endothelial dysfunction and TAG rich lipoproteins and their remnants, which in turn induces apoptosis in endothelial cells by cytokine production [47]. Endothelial EVs have been suggested to impair the integrity and vasorelaxation of endothelium , resulting in the progression of hypertension [48]. In addition,

endothelial EVs have been suggested to impair the release of nitric oxide (a regulator of artificial stiffness) from vascular endothelial cells and participate in the process of arterial stiffness by increasing the apoptosis of endothelial progenitor cells which might impairer vascular reparation, contribute to the increase of vascular tone and arterial stiffness. [48, 49]. Moreover, *in vitro* study demonstrated that activated endothelial cells with different stressors were reported to produce exosomes with different abundances in protein and RNA composition compared to control exosomes (no stress), which suggests the role of exosomes in relaying stress signals to other cells during cell activation and disease [50]. These stressors were also linked to the development endothelial dysfunction and CVD [51], indicating the possible role of exosomes in mediating these effects. Furthermore, Agouni et al. reported an increase in the levels of procoagulant (annexin V-positive), endothelial, erythrocyte and platelet derived microvesicles in patients with metabolic syndrome compared to healthy subjects [8]. These microvesicles were demonstrated to stimulate endothelial dysfunction *in vitro* via the decrease in nitric oxide synthase activity and increased phosphorylation at its inhibitory site, therefore decrease the production of nitric oxide, which is an important molecule for the regulation of endothelial vasomotor tone. The pathophysiological effects of EVs and their influence on cells have been shown to be dependent on the condition in which they are released [52]. Endothelial EVs exposed to high glucose concentrations *in vitro* were found to have higher NADPH oxidase activity and reactive oxygen species (ROS) compared to control EVs, indication of endothelial inflammation and dysfunction. ROS were found to induce the activation of P38 (a protein released in response to extracellular stimuli) in a ROS-dependent manner, and P38 in turn increases the expression of adhesion proteins involved in endothelial inflammation, such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. Additionally, dendritic cells derived exosomes demonstrated to increase endothelial inflammation and atherosclerosis *in vitro* [53]. This is mediated by tumour necrosis factor

(TNF-α) which is an inflammatory cytokine expressed on exosome membrane. TNF-α in turn activate endothelial cells via nuclear factor-κB (NF-κB) pathway (Figure 1.4).

It is important to recognise that not all of the reported effects of EVs are undesirable; a few studies suggest that EVs have some beneficial roles in cardiovascular health, and in particular, that they can carry factors which contribute to cardioprotection and tissue repair [54-56]. Because of EV cargo content, such as RNAs and proteins, and since EVs can be secreted and specifically taken up by other cells, EVs were suggested to have a role in intercellular signal transfer mechanisms in remote ischemic preconditioning (RIPC) [54]. An *in vitro* study has demonstrated that EV release from the heart increased and the infract size was reduced after ischemia-reperfusion (restriction of blood flow to the heart followed by restoration of blood flow and reoxygenation) compared to control, but EV depletion failed to reduce the infarct size [54, 57]. This suggests the potential role of EVs in the transmission of remote conditioning signals for cardioprotection [54].

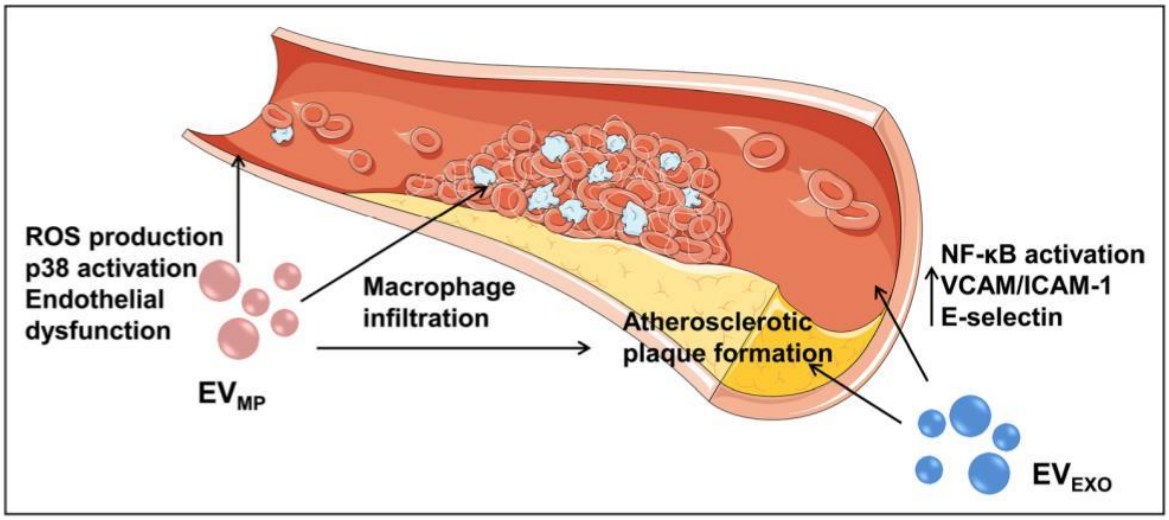

Figure 1.4 EVs contribute to the development of atherosclerotic plaque. Microvesicles from smooth muscle cells (pink) stimulate endothelial dysfunction and macrophage infiltration in the vessel wall through the reactive oxygen species production in endothelial cells. Exosomes derived from dendritic cells (blue) increase endothelial inflammation by activation of nuclear factor- κB (NF-κB) pathway and increasing expression of proinflammatory molecules [58].

1.6 EVs and coagulation

EV exposes PS and tissue factors (TFs), which are important factors involved in initiating coagulation associated with EVs [59]. In normal cells, the plasma membrane has asymmetrical distribution of lipids, where PS presents in the inner membranes [60]. During the process of EV formation, the asymmetry of cell membrane is disturbed, causing PS to flip to the outer membrane of microvesicles, triggering procoagulant activity and stimulating the assembly of the clotting cascade [61]. However, it has been reported that not all EV population exposes PS as measured by annexin-V binding (a protein with high affinity to PS) [62]. This indicate that not all EV particles involved in the coagulation process and suggest that EV negative for PS, might play distinct roles other than haemostasis.

TFs are one of the key triggers of coagulation cascade and they are highly expressed on EV [61, 63]. TFs have high affinity to bind and activate FVII, a factor important in initiating coagulation and therefore, TF-positive EVs bind to FVII and participate in the coagulation cascade [61, 63]. Additionally, the presence of protease enzymes, such as metalloproteinases and urokinase plasminogen activator (uPA) on the EV surface suggest their role in the fibrinolytic system [64]. EVs also express urokinase-type plasminogen activator receptor (uPAR), which is important for plasminogen binding [64]. Plasminogen binds to EVs through its receptor and activated to plasmin, is an important enzyme for blood clot degradation [64, 65].

1.7 Dietary fats and CVD

Dietary fats consist mainly of TAG composed of three fatty acids attached to a glycerol backbone[66]. They can be classified into saturated fatty acids (SFA), and unsaturated fatty acids which include monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) [67]. Saturated fats contain single bonds between individual carbon atoms and are solid at room temperature [66]. On the other hand, unsaturated fats contain one or more double

bond and are liquid at room temperature. It is well established that SFA increase low-density lipoprotein (LDL) cholesterol, a strong risk factor for CVD, while unsaturated fats such as omega-3 and 6 are reported to exert protective effects on CVD by lowering TAG and LDL levels in the circulation [67, 68].

In the food industry, the use of unsaturated and saturated fatty acids is dictated by their chemical composition and physical properties; for instance, unsaturated fats are susceptible to decomposition, rancidity, and unpleasant odour when exposed to air, while SFA are solid at room temperature and not suitable for many applications [69]. To widen the use of these fats, they can be partially hydrogenated to increases their melting point, shelf life and stability [70]. However, during the process of partial hydrogenation, some of the double bonds are rearranged, which alters cis/trans configurations, thereby producing trans fatty acids which are known to be detrimental to health, particularly cardiovascular health [69, 71]. Effort to replace industrially produced trans fatty acids with healthier options has led to the increasing use of IE fatty acids, which are solid fats formulated by the rearrangement of fatty acids within the triacylglycerol [72]. The fatty acids molecules can be interesterified chemically or enzymatically. In chemical interesterification, sodium methoxide used as a catalyst to hydrolyse and randomly redistribute of all fatty acids within a TAG mixture. Enzymatic interesterification uses lipase enzymes and gives either a random or specific redistribution of fatty acids, depending on the specificity of the lipases used [72]. IE fats are now used in food industry to improve the texture and stability of food products, and to replace trans fats in many foods application such as fat spreads, bakery, and confectionery products [73]. Fat interesterification reduces the content of saturated and trans fatty acids in foods, but the impact of industrially relevant IE fats on cardiovascular health is unknown [72]. The postprandial response to IE fat has been investigated in relation to CVD risk markers, but the results have been inconsistent. Non-IE palm oil has been shown to increase plasma TAG when compared

to IE palm oil, but another study reported no difference between native and IE shea butter [74-76]. However, the studies on IE fats and CVD risk markers have used IE fats that are not commonly used for food formulation, which make it difficult to generate a public health advice on IE. There is also limited data on the effects of IE fats on other markers for CVDs.

Omega-3 fatty acids are long chain polyunsaturated fatty acids consisting of more than two double bonds after the third carbon from the methyl end [77]. There are three n-3 fatty acids that have been shown to be important to human health: alpha-linolenic acid (ALA), EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). ALA is the major type of n-3 fatty acid in the diet, is an essential fatty acid, which cannot be synthesised in the body [77]. ALA is the shortest n-3 PUFA, consisting of 18 carbons and found in green plant-based foods. EPA and DHA consist of 20 and 22 carbons respectively and can be consumed from fish and fish oil [78]. ALA can theoretically be converted into the body to synthesize EPA and DHA; however, the converted amount is extremely limited, making these PUFA conditionally essential. According to the Scientific Advisory Committee on Nutrition, it is recommended to consume two portions of fish, including one portion of oily fish, and meeting these recommendations will provide 450 mg of EPA/DHA per day [79].

The potential beneficial effects of fish consumption and CVD first emerged when a study reported low mortality from ischaemic heart disease (IHD) in Greenland, which was suggested to be due to high intake of oily fish [80]. There is a strong evidence from epidemiological, case-control and cohort studies that the consumption of fish, or EPA and DHA is inversely associated with MI, sudden death, sudden cardiac death, CHD and stroke mortality [81-85]. A recent prospective cohort study with 16 years follow up reported that intake of fish and n-3 PUFAs decreased CVD mortality by 10% in men and women [86]. More recent meta-analysis of 13 randomised controlled trials (RCTs) suggests that marine omega-3 supplementation is

associated with lower risk of MI, total CHD, total CVD, and death from CHD or CVD causes [87].

There are a number of suggested mechanisms for modulation of CVD risk by n-3 PUFAs, including EPA and DHA. A meta-analysis of randomised controlled trials reported that oral supplementation with EPA and DHA exerts cardioprotective effects by significantly decreasing blood TAG, heart rate, platelet aggregation, pro-inflammatory cytokines, and systolic and diastolic blood pressure [68, 88, 89]. In addition, omega-3 fatty acids are reported to decrease the production of inflammatory prostaglandins, resulting in a greater decrease in inflammation. This occur by reducing cyclooxygenase, which is an enzyme responsible for formation of prostaglandins [90].

Despite the fact that many meta-analyses and cohort studies have concluded that there is a positive association between n-3 PUFA and cardiovascular health, evidence for primary prevention of CVD through RCTs is limited. In a primary prevention randomised trial, 15,480 patients with diabetes and without evidence of atherosclerotic CVD received either marine n-3 PUFA (840 mg/d EPA + DHA) or olive oil placebo [91]. The primary outcome was the first serious vascular event and after a mean follow-up of 7.4 years, there was no significant difference in the risk of serious vascular events between those who were assigned to receive n−3 PUFA and those who were assigned to receive the placebo. In another primary prevention study investigating the effects of supplementation with vitamin D3 (at a dose of 2000 IU per day) and fish-oil capsules (containing 840 mg/d of EPA+DHA), after a median follow-up of 5.3 years, supplementation with n−3 PUFA had not resulted in a lower incidence of major cardiovascular events compared with the placebo [92].

A number of randomized, controlled, secondary prevention trials have been conducted to explore the effect of EPA and DHA in patients with existing CVD. A randomised, double-blind, placebo-controlled trial explored the effects of 840 mg/d EPA+DHA in patients with chronic heart failure [93], showing a decrease in all-cause mortality and a reduction in admission to hospital for CVD events following supplementation with n-3 PUFA. A meta-analysis of 11 RCTs investigated the effects of n-3 PUFA with a dose range of 0.3–6.0 g/d EPA and 0.6–3.7 g/d DHA in patients with CHD and reported 30% reduction in fatal MI, 30% reduction in sudden death and 20% reduction in overall mortality [94]. In contrast, three trials investigated the effects of n-3 PUFA supplementation on high-risk CVD patients and reported no beneficial effects [95-97], and that inconsistency in results could be related to the short duration of the trial or the low doses of EPA+DHA in these studies [98].

Despite the inconsistency, there are well-documented biological effects of n-3 PUFA on CVD-relevant parameters, as evidenced by their modulation of CVD risk factors and risk markers. Therefore, it remains possible that there are biological effects of n-3 PUFA which contribute to protective effects in CVD, but that intervention studies have so far been inadequately powered to demonstrate these protective effects.

A few studies have evaluated the effects of diet and lifestyle on EVs and demonstrated a decrease in the total number of EVs after intervention with fish oil capsules, a low fat diet, flavonols and weight reduction [63, 99-101]. Wu et al. investigated the influence of 8 weeks supplementation with fish oil (900 mg EPA plus 600 mg DHA) on the numbers of circulating endothelial- and platelet-derived EVs in participants with moderate risk of CVD [99]. The study demonstrated a significant decrease in numbers of endothelial EVs, but no effect on platelet EVs. In healthy individuals, Phang et al. explored the impact of EPA (1000 mg) and DHA (1000 mg) on numbers of platelet EVs 24 hours after supplementation [102].

Interestingly, the study reported gender-dependent effects in which EPA reduced platelet aggregation in men and DHA decreased platelet aggregation in women, but there were no effects on platelet EVs. Another study by Turco et al. evaluated the effects of an acute dose of fish oil (4300 mg of EPA and DHA) on EVs in patients with myocardial infarction [103]. Fish oil decreased numbers of platelet- and monocyte-derived EVs, but did not alter numbers of endothelial EVs. The variation in the results of the above studies could be attributed to the doses provided, duration of consumption or other variation in methodology.

There are no published data on the effects of IE fats on EVs, but the postprandial impact of high fat meals on EV number has been explored. EV number increases postprandially in response to a high fat meal, suggesting a possible relationship between dietary fat, the vesiculation process and EV generation [104, 105]. The levels of circulating EVs were reported to be positively associated with the plasma lipids, particularly, TAG and cholesterol [99]. TAG is suggested to react with platelet endogenous activator, leading to platelet stimulation and impairing the action of prostaglandin (inhibitor of platelet activation) [106, 107], suggesting this stimulation might induce activated platelets to release EVs. In addition, the postprandial increase in EV number could be attributed to the positive correlation between EVs and oxidized low-density lipoprotein (LDL), which is reported to react with platelets through surface receptors, causing their activation and potentially EV production [108, 109]. Postprandial lipemia has been associated with endothelial dysfunction and cell activation [110, 111], and the positive association between EVs and blood lipids could indicate a role of blood lipids in cell activation, membrane blebbing and EV release. In addition, replacing dietary SFAs with MUFAs or n–6 PUFAs has been found to decrease the number of endothelial microparticles in a population at moderate risk of CVD [112]. Overall, these findings suggest the importance of dietary fats in modulating this emerging risk marker for CVD.

1.8 Aims of this thesis

This thesis is comprised of five chapters. Chapter one is a literature review on EVs and their association with CVD, and the impact of dietary fatty acids on EVs as an emerging CVD risk marker. Chapter 2 investigates the extent to which lipoproteins interfere with EV isolation, enumeration and functional analysis, comparing samples taken during fasting and after a high fat meal. The results of this work directly informed Chapter 3, which investigated the acute (postprandial) effects of commercially available IE fats on EV number and procoagulant activity. Chapter 4 examined the chronic effects of fish oil supplements and oily fish meals on EV number, phenotype, composition and procoagulant activity in healthy human volunteers. Each of the three results chapters is presented as a manuscript ready for submission. Chapter 5 is consisted of general discussion, future perspective and conclusion.

Brief communication

Chapter II: EVs isolated following a high-fat meal are not contaminated with lipoproteins

Manuscript prepared for submission to Current Developments in Nutrition (CDN)

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2.1 Abstract

Background:

Extracellular vesicles (EVs) are small membrane-bound vesicles shed from cells upon activation, damage, or death, and are linked to several pathological and physiological conditions. There is growing interest in EVs due to their potential role in disease diagnosis. However, EV analysis is hindered by possible contamination with lipoproteins due to size overlap.

Objective:

The aim of this study was to investigate the extent to which EVs isolated after a high-fat meal were contaminated with lipoproteins, comparing samples taken during the fasting and postprandial state. We hypothesized that EVs can be isolated and analysed with minimal contamination and interference from lipoproteins.

Methods:

Twelve healthy volunteers consumed a high fat test meal. Blood and PFP samples were collected at baseline and 4h. EVs were enumerated using nanoparticle tracking analysis (NTA) and EVs isolated by size exclusion chromatography were investigated for possible contamination with lipoproteins by Apo-B48 and Apo-B100 ELISA applied to EV fractions. Lipoprotein fractions were prepared by density gradient centrifugation, stained with antibodies for EV-related markers and analysed by flow cytometry. They were also subjected to size exclusion chromatography and fractions corresponding to the size range of EVs were analysed.

Results:

EVs isolated by size exclusion chromatography were not contaminated with apoB48 or apoB100. EVs were not detectable in lipoprotein fractions prepared from fasted and postprandial plasma and when the lipoprotein fractions were subjected to size exclusion chromatography, lipoproteins did not co-elute with EVs.

Conclusion:

EVs can be isolated by size exclusion chromatography from plasma after a high-fat meal without significant contamination by lipoproteins.

Keywords:

Chylomicron, extracellular vesicles, lipoprotein, size exclusion chromatography, VLDL

2.2 Introduction

Extracellular vesicles are membrane-bound vesicles derived from cells and classified into three types, exosomes, microvesicles and apoptotic bodies, depending on their size, biogenesis and method of cellular release [28]. There is growing interest in EVs due to their potential role in normal physiological processes, such as tissue repair, immune surveillance and blood coagulation [113]. Circulating EVs may also serve as biomarkers for disease, since they are elevated in CVD, cancer and diabetes [114-116].

EVs are isolated from body fluids using a variety of methods, including differential centrifugation, density gradient ultracentrifugation, size exclusion chromatography (SEC), and precipitation [117-120]. EV isolation methods differ in yield, cost and labour intensity [121]. Ultracentrifugation results in a low yield of EVs, generates protein aggregates and causes physical damage to EVs [122, 123]. SEC, on the other hand, removes most soluble plasma proteins and maintains EV structure and composition [120]. Additionally, SEC produces higher yields compared to other isolation methods and enables removal of >99% of potentially contaminating free proteins [124, 125].

In the postprandial state, the concentration of blood lipoproteins increases substantially, particularly after high fat meal [126]. Given that postprandial lipaemia is associated with an acute inflammatory response [127], it is of interest to examine whether postprandial lipaemia alters the numbers of circulating EVs. However, examining EVs during the postprandial period presents challenges, since the appearance of large chylomicrons in the circulation, whose size overlaps with that of small EVs, could impair reliability and accuracy. The current study therefore investigates whether a high fat meal leads to lipoproteins interfering with analysis of EV number and activity.

2.3 Materials and methods

2.3.1 Subjects and study design

Twelve male and female subjects (females=10, males=2) (aged 22-37y) were recruited from the University of Reading. All subjects were given informed consent. Ethical approval for the study was obtained from the SCFP Ethics Committee at the University of Reading (Study number 17/17) and conducted according to the guidelines laid down in the Declaration of Helsinki. Exclusion criteria included the following: the presence of illness or prescribed medication. Participants were also asked to avoid exercise, alcohol, and fatty meals the day before the study visit and were asked to consume a low-fat meal the evening before the study visit.

The inclusion criteria comprised the following:

- Age range 18-65 y
- Non smoker
- Haemoglobin ≥115 g/l for women and 130 g/l for men
- Total cholesterol < 5 mmol/l
- TAG 0.4-1.5 mmol/l
- No disclosed history of drug or alcohol abuse
- No illness or disease requiring medication (excluding HRT, oral contraceptive, and thyroxine replacement therapy)

Participants attended the Hugh Sinclair Unit of Human Nutrition following a 12 hr overnight fast and a baseline fasting blood sample was taken before consuming a high fat test meal consisting of two all-butter croissants containing 24 g fat, 10.6 g protein and 48 g carbohydrate (composition in Supplemental Table 2.1). A second blood sample was collected 4 hr later, corresponding to the point at which postprandial lipaemia reaches a peak [128, 129].

2.3.2 Sample collection and preparation of platelet free plasma (PFP)

Venous blood samples were drawn into citrated tubes, inverted 4 times, and processed immediately. Citrated tubes were used because citrate binds to free calcium, thereby preventing the degranulation of platelets and leukocytes and the associated vesiculation process [130]. Samples were centrifuged at 1500 x g at room temperature for 15 min to remove larger cells and cellular debris. Further centrifugation at 13000 x g for 2 min at room temperature produced PFP, which was aliquoted and stored at -80^{o}C for further analysis.

2.3.3 Isolation of EVs using SEC

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

The SEC column contains a gel with pores of 70 nm and the principle of this method is to enable particles isolation solely according to their size. The technique works by distributing a mixture of particles into two phases: the stationary phase and mobile phase. In the stationary phase, proteins and other sample components smaller than 70 nm are trapped by the pores delaying their passage down the column so they elute in the later fractions, while in the mobile phase, particles larger than the pore (>70 nm) flow through the column quickly and therefore appear in the earlier fractions (fractions 7-9) [124].

2.3.4 Lipoprotein isolation

Density gradient centrifugation was performed on baseline and postprandial PFP to isolate chylomicrons (100-2000 nm) [131], VLDL-1 (50-80 nm) and VLDL-2 (30-50 nm) [132]. The interior surface of the centrifuge tubes was coated with poly vinyl alcohol (PVA) solution to ensure the smooth flow of the density gradient solutions down the interior side of the tubes. Double distilled water ddH_2O (250 ml) heated to 80-90 °C. PVA powder (10g) was added to the water in the presence of anti-bumping beads for safe boiling. Propan-2-ol (250 ml) was added to the solution until a clear solution is obtained. Once the solution cooled down at room temperature, the centrifuge tubes were filled to the brim with the PVA solution, left for 15 minutes and then the solution poured back into the original flask. Any residues of the solution were removed from the tubes, invert and left to dry overnight. The dry tubes filled with ddH_2O and allowed to stand overnight and then the tubes were flushed with water and left to dry at room temperature.

To avoid the cleavage of lipoproteins, a preservative solution was added to the isolated lipoprotein fractions. The preservative solution was prepared by adding EDTA (2g) into 40 ml of ddH_2O and the pH was adjusted to 7.4 – 8.0. The following chemicals were added in the following order: Sodium azide (100 mg), Aprotinin (4 TIU/mg), Chloramphenicol (80 mg), Benzamidine hydrochloride hydrate (160 mg) Gentamicin sulphate (80 mg). Water (10 ml) was added to the solution and left to mix for 30 – 45 minutes.

The density of baseline and postprandial PFP was adjusted to 1.10 g/ml with sodium bromide and transferred to the bottom of the centrifuge tube. The following density gradient sodium chloride solutions were slowly layered over the sample: 3.0 ml of density = 1.063 kg/l NaCl, 3 ml of density = 1.020 kg/l NaCl, and finally, 2.8 ml of density = 1.006 kg/l NaCl. Three

ultracentrifugation steps were carried out in a Beckman L90K ultracentrifuge; 1) chylomicrons; 40,000 rpm, 15°C for 32 min, 2) VLDL-1; 40,000 rpm, 15°C for 3 h 28 min 3) VLDL-2; 40,000 rpm, 15°C for 16 h. After ultracentrifugation, the top 1 ml of each tube was collected and 50 µl of apoB48 preservative were added (5% v/v) and the fractions were stored at -20°C for later analysis.

2.3.5 Nanoparticle tracking analysis of EVs and lipoprotein classes

The concentration and size distribution of EVs and lipoprotein classes were assessed using Nanoparticle Tracking Analysis (NTA) (NanoSight 300; Malvern, Amesbury, UK), equipped with a 488 nm laser and a high sensitivity CMOS camera. EVs and lipoproteins were diluted with 0.1µm filtered PBS and adjusted to a range of $1x10^8 – 9x10^8$ particles per ml. NTA utilizes the properties of both light scattering and Brownian motion to obtain the size distribution and concentration of particles in liquid suspension. The software tracks particles individually and using the Stokes-Einstein equation calculates their hydrodynamic diameters. The measurements of particle concentration and size were based on the average of five videos, each recorded for 60 s. For EV analysis, the threshold was set at 71 nm to avoid the interference of small lipoproteins, such as HDL, LDL and VLDL.

2.3.6 Flow cytometric analysis of EVs and lipoproteins

Reference beads were used to set live gates based on particle size. These consisted of a mixture of non-fluorescent silica ApogeeMix beads (Apogee flow systems, Hemel Hempstead, UK) with diameters of 180 nm, 240 nm, 300 nm, 590 nm, 880 nm and 1300 nm (with a refractive index of η=1.43, similar to the refractive index of EVs η~1.39) and green fluorescent latex beads with diameters of 110 nm and 500 nm (with refractive index η=1.59). A BD FACSCanto II flow cytometer (BD Biosciences, Oxford UK) was used with a detection threshold set at 200

nm to exclude instrument noise. Because of the unavailability of 1 μm silica beads, the gate was set just above 880 nm silica beads to exclude particles > 1μm. The live gate P1 therefore represents EVs, defined as particles within the size range 240 – 1000 nm, with P3 representing platelets (Supplemental Figure 2.1).

Due to the size overlap between EVs and lipoproteins, particularly when chylomicrons appear in the circulation during the postprandial period, it was considered important to test the specificity of EV markers with respect to flow cytometric labelling of EVs. Three lipoprotein classes (chylomicrons, VLDL-1 and VLDL-2) were isolated as described and three EV markers; Annexin APC (phosphatidylserine-positive EVs), CD41 PE (platelet-derived EVs) and CD105 eFlur450 (endothelial EVs) were used to stain samples of lipoproteins and thawed PFP (for EVs). All markers, reagents and buffers were filtered using 0.1 μm pore size centrifugal filter unit (Millipore UK Limited, Hertfordshire, UK) for 20 min at low speed (0.1xg) to remove aggregates and contaminants. All antibodies were also titrated to determine the optimum concentration for EV staining.

PFP or lipoprotein samples (5 μl) were incubated for 15 min with the following:

- FcR blocking reagent (5 μl) (Miltenyi Biotec Ltd, Surrey, UK).
- Annexin V buffer (Cambridge Bioscience Ltd, Cambridge, UK) containing calcium, which is important for Annexin V binding to PS exposed on the EV surface.
- Argatroban (1 μl) (a synthetic direct thrombin inhibitor), which was used as an anticoagulant to stop plasma clotting caused by the calcium in the Annexin V buffer).

After the first incubation, PFP and lipoprotein samples were labelled with a panel of antibodies and isotype-matched controls for 15 min at room temprature in the dark. Following incubation, samples were diluted with 200 μl filtered Annexin V buffer and analysed by flow cytometry. A summary of the sample tubes analysed by FCM is shown in Supplemental Table 2.2

BD TruCount tubes (BD Biosciences) were used to obtain absolute counts of stained particles in the original sample. Each TruCount tube contains 47,000 beads, which were resuspended in 500 µl of filtered PBS and analysed by FCM for calibration of particle numbers.

2.3.7 Assessment of contamination of EV fractions with apoB48 and apoB100

To determine whether EV fractions prepared from PFP by SEC were contaminated with chylomicrons or VLDL, EV fractions prepared from both baseline and postprandial samples of PFP were subjected to ELISA for ApoB48 (CM) and ApoB100 (VLDL).

The presence of CM was evaluated using the Human Apolipoprotein B48 ELISA kit purchased from ElabScience (Houston, USA) and the presence of VLDL was investigated using the human apolipoprotein B100 ELISA kit. (SIGMA-Aldrich, Saint Louis, USA). The ELISA tests were performed according to the manufacturer's instructions and read on a plate reader (Spark, Tecan, UK).

2.3.8 Assessment of the potential co-isolation of lipoproteins prepared by density gradient centrifugation with EVs

Density gradient centrifugation was performed as described on baseline and postprandial PFP samples to isolate CM, VLDL-1 and VLDL-2. These lipoprotein fractions were then subjected to SEC to determine whether lipoproteins eluted in the same fractions as expected for EVs. Fractions were analysed using NTA (Supplemental Figure 2.2).

2.3.9 Statistical analysis

The results are presented as means ± SEM. Differences were determined using paired t-tests. P-values <0.05 were considered statistically significant. Statistical analyses were performed using IBM SPSS statistics 27.

2.4 Results

2.4.1 Subject characteristics

A total of 12 subjects (females=10, males=2) (aged 22-37y). The clinical and laboratory characteristics of the subjects are shown in Supplemental table 2.4.

2.4.2 Numbers of EVs and lipoprotein particles increase after a high fat meal

The concentration of EVs, CM, VLDL-1 and VLDL-2 increased significantly postprandially (p-value < 0.001). In addition, the concentration of lipoprotein classes collectively outnumbered the concentration of EVs at baseline and in the postprandial state (p-value < 0.001) (Supplemental table 2.3).

The mean size of particles increased postprandially, EV (p-value = 0.014), VLDL-1 and VLDL-2 (p-value < 0.001), but no effects on the mean size of CM (p-value = 0.05). The mean size of EVs was greater than that of lipoproteins (Supplemental table 2.3).

2.4.3 Lipoprotein fractions do not contain significant numbers of EVs

Lipoprotein fractions (CM, VLDL1 and VLDL2), prepared by density gradient centrifugation and stained to visualise annexin V positive EVs, platelet-derived EVs and endothelial derived EVs, contained only minimal EVs (Figure 2.1).

2.4.4 ApoB48 and apoB100 do not co-elute with EVs

When PFP was subjected to SEC, EVs eluted in fractions 7-9. Figure 2.2 (A, B) shows that CM (apoB48) and VLDLs (apoB100) in PFP samples only began to elute from fraction 11 onwards, demonstrating that lipoproteins eluted later than EVs, with little cross-contamination. This is consistent with lipoproteins having a smaller diameter than EVs.

2.4.5 Lipoproteins eluted after EV enriched fractions

Lipoproteins isolated by density gradient centrifugation and subjected to SEC did not elute in EV enriched fractions (fractions 7,8,9) and began to elute in the later fractions (Figure 2.2, C, D, E).

2.5 Discussion:

Separation of EVs from non-EV components, such as soluble proteins, lipoproteins and protein aggregates [133], is essential for any downstream analysis [134]. The current paper demonstrates that concentration and size of EVs, CM, VLDL-1 and VLDL-2 were increased significantly postprandially. Lipoprotein fractions (CM, VLDL-1 and VLDL-2), prepared by density gradient centrifugation and stained to visualise annexin V-positive, platelet-derived and endothelial-derived EVs contained only minimal EVs. When CM (apoB48) and VLDL (apoB100) were applied to SEC-EVs, they only began to elute from fraction 11, demonstrating that lipoproteins eluted later than EVs, with little cross-contamination. In addition, lipoproteins isolated by density gradient centrifugation and subjected to SEC did not elute in EV enriched fractions (fractions 7,8,9) and begun to elute in the later fractions. These findings suggest that SEC is efficient in purifying EV and separating EVs from contaminants such as lipoproteins [131].

Due to size overlap between EV and lipoprotein classes, there is a potential interference of lipoproteins with EV analysis, particularly in postprandial studies [134]. In this paper, lipoprotein fractions (CM, VLDL-1 and VLDL-2), prepared by density gradient centrifugation and stained with EV markers to visualise annexin V-positive, platelet-derived and endothelial-derived EVs contained only minimal EVs. Similarly, Sodat et al, reported that LDL stained for apoB and apoCII, but not for the EV markers, CD9 and CD63 [135]. In the current study, a threshold of refractive index = 1.42 was used to discriminate between EV and lipoprotein populations. This excluded the detection of lipoprotein particles and its contents (lipids and proteins) which have a refractive index of > 1.42, while EVs have a refractive index equal to 1.39 [136]. This suggests that the refractive index setting might help to minimise interference from lipoproteins. In addition, the minimum detection threshold for the FCM was set at 240 nm to avoid background and instrument noise. Consequently, this will exclude any labelling for lipoprotein particles smaller than 240 nm in diameter, which includes the majority.

SEC is reported to effectively remove most high-abundant proteins, such as albumin; however, it may not completely remove particles whose size overlaps with small EVs, such as chylomicron and VLDLs [137]. According to the qEV manufacturer, EVs are predominately eluted in fractions 7,8 and 9, which contain minimum interference with plasma proteins [36]. Although some EVs will elute later than fraction 9 [138], the majority elute within fractions 7-9, and only these fractions were pooled for EV analysis to limit the levels of contaminating proteins [139]. In the current paper, ApoB48 and ApoB100 applied to fractioned PFP have shown that CM and VLDL eluted from fraction 11 later than EV enriched fractions (7-9), and SEC appeared to be more efficient in removing VLDL from EV fractions, since it eluted in the fractions beyond EVs and CM. Similarly, Zhang et al., reported that SEC was effective in removing the majority of apoA1 (HDL marker) and apoB100 from EV fractions [134]. The

efficiency of SEC in removing lipoproteins from EV enriched fractions was also confirmed by isolating lipoproteins by density gradient centrifugation and subjecting them to SEC, where they eluted in the fractions beyond 7-9, and therefore after the majority of EVs. In addition, Other lipoproteins (HDL and LDL) and albumin smaller than 70 nm in size were not investigated. Theoretically, these particles are too small to appear in the earlier fractions and they will be retained by the beads in the column, which means that they would elute much later than EVs and the larger lipoproteins.

In conclusion, EVs can be isolated by size exclusion chromatography from plasma after a high-fat meal without significant contamination by lipoproteins.

2.6 Acknowledgement:

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Author contribution:

Amal Alanzeei performed the experiments and wrote the manuscript

Parveen Yaqoob supervised the work and reviewed the manuscript.

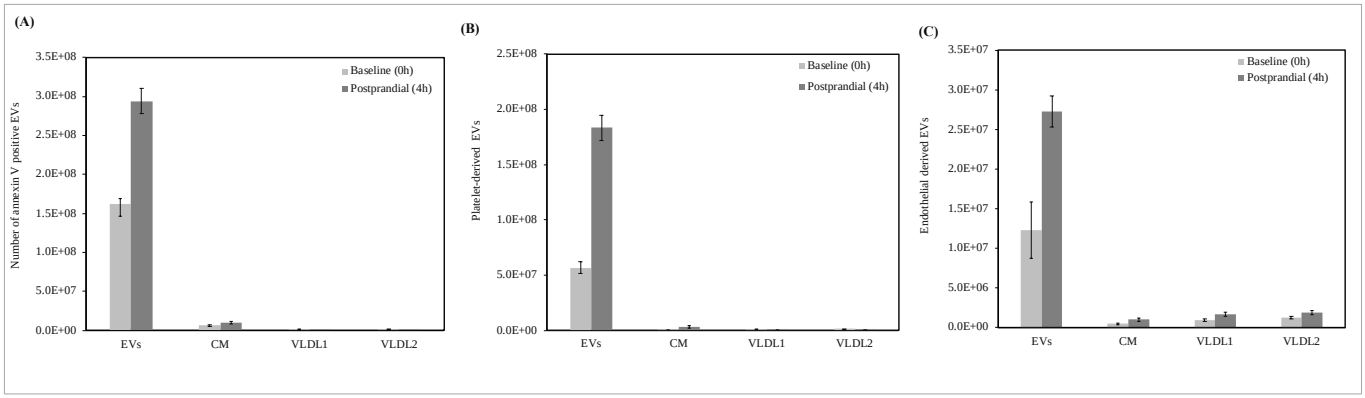


Figure 2.1 Flow cytometric analysis of EV markers (n=12). Samples of PFP were stained with antibodies against EV surface markers, including annexin V (A), CD41(B), and CD105 (C). Lipoprotein fractions were largely devoid of EV markers. Data are means ± SEM.

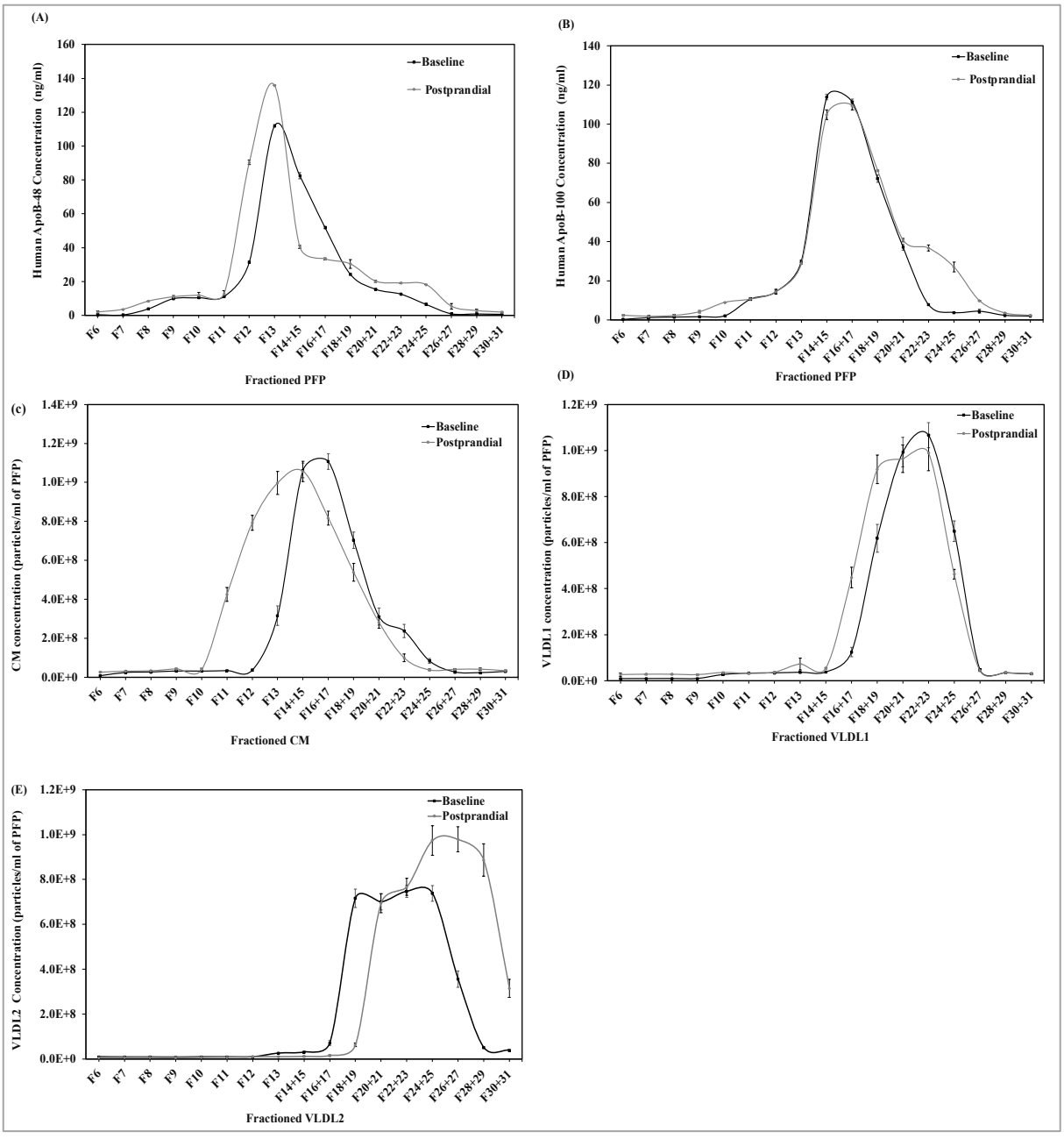


Figure 2.2 (A,B) ApoB-48 and ApoB-100 Elisa of SEC EV fractions (n=2) . CM **(A)** and VLDL **(B)** in PFP samples only began to elute from fraction 11 onwards, indicating that lipoproteins appear later than EVs (fractions 7,8,9), with little cross-contamination. **(C,D,E) Lipoprotein particle numbers in fractions following SEC as assessed by NTA (n=12).** Lipoproteins were isolated sequentially by density gradient centrifugation and subjected to size exclusion chromatography. They did not elute in EV-enriched fractions (7-9), only beginning to elute in the later fractions. Data are means ± SEM.

Supplementary material

Supplemental Table 2.1 Fatty acid composition of all butter croissants

Typical values	Per 100g	Per croissant	Per 2 croissants
Energy (kcal)	405	224	448
Fat (g)	21	12	24
of which saturates (g)	15	8.5	17
Carbohydrate (g)	43	24	48
of which sugars (g)	4.9	2.7	5.4
Protein (g)	9.5	5.3	10.6

Supplemental Table 2.2 Antibody panel for EV and lipoprotein classes labelling using flow cytometry.

Tubes	PFP/lipoprotein classes (µl)	FcR (µl)	AVB (µl)	AV (µl)	CD41-(PE(µl)	IgG1- PE(µl)	CD105-eF450(µl)	IgG1- eF450(µl)
1. Annexin V buffer alone	-	5	45	-	-	-	-	-
2. PFP alone	5	5	40	-	-	-	-	-
3. CD41-PEFMO	5	5	32	2	-	2	4	-
4. CD105-eFluor450 FMO	5	5	35	2	2	-	-	1
5. Key sample tube	5	5	32	2	2	-	4	-
6. AV FMO	5	5	32 (PBS)	2	2	-	4	-

Tube 1 contained AV buffer (a rich calcium buffer to help in AV binding) + FcR blocking reagent only, to assess the level of background contaminating events. Tube 2 contained PFP/lipoprotein classes only and was used to adjust the flow rate (typically 250-500 events/sec). Tube 3 was a CD41-PE fluorescence minus one (FMO) control. Tube 4 was a CD105-eFluor450 FMO control. Tube 5 represented the main tube which had multicolor labelling for EVs. Tube 6 was the AV-APC FMO control. Fluorochromes: Annexin (APC), CD41 (PE), CD105 (eFlur450), IgG1 (PE), IgG1 (eFlur450). Tube 5 represents the main tube which has the multicolor labelled EVs/lipoprotein classes sample. Tube 6 is AV-APC FMO control, a calcium free buffer was used (PBS) which will prevent AV binding. AV, Annexin V; AVB, Annexin V buffer; FCR, blocking reagent; FMO, fluorescence minus one.

Supplemental Table 2.3 Concentration and diameter of SEC-EVs and lipoprotein classes (n=12).

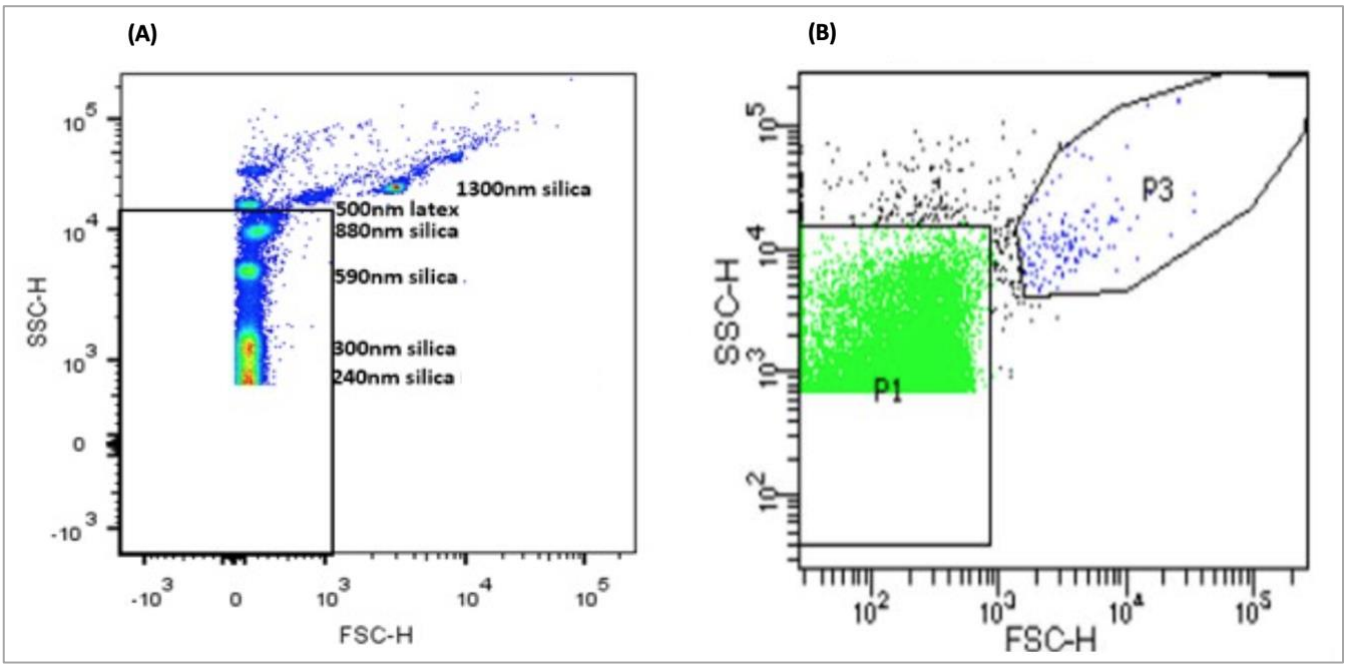
	Concentration (Particles/ml of PFP)		
	Baseline	Postprandial	P-value (paired t-tests)
EVs	5.45E+10	8.04E+10	**< 0.001**
CM	1.94E+11	4.21E+11	**< 0.001**
VLDL1	1.02E+12	1.35E+12	**< 0.001**
VLDL2	1.33E+12	1.61E+12	**< 0.001**
	Size (nm)		
EVs	135.11	140.6	**0.014**
CM	110.62	121.39	0.05
VLDL1	105.83	117.05	**< 0.001**
VLDL2	89.41	101.77	**< 0.001**

EVs: extracellular vesicles, CM, chylomicrons, VLDL-1: very low-density lipoprotein 1, VLDL-2: very low-density lipoprotein 2.

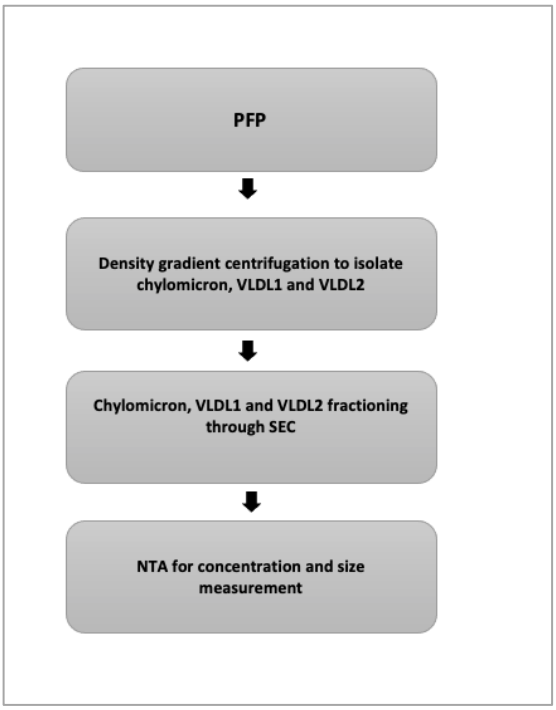
Supplemental Table 2.4 Baseline characteristics of the participants (n=12)

Subject characteristics	Mean±SE
Age (years)	30.0±1.33
Height (m)	1.63±0.015
Weight (kg)	66.5±3.19
BMI (kg/m^2)	24.70±0.96
TAG (mmol/L)	0.66±0.03
CHOL (mmol/L)	3.90±0.08
HDL (mmol/L)	1.79±0.019
Glucose (mmol/L)	4.97±0.12

Results are means ± SEM Abbreviations: BMI, body mass index; TAG, triglycerides; CHL, cholesterol; HDL, high density lipoprotein.



Supplemental Figure 2.1 Setting a size gate for EV detection. (a) ApogeeMix beads were analysed on forward scatter (FSC) and side scatter (SSC) by FACSCanto II to set a size gate from 240 to 1000 nm for EVs detection; **(b)** the gate is applied on plasma EVs on the scattering mode; gate (P1) indicates the gated EV population excluding particles larger than 1000 nm and smaller than 240 nm; gate (P3) contains platelets. The black dots outside gate (P1) are cellular particles larger than 1000 nm.



Supplemental Figure 2.2 Lipoprotein contamination in EV fractions. Density gradient centrifugation was applied on baseline/postprandial PFP for lipoproteins isolation. Lipoprotein classes were then fractioned through SEC and analysed for concentration and size measurement using NTA

Chapter III: Acute effects of interesterified fats on numbers and thrombogenic activity of circulating extracellular vesicles

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Conflict of interest:

The authors declare no conflict of interest.

3.1 Abstract

Background:

Interesterified (IE) fats are used in the food industry as substitutes for saturated and trans fatty acids, particularly in spreads, to improve health. However, the effects of these fats on CVD risk markers are uncertain. Extracellular vesicles (EVs) are small membrane-bound vesicles released from cells during activation, damage, or death. They are reported to have pathophysiological effects in CVD, and are modified by dietary fat intake, suggesting their use as a potential biomarker.

Objective:

To investigate whether commercial IE spreads alter the number and thrombogenic activity of EVs during the postprandial response following a single meal.

Methods:

A randomized controlled, double-blind, crossover study was conducted at the Metabolic Research Unit in Nutritional Sciences Department, Franklin Wilkins Building, King's College London. Thirty-five healthy subjects received four test meals (Lurpak spreadable butter, IE Clover spread, IE Free Retail Spread, Control rapeseed spread) in a random order containing 50 g test fat, 15 g protein and 85 g carbohydrate. Blood was sampled at four postprandial time points (0, 2, 4 and 8h). EV subtypes and total number were analysed using FCM and NTA respectively. The thrombogenic activity of EVs was assessed using TFs based thrombin generation and fibrinolysis assays.

Results:

There was a marked postprandial increase in the number and thrombogenic activity of EVs, which reached a peak at 4h. There was no influence of the IE spreads relative to non-IE spreads on EV number, EV-stimulated thrombin generation or fibrinolytic activity.

Conclusion:

There is a pronounced postprandial increase in the number and thrombogenic activity of circulating EVs following a high-fat meal, but this is not altered by replacing the fat source with commercial IE fats.

Keywords:

Extracellular vesicles, interesterified fats, cardiovascular diseases, thrombosis.

3.2 Introduction

Cardiovascular diseases (CVD) are the primary cause of death in the UK [140, 141]. Diets high in saturated fatty acids (SFA) and trans fatty acids (TFA) have traditionally been associated with higher risk of CVD mortality [142, 143]. Partially hydrogenated fats increase the shelf life and flavour stability of food products [70], but they are known to be detrimental to health and associated with increased risk of CVD [71]. Effort to replace industrially produced trans fatty acids with healthier options has led to the increasing use of interesterified (IE) fatty acids, which are solid fats formulated by the rearrangement of fatty acids within the triacylglycerol [72]. However, to date there has been little characterisation of the effects of IE fats on CVD risk markers [73].

The postprandial effects of IE fat on triacylglycerol (TAG) levels have been investigated, but studies report inconsistent findings. Berry et al. [74] and Sanders et al. [75] reported that non-IE palm oil increased plasma TAG to a greater extent than IE palm oil, but a separate study reported no difference in the plasma TAG response to native vs IE shea butter [76]. However, since the studies examined IE fats which are not commonly used in food production, it is difficult to extrapolate their relevance to public health advice. There is also limited data on the effects of IE fats on other markers for CVDs.

Extracellular vesicles (EVs) are phospholipid bilayer-enclosed vesicles shed from cells during activation, damage and apoptosis and are suggested to have pathophysiological roles in CVD, suggesting their use as potential prognostic markers [144]. Numbers of circulating EVs are positively correlated with plasma TAG and cholesterol concentration [99] and some studies have reported a postprandial increase in EV number after the consumption of a high fat meal, which suggests a role for dietary fat in the process of vesiculation and EV release [104, 105].

There is also some evidence that EVs have the ability to stimulate coagulatory pathways, particularly thrombin generation [145], but it is not clear whether this thrombogenic capacity is modifiable by dietary fatty acids. This study investigated for the first time the acute effects of commercially available IE and non-IE fats on EV number and thrombogenic activity during a postprandial response. We hypothesized that there is no difference in response between IE fats and non-IE fats on EV number and functional activity during the postprandial period.

3.3 Material and methods

3.3.1 Subject and study design

A randomised controlled, double-blind, crossover study was conducted at the Metabolic Research Unit in Nutritional Sciences Department, Franklin Wilkins Building, King's College London. The study was registered at clinicaltrials.gov as NCT03438084 and conducted according to the guidelines laid down in the Declaration of Helsinki. Ethical approval for the study was obtained from the Research Ethics Committee at King's College London (HR-17/18 5499).

Thirty-five subjects (females=19, males=16) (aged 35-75y) were recruited through Kings College London (KCL), staff and student newsletter, social media, posters, and newspaper advertisements. Participants were healthy (free of diagnosed diseases listed in exclusion criteria), able to understand the information sheet, willing to comply with the study protocol, and able to give informed consent. The exclusion criteria comprised the following:

- Medical history of myocardial infarction, angina, thrombosis, stroke, cancer, liver or bowel disease or diabetes
- Body mass index < 20 kg/m^2 or > 35 kg/m^2
- Plasma cholesterol ≥ 7.5 mmol/L

- Plasma triacylglycerol > 3 mmol/L
- Plasma glucose > 7 mmol/L
- Blood pressure ≥140/90 mmHg
- Current use of antihypertensive or lipid lowering medications
- Alcohol intake exceeding a moderate intake (> 28 units per week)
- Current cigarette smoker (or quit within the last 6 months)
- ≥ 20% 10-year risk of CVD as calculated using a risk calculator

3.3.2 Dietary intervention

Participants (n=35) received four test meals consisting of a muffin and a milkshake in a random order and containing 50 g test fat, 15 g protein and 85 g carbohydrate. The test fats were produced by Archer-Daniels-Midland Company (ADM Ltd) and the composition is shown in Table 3.1. Study days were separated by at least 7 days and participants were asked to avoid exercise and alcohol for 24 h before the study visit and were given a standardised low-fat meal for dinner the night before the study visits.

At each study visit, participants attended the clinic at approximately 07:30 am, following a 10 hr overnight fast. A cannula was inserted and a baseline fasting blood sample was taken. The test meal was then consumed by the participant, within 15 minutes. Further blood samples were collected at four postprandial time points (0, 2, 4 and 8h) following the test meal. A second meal challenge as a muffin providing 35 g of rapeseed oil (15% protein, 50% carbohydrate, 35% fat) was given at 5 hours to replicate a realistic meal pattern (Supplemental Figure 3.2).

3.3.3 Sample collection and platelet free plasma (PFP) preparation

Venous blood samples were drawn into citrated tubes, inverted 4 times, and processed immediately. Citrated tubes were used because citrate binds to free calcium, thereby preventing the degranulation of platelets and leukocytes and the associated vesiculation process [130]. Samples were centrifuged at 1500 x g at room temperature for 15 min to remove larger cells and cellular debris. Further centrifugation at 13000 x g for 2 min at room temperature produced platelet-free plasma (PFP), which was aliquoted and stored at -80^{o}C for further analysis.

3.3.4 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.

3.3.5 Nanoparticle tracking analysis

The concentration and size distribution of EVs were assessed using Nanoparticle Tracking Analysis (NTA) (NanoSight 300; Malvern, Amesbury, UK), equipped with a 488 nm laser and a high sensitivity CMOS camera. EVs were isolated by SEC and fractions 7 to 9 were pooled, diluted with 0.1µm filtered PBS and adjusted to a range of $1x10^{8}$ – $9x10^{8}$ particles per ml. NTA utilizes the properties of both light scattering and Brownian motion to obtain the size distribution and concentration of particles in liquid suspension. The software tracks particles individually and using the Stokes-Einstein equation calculates their hydrodynamic diameters. The measurements of particle concentration and size were based on the average of five videos, each recorded for 60 s. To prevent the interference of small lipoproteins, such as HDL, LDL

and VLDL, the threshold was set at 71 nm. Particles ranging in size from 71-1000 nm were identified as EVs.

3.3.6 Flow cytometric analysis of EVs

PFP samples were analysed for EV subtypes (phosphatidylserine-positive EVs, platelet-derived EVs and endothelial EVs) using a BD FACSCanto II FCM (BD Biosciences, Oxford UK). Reference beads were used to set live gates based on particle size. These consisted of a mixture of non-fluorescent silica ApogeeMix beads (Apogee flow systems, Hemel Hempstead, UK) with diameters of 180 nm, 240 nm, 300 nm, 590 nm, 880 nm, and 1300 nm (with a refractive index of η=1.43, similar to the refractive index of EVs η~1.39) and green fluorescent latex beads with diameters of 110 nm and 500 nm (with refractive index η=1.59). The EV detection threshold was set at 240 nm to exclude instrument noise. Because of the unavailability of 1 µm silica beads, the gate was set just above 880 nm silica beads to exclude particles > 1µm. The live gate P1 therefore represents EVs, defined as particles within the size range 240 – 1000 nm, with P3 representing platelets (Supplemental Figure 2.1).

To determine the cellular origin of EVs, three EV markers were used; Annexin APC (PS exposure marker), CD41 PE (platelet-derived EV marker) and CD105 eFlur450 (endothelial-derived EV marker). All reagents and buffers were filtered using 0.1 µm pore size centrifugal filter units (Millipore UK Limited, Hertfordshire, UK) for 20 min at low speed (0.1 x g) to remove debris and aggregates. Antibodies were titrated to determine the optimum concentration for EV staining. Frozen PFP was thawed at room temperature on a sample roller. PFP (5 µl) was incubated for 15 min with FcR blocking reagent (5 µl) (Miltenyi Biotec Ltd, Surrey, UK), to block unwanted binding, Annexin V buffer (Cambridge Bioscience Ltd, Cambridge, UK), which contains the calcium required for Annexin V binding to

phosphatidylserine (PS) exposed on EV surface, and argatroban (1 µl), a synthetic thrombin inhibitor, which is used as an anticoagulant to prevent clotting caused by the calcium in the Annexin V buffer.

After the first incubation, PFP was labelled with a panel of antibodies and isotype-matched controls, incubated for 15 min at room temperature in the dark and analysed by FCM (Supplemental Table 2.2).

BD TruCount tubes (BD Biosciences) were used to obtain the absolute count of EVs in the original sample. Each TruCount tube contains 47,000 beads, which were resuspended in 500 µl of filtered PBS and analysed by FCM to calibrate the numbers of EVs in the samples.

3.3.7 Thrombin generation assay

The procoagulant activity of EVs was assessed using the Technothrombin MP kit (Technoclone, Vienna), which is based on the thrombin-dependent cleavage of a fluorogenic substrate over time, according to the manufacturer's instructions. Results were recorded using a FlexStation 3 microplate reader (Molecular Devices, San Jose, USA) and the data were analysed using TGA evaluation software (Technoclone, Vienna).

The thrombogenicity of EVs was analysed by isolating them from PFP using SEC, adding them to vesicle-free plasma (VFP) and comparing thrombin generation with that in VFP alone. EV fractions eluted by SEC were concentrated using Vivaspin™ 6 Sample Concentrators with 100,000 MWCO (Fisher Scientific, Loughborough) at 1500 x g for 40 min. Aliquots of EVs (10 µl) with final protein concentrations of 10, 5 and 2 µg/ml were added to 30 µl of VFP, followed by 10 µl of phospholipids and 50 µl of fluorogenic substrate before initiating kinetic

reading at 1 min intervals for 60 min at 37 °C, using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

VFP was prepared from pooled plasma from three healthy volunteers. Citrated blood (200 ml) from fasted volunteers was centrifuged at 2,500 x g for 15 min to remove large cells. The resulting plasma was transferred to a clean tube, leaving 1 ml above the buffy coat, and centrifuged at 2,500 x g for 15 min to remove platelets and cellular debris. The supernatant was then centrifuged at 20,000 x g for 1 h, at 4°C to pellet large vesicles. The supernatant was subjected to a final centrifugation at 100,000 x g for 1 h, at 4°C to pellet small vesicles. The final supernatant (VFP) was aliquoted and stored at -80 °C until further use.

To investigate the contribution of PS to thrombin generation, a blocking experiment was performed by incubating Annexin V at final concentrations of 0.20, 0.30, 0.60 mg/ml with EV aliquots (5 µg/ml) for 15 min.

3.3.8 Effect of EVs on clot formation and fibrinolytic activity

The clot-forming and plasmin generation capacity of EVs were assessed by isolating EVs using SEC, adjusting the concentration to 5 µg/ml and applying clot formation and lysis assays adapted from [146] and [147] to compare clot formation and fibrinolytic activity in VFP with VFP 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 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 10 h using a FlexStation 3 microplate reader (Molecular Devices, San Jose, USA).

All data were analysed using an online tool for analysis of clot and lysis using the Shiny App developed by Longstaff [148].

3.3.9 Statistical analysis

The results are presented as the means ± standard error of the mean. Differences between the four treatment groups were determined using a linear mixed model followed by post-hoc analysis using Bonferroni tests where applicable. The model was adjusted for baseline and age. Multivariate linear regression was used to determine the relationship between all independent variables together and one dependent variable. Stepwise regression analysis was performed to determine the independent predictors of dependent variable. P-values <0.05 were considered statistically significant. Statistical analyses were performed using IBM SPSS statistics 27.

3.4 Results

3.4.1 Subject characteristics

A total of 35 participants with a mean age of 55 years (range 35-74) completed the 12-week intervention. The clinical and laboratory characteristics of the participants are shown in Table 3.2.

A multivariate regression model examining relationships between baseline characteristics and circulating EV numbers demonstrated that EV number was significantly associated with plasma TAG concentration and stepwise regression analysis suggested that plasma TAG level explained 79.4% of the variance for EV total concentration (Table 3.3).

3.4.2 High-fat meals increase EV numbers, but no effect of replacing non-IE fats with IE fats

There was a significant effect of time ($P<0.001$) on EV subtypes and total concentration. However, there was no significant effect of the treatment and no time*treatment interaction. The number of total circulating EVs and all EV subtypes (annexin V-positive, platelet-derived and endothelial-derived) increased during the postprandial period, reaching a peak at 4h (Figure 3.1).

Figure (3.1, E) illustrates the proportion of annexin V-positive EVs which were classified as platelet-derived or endothelial-derived over the course of the postprandial period. At 0, 4 and 8h, these EVs comprised approximately 60% of annexin V-positive EVs, but at 2h, they comprised 76%.

3.4.3 EVs support thrombin generation

Isolated EVs added to VFP supported thrombin formation in a dose-dependent manner, resulting in higher peak thrombin concentration and a greater area under the curve (AUC) (Supplemental Figure 3.3). When EVs were added to VFP at a protein concentration of 5 µg/ml, they resulted in thrombin generation equivalent to that in PFP. Addition of Annexin V to the assay decreased thrombin generation in a dose-dependent manner due to its ability to compete with prothrombin for binding to PS, with 0.60 µg/ml Annexin V resulting in a level of thrombin generation equivalent to that in VFP (Supplemental Figure 3.4).

3.4.4 High fat meals increase EV-stimulated thrombin generation and fibrinolysis

There was a significant effect of time ($P < 0.001$) on EV-dependent thrombin generation and fibrinolysis, where peak thrombin concentration and AUC for thrombin generation, clot formation and clot lysis increased during the postprandial period, peaking at 4 hr (Figure 3.2). However, there was no effect of the treatment and no significant time*treatment interaction.

3.5 Discussion:

The relationship between dietary fats and CVD is well-established, particularly with respect to TFA [143]. SFA and TFA have been linked to an abnormal lipid profile, endothelial dysfunction and increased risk of CVD [149]. On the other hand, substituting SFA with PUFAs reduces CVD risk factors [150]. Food manufacturers have historically used hydrogenation to prolong the shelf life of food products [151], but the adverse effects of TFAs generated during this process have meant that hydrogenated fats have been removed from food supplies in 58 countries [152]. IE fats have been introduced as a substitute for partially hydrogenated fats due

to their reduced content of SFA and TFA, while maintaining suitable physical properties [72]. Despite the wide use of IE fats, their impact on human health, and particularly CVD, is unknown. To our knowledge, this is the first study investigating the effects of industrially used IE fats on EV number and functional activity. EVs are small particles derived from cells and have potential as prognostic markers for CVD [153]. This study demonstrates that EV numbers and EV-dependent thrombogenic and fibrinolytic activity increase postprandially, peaking at 4 h, but there is no difference in the response to meals containing IE vs non-IE fats, suggesting that the postprandial response is related to the amount of dietary fats instead of its content.

Consumption of high fat meal is suggested to impair postprandial FMD, induce endothelial function, stimulate postprandial coagulation activation, increases TAG and cytokines concentration, indicating the role of dietary fats in inflammation [154, 155]. A few studies have investigated the postprandial response of blood parameters following IE and non-IE fats and reported inconsistent findings. Berry et al. reported a reduction in plasma TAG concentration following the consumption of IE fat compared to non-IE fats [74]. In contrast, another study reported no difference in response between IE and non IE fats [76]. The inconsistency in the results could be attributed to the differences in the solid contents of the test fats between the two studies, where fats with high solid contents emulsified slowly due to their high melting point resulting in a lower increase in plasma TAG [74, 76]. However, these studies did not test IE fats commonly used in the food industry for food formulation.

Postprandial studies on EVs have reported an increase in EV number associated with hypertriglyceridemia suggesting an association between TAG and endothelial dysfunction [156, 157]. There are no published studies examining the effects of IE fats on EV number or function, but the effects of high fat meals on EV number have been investigated. Tushuizen et al. reported

a postprandial increase in EV total count (comprised of 88-98% platelet-derived EVs) after consuming two high fat meals (containing 50 g of fat, of which 60% was saturated, 55 g of carbohydrates and 30 g of proteins) [109]. The authors proposed a role for oxidized LDL in the mechanisms, whereby interaction of oxidised LDL with platelets via receptors could lead to activation and EV production [108]. In the current study, the results have shown that there is no difference between IE and non-IE fats on EV numbers, where EV count has increased postprandially after all fat spreads peaking at 4 h time point. A preliminary experiment has shown that postprandial EV isolated by SEC are minimally contaminated with lipoproteins as measured by apoB48 or apoB100. The plasma TAG concentration was strongly predictive of EV number, in line with previous studies [156-158], suggesting that postprandial hypertriglyceridemia and elevation of EV numbers may be causally linked. High TAG levels have been shown to induce apoptosis and lipotoxic cell death by stimulating the production of cytosolic calcium and the number of cells exposing PS (annexin V-positive cells) [159]. In addition, the cleavage of two apoptotic markers, caspase 3 and Poly (ADP-ribose) polymerase, have been increased during hypertriglyceridemia [159], indication of cells undergoing apoptosis and the association between high TAG level and apoptosis. Another possible mechanism implicating hypertriglyceridemia in EV production is the association of high TAG level with endothelial dysfunction [160]. Because of the insolubility of TAG particles in the blood, they are transferred into the blood in the lipoprotein-bound form such as LDL. During hypertriglyceridemia, LDL particles are reported to be small and dense and therefore their metabolism is slow and hence they exist in the plasma for a long time, which make them easily contact with endothelial cells and infiltrate into sub-endothelium, causing endothelial dysfunction, [160] which potentially induces EV release. There was also a positive relationship between EV numbers and insulin concentration, in line with previous studies [161, 162]. Elevated levels of insulin have been reported to be involved in the apoptotic process.

Hyperinsulinemia induces the production of high-mobility group box 1 (HMGB1) which is reported to induce apoptosis, a mechanism involved in EV biogenesis [163-165]. In addition, compared to control pancreatic β cells, pancreatic β cells with high insulin concentration were found to induce β cell death by increasing the activity of caspase (proteolytic enzymes trigger cell death), decrease in cellular reducing power and increases the production of lactate dehydrogenase (LDH), an enzyme released when the plasma membrane is damaged [166]. These findings indicate that cells activation and apoptosis mediated by TAG and insulin might play a role in EV generation.

Postprandial hypertriglyceridemia has been implicated in the postprandial activation of blood coagulation and suppressed fibrinolysis [167, 168]. The chronic and acute consumption of dietary fats have been suggested to cause postprandial activation of FVII, a protein initiates the clotting cascade [169]. The underlying mechanism for activation of FVII is uncertain, however, there are several explanations have been suggested. One explanation has been based on the positive association of vitamin K-dependent clotting proteins, such as FVII and FIX, with triglyceride-rich lipoproteins in plasma [169]. Another explanation is that during reverse cholesterol transport (a mechanism activated postprandially to remove excess cholesterol) out of cells, the procoagulant phospholipid (PS) moved from the inner leaflet to the outer leaflet of cell surface membranes leading to the assembly and activation of the vitamin K-dependent clotting factors on the membrane [167, 169, 170]. Postprandial studies have examined the chronic effects of consuming high fat diet on coagulation and reported an increase in thrombin peak and endogenous thrombin potential in rats, while the chronic consumption of Mediterranean and low fat diets decreased endogenous thrombin potential in humans by 21% [171]. In addition, coagulation factors (fibrinogen, FII and FVIII) have been shown to rapidly increased in response to the chronic consumption of high fat meal and that response has

reserved after the chronic consumption of low fat meal [172]. Moreover, the acute consumption of high fat meal has been found to activate thrombin formation in humans [173]. The mechanisms underlying this were not clear. Furthermore, the acute consumption of dietary fats were also found to have a role in the fibrinolytic pathway by decreasing plasminogen activator inhibitor-1 responsible to suppress fibrinolysis regardless of the amount and type of dietary fats provided [174]. A recent study has investigated the effects of the chronic consumption of fat rich meal and reported a delay in plasmin generation and fibrinolysis [168]. The delay in plasmin generation is attributed to the thrombomodulin and TAFI (thrombin activatable fibrinolysis inhibitor) dependant mechanism. The concentration of thrombomodulin increased in response to high fat meal and bind to thrombin [168]. The thrombin- thrombomodulin complex activates the profibrinolytic substrate (TAFI) which reduces the binding of fibrin to plasmin (a proteolytic enzyme) and plasminogen (lyses fibrin clots) leading to decrease the fibrin ability to enhance plasmin generation [168]. A few studies have explored the effects of IE on coagulation. Sanders et al. reported a decrease in postprandial lipemia and FVII activation following consumption of IE fats compared with native fat, with the IE fat being less well absorbed and their release delayed in the circulation [175]. In contrast, Berry et al. reported that IE and non-IE fats did not differ in terms of postprandial plasma TAG and FVII concentrations[176]. Taken together, the composition of dietary fats plays a role in modulating the haemostasis system. However, the data related to IE fat are inconsistent, and majority of IE fat studies have used not commercially relevant IE fat. The inconsistency in results could be explained by that the difference in fats solid content, where fats with high solid content contain crystalline solids at body temperature which might affect micelle formation and retard the process of absorption and consequently result in reduced postprandial effects [176].

EV supports blood coagulation through PS and TF exposure [59]. PS exposure on EVs contributes to coagulation by favouring the assembly and activation of tenase and prothrombinase complexes to thrombin and thus initiate thrombin formation [63]. TFs are key activators of the coagulation cascade and their expression on EVs stimulates coagulation by binding to and activating FVII [63]. Additionally, EVs participate in the fibrinolytic system through the exposure of plasminogen receptors on its surface [64]. Plasminogen activation to plasmin involves the binding of plasminogen to its receptors present on EV surface [64]. To our knowledge, the effects of IE fats on EV procoagulant and fibrinolytic activity have not previously been investigated and there is little data relating to dietary fat modulation of EV procoagulant activity in general. EV postprandial studies reported an increase in EV shedding [109, 156, 177] and the increase in EV levels has been found to be associated with increasing thrombin generation in a concentration dependent manner [109], indicating the importance of EV in the coagulation system.

Postprandial hypertriglyceridemia is reported to activate the coagulation factors, induce endothelial damage, which might trigger EV release [178, 179]. In this study, EV-dependant procoagulant and fibrinolytic activity has increased postprandially following IE and non-IE fats. The studies related to dietary fat modulation of EV procoagulant activity are too limited. Tushuizen et al. has demonstrated that despite the change in EV number and phospholipid composition after consuming high fat meal, there is no difference in EV procoagulant behaviour between fasting and postprandial state [180]. The author has attributed this to the unchanged amount of EV exposing PS at postprandial state compared to baseline, where PS exposure on EV is an important factor to initiate coagulation. In this paper, there was no difference in EV dependant thrombotic and fibrinolytic behaviour between IE and non-IE fats. EV-dependant thrombotic and fibrinolytic activity have postprandially increased after all fat

spreads peaking at 4 hr time point. The increase in EV dependant thrombin generation as results of consuming the four fat spreads could be attributed to the increase in PS exposure on EV, one of the main triggers of coagulation. The increase exposure of PS is evident in this study by the increase of Annexin V positive EV as measured by FCM. Furthermore, the positive correlation between TAG and insulin with thrombin AUC mediated by EVs might play a role in EV thrombogenicity. High levels of TAG have been found to activate coagulation and impair endothelial function [178, 179], which might in turn induces EV production. High levels of insulin is demonstrated to strongly activate platelets [181] stimulating EV release and causing a rapid increases in circulating TFs procoagulant activity associated with thrombin generation and acute cardiovascular events [182, 183]. These findings demonstrate that both IE and non-IE fats modulate EV procoagulant activity regardless of the structure and content, and that increase in PS exposure have a key role in EV coagulatory behaviour. In addition, TAG and insulin might play a role in stimulating the release of procoagulant EV through the elevation in coagulation parameters, cell activation and damage.

In conclusion, this study demonstrated a dramatic increase in the numbers and thrombogenicity of EVs during the postprandial period following a high fat meal, but there was no difference in the response to high-fat meals containing IE vs non-IE fats. Since EV numbers were strongly related to plasma TAG concentration, postprandial hypertriglyceridemia may be causally connected to the postprandial increase in EV number and thrombogenicity.

3.6 Acknowledgement:

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Authors' contributions:

SB, WH and PY designed the study, AsA and PG conducted the intervention and blood parameter analysis, AmA, DT and SS conducted the EV analysis under the supervision of PY. AmA performed the statistical analysis and wrote the manuscript, with contribution from all authors.

Table 3.1 Fatty acid composition of the four fat spreads

Fatty acids	Lurpak spreadable butter (wt %)	IE Clover spread (wt %)	IE Free Retail Spread (wt %)	Control rapeseed spread (wt %)
Butyric acid	2.33	0.00	0.00	0.00
Caproic acid	1.33	0.06	0.00	0.00
Caprylic acid	0.67	0.74	0.80	0.00
Capric acid	1.67	0.74	0.30	0.00
Lauric acid	2.00	10.24	4.10	0.00
Myristic acid	8.03	3.78	1.90	0.10
Pentadecylic acid	1.33	0.00	0.00	0.00
Palmitic acid	19.33	16.73	27.90	4.00
Margaric acid	0.37	0.06	0.10	0.10
Stearic acid	8.50	2.51	3.50	1.50
Arachidic acid	0.17	0.39	0.50	0.50
Behenic acid	0.10	0.19	0.20	0.30
C18:1 Elaidic acid	2.00	0.00	0.00	0.00
C18:2 trans Linolelaidic acid	0.13	0.00	0.10	0.00
C18:3 trans gamma-Linolenic acid	0.00	0.00	0.30	0.00
C16:1-cis Palmitoleic acid	1.43	0.17	0.10	0.30
C18:1-cis Oleic acid	37.83	44.47	44.30	61.50
C18:2-cis Linolelaidic acid	8.20	13.33	11.70	20.00

C18:3-cis gamma-Linolenic acid	3.67	5.62	4.00	10.00
C20:1-cis Gondoic acid	0.67	0.58	0.50	1.00
C22:1-cis Erucic acid	0.17	0.28	0.00	0.50

Table 3.2 Baseline characteristics of the study participants

Subject characteristics	Mean ± SE
Age (years)	55.26±1.93
Height (m)	1.71±0.01
Weight (kg)	72.05±2.04
BMI (kg/m^2)	24.52±0.60
Waist (cm)	86.36±1.83
Hip (cm)	93.90±1.58
Waist-Hip ratio	0.92±0.01
% Fat	25.22±1.71
Fat mass (kg)	18.29±1.60
Fat free mass (kg)	53.67±1.71
TAG (mmol/L)	1.00±0.08
CHOL (mmol/L)	5.14±0.15
LDL (mmol/L)	3.14±0.12
HDL (mmol/L)	1.80±0.07
Glucose (mmol/L)	5.44±0.01
SBP (mm Hg)	112.26±1.78
DBP (mm Hg)	74.65±1.32
RBC 10^12/L	4.48±0.06
WBC 10^9/L	4.99±0.014
NEUT 10^9/L	2.75±0.14
Lymph 10^9/L	1.57±0.07
MONO 10^9/L	0.46±0.03
FMD	4.89±0.33

Results are means ± SE Abbreviations: BMI, body mass index; TAG, triglycerides; CHL, cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; RBC, red blood cell; WBC, white blood cell; NEUT, neutrophils; Lymph, lymphocytes; MONO, monocytes; FMD, flow-mediated dilation

Table 3.3 Multivariate regression analysis for the association of EV total number and AUC of thrombin mediated by EV with TAG, insulin, and other markers. Independent predictors of Total EV number and AUC of thrombin mediated by EV determined by stepwise regression

Multivariate regression

	Log total EV (Per ml blood)		AUC of thrombin mediated by EV	
	R^2=0.814 *p<0.001*		R^2=0.251 *p=0.010*	
	B	***p***	**B**	***p***
Log TAG (mmol/l)	1.50	**<0.001**	598.5	0.114
HDL (mmol/l)	-0.091	0.216	-	-
Insulin	-0.090	0.537	424.8	0.101
RBC (10^9/L)	1.70	0.150	-	-
Weight (kg)	0.00	0.974	-	-
Waist (cm)	0.002	0.681	-	-
SBP (mmHg)	-	-	-	-
DBP (mmHg)	-	-	-	-

stepwise regression

		Model	B (95% CI)	SE (B)	β	*p*-value
Total EV number	1	(Constant)	9.81 (9.74, 9.87)	0.030		**<0.001**
		Log TAG	1.64	0.166	0.865	**<0.001**
		R^2=0.748	(1.30, 1.97)			
		(Constant)	8.25 (7.18, 9.32)	0.52		**<0.001**
	2	Log TAG	1.50	0.156	0.794	**<0.001**
		Log RBC	(1.18, 1.82) 2.39	0.807	0.244	**0.006**
		R^2=0.802	(0.748, 4.03)			
AUC of thrombin mediated by EV	1	(Constant)	2872.3 (2508.6, 3236.1)	178.7		**<0.001**
		Log insulin	625.01	225.1		**0.009**
		R^2=0.435	(166.9,1083.1)			

In multivariate model, only TAG was still significantly correlated with EV total number. TAG, triglycerides; HDL, high density lipoproteins; RBC, Red blood cells; SBP, systolic blood pressure; DBP, diastolic blood pressure. Unstandardized coefficients (B) indicates that as the independent variables change by one unit, the dependent changes by B units. Regression coefficient (β) indicates that as the independent variables change by 1 SD, the dependent variable changes by β SD. CI, confidence interval; SD, standard deviation; SE, standard error.

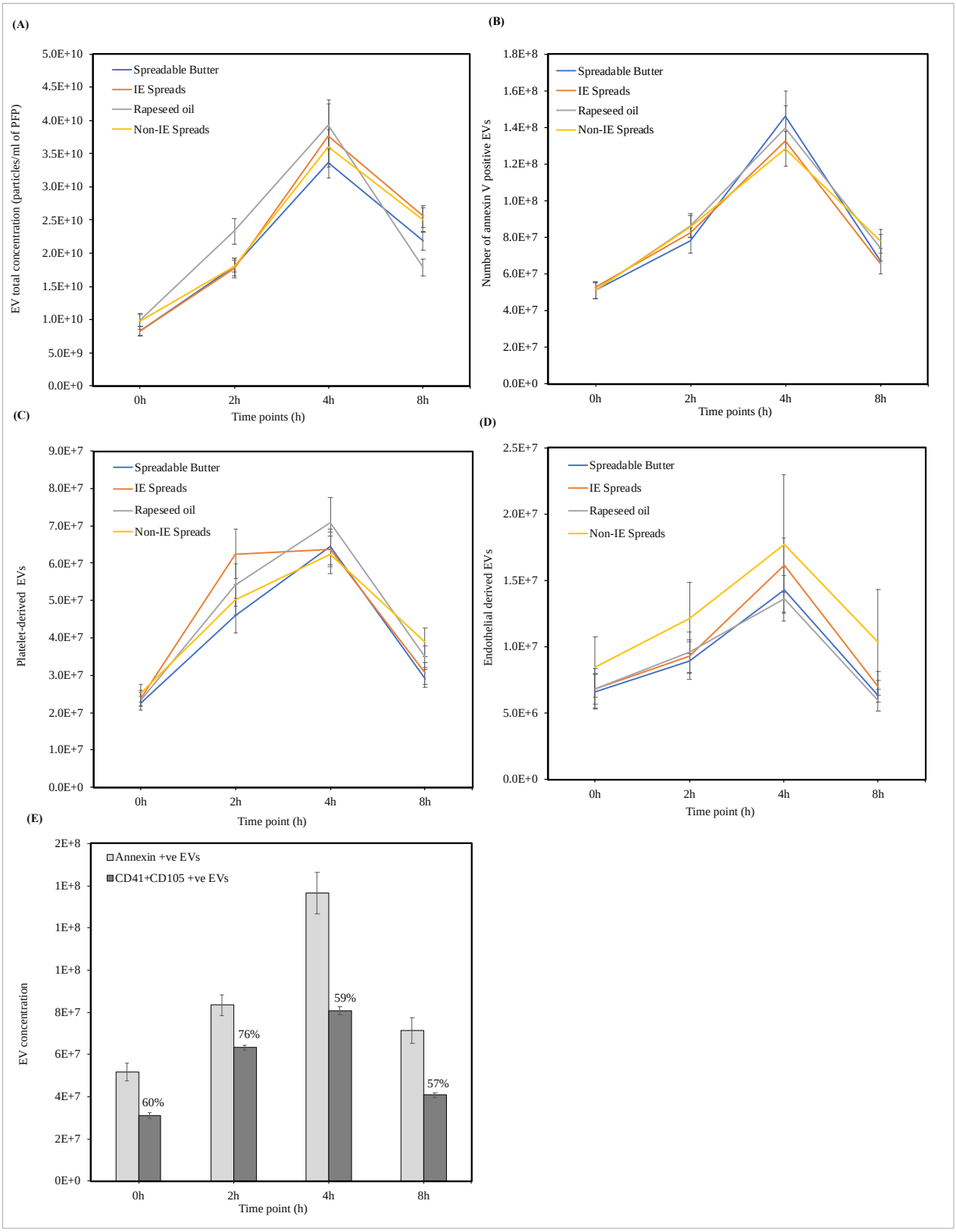


Figure 3.1. Data were analysed using a linear mixed model (n=35). **(A)** There were no significant treatment effects or time*treatment interaction on EV total concentration (P=0.243). However, there was a significant effect of time (P-value < 0.001) on EV concentration, where EV number peak at time point 4 compared to 2 & 8 (P-value < 0.001), but there was no significant difference between time points 2 & 8 (P-value = 0.768). **(B, C, D)** There were no significant treatment effects on EV subtypes (annexin +ve EV p-value= 0.271; platelets-derived EV p-value = 0.437; endothelial-derived EV p-value = 0.313) and no time*treatment interaction. However, there was a significant effect of time on EV subtypes number where the number peak at 4 hr time point. **(B)** The number of annexin V positive EV has increased at 4 hr time point compared to time points 2 and 8 (P-value < 0.001, where there was no significant difference between time point 2 and 8 (P-value = 0.358). **(C, D)** Platelets and endothelial derived EVs have increased at 4 hr time point compared to time points 2 & 8 (P-value < 0.001) and increased in time point 2 compared to time point 8 (P-value < 0.001). **(E)** The figure illustrates the proportion of annexin V-positive EVs which were classified as platelet-derived or endothelial-derived over the course of the postprandial period. At 0, 2, and 8h, these EVs comprised approximately 60% of annexin V-positive EVs, but at 2h, they comprised 76%. Data are means ± SEM.

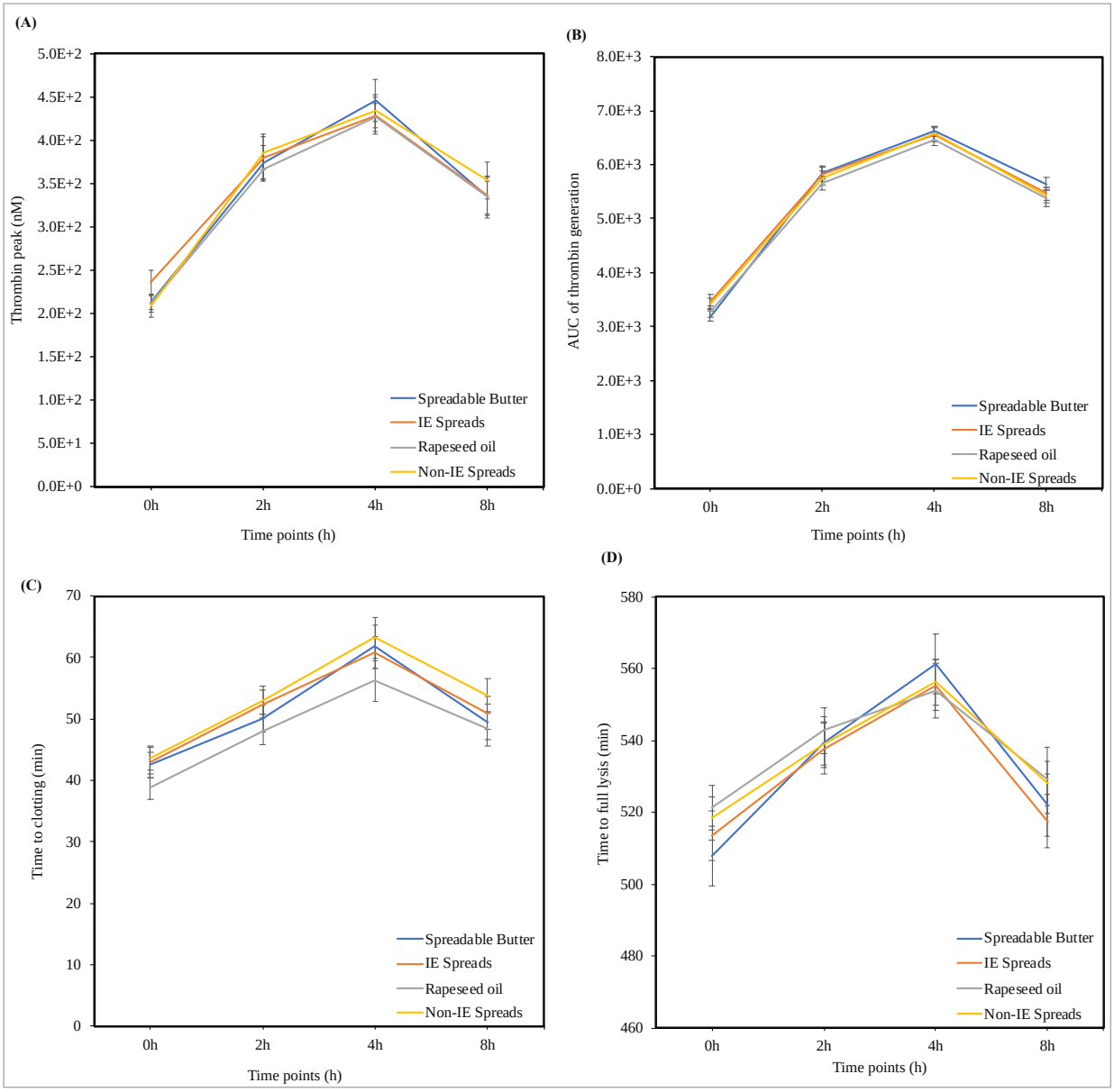
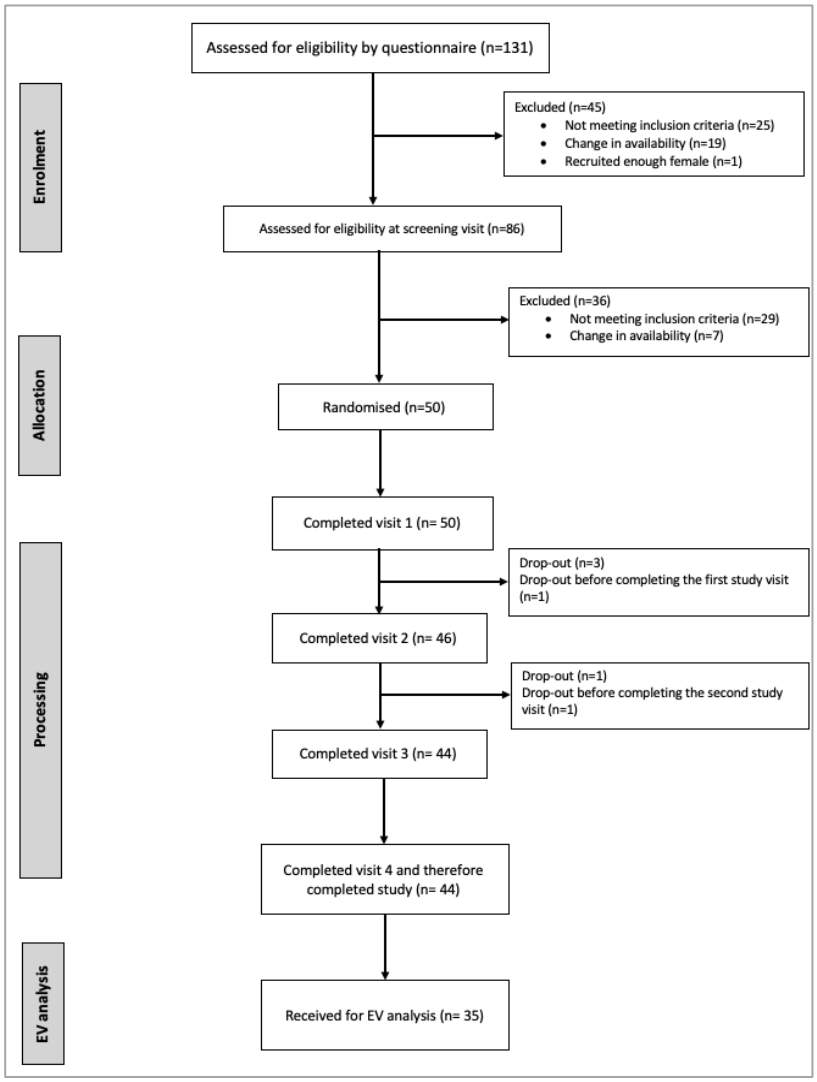
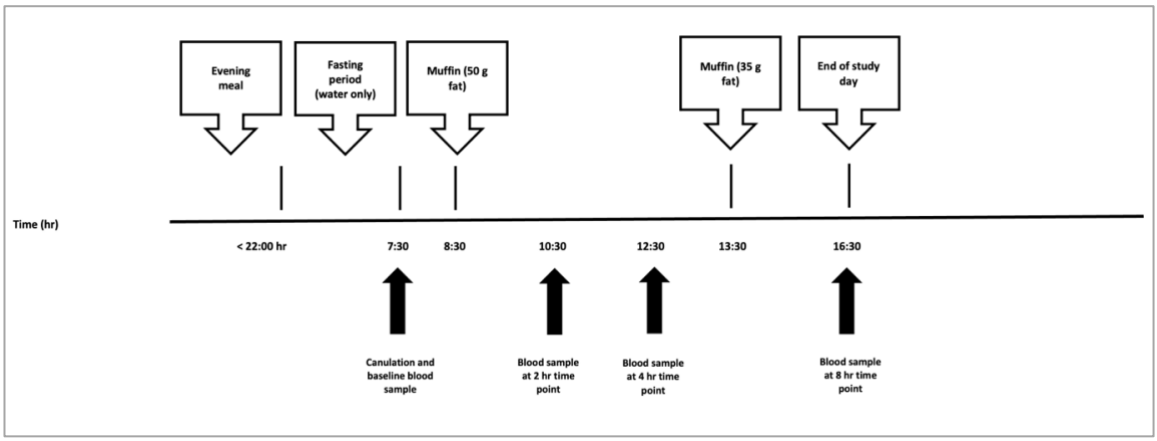


Figure 3.2. Data were analysed using a linear mixed model (n=35). **(A, B, C, D)** There were no significant treatment effects on EV dependant thrombin generation and fibrinolytic activity; thrombin peak (p-value= 0.48), AUC (p-value= 0.33), time to clotting (p-value= 0.94), and time to full lysis (p-value = 0.06), and no time*treatment interaction. However, there was a significant effect of time on EV dependant thrombin generation and fibrinolytic activity (P-value < 0.001), where EV dependant thrombin peak, AUC, time to clotting, and time to full lysis have significantly increased at time point 4 compared to time points 2 & 8 (P-value < 0.001). Thrombin peak, AUC and time to full lysis were higher in time point 2 compared to time point 8 with p-values 0.03, 0.004, and < 0.001 respectively. Time points 2 & 8 did not differ significantly for time to clotting. Data are means ± SEM.

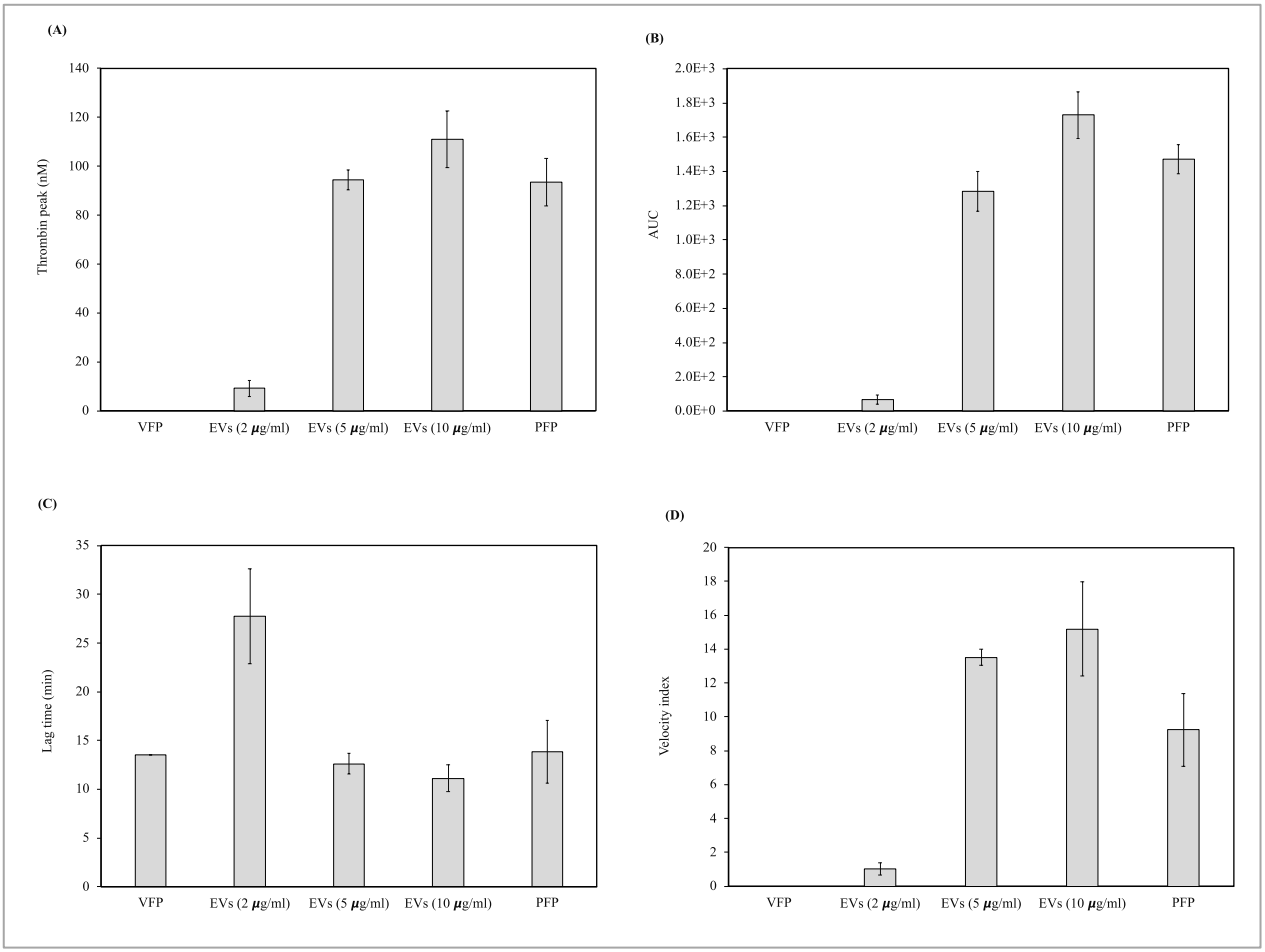
Supplementary Material



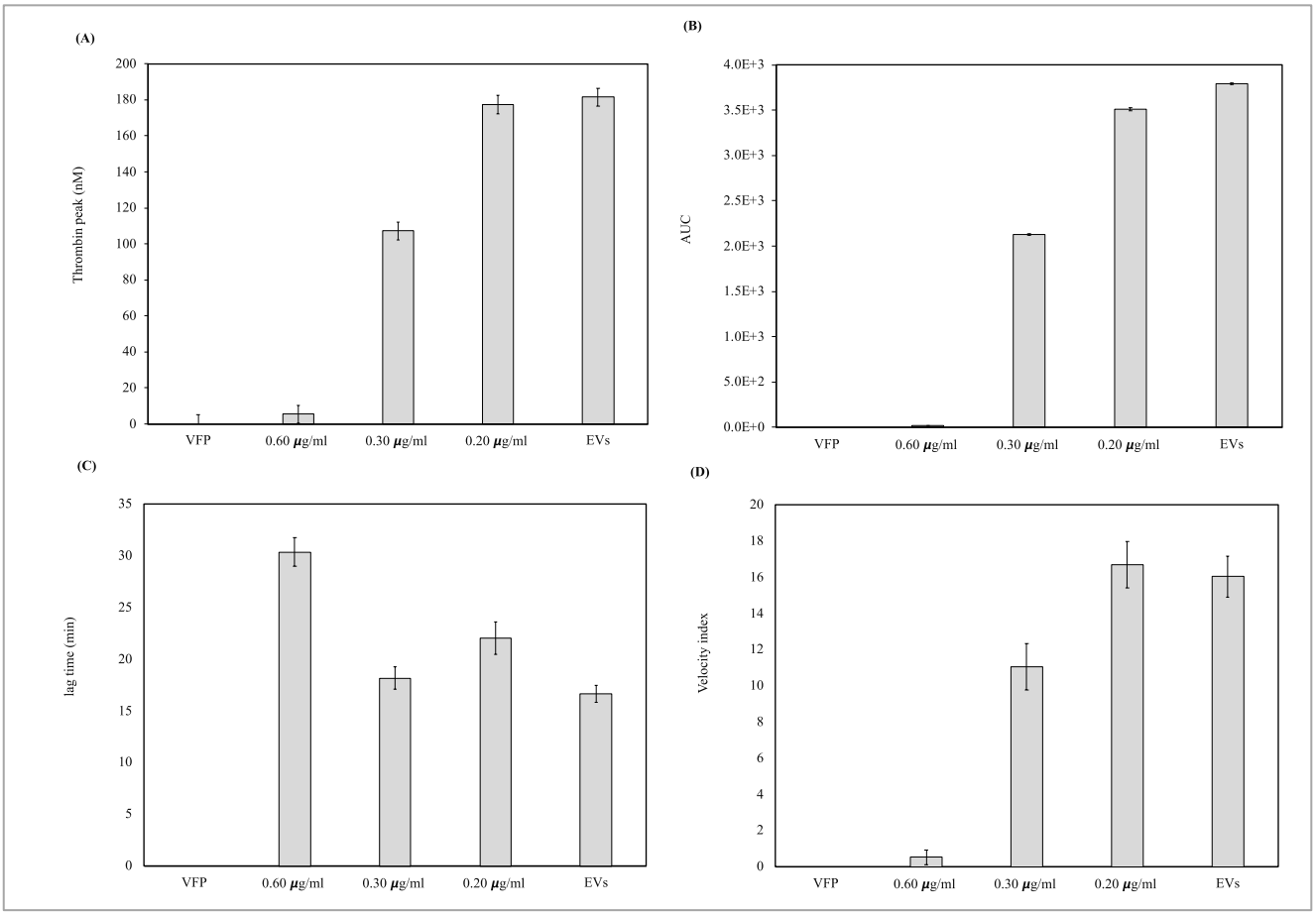
Supplemental Figure 3.1 Flow chart of study participants in the intervention study. Participants drop out was due to schedule conflicts.



Supplemental Figure 3.2 Timeline of subject flow through each visit.



Supplemental Figure 3.3. **Effect of increasing concentrations of EVs on in vitro thrombin generation (n=4).** Data are mean ± SE for n=4 replicates. Higher concentration of EVs contributed to **(A)** higher thrombin peak, **(B)** AUC, **(D)** velocity and **(C)** shorten the lag time. Data are means ± SEM.



Supplemental Figure 3.4. **Annexin blockage of thrombin generation (n=3). (A,B)** Annexin V has stopped thrombin generation in a dose-dependent manner. **(C,D)** Higher concentration of annexin V increased the lag time and decreased speed to form thrombin. Data are means ± SEM.

Chapter IV: Fish oil supplements, but not oily fish, alter the number and function of extracellular vesicles in healthy human subjects.

Manuscript prepared for submission to Journal of Nutrition

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4.1 Abstract

Background: Evidence suggests that n-3 polyunsaturated fatty acids (n-3 PUFA) alter numbers of circulating extracellular vesicles (EVs), which may play a role in cardiovascular diseases (CVDs), but data is limited and relates only to n-3 PUFA supplements. There is no information about whether consumption of oily fish has a similar effect and there is little information about the effects of dietary n-3 PUFA, regardless of the source, on EV function.

Objective: To assess the effects of fish oil supplements and oily fish, at a level achievable in the diet, on EV number, phenotype, composition and procoagulant activity in healthy human volunteers.

Methods: A randomized, double-blind, placebo-controlled, parallel trial was conducted at the School of Sport and Exercise Sciences, Liverpool John Moores University. 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 a period of 12 weeks. Nanoparticle tracking analysis (NTA) and FCM were used to enumerate and characterise EVs and the procoagulant activity of EV was assessed using thrombin generation and fibrinolysis assays.

Results: Consumption of both oily fish and fish oil supplements significantly increased the EV content of EPA and DHA. Supplementation with fish oil decreased the number of EVs in the circulation, decreased EV-stimulated thrombin generation and modestly altered clot formation, but consumption of oily fish providing half the dose of n-3 PUFA compared with that in the supplements had no effect on any of these parameters. There was no effect of either oily fish or fish oil supplements on EV subtypes or fibrinolytic activity.

Conclusion:

Supplementation with fish oil significantly decreased both number and thrombogenic capacity of EVs, while consumption of oily fish at a level achievable in the diet had no effect on either EV number or thrombogenic capacity.

Keywords:

Extracellular vesicles, n-3 polyunsaturated fatty acids, cardiovascular diseases, thrombosis, eicosapentaenoic acid, docosahexaenoic acid.

4.2 Introduction

Almost all types of cells release extracellular vesicles (EVs), which are very small, membrane-derived vesicles enclosed by a lipid bilayer and which carry cargo, such as nucleic acids, proteins and lipids, reflecting the cells of their origin [1]. EVs are naturally present in the bodily fluids of healthy individuals, reported to contribute to the regulation of normal physiological processes, such as blood coagulation, intercellular communication and tissue repair [184, 185], and may serve as potential biomarkers for diseases, such as cancer, cardiovascular diseases (CVDs) and type 2 diabetes [186-188]**.** There is particular interest in the association of EV number with CVDs and metabolic syndrome [8, 189], and with aspects of blood coagulation pathways [145]. Increased numbers of EVs in the circulation are also associated with endothelial dysfunction and vascular inflammation [190].

Dietary n-3 polyunsaturated fatty acids (n-3 PUFA), 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 [191, 192]. 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 PUFA [193].

A range of potential mechanisms have been considered [194], but to date, very few studies have explored the effects of diet and lifestyle on EV numbers or function. A small number of studies have demonstrated a decrease in the total number of EVs after intervention with fish oil capsules, a low fat diet, flavanols or weight reduction [63, 100, 101, 103]. We have previously demonstrated that supplementation with n-3 PUFA decreased the number of endothelial EVs in individuals with moderate risk of CVD [99]. However, there is no

information about whether oily fish, consumed at a level that is achievable through the diet, affects either EV number or function. The current study therefore examines, for the first time, whether n-3 PUFA delivered in the form of oily fish are able to modify the profile and coagulatory behaviour of EVs in the circulation in the same way as fish oil supplements, consumed at a dose sufficient to lower numbers of EVs. We hypothesized that fish oil supplements and oily fish are equally as effective in reducing EV number and functional activity.

4.3 Material and methods

4.3.1 Subject and study design

A randomized, double-blind, placebo-controlled, parallel trial was conducted at the School of Sport and Exercise Sciences, Liverpool John Moores University and carried out according to the guidelines in the Declaration of Helsinki, with ethical approval from the National Research Ethics Service (S16SPS041). All participants provided written consent prior to the start of the study.

Forty-two subjects (females=28, males=10, unspecified=4) were recruited through the media (BBC local and national radio, online forums, social media, societies, and flyers in the street) (Figure 4.1). The Framingham Risk Score was used to identify participants who were at above average risk of developing CVD [195], 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 4.1, which includes family history of myocardial infraction or type 2 diabetes [196]. Exclusion criteria included the following: infection, immune disorder including HIV, autoimmune disease, or fever of unknown origin; unstable medical conditions requiring immediate intervention, unstable or rapidly progressive neurological diseases; a history of haemorrhagic or ischemic stroke within the last 3 months; consuming oily fish more than once per week on

average; allergy, hypersensitivity, or intolerance to fish, fish oils or omega-3 fats; any known food allergies and pregnant or breastfeeding.

4.3.2 Dietary intervention

Participants (n=42) were allocated randomly (block randomisation 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 (Table 4.1) plus two white fish meals per week, (ii) control capsules containing refined olive oil (Table 4.1) plus two oily fish meals containing 1.44 g/d of n-3 PUFA (one meal containing salmon and the other mackerel) and (iii) control capsules plus two white fish meals. Although the doses of n-3 PUFA 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 [99]. 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). Portions of mackerel, salmon and white fish contained within the ready meals were 229 g, 240 g and 110 g respectively. The fatty acid composition of the fish meals was analysed by the West Yorkshire analytical services (Leeds, UK) and is shown in Table 4.2. All meals were delivered chilled and distributed to participants twice a week, with storage and heating instructions provided by the supplier**.** Volunteers attended three visits during the intervention: at screening, baseline, and end of intervention. Compliance to the intervention was confirmed verbally during each week of the trial.

4.3.3 Sample collection and platelet free plasma (PFP) preparation

Venous blood samples were drawn into citrated tubes, inverted 4 times and processed immediately. Citrated tubes were used because citrate binds to free calcium, thereby preventing

the degranulation of platelets and leukocytes and the associated vesiculation process [130]. Samples were centrifuged at 1500 x g at room temperature for 15 min to remove larger cells and cellular debris. Further centrifugation at 13000 x g for 2 min at room temperature produced PFP, which was aliquoted and stored at -80ºC for further analysis.

4.3.4 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.

4.3.5 Plasma lipid analysis

The plasma lipid profile before and after the treatment was assessed using a Daytona Plus clinical chemistry analyser (Randox). Plasma cholesterol and triacylglycerol were analysed using enzyme-based assays and HDL-C was analysed using a clearance assay. LDL-C was estimated using the Friedewald formula.

4.3.6 Analysis of the fatty acid composition 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 extracted with 5 ml of chloroform/methanol (2/1) containing 50 mg/l butylated hydroxytoluene antioxidant and 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).

4.3.7 Nanoparticle tracking analysis

The concentration and size distribution of EVs was assessed using Nanoparticle Tracking Analysis (NTA) (NanoSight 300; Malvern, Amesbury, UK), equipped with a 488 nm laser and a high sensitivity CMOS camera. EVs were isolated by SEC and fractions 7 to 9 were pooled, diluted with 0.1µm filtered PBS and adjusted to a range of $1x10^8 – 9x10^8$ particles per ml. NTA utilizes the properties of both light scattering and Brownian motion in order to obtain the size distribution and concentration of particles in liquid suspension. The software tracks particles individually and using the Stokes-Einstein equation calculates their hydrodynamic diameters.

The measurements of particle concentration and size were based on the average of five videos, each recorded for 60 s. To prevent the interference of small lipoproteins, such as HDL, LDL and VLDL, the threshold was set at 71 nm. Particles of size range from 71-1000 nm were identified as EVs.

4.3.8 Flow cytometric analysis of EVs

PFP samples were analysed for EV subtypes (phosphatidylserine-positive EVs, platelet-derived EVs and endothelial EVs) using a BD FACSCanto II flow cytometer (BD Biosciences, Oxford UK). Reference beads were used to set live gates based on particle size. These consisted of a mixture of non-fluorescent silica ApogeeMix beads (Apogee flow systems, Hemel Hempstead, UK) with diameters of 180 nm, 240 nm, 300 nm, 590 nm, 880 nm and 1300 nm (with a refractive index of η=1.43, similar to the refractive index of EVs η~1.39) and green fluorescent latex beads with diameters of 110 nm and 500 nm (with refractive index η=1.59). The EV detection threshold was set at 240 nm to exclude instrument noise. Because of the unavailability of 1 µm silica beads, the gate was set just above 880 nm silica beads in order to exclude particles > 1µm. The live gate P1 therefore represents EVs, defined as particles within the size range 240 – 1000 nm, with P3 representing platelets (Supplemental Figure 2.1).

To determine the cellular origin of EVs, three EV markers were used; Annexin APC, CD41 PE and CD105 eFlur450. All reagents and buffers were filtered using 0.1 µm pore size centrifugal filter units (Millipore UK Limited, Hertfordshire, UK) for 20 min at low speed (0.1 x g) to remove debris and aggregates. Antibodies were titrated to determine the optimum concentration for EV staining. Frozen PFP was thawed at room temperature on a sample roller. PFP (5 µl) was incubated for 15 min with FcR blocking reagent (5 µl) (Miltenyi Biotec Ltd, Surrey, UK), Annexin V buffer (Cambridge Bioscience Ltd, Cambridge, UK), which contains

the calcium required for Annexin V binding to phosphatidylserine (PS) exposed on the EV surface, and argatroban (1 µl), a synthetic thrombin inhibitor, which is used as an anticoagulant to prevent clotting caused by the calcium in the Annexin V buffer. After the first incubation, PFP was labeled with a panel of antibodies and isotype-matched controls, incubated for 15 min at room temperature in the dark and analysed by FCM (Supplemental Table 2.2). After staining gate (P1) represents annexin positive EVs; gates (P2) and (P3) are subsets of gate (P1) and shows CD41 positive EVs (platelets-derived EVs) and CD105 positive EVs (endothelial-derived EVs) respectively Supplemental Figure 4.1.

BD TruCount tubes (BD Biosciences) containing 47,000 beads were resuspended in 500 µl of filtered PBS and analysed by FCM in order to calibrate the numbers of EVs in the samples.

4.3.9 Thrombin generation assay

The procoagulant activity of EVs was assessed using the Technothrombin MP kit (Technoclone, Vienna), which is based on the thrombin-dependent cleavage of a fluorogenic substrate over time, according to the manufacturer's instructions. Results were recorded using a FlexStation 3 microplate reader (Molecular Devices, San Jose, USA) and the data were analysed using TGA evaluation software (Technoclone, Vienna). The thrombogenicity of EVs was analysed by using two approaches: (i) thrombin generation in PFP compared with that in vesicle-free plasma (VFP) and (ii) thrombin generation in VFP plus EVs isolated by SEC compared with that in VFP alone.

VFP was prepared from pooled plasma from three healthy volunteers. Citrated blood (90 ml) from fasted volunteers was centrifuged at 2,500 x g for 15 min to remove large cells. The resulting plasma was transferred to a clean tube, leaving 1 ml above the buffy coat and centrifuged at 2,500 x g for 15 min to remove platelets and cellular debris. The supernatant was

then centrifuged at 20,000 x g for 1 h, at 4°C in order to pellet large vesicles. The supernatant was subjected to a final centrifugation at 100,000 x g for 1 h, at 4°C to pellet small vesicles. The supernatant (VFP) was aliquoted and stored at -80 °C until further use.

For the second approach (comparison of thrombin generation in VFP with and without EVs), EV fractions eluted by SEC were concentrated using Vivaspin™ 6 Sample Concentrators with 100,000 MWCO (Fisher Scientific, Loughborough) at 1500 x g for 40 min. EV aliquot (10 µl) with final protein concentration of 5 µg/ml was added to 30 µl of VFP, followed by 10 µl of phospholipids and 50 µl of fluorogenic substrate before initiating kinetic reading at 1 min intervals for 60 min at 37 °C, using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

4.3.10 Effect of EVs on clot formation and fibrinolytic activity

The clot-forming and plasmin generation capacity of EVs were assessed by isolating EVs using SEC, adjusting the concentration to 5 µg/ml and applying clot formation and lysis assays adapted from [146] and [147] to compare clot formation and fibrinolytic activity in VFP with VFP 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 analysed using an online tool for analysis of clot and lysis using the Shiny App developed by Longstaff [148].

4.3.11 Statistical analysis

The results are presented as the means ± standard error of the mean. Differences between the three groups were determined using a general linear model followed by post-hoc analysis using Bonferroni tests where applicable. P-values <0.05 were considered statistically significant. Statistical analyses were performed using IBM SPSS statistics 27.

4.4 Results

4.4.1 Subject characteristics

A total of 42 participants with a mean age of 55 years (range 36-73) completed the 12-week intervention. The three groups did not differ significantly with regard to age, gender, weight or a range of physiological parameters (Table 4.3).

4.4.2 Effect of n-3 PUFA on blood lipid profile

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 TAG concentration and an increase in LDL cholesterol concentration following intervention with both oily fish and fish oil supplements (Supplemental Table 4.2).

4.4.3 Consumption of oily fish and fish oil supplements increase EV and RBC content of n-3 PUFA to a similar degree

The EV and RBC content of both EPA and DHA increased significantly and to a similar degree following intervention with oily fish and fish oil supplements, even though the ratio of EPA:DHA was 1:1 in the oily fish meals and 3:1 in the supplements and, the fish oil supplements had a higher content of EPA+DHA compared with the oily fish (Tables 4.4 & 4.5). There were no accompanying changes in the content of any other fatty acids, although there was a trend for a decrease in the proportion of arachidonic acid in EVs from subjects on both the oily fish and the fish oil interventions. In addition, there was a trend for a reduction in the proportion of arachidonic acid in RBCs in the fish oil group only and a trend for a reduction in the proportion of linoleic acid in RBCs in both intervention groups.

4.4.4 Effects of oily fish and fish oil supplements on numbers of circulating EVs

There was a significant effect of the treatment (P=0.004) on numbers of circulating EVs and significant time*treatment interaction ($p<0.001$). EV number has significantly decreased after supplementation with fish oil, while the consumption of oily fish had no effect. Also, there was a significant effect of time ($P<0.001$) (Figure 4.2).

There was no effect of either oily fish or fish oil supplements on the mean size of the EV population (Figure 4.2) or on numbers of annexin V positive or platelet/endothelial-derived EVs (Supplemental Table 4.3).

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

The thrombogenicity of EVs following intervention was analysed using two approaches. In the first approach, the difference in thrombin generation between PFP and VFP indicated the degree to which EVs influenced TF-stimulated thrombin generation in plasma before and after the intervention. Figure 4.3 illustrates the influence of dietary n-3 PUFA on this EV-supported thrombin generation. EVs from subjects supplemented with fish oil had significantly lower thrombogenic capacity than those from subjects consuming oily fish or the control meals and capsules, as determined by peak thrombin concentration, area under the curve (AUC), lag time and velocity index (Figure 4.3). Therefore, only the fish oil supplement influenced the thrombogenicity of EVs, with oily fish having no effect.

The second approach, where the effects of SEC-isolated EVs from subjects who had completed the intervention were added to pooled VFP from healthy subjects, yielded identical results. Once again, only the EVs from the fish oil group decreased TF-stimulated thrombin generation compared with both baseline and with the other intervention groups (Figure 4.3).

4.4.6 Effect of oily fish and fish oil supplements on ability of EVs to induce clot formation and lysis

Just as the addition of isolated EVs to VFP supported TF-stimulated thrombin generation, it also supported clot formation in the presence of calcium. Intervention with oily fish or fish oil supplements did not affect the majority of clot formation parameters, apart from a reduction in the area under the curve (AUC) for clot formation in the fish oil group (Figure

4.4). EVs also supported fibrinolytic activity, but there was no effect of either of the interventions. (Figure 4.4).

4.5 Discussion:

Recent reviews and meta-analyses support evidence for cardioprotective effects of n-3 PUFA [98, 191, 197], which may be manifested through anti-inflammatory effects [198], improvement in endothelial and vascular function [199], contribution to plaque stability [200], lowering of plasma triacylglycerol concentration [201] and anti-thrombotic effects [202]. To our knowledge, this is the first study examining the effects of n-3 PUFA in the form of oily fish, at a level achievable in the diet, in comparison to fish oil supplements on EV number and functional activity. EVs are emerging risk markers for CVDs and high numbers of circulating EVs are associated with conventional CVD risk markers [203] and patients with CVDs [204]. This study demonstrates for the first time that 12 weeks of intervention with n-3 PUFA in the form of oily fish or fish oil supplements resulted in similar incorporation of EPA and DHA into EVs, despite a difference in dose of n-3 PUFA but had different effects on EV numbers and procoagulant activity. Fish oil supplements, but not oily fish, decreased numbers of total circulating EVs and decreased the procoagulant activity of EVs, but there was no effect of either oily fish or supplements on EV subtypes or fibrinolytic activity. These findings suggest that supplementation with fish oil supplements is more effective than oily fish in terms of altering the number and procoagulant activity of EVs.

The dramatic reduction in numbers of circulating EVs in only the fish oil group confirms previous observations that n-3 PUFA at a dose of approximately 2 g/d alters numbers of circulating EVs in subjects at moderate risk of CVDs [99, 103]. The lack of effect in the oily fish group could be due to the lower dose of n-3 PUFA and/or the difference in ratio of EPA:DHA, which was approximately 1:1 in the oily fish meals and 3:1 in the supplements.

Overall, the supplements delivered 2g/d EPA+DHA, whereas the oily fish meals delivered 1 g/d EPA+DHA, with the dose of DHA being identical in the two groups, but the fish oil supplements delivering three times as much EPA and the oily fish meals. Despite this, the fatty acid composition changes in RBCs and EVs were comparable in the two groups. In addition, the provided dose of EPA+DHA from the oily fish group in this study could be too low to reduce EV number, where a dose of 1.5 g/d EPA+DHA has been found to decrease the number of endothelial-derived EVs but no effect on platelet derived EVs. This raises the question of the threshold dose of n-3 PUFA required to significantly reduce numbers of circulating EVs.

A small number of studies have compared intervention with n-3 PUFA in the form of oily fish and fish oil capsules on incorporation of EPA and DHA into plasma and cellular lipids. Harris et al. [205] demonstrated equivalent incorporation 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. 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 [206]. In the current study, the ratio of EPA:DHA was dictated by the availability of intervention materials and by logistical constraints but is clearly an important consideration for comparative interventions of this type. It is interesting to note that the degree of incorporation of n-3 PUFA in the two groups relative to baseline and to the control group was similar and that the fatty acid composition of EVs and RBCs at the end of the intervention was not different between the two groups despite the difference in EPA:DHA ratio, although there was a trend towards an increase in the EPA content of RBCs in the fish oil group compared to the oily fish group. It is possible that the lack of significant

difference is due to the relatively small sample size, particularly for EVs, where detection of small differences in the content of minor fatty acids in these cell-derived particles is challenging.

Nevertheless, the fact that only the fish oil supplements altered EV number and function suggests that EPA rather than DHA may have been driving the effects. It has been demonstrated elsewhere that EPA and DHA have differential effects on membrane structure and lipid rafts, with DHA showing a greater ability to modify lipid rafts, which might lead to differential effects on membrane blebbing, membrane fluidity and cytosolic Ca2+ concentration [207], and therefore subsequently on EV production. Browning et al. [208] compared weekly vs daily consumption of matched doses of EPA and DHA (provided in the form of capsules at a dose of approximately 1 g/d) and reported that daily supplementation resulted in greater enrichment of EPA and DHA into cells compared with weekly supplementation. This implies that consuming oily fish twice per week may have been expected to result in a lower degree of incorporation of n-3 PUFA into cells than fish oil supplements simply due to frequency of consumption. However, this did not appear to be the case in the current study, where incorporation of n-3 PUFA in RBCs and EVs was approximately equivalent for the supplements and oily fish meals.

Both fish oil supplementation and consumption of oily fish are widely reported to reduce plasma triacylglycerol concentration [98], but the current study demonstrated only a trend, despite a significant effect having been reported previously at a dose of 1.8 g/d n-3 PUFA, albeit with a slightly greater sample size [99]. This suggests that the impact of n-3 PUFA on EV number and function appears to be greater than the well-characterised effects on plasma triacylglycerol concentration.

In the current study, although fish oil significantly decreased total numbers of circulating EVs, neither fish oil nor oily fish altered numbers of EV subtypes, including annexin V positive or annexin V positive platelet/endothelial EVs. In contrast, Del Turco et al [103] reported a significant decrease in both platelet- and monocyte-derived EVs, but not endothelial EVs, following intervention with fish oil supplements providing a dose of 4.3 g/d n-3 PUFA for 12 weeks in patients who had experienced a previous myocardial infarction. The authors attributed this to the ability of n-3 PUFA to modulate platelet and monocyte function through modification of membrane composition, fluidity and cytosolic calcium concentration, which they suggested would reduce cell activation and apoptosis, resulting in lower EV release [103]. Unlike the Del Turco study [103], Wu et al., [99] demonstrated that an 8 week intervention with n-3 PUFA in the form of supplements (providing 0.9 g EPA and 0.6 g DHA) decreased the number of endothelial EVs, but had no effect on numbers of circulating platelet-derived EVs. The reasons for the inconsistency are not clear, but perhaps represent the challenges associated with comparative analysis of studies employing different subjects, doses, study designs and analytical techniques.

EVs supported thrombin generation in a dose-dependent manner and blocking PS with annexin V reduced thrombin generation in a dose-dependent manner, illustrating the importance of PS exposure in the thrombogenicity of EVs. PS exposure on the outer leaflet of plasma membrane is regarded as the key regulator of TF-mediated procoagulant activity at the cell surface [209] and PS positive EVs have been shown to contribute to thrombin generation [210]. A procoagulant role for PS is well-characterised [145, 211] and EVs have been reported to possess procoagulant and fibrinolytic activities through the exposure of TF, one of several possible coagulation pathways [212, 213]. Furthermore, inhibition of TF reduces thrombin generation [214]. Adding EVs to PFP resulted in increased clot formation through the TF

pathway, with PS providing a catalytic surface for the formation of tenase and prothrombinase complexes and triggering conversion of TF to its biologically active form [215]. EVs are also reported to participate in fibrinolysis by producing plasmin [216]. Endothelial and leukocyte-derived EVs express the urokinase-type plasminogen activator and its receptor on their surface, which is involved in the conversion of plasminogen to plasmin [217].

N-3 PUFA are suggested to play an important role in haemostasis and exhibit anticoagulant properties, for example by altering platelet lipid composition, inhibiting collagen-mediated platelet activation and modifying the release of thrombotic substances that employ activated platelets to form a clot [218, 219]. Moreover, an increase in the EPA and DHA content of platelets leads to the reduction of PAC-1 (marker of α-granule secretion) binding, P-selectin exposure and Annexin-V binding, which indicates inhibition of the binding of platelets to fibrinogen and of their ability to generate thrombin [219, 220].

In the current study, n-3 PUFA in the form of fish oil dramatically reduced TF-stimulated thrombin generation but had no effect when delivered in the form of oily fish. Del Turco et al [103] also reported a decrease in the TF-dependent procoagulant activity of EVs following fish oil supplementation at a dose of 4.3 g/d in patients with post-myocardial infarction. The authors attributed this reduction in EV thrombogenicity to a reduction in TF activity on the EV surface and proposed that this may be associated with the modulation of phospholipids involved in initiation of coagulation, particularly anionic phospholipids such as PS. There are too few published studies to ascertain whether the lack of effect of the oily fish intervention was due to the lower dose of n-3 PUFA or the greater proportion of EPA compared with the fish oil supplements. It has been reported that EPA, but not DHA, reduces mean platelet volume, which is a marker of platelet activation and blood coagulation [221]. EPA has been also shown to

lower circulating levels of apolipoprotein B, which is associated with lower levels of PAI-1, and therefore atherothrombotic events [213]. There are also differential effects of EPA and DHA on pathways for lipid mediator synthesis [222]. It would be important to conduct dose-response studies of the effects of fish oil containing different ratios of EPA and DHA on both EV number and thrombogenicity to clarify the likely thresholds for the effects and the influence of the ratio of EPA:DHA. This would also be important in evaluating implications for dietary guidance.

In conclusion, this study demonstrates that fish oil alters the number and thrombogenicity of circulating EVs and this cannot be replicated by a relatively high dose of n-3 PUFA delivered through oily fish meals. The greater proportion of EPA in the fish oil supplements may be a key factor in these differential effects and further work to understand the influence of dose and ratio of EPA and DHA on EV number and function is warranted.

4.6 Acknowledgement:

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Author contribution:

GC, JP, PY and PC designed the study together with the sponsors. GC and JP ran the intervention study, HF and PC conducted the fatty acid composition analysis, and AA and DT conducted the remaining experimental work, under the supervision of PY.

Table 4.1 Fatty acid composition of fish oil and control oil capsules

	Fish oil capsules		Control capsules (olive oil)	
Fatty acids	g/capsule	(wt%)	g/capsule	(wt%)
Myristic Acid C14:0	0.0003	0.0375	0.0001	0.0198
Tetracosanoic C24:0	0.0038	0.3875	0.0007	0.0761
Oleic acid C18:1n9	0.0042	0.425	0.6694	66.945
Linoleic Acid C18:2n6	0.0016	0.1625	0.0871	8.7133
Dihomo-gamma-linolenic C20:3n6	0.0018	0.1875	0	0
Arachidonic Acid C20:4n6	0.0195	1.95	0	0
Alpha Linolenic Acid (ALA) C18:3n3	0.002	0.2	0.0065	0.6567
Eicosatetraenoic (ETA) C20:4n3	0.0206	2.0625	0	0
Eicosapentaenoic (EPA) C20:5n3	0.7543	75.437	0.0003	0.03208
Heneicosapentaenoic (HPA) C21:5n3	0.0197	1.975	0	0
Docosapentaenoic (DPA) C22:5n3	0.0166	1.6625	0.0005	0.0506
Docosahexaenoic (DHA) C22:6n3	0.2671	26.712	0.0006	0.0604
Total Saturated fatty acids	0.0042	0.425	0.1401	14.011
Total Monounsaturated fatty acids	0.0108	1.0875	0.6771	67.718
Total n-3 PUFA/capsule	1.1038	110.38	0.0079	0.7998
	g per 2 fish oil capsules/day			
Total n-3 PUFA	2.2			
EPA	1.5			
DHA	0.53			
EPA+DHA	2			

Table 4.2 Fatty acid composition of the whole homogenized fish meals

parameter	Oily fish		Control fish
	Piri Piri Fish (Salmon) g/portion	Katsu Fish Curry (Mackerel) g/portion	Piri Piri Fish (White Fish) g/portion
Meal weight	534 g	586 g	378 g
Fish weight	226 g	229 g	110 g
Total calories	837 kcal	845 kcal	861 kcal
Protein	69	70	72
Carbohydrates	56	57	58
Fat	36	36	37
Alpha Linolenic Acid (ALA) C18:3n3	0.7	0.9	0.6
Eicosapentaenoic (EPA) C20:5n3	0.9	2.2	0.04
Docosapentaenoic (DPA) C22:5n3	0.2	0.4	<0.04
Docosahexaenoic (DHA) C22:6n3	0.9	3.2	0.1
Total n-3 PUFAs	2.7	6.7	0.94

	g/day (oily fish)
Total n-3 PUFA	1.44
EPA	0.4
DHA	0.58
EPA+DHA	1

Table 4.3 Baseline subject characteristics

	Oily fish (n=14)	Control (n=13)	Fish oil supplement (n=15)	p-value
Subject characteristics				
Age (years)	56.14±2.50	51.16±2.00	57.23±2.76	0.209
Gender	1.36±0.13	1.18±0.12	1.23±0.12	0.603
Height (m)	1.65±0.02	1.68±0.02	1.69±0.02	0.561
Weight (kg)	68.85±2.83	73.63±4.64	75.86±3.40	0.370
BMI (kg/m^2)	25.16±0.98	25.83±1.50	27.10±1.45	0.675
SBP (mm Hg)	128.15±3.96	125.18±6.13	127.91±4.67	0.855
DBP (mm Hg)	72.30±2.88	72.81±2.35	69.66±2.25	0.586
Biochemical values (mmol/L)				
Total cholesterol	4.30±0.17	4.57±0.30	4.90±0.28	0.079
Triglycerides	1.31±0.29	0.95±0.15	1.21±0.15	0.769
HDL	1.15±0.08	1.48±0.08	1.41±0.05	0.062
LDL	2.55±0.18	2.66±0.23	2.94±0.23	0.106
Glucose	4.93±0.09	4.84±0.13	5.20±0.21	0.060
FMD measures				
MAP (mm Hg)	90.92±2.99	90.27±3.38	89.08±2.55	0.904
HR (beats/pm)	68.38±3.40	62.36±4.02	64.91±2.49	0.419
Baseline diameter (mm)	0.37±0.01	0.34±0.02	0.38±0.01	0.317
Peak diameter (mm)	0.40±0.01	0.35±0.02	0.40±0.02	0.159
FMD%	7.30±1.14	4.39±0.53	7.18±0.94	0.068
TTP (s)	53.43±5.14	67.81±11.2	56.87±7.17	0.423
SRAUC	5.1E+4±2.1E+3	2.1E+4±3.3E+3	2E+4±3.2E+3	0.985

Values are mean ± SEM. SBP: FMD, Flow- mediated dilation, BMI: body mass index, systolic blood pressure, SBP: systolic blood pressure, HDL: high-density lipoprotein, LDL: low-density lipoprotein, MAP: mean atrial pressure, HR: heart rate, TTP: time to peak, SRAUC: shear rate area under the curve. There were no significant differences observed between the treatment groups (General Linear Model).

Table 4.4 Fatty acid composition of EVs before and after intervention.

	Oily fish		Placebo		Fish oil supplement		P value
Fatty acids	PRE (%)	POST (%)	PRE (%)	POST (%)	PRE (%)	POST (%)	
SFA							
Palmitic acid (16:0)	25.86±0.62	25.15±0.55	24.50±0.38	24.75±0.52	24.83±0.64	26.05±0.61	0.372
Stearic acid (18:0)	8.93±0.75	8.56±0.57	9.83±0.74	9.60±0.80	9.66±0.91	8.71±0.55	0.570
MUFA							
Oleic acid (18:1, n-9)	30.34±1.08	29.21±1.07	30.52±1.08	29.89±0.93	30.28±1.03	28.43±0.66	0.805
n-3 PUFA							
DPA (22:5, n-3)	0.44±0.02	0.42±0.02	0.36±0.02	0.31±0.01	0.36±0.02	0.39±0.02	0.249
DHA (22:6, n-3)	**1.10±0.08**	**1.53±0.06**	**0.91±0.07**	**0.94±0.10**	**1.06±0.06**	**1.45±0.07**	**<0.001**
EPA (20:5, n-3)	**0.45±0.03**	**1.03±0.06**	**0.44±0.03**	**0.48±0.04**	**0.43±0.03**	**1.07±0.06**	**<0.001**
ALA (18:3, n-3)	0.25±0.02	0.20±0.02	0.23±0.01	0.23±0.02	0.25±0.02	0.25±0.01	0.386
n-6 PUFA							
Linoleic acid (18:2, n-6)	17.56±0.77	18.58±0.96	17.44±1.05	17.97±1.08	17.15±0.44	17.48±0.44	0.740
Arachidonic acid (20:4, n-6)	3.68±0.20	3.05±0.23	3.69±0.16	3.44±0.23	3.41±0.21	2.90±0.13	0.247
Total							
Total SFA	35.44±1.03	34.32±0.93	35.28±0.70	35.37±1.07	35.25±1.06	35.54±0.87	0.883
Total MUFA	33.72±1.08	32.47±1.03	33.93±1.11	33.33±0.95	33.34±1.11	31.84±0.76	0.735
Total n-3 PUFA	**2.98±0.13**	**4.17±0.14**	**2.98±0.19**	**2.88±0.17**	**2.76±0.13**	**4.11±0.17**	**0.003**
Total n-6 PUFA	23.54±0.86	24.14±1.04	23.59±1.07	24.02±1.20	22.79±0.52	22.60±0.50	0.465

Values are expressed as mean ± SEM. DPA: Docosapentaenoic acid, DHA: Docosahexaenoic acid, EPA: Eicosapentaenoic acid, ALA: α-Linolenic acid, SFA: saturated fatty acids, MUFA: mono-unsaturated fatty acids, n-3 PUFA: n-3polyunsaturated fatty acids, n-6 PUFA: n-6 polyunsaturated fatty acids. Data were analysed using general linear model**. DHA:** There was a significant effect of the treatment on EV composition of DHA ($p<0.001$) and significant time*treatment interaction ($p=0.004$). DHA concentration has increased after fish oil and oily fish groups compared to the control group (p-values are $p=0.004$ and $p<0.001$, respectively). However, there was no difference between fish oil and oily fish groups. There was also a significant effect of time ($p<0.001$). **EPA:** There was a significant effect of the treatment on EV composition of EPA ($p<0.001$) and significant time*treatment interaction ($p<0.001$). EPA concentration has increased after fish oil and oily fish groups compared to the control group ($p<0.001$). However, there was no difference between fish oil and oily fish groups. There was also a significant effect of time ($p<0.001$). **Total n-3 PUFA:** There was a significant effect of the treatment on EV composition of total n-3 PUFA ($p=0.003$) and significant time*treatment interaction ($p<0.001$). Total n-3 PUFA concentration has increased after fish oil and oily fish groups compared to the control group (p-values are $p=0.004$ and $p=0.024$, respectively). However, there was no difference between fish oil and oily fish groups. There was also a significant effect of time ($p<0.001$).

Table 4.5 Fatty acid composition of RBCs before and after intervention.

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

Values are expressed as mean ± SEM. DPA: Docosapentaenoic acid, DHA: Docosahexaenoic acid, EPA: Eicosapentaenoic acid, ALA: α-Linolenic acid, SFA: saturated fatty acids, MUFA: mono-unsaturated fatty acids, n-3 PUFA: n-3polyunsaturated fatty acids, n-6 PUFA: n-6 polyunsaturated fatty acids ($p<0.001$). **DHA:** There was a significant effect of the treatment on RBC composition of DHA ($p=0.005$) and significant time*treatment interaction ($p=0.001$). DHA concentration has increased after fish oil and oily fish groups compared to the control group (p-values are $p=0.022$ and $p=0.007$, respectively). However, there was no difference between fish oil and oily fish groups. There was also a significant effect of time ($p<0.001$). **EPA:** There was a significant effect of the treatment on RBC composition of EPA ($p<0.001$) and significant time*treatment interaction ($p<0.001$). EPA concentration has increased after fish oil and oily fish groups compared to the control group ($p<0.001$). However, there was no difference between fish oil and oily fish groups. There was also a significant effect of time ($p<0.001$). **Total n-3 PUFA:** There was a significant effect of the treatment on RBC composition of total n-3 PUFA ($p=0.003$) and significant time*treatment interaction ($p<0.001$). Total n-3 PUFA concentration has increased after fish oil and oily fish groups compared to the control group (p-values are $p=0.004$ and $p=0.024$, respectively). However, there was no difference between fish oil and oily fish groups. There was also a significant effect of time ($p<0.001$). **Total n-6 PUFA:** There was a significant effect of the treatment on RBC composition of total n-6 PUFA ($p=0.037$) and significant time*treatment interaction ($p=0.008$). Total n-6 PUFA concentration has increased after oily fish group compared to the control group (p-values =0.043). However, there was no difference between fish oil and oily fish groups, and no difference between fish oil and control. There was also a significant effect of time ($p<0.001$).

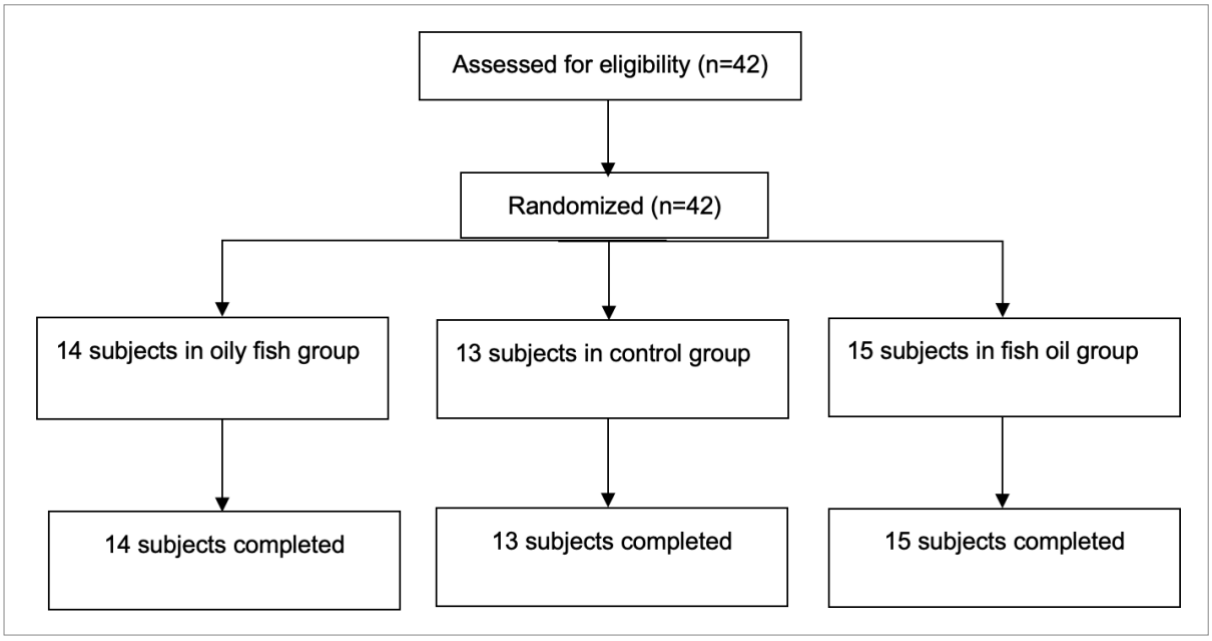


Figure 4.1 Participant flow diagram for the intervention study

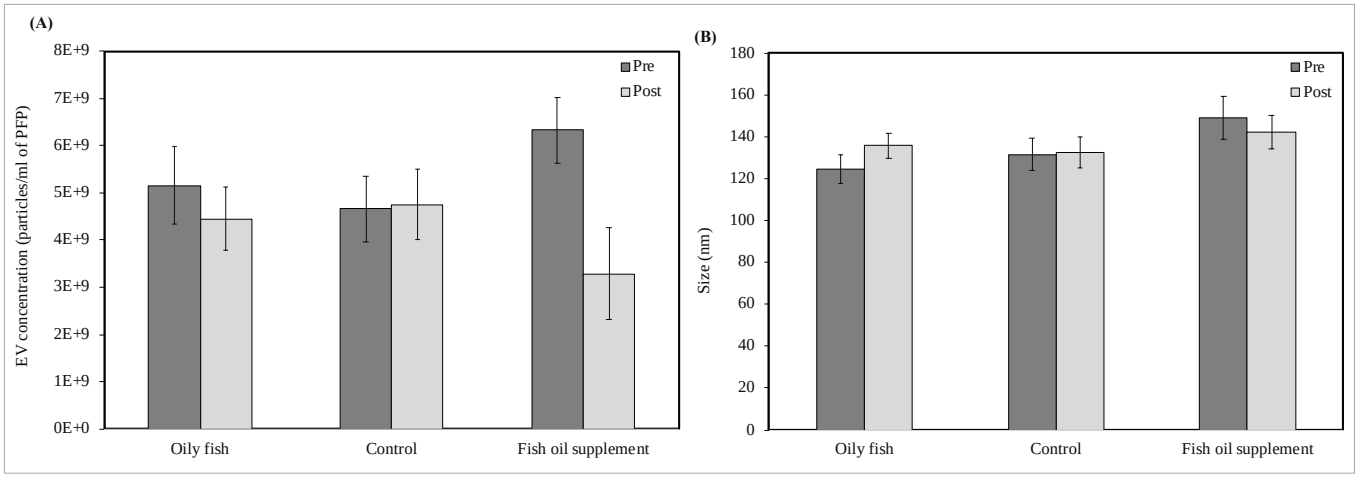


Figure 4.2 Effect of oily fish and fish oil supplements on the number (A) and mean size (B) of circulating EVs as measured by general linear model (n=42). (A) There is a significant effect of the treatment on EV number ($p=0.004$) and significant time*treatment interaction ($p<0.001$). EV number has significantly decreased after fish oil supplement compared to oily fish ($p=0.023$) and control ($p=0.007$). Also, there was a significant effect of time on EV total concentration ($p<0.001$). (B) There were no statistically significant effects of oily fish and fish oil on EV size ($p=0.299$) and no significant effect of time on EV size ($p=0.389$). Data are means ± SEM.

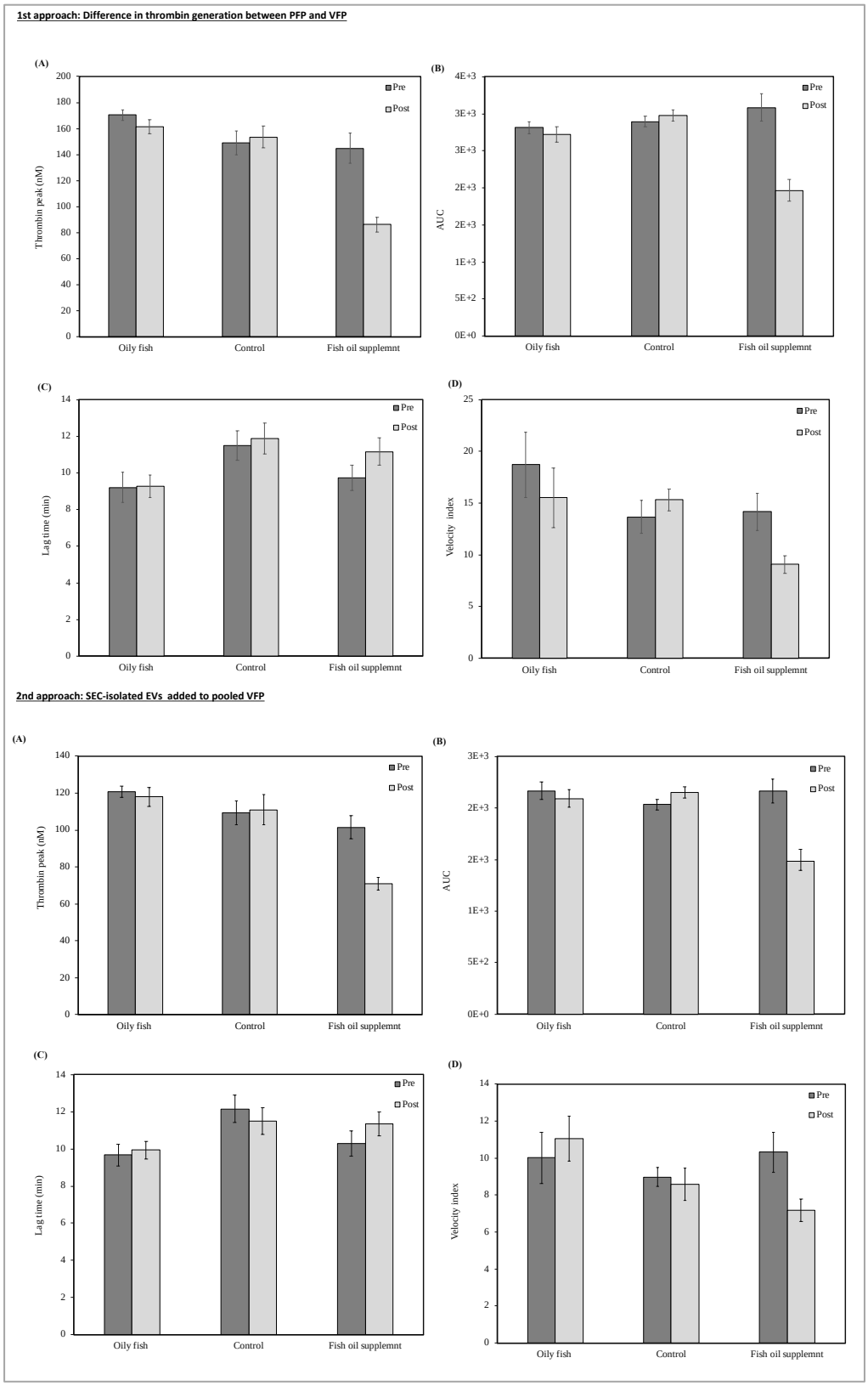


Figure 4.3 Effect of intervention with oily fish or fish oil supplements on EV-supported thrombin generation as measured by general linear model (n=42). In the 1st approach (PFP vs VFP), **(A, B, D)** there were significant effects of the treatment on EV-dependant thrombin peak ($p<0.001$), AUC ($p<0.001$) and velocity index ($p=0.015$), where thrombin peak, AUC and velocity index have decreased after fish oil compared to oily fish and control. Also, there were significant time*treatment interaction and significant effect of time ($p<0.001$). **(C)** However, there were no statistically significant effects of the treatment and time on lag time.
In the 2nd approach (isolated EVs added to VFP). **(A, B, D)** There were a significant effect of the treatment on EV-supported thrombin peak ($p<0.001$), AUC ($p<0.001$) and velocity index (0.017), where thrombin peak, AUC and velocity index have decreased after fish oil compared to oily fish and control. Also, there were significant time*treatment interaction ($p=0.001$) and significant effect of time ($p=0.010$). **(C)** However, there were no statistically significant effects of the treatment and time on lag time. Data are means ± SEM.

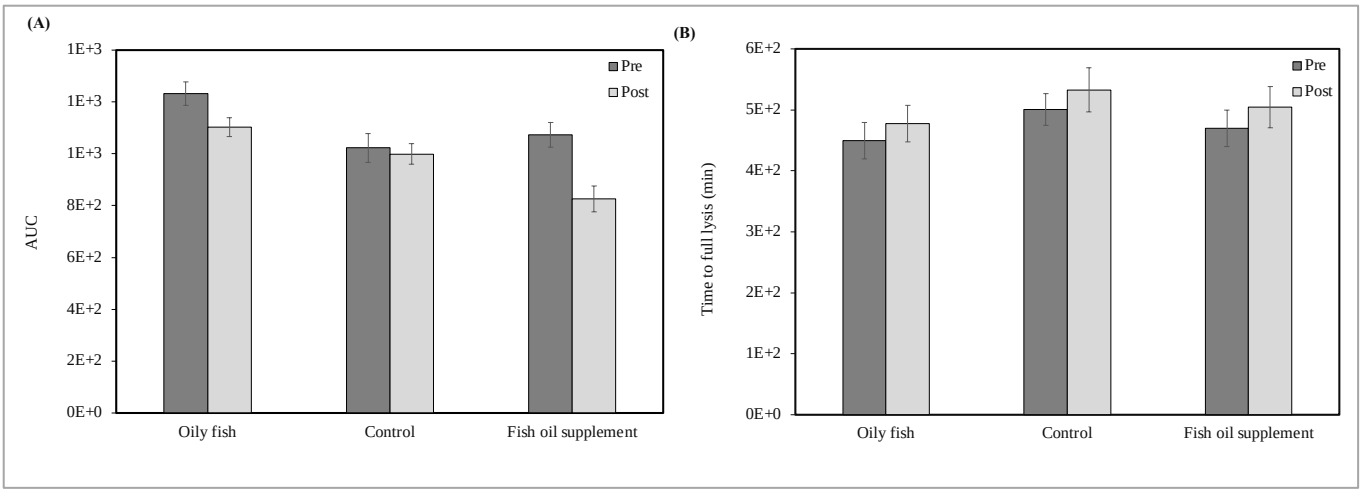


Figure 4.4 Effect of intervention with oily fish or fish oil supplements on clot formation and lysis supported by EVs as measured by general linear model (n=42). (A) There was a significant effect of the treatment on EV dependant clot formation as indicated by AUC (p=0.002), where fish oil supplement significantly decreased the clot formation capacity as indicated by AUC compared to oily fish group and control group (p=0.002). There are also significant time*treatment interaction and significant effect of time (p<0.001). **(B)** There is no significant effect of the treatment and time on EV dependant clot lysis. Data are means ± SEM.

Supplementary material

Supplemental Table 4.1 Scoring tool for recruitment. The scoring tool was based on the Framingham Risk Score system [195], adapted by Chong et al [196] to include a score for family history of MI or T2D [223]. Participants were required to score ≥ 2 points to have an RR ≥ 1.5 of developing CVD, which could be achieved through a combination of CVD risk factors. MI, myocardial infraction; T2D, type 2 diabetes; NA, not applicable.

CVD risk factors	1 point		2 points	
	Men	Women	Men	Women
Total Cholesterol (mmol/L)	5.18-6.21	5.18-6.21	6.22-7.99	6.22-7.99
HDL Cholesterol (mmol/L)	0.91-1.16	1.17-1.29	<90	<1.16
Glucose (mmol/L)	6.00-6.99	6.00-6.99	NA	NA
BMI (kg/m^2)	25.5-29.9	25.5-29.9	30.0-39.9	30.0-39.9
Waist circumference (cm)	>94	>80	>102	>88
Systolic blood pressure (mm Hg)	130-139	130-139	140-159	140-159
Diastolic blood pressure (mm Hg)	NA	NA	90-99	90-99
First degree relative diagnosed with MI or T2D (age of diagnosis) (y)	NA	NA	<55 y in male relatives; <65 y in female relatives	<55 y in male relatives; <65 y in female relatives

Supplemental Table 4.2 Plasma lipid profile pre and post intervention (n=42).

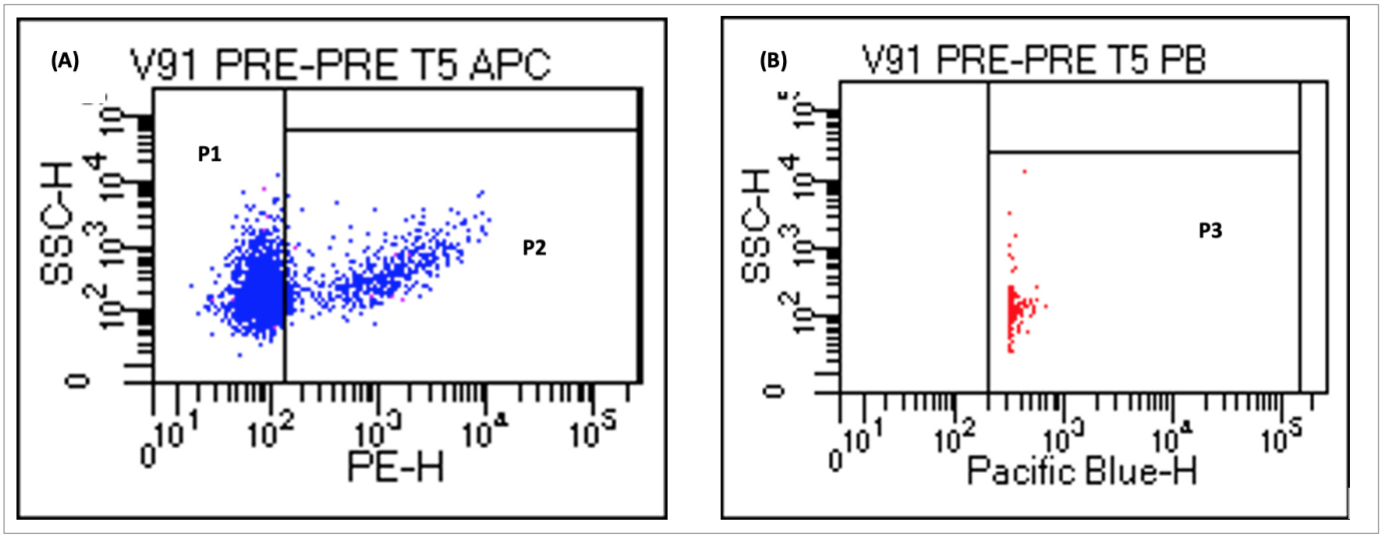
Plasma lipids (mmol/L)	Oily fish		Control		Fish oil supplement		P value
	PRE	POST	PRE	POST	PRE	POST	
Total cholesterol	4.30±0.17	4.70±0.17	4.57±0.30	4.79±0.35	4.90±0.28	4.99±0.25	0.218
LDL cholesterol	2.55±0.18	2.99±0.15	2.66±0.23	2.84±0.30	2.94±0.23	3.11±0.21	0.218
HDL cholesterol	1.15±0.08	1.30±0.08	1.48±0.08	1.54±0.06	1.41±0.05	1.43±0.10	0.173
Triglycerides	1.31±0.29	0.89±0.15	0.95±0.15	0.90±0.19	1.21±0.15	0.99±0.07	0.988

Values are expressed as mean ± SEM. LDL: low density lipoprotein, HDL: high density lipoprotein.

Supplemental Table 4.3 Effects of fish oil or oily fish on numbers of EV subtypes (n=42).

Group	Annexin V positive EVs		Platelets-derived EVs		Endothelial- derived EVs	
	Pre Mean ± SE	Post Mean ± SE	Pre Mean ± SE	Post Mean ± SE	Pre Mean ± SE	Post Mean ± SE
Oily fish	1.19E+8±1.43E+7	6.90E+7±1.31E+7	7.86E+7±1.33E+7	3.96E+7± 7.11E+6	1.43E+7±4.33E+6	8.14E+6 ±1.90E+6
Placebo	1.16E+8±4.12E+7	6.81E+7±1.63E+7	8.63E+7±3.97E+7	4.22E+7± 7.43E+6	1.36E+7±4.54E+6	8.26E+6±1.21E+6
Fish oil	1.16E+8 ±1.61E+7	6.22E+07±9.30E+6	5.59E+7±9.76E+6	3.34E+7±9.89E+6	1.75E+7±2.08E+6	1.23E+7±1.46E+6
P-value	*P*= 0.976		*P*= 0.567		*P*= 0.387	

Data are shown as mean ± SE. Difference in means was analysed using general linear model.



Supplemental Figure 4.1. EV gating strategy. (A) gate (P1) shows annexin positive EVs; **(A, B)** gates (P2) and (P3) are subsets of gate (P1) and shows CD41 positive EVs (platelets-derived EVs) and CD105 positive EVs (endothelial-derived EVs) respectively.

Chapter V: General discussion, future perspectives, and conclusion

This chapter summarises and reflects on the key findings of the studies presented in this thesis and makes some recommendations for future research.

5.1 introduction

Cardiovascular diseases are the leading cause of death globally accounting for almost 18 million deaths [42]. EVs are small vesicles released from activated cells and their number has been reported to increase in patients with CVD compared to healthy individuals [224]. Therefore, measuring EV concentration can provide useful information when making comparisons between health and disease. Despite the availability of various techniques for EV isolation, separating EVs from biological fluids is potentially hampered by the presence of non-EV particles such as lipoproteins. There is a lack of information about whether lipoproteins co-isolate with EV and interfere with its quantification and labelling in the postprandial state. The aim of chapter 2 was therefore to test the efficiency of SEC in removing lipoprotein particles from EV enriched fractions, and to ascertain the reliability of the reported findings in relation to EV quantification and function.

IE were introduced in food industries to replace hydrogenated fats due to their lower content of TFA and SFA [72, 73]. Despite the fact that dietary fats have been widely investigated in relation CVD, there is very little information on IE fats and CVD. Postprandial lipemia has been associated with endothelial dysfunction, cell activation, blood coagulation and EV release [109-111, 167, 168], but there has been no study investigating the effects of IE fats on EV number and its procoagulant activity. Therefore, chapter 3 focused on investigating the effects of IE fats on the number and procoagulant behaviour of EVs in healthy subjects.

N-3 PUFAs have been demonstrated to have cardioprotective effects on CVD and although the strength of evidence has been questioned, most of the recent meta-analyses and systematic reviews support the cardioprotective role of n-3 PUFA [191, 192]. N-3 PUFA have also been demonstrated to protect against CVD by improving endothelial and vascular function [199], contributing to plaque stability [200], reducing plasma TAG [201] and exerting anti-thrombotic effects [202]. The literature on n-3 PUFA has focused mainly on studying the effects of n-3 PUFA capsules on CVD markers, but there is limited data on oily fish and CVD. In addition, a few studies have investigated the effects of n-3 PUFA capsules on EVs and reported a decrease in EV number and thrombotic behaviour [99, 103]. However, there is no study examining the effects of n-3 PUFA in the form of oily fish, at a level achievable in the diet, in comparison to fish oil supplements on EV number and thrombogenic activity. Chapter 4 therefore examines, for the first time, whether n-3 PUFA delivered in the form of oily fish are able to modify the profile and coagulatory behaviour of EVs in the circulation in the same way as fish oil supplements.

The key findings of this thesis are summarised below:

- SEC is an efficient method for purifying EV free from contamination with lipoproteins, such as CM and VLDLs.

- In healthy human volunteers, there was no difference in the number and thrombogenic activity of circulating EV following the consumption of industrially produced IE fats and non-IE fats, but there was a marked postprandial effect on the number and thrombogenicity of EVs following consumption of a high fat meal, regardless of the type of fat.

- In subjects with moderate CVD risk, fish oil supplements reduced the number and thrombogenic activity of circulating EVs compared to oily fish. The higher content of EPA in the fish oil capsules might be the important element in driving the protective effects on EVs.

5.2 Do lipoproteins co-isolate with EVs in the postprandial state?

Although different techniques have been developed for EV isolation, it was unknown whether lipoproteins particles co-isolate with EVs in the postprandial state after consuming a high fat meal, particularly when using size-based isolation method. To address this gap in knowledge, the first trial (chapter 2) investigated the presence of non-EV components, whose size overlaps with EVs. The results in chapter 2 demonstrated that SEC is efficient in separating EV enriched fractions (7,8,9) free from contamination with lipoproteins, since ApoB48 and ApoB100 eluted after the EV fractions, indicating that lipoproteins eluted later than EVs. This is consistent with lipoproteins generally having a smaller diameter than EVs. The efficacy of SEC in removing contaminants from EV fractions was also confirmed by the fact that purified lipoproteins (CM, VLDL1 and VLDL2) subjected to SEC did not elute in EV enriched fractions and only eluted in the later fractions. The majority of EVs are reported to elute in fractions 7,8 and 9, which contain minimum interference with plasma proteins and although some EVs will appear later than fraction 9, only fractions 7,8 and 9 were collected for EV analysis to avoid collecting any contaminating proteins [36, 138, 139]. It has also been reported that SEC is efficient in removing HDL and VLDLs particles from EV fractions [134]. Other particles such as LDL and albumin were not investigated in chapter 2, since their size is less than 70 nm and therefore they would be retained by the beads in the column, and elute much later than EVs and the larger lipoproteins.

Since FCM can detect all EVs and non-EV particles above the detection threshold, fluorescent markers used to stain EVs may also stain lipoproteins due to non-specific binding and cross-reactivity, leading to misidentification of EVs [136]. There was very little information about the potential interference of lipoproteins with EV labelling. Chapter 2 investigated whether lipoproteins interfere with the staining specificity of EV by FCM. Lipoprotein fractions (CM, VLDL1 and VLDL2), prepared by density gradient centrifugation and stained using EV specific markers to detect annexin V positive EVs, platelet-derived EVs and endothelial derived EVs contained very small amounts of EVs. The use of ApogeeMix reference beads in this project for EV gating with a threshold refractive index of η=1.43, similar to the refractive index of EVs η~1.39 helps to discriminate between EV and lipoprotein populations. This consequently prevent the detection of lipoprotein particles and its contents (lipids and proteins) which have a refractive index of > 1.42, while EVs have a refractive index equal to 1.39 [136]. In addition, the minimum detection threshold for the FCM was set at 240 nm to avoid background and instrument noise. Consequently, this will exclude any labelling for lipoprotein particles smaller than 240 nm in diameter, which includes the majority.

Taken together, SEC is an effective method to separate EVs from potential contaminating particles and collecting only the purest EV fractions helps to avoid the interference of contaminants. Additionally, applying reference beads with refractive index similar to EVs for EV gating helps to prevent the detection of lipoproteins within the EV gate.

5.3 Comparing the effects of commercially available IE fats and non-IE fats on number and procoagulant activity of EVs

Despite the wide use of industrially relevant IE fats in food products, their effect on cardiovascular health is unknown [73]. There are very few studies on IE fats and CVD markers. However, these studies have used non commercially available IE fats and reported inconsistent results. Some studies reported an increase in plasma TAG after consuming non-IE fat compared to IE fats [74, 75], while others reported no difference in the plasma TAG between IE fat and non-IE fats [76]. The inconsistency may be due to variations in the solid contents of the test fats, where the digestion of fats with high solid contents is delayed due to their high melting point leading to a slow release of the plasma TAG in the circulation [74, 76]. Chapter 4 compared the effects of commercially available IE fats and non-IE fats on the concentration and procoagulant activity of EVs and demonstrated for the first time that there was no difference in response between IE fat and non-IE fats, where all fat spreads increased the number and procoagulant activity of EVs postprandially. Dietary fats have been studied in relation to EVs and found to increase EV number postprandially [109], but this was the first study to have investigated the effects of industrially available IE fats on EVs. The mechanisms explaining the postprandial increase in EV number and TF-dependent thrombin generation following IE fats are not clear. Postprandial hypertriglyceridemia could be implicated in the elevation of EV numbers, since plasma TAG concentration was strongly associated with EV number, consistent with previous findings [156-158]. Hypertriglyceridemia has been demonstrated to induce the release of cytosolic calcium, stimulate PS exposure into cells and the cleavage of apoptotic markers, indication of cells undergoing apoptosis [159]. Additionally, during hypertriglyceridemia, TAG particles circulate into the blood attached to LDLs, which are small and dense particles, and their metabolism is slow, therefore they exist in the blood for a long time, placing them in direct contact with endothelial cells, where they accumulate

into the sub-endothelium, causing endothelial dysfunction and potentially EV release [160]. Moreover, there was also a positive relationship between EV numbers and insulin concentration, in line with previous studies [161, 162]. High insulin concentrations have been implicated in the apoptotic process and EV production, through the increased production of caspase and lactate dehydrogenase, enzymes released during plasma membrane damage and cell death [166].

Chapter 3 demonstrated a strong postprandial effect, but no difference in EV dependent thrombotic behaviour between IE and non-IE fats. Studies on dietary fat modulation of EV procoagulant activity are limited. The increase in EV procoagulant activity following the consumption of the fat spreads might be due to the increase in PS exposure on EVs (one of the main triggers of coagulation) as measured by FCM. A previous study reported no difference in EV procoagulant behaviour between baseline and the postprandial state due to the unchanged amount of PS exposure on EV at postprandial state compared to baseline, indicating the importance of PS exposure in initiating coagulation [180]. Furthermore, a positive correlation between plasma TAG and insulin concentrations with thrombin AUC mediated by EVs might play a role in EV thrombogenicity. High levels of TAG have been reported to induce coagulation and endothelial dysfunction [178, 179], which might stimulate EV production. In addition, high insulin levels trigger platelet activation [181], stimulate EV release and cause rapid increases in circulating TFs procoagulant activity associated with thrombin generation and acute cardiovascular events [182, 183].

Altogether, both commercially relevant IE and non-IE fat increases the number and procoagulant activity of EVs during the postprandial period, regardless of the structure, type, and content of the consumed fats. In addition, since EV numbers and procoagulant activity

were positively associated with plasma levels of TAG and insulin, postprandial hypertriglyceridemia and hyperinsulinemia might be causally connected to the postprandial increase in EV number and thrombogenicity.

5.4 Comparing the effects of n-3 PUFAs in the form of fish oil capsules and oily fish on number and procoagulant activity of EVs

We have previously demonstrated that supplementation with n-3 PUFA decreased the number of endothelial derived EVs in individuals with moderate risk of CVD [99]. It has been also reported that the consumption of fish oil supplements decreased the number and procoagulant activity of circulating EVs in patients with a previous myocardial infarction [103]. However, the studies relating to n-3 PUFA and EVs focused only on fish oil supplements and there are no studies investigating whether n-3 PUFAs in the form of oily fish, at a level achievable in the diet, alter EV number and procoagulant activity in the same way as fish oil supplements. Chapter 4 demonstrated for the first time that 12 weeks of intervention with fish oil supplements decreased the total number of circulating EVs and TF-dependent thrombin generation, whereas oily fish had no effect. The mechanisms by which only fish oil supplements decreased circulating EV number and procoagulant behaviour are unknown. The lack of effect in the oily fish group could be attributed to the lower dose of n-3 PUFAs delivered in the oily fish group (1.5 g/d) compared to the supplement group (2 g/d), and/or to the difference in ratio of EPA:DHA (1:1 in the oily fish and 3:1 in the fish oil capsules). In addition, the amount of EPA+DHA from the oily fish group (1 g/d), could be too low to exert a favourable effect on EV number and activity, since a dose of 1.5 g/d EPA+DHA has been reported to decrease the number of endothelial-derived EVs, but had no effect on platelet derived EVs. This brings into question the threshold dose of n-3 PUFA sufficient to significantly reduce numbers of circulating EVs.

EPA and DHA have been reported to incorporate into cells following the consumption of oily fish or fish oil capsules in a manner dependent on their ratio [205, 206]. However, chapter 4 demonstrated that there was no difference in the incorporation of EPA and DHA into EVs and RBCs between the fish oil and oily fish groups, despite the difference in EPA:DHA ratio, although there was a trend towards an increase in the EPA content of RBCs in the fish oil group compared to the oily fish group. The lack of significant difference could be due to the relatively small sample size, particularly for EVs, where detection of small differences in the content of minor fatty acids is challenging.

The decrease in the number and function of circulating EVs only in the fish oil supplements group could be due to EPA rather than DHA, since EPA and DHA have been demonstrated to have different effects on membrane structure and lipid rafts, with DHA showing a greater ability to modify lipid rafts, which might lead to differential effects on membrane blebbing, membrane fluidity and cytosolic Ca2+ concentration [207], and therefore subsequently on EV production.

Although fish oil significantly decreased total numbers of circulating EVs, neither fish oil nor oily fish altered numbers of EV subtypes, including annexin V positive or annexin V positive platelet/endothelial EVs. In contrast, fish oil supplements were reported to decrease the number of platelet- and monocyte-derived EVs, but not endothelial EVs [103]. In another study, fish oil supplements reduced the number of endothelial derived EVs, but had no effect on numbers of circulating platelet-derived EVs [99] The inconsistency in findings is unexplained, but indicates the challenges associated with comparative analysis of studies employing different subjects, doses, study designs and analytical techniques.

Furthermore, n-3 PUFA in the form of fish oil supplements reduced TF-stimulated thrombin generation, but had no effect when delivered in the form of oily fish. A previous study has also reported a decrease in the TF-dependent procoagulant activity of EVs following the consumption of fish oil supplements [103]. There are very few studies comparing fish oil and oily fish, which makes it difficult to ascertain whether the lack of effect in the oily fish group was due to the lower dose of n-3 PUFA or lower proportion of EPA compared with the fish oil supplements. However, the decrease in EV procoagulant activity in the fish oil group could be due to the higher proportion of EPA, since EPA, but not DHA, is reported to reduce mean platelet volume, a marker of platelet activation and blood coagulation [221]. EPA has been also reported to reduce the production of apolipoprotein B, which is associated with lower levels of PAI-1 and atherothrombotic events [213].

These findings together demonstrate that n-3 PUFA from fish oil is effective in reducing the number and thrombogenicity of circulating EVs compared to n-3 PUFA from oily fish meals. The higher ratio of EPA in the fish oil supplements may be the important element that drives the favourable effects on the number and procoagulant activity of EVs.

5.5 Future perspectives

This thesis describes some of the current limitations and challenges in EV isolation methods and addresses some of the gaps in relation to EV isolation and characterisation, particularly in the postprandial state, where the number of EVs increases postprandially. Also, it discusses the role of EVs in relation to CVD and the modulation of EVs by dietary fats.

Although, using qEV/70nm columns to isolate EVs is efficient in removing contaminating particles, qEV/70nm column allow EV isolation in the range 70nm-1000nm, excluding those

smaller than 70 nm. Recent availability of qEV/35nm columns will now allow particle isolation in the range 35nm-350nm and is recommended for future studies in combination with the traditional qEV columns to enable the isolation of both small and large EVs. To resolve the possible co-isolation of small lipoproteins (HDL and VLDLs) when using the qEV/35nm columns, magnetic beads loaded with lipoprotein antibodies might be used to pull out the contaminants from EV samples, although this would be very costly.

In this thesis, the detection threshold for the FCM was set at 240 nm to exclude the interference of background noise and this consequently excluded the detection of small EVs. It is recommended to wash the stained EV sample with wash buffer to ensure the removal of any excess antibody [225], which will decrease the background noise and therefore reduce the threshold to detect smaller EVs. In our lab, washing the stained EV sample dramatically reduced the number of positive events, which could be due to the loss of fluorescence intensity. Future work could focus on developing a protocol for removing unbound antibody from EV sample without affecting the fluorescence intensity of the antibodies.

The characterisation protocol used in this thesis included the detection of PS exposing EVs, platelets and endothelial derived EVs. There is a need for the development of a complete labelling protocol to include the labelling of the majority of circulating EVs. It is still challenging to identify a universal EV marker for staining of the majority of the EV population, and the light scatter intensities of most EVs are often below the background noise of FCM. There is a demand to develop a method for the fluorescent labelling of EVs using NTA to provide a reliable detection of EV concentration, phenotyping, and size distribution. Current NTA techniques cannot discriminate between EV subtypes, and this work is still under development.

Dietary fat modulation (including n-3 PUFA and IE fats) of EV number and activity has provided important insight into the important role of dietary fats on EVs and CVD risk. Future work could study in vitro models by incubating these fats with cells and investigate their effects on EV production, composition, and functional activity. The generated EVs could also be injected into animal models to obtain information about the role of those EVs in haemostasis and blood coagulation.

Future work could also focus on investigating the effects of matched doses of n-3 PUFA from fish oil capsules and oily fish on EV production and functional activity. Additionally, further work is required to understand the effects of fish oil containing different ratios of EPA and DHA on both EV number and thrombogenicity to clarify the likely thresholds for the effects and the influence of the ratio of EPA:DHA. This would also be important in evaluating implications for dietary guidance.

5.6 Conclusions

This thesis demonstrated that SEC is an efficient method for purifying EV free from contamination with lipoproteins. It demonstrated that there was a dramatic increase in numbers of EVs during the postprandial period following consumption of a high fat meal, but there were no differences in EV production or thrombogenic activity following acute consumption of industrially produced IE fats and non-IE fats. Fish oil reduced the number and thrombogenic activity of circulating EVs compared to oily fish and the higher content of EPA in the fish oil capsules was likely to be a key factor in the effects.

References:

1. Vader, P., X.O. Breakefield, and M.J. Wood, *Extracellular vesicles: emerging targets for cancer therapy.* Trends Mol Med, 2014. **20**(7): p. 385-93.
2. Andreu, Z. and M. Yáñez-Mó, *Tetraspanins in Extracellular Vesicle Formation and Function.* Frontiers in Immunology, 2014. **5**.
3. Chargaff, E. and R. West, *The biological significance of the thromboplastic protein of blood.* J Biol Chem, 1946. **166**(1): p. 189-97.
4. Hargett, L.A. and N.N. Bauer, *On the origin of microparticles: From "platelet dust" to mediators of intercellular communication.* Pulmonary circulation, 2013. **3**(2): p. 329-340.
5. Raposo, G., et al., *B lymphocytes secrete antigen-presenting vesicles.* J Exp Med, 1996. **183**(3): p. 1161-72.
6. van der Pol, E., et al., *Classification, functions, and clinical relevance of extracellular vesicles.* Pharmacol Rev, 2012. **64**(3): p. 676-705.
7. Heijnen, H.F., et al., *Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules.* Blood, 1999. **94**(11): p. 3791-9.
8. Agouni, A., et al., *Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome.* Am J Pathol, 2008. **173**(4): p. 1210-9.
9. Leroyer, A.S., A. Tedgui, and C.M. Boulanger, *Microparticles and type 2 diabetes.* Diabetes & Metabolism, 2008. **34**: p. 27-32.
10. Lee, T.H., et al., *Microvesicles as mediators of intercellular communication in cancer--the emerging science of cellular 'debris'.* Semin Immunopathol, 2011. **33**(5): p. 455-67.
11. WHO, *International Programme on Chemical Safety Biomarkers in Risk Assessment: Validity and Validation.* . 2001.
12. Strimbu, K. and J.A. Tavel, *What are biomarkers?* Curr Opin HIV AIDS, 2010. **5**(6): p. 463-6.
13. Simpson, R. and S. Mathivanan, *Extracellular Microvesicles: The Need for Internationally Recognised Nomenclature and Stringent Purification Criteria.* Journal of Proteomics & Bioinformatics, 2012. **05**.
14. Kalra, H., G.P. Drummen, and S. Mathivanan, *Focus on Extracellular Vesicles: Introducing the Next Small Big Thing.* Int J Mol Sci, 2016. **17**(2): p. 170.
15. Ailawadi, S., et al., *Pathologic function and therapeutic potential of exosomes in cardiovascular disease.* Biochim Biophys Acta, 2015. **1852**(1): p. 1-11.
16. Wollert, T. and J.H. Hurley, *Molecular mechanism of multivesicular body biogenesis by ESCRT complexes.* Nature, 2010. **464**(7290): p. 864-9.
17. Zwaal, R.F. and A.J. Schroit, *Pathophysiologic implications of membrane phospholipid asymmetry in blood cells.* Blood, 1997. **89**(4): p. 1121-32.
18. Akers, J.C., et al., *Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies.* J Neurooncol, 2013. **113**(1): p. 1-11.
19. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* British journal of cancer, 1972. **26**(4): p. 239-257.
20. Mariño, G. and G. Kroemer, *Mechanisms of apoptotic phosphatidylserine exposure.* Cell Research, 2013. **23**(11): p. 1247-1248.

21. Sunkara, V., H.-K. Woo, and Y. Cho, *Emerging techniques in isolation and characterization of extracellular vesicles and their roles in cancer diagnostics and prognostics.* The Analyst, 2015. **141**.
22. Wubbolts, R., et al., *Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation.* J Biol Chem, 2003. **278**(13): p. 10963-72.
23. Crescitelli, R., et al., *Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes.* Journal of extracellular vesicles, 2013. **2**: p. 10.3402/jev.v2i0.20677.
24. Keerthikumar, S., et al., *ExoCarta: A Web-Based Compendium of Exosomal Cargo.* J Mol Biol, 2016. **428**(4): p. 688-692.
25. Zaborowski, M.P., et al., *Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study.* Bioscience, 2015. **65**(8): p. 783-797.
26. Calderwood, S.K., et al., *Extracellular heat shock proteins in cell signaling.* FEBS Lett, 2007. **581**(19): p. 3689-94.
27. Weerheim, A.M., et al., *Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography.* Anal Biochem, 2002. **302**(2): p. 191-8.
28. Doyle, L.M. and M.Z. Wang, *Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis.* Cells, 2019. **8**(7): p. 727.
29. Coumans, F.A.W., et al., *Methodological Guidelines to Study Extracellular Vesicles.* Circ Res, 2017. **120**(10): p. 1632-1648.
30. Witwer, K.W., et al., *Standardization of sample collection, isolation and analysis methods in extracellular vesicle research.* J Extracell Vesicles, 2013. **2**.
31. Yu, L.-L., et al., *A Comparison of Traditional and Novel Methods for the Separation of Exosomes from Human Samples.* BioMed Research International, 2018. **2018**: p. 3634563.
32. Gallart-Palau, X., et al., *Extracellular vesicles are rapidly purified from human plasma by PRotein Organic Solvent PRecipitation (PROSPR).* Sci Rep, 2015. **5**: p. 14664.
33. Gámez-Valero, A., et al., *Size-Exclusion Chromatography-based isolation minimally alters Extracellular Vesicles' characteristics compared to precipitating agents.* Sci Rep, 2016. **6**: p. 33641.
34. Gieseler, F., et al., *Using annexin V-coated magnetic beads to capture active tissue factor-bearing microparticles from body fluids.* Cell Biol Int, 2014. **38**(2): p. 277-81.
35. Shih, C.L., et al., *Development of a magnetic bead-based method for the collection of circulating extracellular vesicles.* N Biotechnol, 2016. **33**(1): p. 116-22.
36. Izon Science, *qEVoriginal / 70 nm* 2021.
37. van der Pol, E., et al., *Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing.* J Thromb Haemost, 2014. **12**(7): p. 1182-92.
38. Botha, J., H.R. Pugsley, and A. Handberg, *Conventional, High-Resolution and Imaging Flow Cytometry: Benchmarking Performance in Characterisation of Extracellular Vesicles.* Biomedicines, 2021. **9**(2).
39. Zhao, Z., et al., *Isolation and analysis methods of extracellular vesicles (EVs).* Extracellular vesicles and circulating nucleic acids, 2021. **2**: p. 80-103.
40. Gandham, S., et al., *Technologies and Standardization in Research on Extracellular Vesicles.* Trends in biotechnology, 2020. **38**(10): p. 1066-1098.

41. Chuo, S.T.-Y., J.C.-Y. Chien, and C.P.-K. Lai, *Imaging extracellular vesicles: current and emerging methods.* Journal of Biomedical Science, 2018. **25**(1): p. 91.
42. World Health Organization, *Cardiovascular diseases.* 17 May 2017.
43. National Health Services, *Cardiovascular disease.* 2018.
44. Osteikoetxea, X., et al., *Extracellular vesicles in cardiovascular disease: are they Jedi or Sith?* J Physiol, 2016. **594**(11): p. 2881-94.
45. Fu, S., et al., *Extracellular vesicles in cardiovascular diseases.* Cell Death Discovery, 2020. **6**(1): p. 68.
46. Amabile, N., et al., *Association of circulating endothelial microparticles with cardiometabolic risk factors in the Framingham Heart Study.* Eur Heart J, 2014. **35**(42): p. 2972-9.
47. Takaeko, Y., et al., *Lower triglyceride levels are associated with better endothelial function.* Journal of Clinical Lipidology, 2021. **15**(3): p. 500-511.
48. Chen, Y., et al., *Plasma endothelial microparticles and their correlation with the presence of hypertension and arterial stiffness in patients with type 2 diabetes.* J Clin Hypertens (Greenwich), 2012. **14**(7): p. 455-60.
49. Bellien, J., et al., *Arterial Stiffness Is Regulated by Nitric Oxide and Endothelium-Derived Hyperpolarizing Factor During Changes in Blood Flow in Humans.* Hypertension, 2010. **55**(3): p. 674-680.
50. de Jong, O.G., et al., *Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes.* Journal of extracellular vesicles, 2012. **1**: p. 10.3402/jev.v1i0.18396.
51. Janaszak-Jasiecka, A., et al., *Endothelial Dysfunction Driven by Hypoxia-The Influence of Oxygen Deficiency on NO Bioavailability.* Biomolecules, 2021. **11**(7): p. 982.
52. Jansen, F., et al., *High glucose condition increases NADPH oxidase activity in endothelial microparticles that promote vascular inflammation.* Cardiovascular Research, 2013. **98**(1): p. 94-106.
53. Gao, W., et al., *Exosomes derived from mature dendritic cells increase endothelial inflammation and atherosclerosis via membrane TNF-α mediated NF-κB pathway.* J Cell Mol Med, 2016. **20**(12): p. 2318-2327.
54. Giricz, Z., et al., *Cardioprotection by remote ischemic preconditioning of the rat heart is mediated by extracellular vesicles.* J Mol Cell Cardiol, 2014. **68**: p. 75-8.
55. Barile, L., et al., *Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction.* Cardiovasc Res, 2014. **103**(4): p. 530-41.
56. Chistiakov, D.A., A.N. Orekhov, and Y.V. Bobryshev, *Cardiac Extracellular Vesicles in Normal and Infarcted Heart.* Int J Mol Sci, 2016. **17**(1).
57. Eltzschig, H.K. and T. Eckle, *Ischemia and reperfusion--from mechanism to translation.* Nature medicine, 2011. **17**(11): p. 1391-1401.
58. Martínez, M.C. and R. Andriantsitohaina, *Extracellular Vesicles in Metabolic Syndrome.* Circulation Research, 2017. **120**(10): p. 1674-1686.
59. Zarà, M., et al., *Biology and Role of Extracellular Vesicles (EVs) in the Pathogenesis of Thrombosis.* International Journal of Molecular Sciences, 2019. **20**(11): p. 2840.
60. Owens, A.P., 3rd and N. Mackman, *Microparticles in hemostasis and thrombosis.* Circ Res, 2011. **108**(10): p. 1284-97.
61. Vallier, L., et al., *Microparticles and Fibrinolysis.* Seminars in Thrombosis and Hemostasis, 2017. **43**: p. 129 134.
62. Boilard, E., A.-C. Duchez, and A. Brisson, *The diversity of platelet microparticles.* Current Opinion in Hematology, 2015. **22**(5): p. 437-444.

63. Morel, O., et al., *Short-term very low-calorie diet in obese females improves the haemostatic balance through the reduction of leptin levels, PAI-1 concentrations and a diminished release of platelet and leukocyte-derived microparticles.* Int J Obes (Lond), 2011. **35**(12): p. 1479-86.
64. Vallier, L., et al., *Microparticles and Fibrinolysis.* Semin Thromb Hemost, 2017. **43**(2): p. 129-134.
65. Chapin, J.C. and K.A. Hajjar, *Fibrinolysis and the control of blood coagulation.* Blood reviews, 2015. **29**(1): p. 17-24.
66. The Institute of Food Science and Technology (IFST), *Oils and Fats.* December 2017.
67. NHS, *Fat: the facts.* 2020.
68. AbuMweis, S., et al., *Eicosapentaenoic acid and docosahexaenoic acid containing supplements modulate risk factors for cardiovascular disease: a meta-analysis of randomised placebo-control human clinical trials.* J Hum Nutr Diet, 2018. **31**(1): p. 67-84.
69. Asif, M., *Process advantages and product benefits of interesterification in oils and fats.* International journal of Nutrition, Pharmacology, Neurological Diseases, 2011. **1**: p. 134.
70. Klonoff, D.C., *Replacements for trans fats-will there be an oil shortage?* Journal of diabetes science and technology, 2007. **1**(3): p. 415-422.
71. Oteng, A.-B. and S. Kersten, *Mechanisms of Action of trans Fatty Acids.* Advances in Nutrition, 2019. **11**(3): p. 697-708.
72. Berry, S.E., et al., *Interesterified fats: What are they and why are they used? A briefing report from the Roundtable on Interesterified Fats in Foods.* Nutrition Bulletin, 2019. **44**(4): p. 363-380.
73. Mills, C.E., W.L. Hall, and S.E.E. Berry, *What are interesterified fats and should we be worried about them in our diet?* Nutrition bulletin, 2017. **42**(2): p. 153-158.
74. Berry, S.E., et al., *Effect of interesterification of palmitic acid-rich triacylglycerol on postprandial lipid and factor VII response.* Lipids, 2007. **42**(4): p. 315-23.
75. Sanders, T.A., et al., *Palmitic acid in the sn-2 position of triacylglycerols acutely influences postprandial lipid metabolism.* The American Journal of Clinical Nutrition, 2011. **94**(6): p. 1433-1441.
76. Berry, S.E., G.J. Miller, and T.A. Sanders, *The solid fat content of stearic acid–rich fats determines their postprandial effects.* The American Journal of Clinical Nutrition, 2007. **85**(6): p. 1486-1494.
77. Covington, M.B., *Omega-3 fatty acids.* Am Fam Physician, 2004. **70**(1): p. 133-40.
78. Flock, M.R., W.S. Harris, and P.M. Kris-Etherton, *Long-chain omega-3 fatty acids: time to establish a dietary reference intake.* Nutr Rev, 2013. **71**(10): p. 692-707.
79. SACN, *Advice on fish consumption: benefits & risks.* 2004.
80. BJERREGAARD, P. and J. DYERBERG, *Mortality from Ischaemic Heart Disease and Cerebrovascular Disease in Greenland.* International Journal of Epidemiology, 1988. **17**(3): p. 514-519.
81. Zhang, J., et al., *Fish Consumption and Mortality from All Causes, Ischemic Heart Disease, and Stroke: An Ecological Study.* Preventive Medicine, 1999. **28**(5): p. 520-529.
82. Pedersen, J.I., et al., *Adipose tissue fatty acids and risk of myocardial infarction--a case-control study.* Eur J Clin Nutr, 2000. **54**(8): p. 618-25.
83. Albert, C.M., et al., *Blood levels of long-chain n-3 fatty acids and the risk of sudden death.* N Engl J Med, 2002. **346**(15): p. 1113-8.

84. He, K., et al., *Fish Consumption and Risk of Stroke in Men.* JAMA, 2002. **288**(24): p. 3130-3136.
85. Albert, C.M., et al., *Fish Consumption and Risk of Sudden Cardiac Death.* JAMA, 1998. **279**(1): p. 23-28.
86. Zhang, Y., et al., *Association of fish and long-chain omega-3 fatty acids intakes with total and cause-specific mortality: prospective analysis of 421 309 individuals.* J Intern Med, 2018. **284**(4): p. 399-417.
87. Hu, Y., F.B. Hu, and J.E. Manson, *Marine Omega‐3 Supplementation and Cardiovascular Disease: An Updated Meta‐Analysis of 13 Randomized Controlled Trials Involving 127 477 Participants.* Journal of the American Heart Association, 2019. **8**(19): p. e013543.
88. Gao, L.G., et al., *Influence of omega-3 polyunsaturated fatty acid-supplementation on platelet aggregation in humans: a meta-analysis of randomized controlled trials.* Atherosclerosis, 2013. **226**(2): p. 328-34.
89. Li, K., et al., *Effect of marine-derived n-3 polyunsaturated fatty acids on C-reactive protein, interleukin 6 and tumor necrosis factor α: a meta-analysis.* PLoS One, 2014. **9**(2): p. e88103.
90. Calder, P.C., *Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance.* Biochim Biophys Acta, 2015. **1851**(4): p. 469-84.
91. *Effects of n−3 Fatty Acid Supplements in Diabetes Mellitus.* New England Journal of Medicine, 2018. **379**(16): p. 1540-1550.
92. Manson, J.E., et al., *Marine n−3 Fatty Acids and Prevention of Cardiovascular Disease and Cancer.* New England Journal of Medicine, 2018. **380**(1): p. 23-32.
93. Tavazzi, L., et al., *Effect of n-3 polyunsaturated fatty acids in patients with chronic heart failure (the GISSI-HF trial): a randomised, double-blind, placebo-controlled trial.* Lancet, 2008. **372**(9645): p. 1223-30.
94. Bucher, H.C., et al., *N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials.* Am J Med, 2002. **112**(4): p. 298-304.
95. Rauch, B., et al., *OMEGA, a randomized, placebo-controlled trial to test the effect of highly purified omega-3 fatty acids on top of modern guideline-adjusted therapy after myocardial infarction.* Circulation, 2010. **122**(21): p. 2152-9.
96. Galan, P., et al., *Effects of B vitamins and omega 3 fatty acids on cardiovascular diseases: a randomised placebo controlled trial.* BMJ, 2010. **341**: p. c6273.
97. Kromhout, D., E.J. Giltay, and J.M. Geleijnse, *n–3 Fatty Acids and Cardiovascular Events after Myocardial Infarction.* New England Journal of Medicine, 2010. **363**(21): p. 2015-2026.
98. Innes, J.K. and P.C. Calder, *Marine Omega-3 (N-3) Fatty Acids for Cardiovascular Health: An Update for 2020.* International Journal of Molecular Sciences, 2020. **21**(4): p. 1362.
99. Wu, S.-Y., et al., *Fish-oil supplementation alters numbers of circulating endothelial progenitor cells and microparticles independently of eNOS genotype.* The American Journal of Clinical Nutrition, 2014. **100**(5): p. 1232-1243.
100. Horn, P., et al., *Dietary flavanol intervention lowers the levels of endothelial microparticles in coronary artery disease patients.* Br J Nutr, 2014. **111**(7): p. 1245-52.
101. Murakami, T., et al., *Impact of weight reduction on production of platelet-derived microparticles and fibrinolytic parameters in obesity.* Thromb Res, 2007. **119**(1): p. 45-53.

102. Phang, M., et al., *Acute supplementation with eicosapentaenoic acid reduces platelet microparticle activity in healthy subjects.* J Nutr Biochem, 2012. **23**(9): p. 1128-33.
103. Serena Del, T., et al., *Effect of the administration of n-3 polyunsaturated fatty acids on circulating levels of microparticles in patients with a previous myocardial infarction.* Haematologica, 2008. **93**(6): p. 892-899.
104. Sustar, V., et al., *Post - prandial rise of microvesicles in peripheral blood of healthy human donors.* Lipids in Health and Disease, 2011. **10**(1): p. 47.
105. Harrison, M., et al., *The endothelial microparticle response to a high fat meal is not attenuated by prior exercise.* European Journal of Applied Physiology, 2009. **106**(4): p. 555.
106. Wang, T., et al., *Hypertriglyceridemia is associated with platelet hyperactivation in metabolic syndrome patients.* International Journal of Clinical Practice, 2020. **74**(7): p. e13508.
107. Aye, M.M., et al., *Acute hypertriglyceridemia induces platelet hyperactivity that is not attenuated by insulin in polycystic ovary syndrome.* J Am Heart Assoc, 2014. **3**(1): p. e000706.
108. Gasecka, A., et al., *LDL-Cholesterol and Platelets: Insights into Their Interactions in Atherosclerosis.* Life, 2021. **11**(1): p. 39.
109. Tushuizen, M.E., et al., *Two consecutive high-fat meals affect endothelial-dependent vasodilation, oxidative stress and cellular microparticles in healthy men.* J Thromb Haemost, 2006. **4**(5): p. 1003-10.
110. Ramírez-Vélez, R., *[Postprandial lipemia induces endothelial dysfunction and higher insulin resistance in healthy subjects].* Endocrinol Nutr, 2011. **58**(10): p. 529-35.
111. Alipour, A., et al., *Leukocyte activation by triglyceride-rich lipoproteins.* Arterioscler Thromb Vasc Biol, 2008. **28**(4): p. 792-7.
112. Weech, M., et al., *Replacement of dietary saturated fat with unsaturated fats increases numbers of circulating endothelial progenitor cells and decreases numbers of microparticles: findings from the randomized, controlled Dietary Intervention and VAScular function (DIVAS) study.* Am J Clin Nutr, 2018. **107**(6): p. 876-882.
113. Zhao, Y., et al., *Trends in the biological functions and medical applications of extracellular vesicles and analogues.* Acta Pharmaceutica Sinica B, 2021. **11**(8): p. 2114-2135.
114. Tramontano, A.F., et al., *Circulating Endothelial Microparticles in Diabetes Mellitus.* Mediators of Inflammation, 2010. **2010**: p. 250476.
115. Bernal-Mizrachi, L., et al., *High levels of circulating endothelial microparticles in patients with acute coronary syndromes.* American Heart Journal, 2003. **145**(6): p. 962-970.
116. Yamanaka, Y., Y. Sawai, and S. Nomura, *Platelet-Derived Microparticles are an Important Biomarker in Patients with Cancer-Associated Thrombosis.* International journal of general medicine, 2019. **12**: p. 491-497.
117. Karttunen, J., et al., *Precipitation-based extracellular vesicle isolation from rat plasma co-precipitate vesicle-free microRNAs.* Journal of extracellular vesicles, 2018. **8**(1): p. 1555410-1555410.
118. Livshits, M.A., et al., *Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol.* Scientific Reports, 2015. **5**(1): p. 17319.
119. Onódi, Z., et al., *Isolation of High-Purity Extracellular Vesicles by the Combination of Iodixanol Density Gradient Ultracentrifugation and Bind-Elute Chromatography From Blood Plasma.* Frontiers in Physiology, 2018. **9**(1479).

120. Gámez-Valero, A., et al., *Size-Exclusion Chromatography-based isolation minimally alters Extracellular Vesicles' characteristics compared to precipitating agents.* Scientific Reports, 2016. **6**(1): p. 33641.
121. Stranska, R., et al., *Comparison of membrane affinity-based method with size-exclusion chromatography for isolation of exosome-like vesicles from human plasma.* J Transl Med, 2018. **16**(1): p. 1.
122. Duong, P., et al., *Cushioned-Density Gradient Ultracentrifugation (C-DGUC) improves the isolation efficiency of extracellular vesicles.* PLOS ONE, 2019. **14**(4): p. e0215324.
123. Heath, N., et al., *Rapid isolation and enrichment of extracellular vesicle preparations using anion exchange chromatography.* Scientific Reports, 2018. **8**(1): p. 5730.
124. Science., I., *qEVoriginal / 70nm SEC Columns for Exosomes Separation and Purification.* . 2021.
125. Sidhom, K., P.O. Obi, and A. Saleem, *A Review of Exosomal Isolation Methods: Is Size Exclusion Chromatography the Best Option?* Int J Mol Sci, 2020. **21**(18).
126. Bozzetto, L., et al., *Dietary Impact on Postprandial Lipemia.* Frontiers in endocrinology, 2020. **11**: p. 337-337.
127. Berry, S., et al., *Impact of Postprandial Lipemia and Glycemia on Inflammatory Factors in over 1000 Individuals in the US and UK: Insights from the PREDICT 1 and InterCardio Studies.* Current Developments in Nutrition, 2020. **4**(Supplement_2): p. 1518-1518.
128. Wojczynski, M.K., et al., *High-fat meal effect on LDL, HDL, and VLDL particle size and number in the Genetics of Lipid-Lowering Drugs and Diet Network (GOLDN): an interventional study.* Lipids in health and disease, 2011. **10**: p. 181-181.
129. Della Pepa, G., et al., *Effects of a diet naturally rich in polyphenols on lipid composition of postprandial lipoproteins in high cardiometabolic risk individuals: an ancillary analysis of a randomized controlled trial.* European Journal of Clinical Nutrition, 2020. **74**(1): p. 183-192.
130. Gyorgy, B., et al., *Improved circulating microparticle analysis in acid-citrate dextrose (ACD) anticoagulant tube.* Thromb Res, 2014. **133**(2): p. 285-92.
131. Böing, A.N., et al., *Single-step isolation of extracellular vesicles by size-exclusion chromatography.* Journal of Extracellular Vesicles, 2014. **3**(1): p. 23430.
132. Packard, C.J., J. Boren, and M.-R. Taskinen, *Causes and Consequences of Hypertriglyceridemia.* Frontiers in Endocrinology, 2020. **11**(252).
133. Ramirez, M.I., et al., *Technical challenges of working with extracellular vesicles.* Nanoscale, 2018. **10**(3): p. 881-906.
134. Zhang, X., et al., *A novel three step protocol to isolate extracellular vesicles from plasma or cell culture medium with both high yield and purity.* Journal of Extracellular Vesicles, 2020. **9**(1): p. 1791450.
135. Sódar, B.W., et al., *Low-density lipoprotein mimics blood plasma-derived exosomes and microvesicles during isolation and detection.* Scientific Reports, 2016. **6**(1): p. 24316.
136. de Rond, L., et al., *Refractive index to evaluate staining specificity of extracellular vesicles by flow cytometry.* J Extracell Vesicles, 2019. **8**(1): p. 1643671.
137. Guo, J., et al., *Establishment of a simplified dichotomic size-exclusion chromatography for isolating extracellular vesicles toward clinical applications.* Journal of Extracellular Vesicles, 2021. **10**(11): p. e12145.
138. Izon Science, *Are samples diluted when passed through the qEV columns?*

139. Stranska, R., et al., *Comparison of membrane affinity-based method with size-exclusion chromatography for isolation of exosome-like vesicles from human plasma.* Journal of Translational Medicine, 2018. **16**(1): p. 1.
140. The National Health Service, *Cardiovascular disease.* 2018.
141. British Heart Foundation, *BHF Statistics Factsheet - UK* 2021.
142. Iqbal, M.P., *Trans fatty acids - A risk factor for cardiovascular disease.* Pakistan journal of medical sciences, 2014. **30**(1): p. 194-197.
143. Sacks, F.M., et al., *Dietary Fats and Cardiovascular Disease: A Presidential Advisory From the American Heart Association.* Circulation, 2017. **136**(3): p. e1-e23.
144. Yuana, Y., A. Sturk, and R. Nieuwland, *Extracellular vesicles in physiological and pathological conditions.* Blood Reviews, 2013. **27**(1): p. 31-39.
145. Tripisciano, C., et al., *Different Potential of Extracellular Vesicles to Support Thrombin Generation: Contributions of Phosphatidylserine, Tissue Factor, and Cellular Origin.* Scientific Reports, 2017. **7**(1): p. 6522.
146. Konings, J., et al., *Factor XIIa regulates the structure of the fibrin clot independently of thrombin generation through direct interaction with fibrin.* Blood, 2011. **118**(14): p. 3942-51.
147. Mitchell, J.L., et al., *Polyphosphate colocalizes with factor XII on platelet-bound fibrin and augments its plasminogen activator activity.* Blood, 2016. **128**(24): p. 2834-2845.
148. Longstaff, C. and t.s.o. fibrinolysis, *Development of Shiny app tools to simplify and standardize the analysis of hemostasis assay data: communication from the SSC of the ISTH.* Journal of Thrombosis and Haemostasis, 2017. **15**(5): p. 1044-1046.
149. Hammad, S., S. Pu, and P.J. Jones, *Current Evidence Supporting the Link Between Dietary Fatty Acids and Cardiovascular Disease.* Lipids, 2016. **51**(5): p. 507-517.
150. Briggs, M.A., K.S. Petersen, and P.M. Kris-Etherton, *Saturated Fatty Acids and Cardiovascular Disease: Replacements for Saturated Fat to Reduce Cardiovascular Risk.* Healthcare (Basel, Switzerland), 2017. **5**(2): p. 29.
151. Bhandari, S.D., et al., *Regulatory Changes Affecting the Production and Use of Fats and Oils: Focus on Partially Hydrogenated Oils.* Journal of the American Oil Chemists' Society, 2020. **97**(8): p. 797-815.
152. The World Health Organization, *More than 3 billion people protected from harmful trans fat in their food.* 2020.
153. Dickhout, A. and R.R. Koenen, *Extracellular Vesicles as Biomarkers in Cardiovascular Disease; Chances and Risks.* Frontiers in cardiovascular medicine, 2018. **5**: p. 113-113.
154. Rathnayake, K.M., et al., *Impact of meal fatty acid composition on postprandial lipaemia, vascular function and blood pressure in postmenopausal women.* Nutr Res Rev, 2018. **31**(2): p. 193-203.
155. Bladbjerg, E.-M., et al., *Postprandial coagulation activation in overweight individuals after weight loss: Acute and long-term effects of a high-monounsaturated fat diet and a low-fat diet.* Thrombosis Research, 2014. **133**(3): p. 327-333.
156. Michelsen, A.E., et al., *Elevated levels of platelet microparticles in carotid atherosclerosis and during the postprandial state.* Thrombosis Research, 2009. **123**(6): p. 881-886.
157. Strohacker, K., et al., *Moderate-intensity, premeal cycling blunts postprandial increases in monocyte cell surface CD18 and CD11a and endothelial microparticles following a high-fat meal in young adults.* Appl Physiol Nutr Metab, 2012. **37**(3): p. 530-9.

158. Koo, B.K., et al., *Hypertriglyceridemia Is an Independent Risk Factor for Cardiovascular Diseases in Korean Adults Aged 30-49 Years: a Nationwide Population-Based Study.* Journal of lipid and atherosclerosis, 2021. **10**(1): p. 88-98.
159. Aflaki, E., et al., *Triacylglycerol accumulation activates the mitochondrial apoptosis pathway in macrophages.* J Biol Chem, 2011. **286**(9): p. 7418-28.
160. Matsumoto, S., et al., *The role of hypertriglyceridemia in the development of atherosclerosis and endothelial dysfunction.* Nutrients, 2014. **6**(3): p. 1236-50.
161. Kobayashi, Y., et al., *Circulating extracellular vesicles are associated with lipid and insulin metabolism.* American Journal of Physiology-Endocrinology and Metabolism, 2018. **315**(4): p. E574-E582.
162. Eguchi, A., et al., *Circulating adipocyte-derived extracellular vesicles are novel markers of metabolic stress.* J Mol Med (Berl), 2016. **94**(11): p. 1241-1253.
163. Ni, X.-R., et al., *High Concentration of Insulin Promotes Apoptosis of Primary Cultured Rat Ovarian Granulosa Cells Via Its Increase in Extracellular HMGB1*. Reproductive Sciences, 2015. **22**(3): p. 271-277.
164. Kim, S.-W., et al., *Extracellular HMGB1 Released by NMDA Treatment Confers Neuronal Apoptosis via RAGE-p38 MAPK/ERK Signaling Pathway.* Neurotoxicity Research, 2011. **20**(2): p. 159-169.
165. Park, S.J., et al., *Molecular mechanisms of biogenesis of apoptotic exosome-like vesicles and their roles as damage-associated molecular patterns.* Proceedings of the National Academy of Sciences of the United States of America, 2018. **115**(50): p. E11721-E11730.
166. Bucris, E., et al., *Prolonged insulin treatment sensitizes apoptosis pathways in pancreatic β cells.* J Endocrinol, 2016. **230**(3): p. 291-307.
167. Tholstrup, T., et al., *Effect of individual dietary fatty acids on postprandial activation of blood coagulation factor VII and fibrinolysis in healthy young men.* The American Journal of Clinical Nutrition, 2003. **77**(5): p. 1125-1132.
168. Miszta, A., et al., *A high-fat diet delays plasmin generation in a thrombomodulin-dependent manner in mice.* Blood, 2020. **135**(19): p. 1704-1717.
169. *The Haemostatic System*, in *Cardiovascular Disease*. 2019. p. 179-211.
170. Spronk, H.M.H., H. ten Cate, and P.E.J. van der Meijden, *Differential roles of Tissue Factor and Phosphatidylserine in activation of coagulation.* Thrombosis Research, 2014. **133**: p. S54-S56.
171. Sanchez, C., et al., *Diet Modulates Endogenous Thrombin Generation, A Biological Estimate of Thrombosis Risk, Independently of the Metabolic Status.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2012. **32**(10): p. 2394-2404.
172. Cleuren, A.C.A., et al., *Changes in Dietary Fat Content Rapidly Alters the Mouse Plasma Coagulation Profile without Affecting Relative Transcript Levels of Coagulation Factors.* PLOS ONE, 2015. **10**(7): p. e0131859.
173. Esposito, K., et al., *Effect of dietary antioxidants on postprandial endothelial dysfunction induced by a high-fat meal in healthy subjects.* Am J Clin Nutr, 2003. **77**(1): p. 139-43.
174. Teng, K.T., et al., *Effects of amount and type of dietary fats on postprandial lipemia and thrombogenic markers in individuals with metabolic syndrome.* Atherosclerosis, 2015. **242**(1): p. 281-7.
175. Sanders, T.A., S.E. Berry, and G.J. Miller, *Influence of triacylglycerol structure on the postprandial response of factor VII to stearic acid-rich fats.* Am J Clin Nutr, 2003. **77**(4): p. 777-82.
176. Berry, S.E., G.J. Miller, and T.A. Sanders, *The solid fat content of stearic acid-rich fats determines their postprandial effects.* Am J Clin Nutr, 2007. **85**(6): p. 1486-94.

177. Heinrich, L.F., et al., *Long-term high fat feeding of rats results in increased numbers of circulating microvesicles with pro-inflammatory effects on endothelial cells.* British Journal of Nutrition, 2015. **113**(11): p. 1704-1711.
178. Yigit, Y., et al., *The Acute Effects of Postprandial Hypertriglyceridemia on Coagulation Parameters in Normal and Overweight Individuals.* Indian Journal of Clinical Biochemistry, 2014. **30**: p. 424-429.
179. Ferreira, A.C., et al., *Postprandial hypertriglyceridemia increases circulating levels of endothelial cell microparticles.* Circulation, 2004. **110**(23): p. 3599-603.
180. Tushuizen, M.E., et al., *Postprandial changes in the phospholipid composition of circulating microparticles are not associated with coagulation activation.* Thrombosis Research, 2012. **130**(1): p. 115-121.
181. Vaidyula, V.R., G. Boden, and A.K. Rao, *Platelet and monocyte activation by hyperglycemia and hyperinsulinemia in healthy subjects.* Platelets, 2006. **17**(8): p. 577-585.
182. Boden, G., et al., *Circulating Tissue Factor Procoagulant Activity and Thrombin Generation in Patients with Type 2 Diabetes: Effects of Insulin and Glucose.* The Journal of Clinical Endocrinology & Metabolism, 2007. **92**(11): p. 4352-4358.
183. Taus, F., et al., *Platelet-Derived Extracellular Vesicles as Target of Antiplatelet Agents. What Is the Evidence?* Frontiers in Pharmacology, 2019. **10**(1256).
184. Yáñez-Mó, M., et al., *Biological properties of extracellular vesicles and their physiological functions.* J Extracell Vesicles, 2015. **4**: p. 27066.
185. Villa, F., R. Quarto, and R. Tasso, *Extracellular Vesicles as Natural, Safe and Efficient Drug Delivery Systems.* Pharmaceutics, 2019. **11**(11): p. 557.
186. Wang, J., et al., *Abstract 297: Increase in Total Number/cargo of Extracellular Vesicles Secreted by Atherosclerotic Plaque Associated Foam Cells and in Serum of Cvd Subjects.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2020. **40**(Suppl_1): p. A297-A297.
187. Hooten, N.N. and M.K. Evans, *Extracellular vesicles as signaling mediators in type 2 diabetes mellitus.* American Journal of Physiology-Cell Physiology, 2020. **318**(6): p. C1189-C1199.
188. Urabe, F., et al., *Extracellular vesicles as biomarkers and therapeutic targets for cancer.* American Journal of Physiology-Cell Physiology, 2020. **318**(1): p. C29-C39.
189. Kranendonk, M.E.G., et al., *Extracellular vesicle markers in relation to obesity and metabolic complications in patients with manifest cardiovascular disease.* Cardiovascular Diabetology, 2014. **13**(1): p. 37.
190. Oggero, S., S. Austin-Williams, and L.V. Norling, *The Contrasting Role of Extracellular Vesicles in Vascular Inflammation and Tissue Repair*. Frontiers in Pharmacology, 2019. **10**(1479).
191. Hu, Y., F.B. Hu, and J.E. Manson, *Marine Omega-3 Supplementation and Cardiovascular Disease: An Updated Meta-Analysis of 13 Randomized Controlled Trials Involving 127 477 Participants.* J Am Heart Assoc, 2019. **8**(19): p. e013543.
192. Sekikawa, A., et al., *Effect of High-Dose Marine Omega-3 Fatty Acids on Atherosclerosis: A Systematic Review and Meta-Analysis of Randomized Clinical Trials.* Nutrients, 2019. **11**(11).
193. Harris, W.S., et al., *Blood n-3 fatty acid levels and total and cause-specific mortality from 17 prospective studies.* Nature Communications, 2021. **12**(1): p. 2329.
194. Calder, P.C. and P. Yaqoob, *Understanding omega-3 polyunsaturated fatty acids.* Postgrad Med, 2009. **121**(6): p. 148-57.
195. Wilson, P.W., et al., *Prediction of coronary heart disease using risk factor categories.* Circulation, 1998. **97**(18): p. 1837-47.

196. Chong, M.F., et al., *Impact of the quantity and flavonoid content of fruits and vegetables on markers of intake in adults with an increased risk of cardiovascular disease: the FLAVURS trial.* Eur J Nutr, 2013. **52**(1): p. 361-78.
197. Li, Z.-H., et al., *Associations of habitual fish oil supplementation with cardiovascular outcomes and all cause mortality: evidence from a large population based cohort study.* BMJ, 2020. **368**: p. m456.
198. Endo, J. and M. Arita, *Cardioprotective mechanism of omega-3 polyunsaturated fatty acids.* Journal of Cardiology, 2016. **67**(1): p. 22-27.
199. Omura, M., et al., *Eicosapentaenoic acid (EPA) induces Ca(2+)-independent activation and translocation of endothelial nitric oxide synthase and endothelium-dependent vasorelaxation.* FEBS Lett, 2001. **487**(3): p. 361-6.
200. Calder, P.C., *The role of marine omega-3 (n-3) fatty acids in inflammatory processes, atherosclerosis and plaque stability.* Molecular Nutrition & Food Research, 2012. **56**(7): p. 1073-1080.
201. Kromhout, D., et al., *Fish oil and omega-3 fatty acids in cardiovascular disease: do they really work?* European heart journal, 2012. **33**(4): p. 436-443.
202. Gajos, G., et al., *Reduced thrombin formation and altered fibrin clot properties induced by polyunsaturated omega-3 fatty acids on top of dual antiplatelet therapy in patients undergoing percutaneous coronary intervention (OMEGA-PCI clot).* Arterioscler Thromb Vasc Biol, 2011. **31**(7): p. 1696-702.
203. Landers-Ramos, R.Q., et al., *Circulating microparticle concentrations across acute and chronic cardiovascular disease conditions.* Physiological reports, 2020. **8**(15): p. e14534-e14534.
204. Loyer, X., et al., *Microvesicles as Cell–Cell Messengers in Cardiovascular Diseases.* Circulation Research, 2014. **114**(2): p. 345-353.
205. Harris, W.S., et al., *Comparison of the effects of fish and fish-oil capsules on the n–3 fatty acid content of blood cells and plasma phospholipids.* The American Journal of Clinical Nutrition, 2007. **86**(6): p. 1621-1625.
206. Djuricic, I.D., et al., *Long-chain n-3 polyunsaturated fatty acid dietary recommendations are moderately efficient in optimizing their status in healthy middle-aged subjects with low fish consumption: a cross-over study.* Nutr Res, 2014. **34**(3): p. 210-8.
207. Williams, Justin A., et al., *Docosahexaenoic and Eicosapentaenoic Acids Segregate Differently between Raft and Nonraft Domains.* Biophysical Journal, 2012. **103**(2): p. 228-237.
208. Browning, L.M., et al., *Compared with daily, weekly n-3 PUFA intake affects the incorporation of eicosapentaenoic acid and docosahexaenoic acid into platelets and mononuclear cells in humans.* J Nutr, 2014. **144**(5): p. 667-72.
209. Rao, L.V.M. and U.R. Pendurthi, *Regulation of tissue factor coagulant activity on cell surfaces.* Journal of thrombosis and haemostasis : JTH, 2012. **10**(11): p. 2242-2253.
210. Zhao, L., et al., *Phosphatidylserine exposing-platelets and microparticles promote procoagulant activity in colon cancer patients.* Journal of Experimental & Clinical Cancer Research, 2016. **35**(1): p. 54.
211. Reddy, E.C. and M.L. Rand, *Procoagulant Phosphatidylserine-Exposing Platelets in vitro and in vivo.* Frontiers in Cardiovascular Medicine, 2020. **7**(15).
212. Iba, T. and H. Ogura, *Role of extracellular vesicles in the development of sepsis-induced coagulopathy.* Journal of Intensive Care, 2018. **6**(1): p. 68.
213. Durrieu, L., A. Bharadwaj, and D.M. Waisman, *Analysis of the thrombotic and fibrinolytic activities of tumor cell–derived extracellular vesicles.* Blood Advances, 2018. **2**(10): p. 1054-1065.

214. Gardiner, C., et al., *Extracellular vesicles, tissue factor, cancer and thrombosis - discussion themes of the ISEV 2014 Educational Day.* J Extracell Vesicles, 2015. **4**: p. 26901.
215. Silachev, D.N., et al., *Effect of MSCs and MSC-Derived Extracellular Vesicles on Human Blood Coagulation.* Cells, 2019. **8**(3): p. 258.
216. Lacroix, R., et al., *Activation of plasminogen into plasmin at the surface of endothelial microparticles: a mechanism that modulates angiogenic properties of endothelial progenitor cells in vitro.* Blood, 2007. **110**(7): p. 2432-2439.
217. Lacroix, R., et al., *Leukocyte- and endothelial-derived microparticles: a circulating source for fibrinolysis.* Haematologica, 2012. **97**(12): p. 1864-72.
218. Moog, S., et al., *Platelet glycoprotein V binds to collagen and participates in platelet adhesion and aggregation.* Blood, 2001. **98**(4): p. 1038-1046.
219. Larson, M.K., et al., *Omega-3 fatty acids modulate collagen signaling in human platelets.* Prostaglandins, leukotrienes, and essential fatty acids, 2011. **84**(3-4): p. 93-98.
220. Lu, Q. and R.A. Malinauskas, *Comparison of Two Platelet Activation Markers Using Flow Cytometry After In Vitro Shear Stress Exposure of Whole Human Blood.* Artificial Organs, 2011. **35**(2): p. 137-144.
221. Park, Y. and W. Harris, *EPA, but not DHA, decreases mean platelet volume in normal subjects.* Lipids, 2002. **37**(10): p. 941-6.
222. So, J., et al., *EPA and DHA differentially modulate monocyte inflammatory response in subjects with chronic inflammation in part via plasma specialized pro-resolving lipid mediators: A randomized, double-blind, crossover study.* Atherosclerosis, 2021. **316**: p. 90-98.
223. Weech, M., et al., *Development of a food-exchange model to replace saturated fat with MUFAs and n-6 PUFAs in adults at moderate cardiovascular risk.* J Nutr, 2014. **144**(6): p. 846-55.
224. Zaldivia, M.T.K., et al., *Platelet-Derived Microvesicles in Cardiovascular Diseases.* Frontiers in Cardiovascular Medicine, 2017. **4**.
225. Inglis, H.C., et al., *Techniques to improve detection and analysis of extracellular vesicles using flow cytometry.* Cytometry. Part A : the journal of the International Society for Analytical Cytology, 2015. **87**(11): p. 1052-1063.

Appendix:

SCHOOL OF CHEMISTRY, FOOD & PHARMACY

ETHICS APPLICATION INTERNAL REVIEW
HEAD OF DEPARTMENT APPROVAL

Approval for projects which are exempt from requirement for full submission to the University Ethics and Research Committee

Title of Project … Effects of a meal on extracellular vesicles in blood

Investigator(s) …Professor Parveen Yaqoob.

Department …Food and Nutritional Sciences….

1. Has this application been read by your Supervisor/PI ? (where applicable)

YES ✓
NO ☐
NOT APPLICABLE ☐

2. Has your Group Internal Reviewer read the application and any suggested revisions been undertaken ?

YES ✓
NO ☐

This project has been subject to the SCFP Internal Ethics Review procedure and is allowed to proceed.

Internal Reviewer Signature ..... Date 18/9/17

Head of Department Signature . ... Date 22/9/17

Ethics Administrator Signature ..... Date.....

SCFP Cover Sheet Exempt V5 05.10.2016

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School of Chemistry, Food and Pharmacy
Research Ethics Committee

Application Form for Internal Approval

SECTION 1: APPLICATION DETAILS

1.1

Project Title: Effects of a meal on extracellular vesicles in blood

Date of Submission: September 2017 Proposed start date: September 2017 Proposed End Date: September 2020

1.2

Principal Investigator: Prof Parveen Yaqoob

Office room number: 2-39 Internal telephone: 8720
Email address: p.yaqoob@reading.ac.uk Alternative contact telephone:
(Please note that an undergraduate or postgraduate student cannot be a named principal investigator for research ethics purposes. The supervisor must be declared as Principal Investigator)

Other applicants
Name: Amal Nadi M. Alanzeei (Student) Food and Nutritional Sciences
Email: a.n.m.alanzeei@pgr.reading.ac.uk

....

1.3

Project Submission Declaration

I confirm that to the best of my knowledge I have made known all information relevant to the SCFP Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I understand that it is a legal requirement that both staff and students undergo Criminal Records Checks when in a position of trust (i.e. when working with children or vulnerable adults).

I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed..... ..... (Principal Investigator) Date: 8/9/17

(Other named investigators) Date: 07/09/17

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1.4

SCFP (Internal Approval) Ethics Committee Applications

Projects expected to require review by the SCFP Ethics Committee must be reviewed by a member of the School research ethics committee and the Head of School before submission.

Signed..... (Chair/Deputy Chair of School Committee) Date:.....

Signed. (Head of Department) Date: 27/9/17

Signed..... (SCFP Ethics Administrator) Date:.....

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SECTION 2: PROJECT DETAILS

2.1

Please provide a summary of the project in **non-specialist terms** that could be understood by **non-scientist members of the public**, which includes a description of the scientific background to the study (existing knowledge), the scientific questions the project will address and a justification of these. Please note that the description must be sufficient for the committee to take a reasonable view on the likely scientific rigour and value of the project

Extracellular vesicles (EVs) are small particles shed from cells, with a role in health and disease and potential as therapeutic targets or markers of disease in the future. Circulating EVs can be isolated from blood and their size and number measured, composition and function studied and their response to drugs and dietary components evaluated.

When analysing circulating EVs, blood samples tend to be taken from fasted volunteers to avoid interference by fat-containing particles in the bloodstream, which have a size range that overlaps with EVs and whose levels increase in the blood following consumption of a meal containing fat. However, this is not a 'typical' situation as most people spend most of the day in a non-fasted state and it is very important not only to resolve the overlap between fat particles and EVs, but also to understand the effects of a meal on numbers, size and behaviour of EVs. We plan to carry out human intervention studies testing the effects of meals containing different types of fat on the ability to isolate EVs and on their number, size, composition and function. This requires isolation of blood EVs from volunteers prior to and following consumption of a meal.

This research will enable us to further our understanding of EV biology, but cannot take place without access to fresh blood samples. We are therefore applying for ethical permission to enable the recruitment of volunteers for blood donation for this basic research on EVs in the Department of Food and Nutritional Sciences.

(*This box may be expanded as required – **Word Limit Maximum 250***)

2.2

Procedure
Please describe concisely what the study will involve for your participants and the procedures and methodology to be undertaken (*you may expand this box as required*).

Volunteer suitability will initially be assessed by answers to the medical and lifestyle questionnaire (Appendix D). All volunteers asked to attend the unit will be given a participant information sheet (Appendix E) which will be explained to the volunteer, and any questions answered before they are given the consent form (Appendix C) to sign if they are happy to participate. All volunteers will need to have fasted overnight (i.e. not eaten food or consumed alcohol since 8 pm the previous evening and only drank water during this time) prior to this screening visit. They will then have their BMI calculated and haemoglobin levels checked, using a thumb prick of blood tested using a hemocue device, before donating a

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blood sample of 15 ml for routine screening, including full blood count, cholesterol, glucose and triacylglycerol levels. Volunteers will be provided with a light breakfast once the blood sample has been taken. If a volunteer is found to have a low hemocue reading, we will suggest that they see their GP for a re-test. The volunteers will then attend the unit for a second time when 2 blood samples will be taken for research purposes, prior to and 3h after eating a set breakfast consisting of two croissants and a warm chocolate drink. Again, volunteers will need to have fasted overnight. Blood sample volumes will be in the range of 20-50 ml.

Recruitment will be from healthy adult non-smokers (age range 18-65 y), with no illnesses requiring medication, such as high blood pressure needing antihypertensive treatment or high cholesterol requiring statin treatment. Recruitment will deliberately be kept broad so as not to bias the methods developed to a particular subgroup of the population.

Inclusion criteria will be:

- Age range 18-65 y
- Non smoker
- Haemoglobin ≥115 g/l for women and 130g/l for men
- No disclosed history of drug or alcohol abuse
- No illness or disease requiring medication (excluding HRT, oral contraceptive and thyroxine replacement therapy)

Blood samples collected during the study visit and screening will be stored under the authority of the School's HTA research licence (currently held by Professor Glenn Gibson).

(Note: All questionnaires or interviews should be appended to this application)

2.3

Where will the project take place? Hugh Sinclair Unit of Human Nutrition

If the project is to take place in Hugh Sinclair Unit of Human Nutrition, projects must be reviewed and approved by the Hugh Sinclair Manager (Sarah Hargreaves s.e.hargreaves@reading.ac.uk)

Signed.. (Hugh Sinclair Unit Manager) Date:...7/9/17

2.4

Funding

Is the research supported by funding from a research council or other *external* sources (e.g. charities, business)? Yes

If Yes, please give details:

This work is funded by a studentship from the Saudi Cultural Bureau for 4 years, starting February 2017 and finishing January 2021.

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Please note that *all* projects (except those considered as low risk, which would be the decision of the School's internal review committee and require Head of Department approval) require approval from the University Research Ethics Committee.

2.5

Ethical Issues
Could this research lead to any risk of harm or distress to the researcher, participant or immediate others? Please explain why this is necessary and how any risk will be managed.

Most people feel no effects during or after blood donation, but occasionally donors may develop some mild bruising. Rarely, some people may feel a little queasy during or after donation. If the volunteer suffers from this, blood collection will cease immediately. Most people recover spontaneously once donation ceases. If there is a continuing problem, first aid is readily available in the Hugh Sinclair Unit of Human Nutrition. An adverse events form will also be completed and then followed up by the units research nurses.

(*this box may be expanded as required*)

2.6

Deception
Will the research involve any element of intentional deception at any stage (i.e. providing false or misleading information about the study, or omitting information)? **No**
[If so, this should be justified. You should also consider including debriefing materials for participants, which outline the nature and the justification of the deception used]

2.7

Payment
Will you be paying your participants for their involvement in the study? **No**
If yes, please specify and justify the amount paid

Note: excessive payment may be considered coercive and therefore unethical. Travel expenses need not to be declared.

2.8

Data protection and confidentiality
What steps will be taken to ensure participant confidentiality? How will the data be stored?

Participants will be identified using codes at all stages of the analysis. All their personal data will be stored on university computers and kept separate from the actual study data. The information on these computers is only accessible by password, known only by the key researcher. Each university computer is equipped with access control to ensure that access to confidential information is restricted to the project researchers only. The consent forms will be stored at the Hugh Sinclair Unit of Human Nutrition for 5 years, or until all final reports and scientific publications that arise from the study are completed. Only the Principal Investigator and members of the research team will have access to the data. All paperwork will be kept securely by the

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investigators in a locked cabinet and locked office. No material is referenced to its source and names will not be used in any publications.

2.9

Consent

Please describe the process by which participants will be informed about the nature of the study and the process by which you will obtain consent

All participants will be given an information sheet (Appendix E: Participant Information Sheets), which explains the basis for our research. Participants will give their consent by completion of a written consent form, in the presence of the researcher, who will also sign (Appendix C: Consent Forms). A copy of the signed consent will be kept by the participants. For each volunteer, the date, time and volume of blood donated will be recorded. These records contain no other information about the blood provided.

Please note that a copy of consent forms and information letters for all participants must be appended to this application.

2.10

Genotyping

Are you intending to genotype the participants? Which genotypes will be determined? **No**

Please note that a copy of all information sheets on the implications of determining the specific genotype(s) to be undertaken must be appended to this application.

SECTION 3: PARTICIPANT DETAILS

3.1

Sample Size

How many participants do you plan to recruit? Please provide a suitable power calculation demonstrating how the sample size has been arrived at or a suitable justification explaining why this is not possible/appropriate for the study.

This is work which will take place over a 3 year period and will involve multiple experiments and refining of analytical techniques, with each experiment being informed by the results of the previous experiment. For this reason, it is impossible to put a number on the sample size, except to say that blood samples from about 1-3 different volunteers per week will be required.

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3.2	Will the research involve children or vulnerable adults (e.g. adults with mental health problems or neurological conditions)? **No** If yes, how will you ensure these participants fully understand the study and the nature of their involvement in it and freely consent to participate? (Please append letters and, if relevant, consent forms, for parents, guardians or carers). Please note: information letters must be supplied for all participants wherever possible, including children. Written consent should be obtained from children wherever possible in addition to that required from parents.
3.3	Will your research involve children under the age of 18 years? **No** Will your research involve children under the age of 5 years? **No**
3.4	Will your research involve NHS patients, Clients of Social Services or will GP or NHS databases be used for recruitment purposes? **No** Please note that if your research involves NHS patients or Clients of Social Services your application will have to be reviewed by the University Research Ethics Committee and by an NHS research ethics committee.
3.5	**Recruitment** Please describe the recruitment process and append all advertising and letters of recruitment. Potential participants will be recruited by the student conducting the study by e mailing staff and students at the University (Appendix F: script for e mail) and displaying posters on noticeboards (Appendix G: poster). The student will also search the Hugh Sinclair Unit of Human Nutrition database for eligible volunteers, who will be contacted directly by email.

Important Notes

1. The Principal Investigator must complete the Checklist in Appendix A to ensure that all the relevant steps have been taken and all the appropriate documentation has been appended.

2. If you expect that your application will need to be reviewed by the University Research Ethics Committee you must also complete the correct Application form including the Form Appendix B.

3. For template consent forms, please see Appendix C.

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Appendix A: Application checklist

This must be completed by an academic staff member (e.g. supervisor)

Please tick to confirm that the following information has been included and is correct.
Indicate (N/A) if not applicable:

Information Sheet

Is on headed notepaper	☒	
Includes Investigator's name and email / telephone number	☒	
Includes Supervisor's name and email / telephone number	☒	
Statement that participation is voluntary	☒	
Statement that participants are free to withdraw their co-operation	☒	
Reference to the ethical process	☒	
Reference to Disclosure	☐	N/A ☒
Reference to confidentiality, storage and disposal of personal information collected	☒	
Consent form(s)	☒	

Other relevant material

Questionnaires	☒	N/A ☐
Advertisement/leaflets	☒	N/A ☐
Letters	☐	N/A ☒
Other (Email script)	☒	N/A ☐

Expected duration of the project (months) 36

Name (print) Parveen Yaqoob Signature … **……**

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Appendix B

Project Submission Form

Note All sections of this form should be completed. Please continue on separate sheets if necessary.

Principal Investigator: Prof Parveen Yaqoob

School: SCFP

Title of Project: Effects of a meal on extracellular vesicles in blood

Proposed starting date: September 2017

Brief description of Project:

Circulating extracellular vesicles (EVs) are small particles shed from all cells, with promise as new biomarkers and therapeutic targets in numerous diseases. When analysing circulating EVs, blood samples tend to be taken from fasted volunteers to avoid interference by fat-containing particles in the bloodstream, which have a size range that overlaps with EVs and whose levels increase in the blood following consumption of a meal containing fat. However, this is not a 'typical' situation as most people spend most of the day in a non-fasted state and it is very important not only to resolve the overlap between fat particles and EVs, but also to understand the effects of a meal on numbers, size and behaviour of EVs. We plan to carry out human intervention studies testing the effects of meals containing different types of fat on the ability to isolate EVs and on their number, size, composition and function. This requires isolation of blood EVs from volunteers prior to and following consumption of a meal. We are therefore applying for ethical permission to recruit volunteers for blood donation for method optimisation and development in the Department of Food and Nutritional Sciences.

I confirm that to the best of my knowledge I have made known all information relevant to the SCFP Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed… .....(Investigator) Date…8/9/17

.. ..(Head of Department) Date…22/9/17…

(Other named investigator Date: 07/09/17 (Where applicable)

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Checklist

1. This form is signed by my Head of Department ☑

2. The Consent form includes a statement to the effect that the project has been subject to ethical review, according to the procedures specified by the University Research Ethics Committee, and has been allowed to proceed ☑

3. I have made, and explained within this application, arrangements for any confidential material generated by the research to be stored securely within the University and, where appropriate, subsequently disposed of securely. ☑

4. I have made arrangements for expenses to be paid to participants in the research, if any, OR, if not, I have explained why not. ☑

5. Tick EITHER **(a) OR (b) - Head of School to sign if (b) ticked**

   (a) The proposed research does **NOT** involve the taking of blood samples; ☐

   OR

   (b) For anyone whose proximity to the blood samples brings a risk of Hepatitis B, documentary evidence of protection prior to the risk of exposure will be retained by the Head of School. ☒

   Signed. .(Head of Department) Date.....22/9/17.

6. Tick **EITHER (a) OR (b)**

   (a) The proposed research does **NOT** involve the storage of human tissue, as defined by the Human Tissue Act 2004; ☒

   OR

   (b) I have explained within the application how the requirements of the Human Tissue Act 2004 will be met. ☐

7. Tick **EITHER (a), (b) OR (c)**

   (a) The proposed research will not generate any information about the health of participants; ☐

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OR

(c) In the circumstance that any test reveals an abnormal result, I will inform the participant and, with the participant's consent, also inform their GP, providing a copy of those results to each; ☒

OR

(c) I have explained within the application why (b) above is not appropriate. ☐

8. Tick **EITHER (a) OR (b) - Head of School to sign if (b) ticked**

(a) The proposed research does not involve children under the age of 5; ☒

OR

(b) My Head of School has given details of the proposed research to the University's insurance officer, and the research will not proceed until I have confirmation that insurance cover is in place. ☐

Signed.....(Head of Department) Date.....

This form and further relevant information (see Sections 5 (b)-(e) of the Notes for Guidance) should be returned to, Barbara Parr, SCFP Ethics Administrator. You will be notified of the Committee's decision as quickly as possible, and you should not proceed with the project until then.

University of Reading

Appendix C

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Consent Form for Effects of a meal on extracellular vesicles in blood

Please initial boxes

1. I confirm that I have read and understand the Participant Information Sheet dated _____for the above study, which was explained by _____. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that confidentiality and anonymity will be maintained and it will not be possible to identify me in any publications. ☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason. ☐
4. I have received a copy of this Consent Form and of the accompanying Participant Information Sheet. This study has been reviewed by the School of Chemistry, Food & Pharmacy Ethics Committee and has been given a favourable ethical opinion for conduct. ☐
5. I consent to an initial finger prick blood sample being taken for screening purposes, followed by a single blood sample as explained on the accompanying Participant Information Sheet. ☐
6. I have had explained to me that consent for my contact details and personal information to be added to the Hugh Sinclair Unit of Human Nutrition Volunteer Database is entirely voluntary.
   Accordingly I consent as indicated below: ☐

- I consent to my contact details being stored on the Nutrition Unit Volunteer Database. ☐ Yes ☐ No
- I consent to my screening information (including date of birth, height, weight, blood pressure, smoking status, long-term use of medication, and haemoglobin test result being stored on the Nutrition Unit Volunteer Database. ☐ Yes ☐ No

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Participant details

Name of Participant: _____ Date of Birth: _____

Signature: _____ Date: _____

Address of Participant:

(Please add if you wish to receive the overall results of the study, and/or you consent to be part of the Hugh Sinclair Unit of Human Nutrition Volunteer Database)

_____

_____

Telephone number: _____

Witnessed by

Name of researcher taking consent: _____

Signature: _____ Date: _____

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Appendix D

Effects of a meal on extracellular vesicles in blood

Medical and Lifestyle Questionnaire

Name:		Title:
Address:		Date of Birth: Age:
Daytime Telephone:	Evening Telephone:	Best time to call:
Weight (Kg)	Height (m)	BMI
E-mail: Do you use emails on a regular basis? YES/NO		Occupation:

How did you hear about the study? _____

Medical questions (*Please circle as appropriate*)

1. Have you been diagnosed as having any of the following?

a) High blood cholesterol YES/NO
b) High blood pressure YES/NO
c) Thyroid disorder YES/NO
d) Diabetes or other endocrine disorders YES/NO
e) Heart problems, stroke or any vascular disease in the past 12 months YES/NO
f) Inflammatory diseases (e.g. rheumatoid arthritis) YES/NO
g) Renal, gastrointestinal, respiratory or liver disease YES/NO
h) Cancer YES/NO

2. Have you been diagnosed as suffering from any other illness? YES/NO
If 'YES', please give details

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3. Within the past 3 months, have you taken any medication (prescription or non-prescription)?
YES/NO
If 'YES', what are they and for what reasons?

4. Have you had any surgery within the past 3 months or do you have surgery planned?
YES/NO
If 'YES', please give details

5. Do you have a pacemaker? YES/NO

Lifestyle questions

6. Are you currently taking part in, or within the last 3 months been involved in a clinical trial or a research study? YES/NO
If 'YES', please give details:

7. Have you been screened or contacted recently about a study? YES/NO
If 'YES', please give details

8. Are you a blood donor? YES/NO
If 'YES', when was the last time you gave blood?

9. Do you use any of the following?
a) Dietary supplements, e.g. fish oils, evening primrose oil, vitamins or minerals (such as iron or calcium) YES/NO
b) Probiotics, e.g. Actimel, Yakult, Activia yoghurts or capsules YES/NO
c) Cholesterol-lowering products, e.g. Flora Pro-Activ or Benecol YES/NO
If 'YES' to any, please give details

10. Are you vegetarian or vegan? YES/NO
If 'YES', please specify

11. Do you drink alcohol? YES/NO
If 'YES', approximately how many units do you drink per week? _____ *Units*
One unit of alcohol is half a pint of beer/lager, a single pub measure of spirits e.g. gin/vodka, or a small glass of wine (125 ml).

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12. Do you exercise more than three times a week, including walking? YES/NO
If 'YES', please specify the type of exercise, frequency and intensity

13. Do you smoke? YES/NO
If 'YES, please give details

This is the end of the questionnaire - thank you for your time.
All information provided will remain confidential at all times.

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Researcher (principal): Prof. Parveen Yaqoob
Researcher (study coordinator): Dr Dionne Tannetta; Dr Plinio Minghin Freitas Ferreira
Email: d.s.tannetta@reading.ac.uk; p.minghinfreitasferreira@reading.ac.uk
Phone: 0118 378 5360

Appendix E: INFORMATION SHEET for Effects of a meal on extracellular vesicles in blood

Why are we doing this study?
Extracellular vesicles (EVs) are tiny particles shed from all cells and can be found in the blood. EVs are emerging as potential new biomarkers in a number of diseases, such as cancer, cardiovascular disease and neurodegenerative diseases. They may also be potential targets for drug treatments.

What is the purpose of the study?
The purpose of the study is to collect blood samples from healthy donors in order to test the effects of a meal on the isolation, characteristics and behaviour of EVs.

Who is eligible, to participate in the study?
We are looking to recruit healthy adult non-smokers (age range 18-65 y) with no diagnosed disease and no illnesses requiring long term medication.

How can you be involved?
You can help by donating a blood sample before and after eating a breakfast consisting of two croissants and a warm chocolate drink. Your blood will be used by the research team to test our methods for the isolation and analysis of EVs in blood. This project has been approved by the School of Chemistry, Food and Pharmacy Internal Research Ethics Committee. You are under no obligation to participate, and can withdraw from the study at any time. Your decision to drop-out will not influence your participation in future studies in the High Sinclair Unit of Human Nutrition.

What will be involved if you take part?
This research will require the completion of a medical and lifestyle questionnaire in the first instance to see if you are suitable to participate in this study. You will then need to make two visits to the Hugh Sinclair Unit of Human Nutrition in the Dept of Food & Nutritional Sciences at the University of Reading, where you will be given the opportunity to ask any questions and a consent form to sign if you are happy to participate. Your BMI will then be measured and a finger prick blood sample will be taken to check haemoglobin levels, followed by the blood sample of 15 ml for routine screening, including full blood count, cholesterol, glucose and triacylglycerol levels. All blood samples will be taken by our research nurses or qualified, experienced investigators (who are not medically qualified but have been trained in venepuncture and first aid). You will need to be fasted for this visit (i.e. not eaten food or consumed alcohol since 8 pm the previous evening and only drank water during this time). You will be provided with a light breakfast once your blood sample has been taken. You will then attend the unit for a second time when 2 blood samples will be taken for research purposes, one prior to and one 3h after eating a set breakfast consisting of two croissants and a warm chocolate drink. Again, you will need to have fasted overnight. Blood sample volumes will be in the range of 20-50 ml.

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Confidentiality, storage and disposal of information
You will be identified using randomised numerical codes throughout this study. All of your personal data will be stored in computers specifically allocated to this study and accessible by password, known only by the study researchers. The consent forms will be stored at the Hugh Sinclair Unit of Human Nutrition for a maximum of 5 years. Only the Principal Investigator and members of the research team will have access to the data. All data and records of all blood donations will be kept securely on password protected computers and paperwork in locked cabinets and locked offices. No material is referenced to its source and names will not be used in any publications.

Are there any benefits/risks to taking part [e.g. health]?
Most people feel no effects during or after blood donation, although occasionally donors may develop some mild bruising. Rarely, some people may feel a little queasy during or after donation. If this happens to you blood collection will cease immediately. Most people recover spontaneously once donation ceases. If there is a continuing problem, first aid is readily available in the Hugh Sinclair Unit of Human Nutrition.

What if there is a problem?
Complaints
If you have a concern about any aspect of this study, you should ask to speak to the investigators who will do their best to answer your questions (see contact details at the end of this Participant Information Sheet). If you remain unhappy and wish to complain formally, you can do this through the Head of the Department of Food & Nutritional Sciences, Professor Richard Frazier (tel: 0118 378 8709; email: r.a.frazier@reading.ac.uk)..

Harm
In the event that something does go wrong and you are harmed during the study the University of Reading has in place Professional Indemnity Insurances.

What expenses and/or payment or equivalent will be made for participation in the study?
We are unable to pay for blood donations, but volunteers who need to travel to the University to provide a blood sample can request reimbursement of their travel expenses.

What will the results of the study be used for?
The results will contribute to ongoing research activities of the group and will underpin studies published in scientific journals.

Contact details for further questions, or in the event of a complaint
For further details please contact the study researcher in the first instance Amal Nadi M. Alanzeei phone: and email: a.n.m.alanzeei@pgr.reading.ac.uk. The study Principal Investigator is Prof Parveen Yaqoob phone: 0118 378 8720 and email: p.yaqoob@reading.ac.uk. The Head of Department is Prof Richard Frazier phone: 0118 378 8709 and email: r.a.frazier@reading.ac.uk.

Thank you for your help.

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Appendix F

Script for e-mail for Effects of a meal on extracellular vesicles in blood
E-mail

Re: Volunteers needed to donate blood for research involving characterisation of blood extracellular vesicles

Extracellular vesicles are released by cells and play an important role in both health and disease. We are investigating the effects of a meal on extracellular vesicles in blood.

If you are a healthy adult non-smoker (age range 18-65 y) with no diagnosed disease, no illnesses requiring long term medication and are willing to donate blood, we need you.

For more information, please contact Amal Nadi M. Alanzeei
email: a.n.m.alanzeei@pgr.reading.ac.uk or phone:

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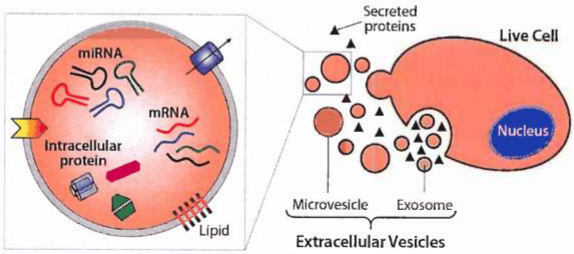
Appendix G

VOLUNTEERS REQUIRED

FOR RESEARCH ON EXTRACELLULAR VESICLES



We require small blood samples to help us understand the effects of a meal on extracellular vesicles in blood

If you are a healthy adult non-smoker (18-65 years) and are willing to help by donating blood samples before and after a breakfast, we need you.

Travel expenses will be reimbursed.

TO FIND OUT MORE:
contact Amal Nadi M. Alanzeei
(Food and Nutritional Sciences)
phone:
& e-mail: a.n.m.alanzeei@pgr.reading.ac.uk

Full Application Form

Filter Questions

1 Is your study considered research as defined in the guidance icon information? (•) Yes ( ) No

2 Does the research fall under the requirements of the HRA and therefore require ethical review by an NHS REC, Social Care REC or MoDREC? Please refer to the information in the guidance icon for further details. ( ) Yes (•) No

Data Collection

3 Select one category from the list below (categories are defined in the guidance icon).

My study involves:

- (•) a) Only primary data collection involving human subjects.
- ( ) b) Only further analysis of pre-existing data (originally obtained from human participants) which is identifiable and not in the public domain.
- ( ) c) Both primary data collection involving human subjects and further analysis of pre-existing data (originally obtained from human participants) which is identifiable and not in the public domain
- ( ) d) Data collection not involving any of the above but presenting sensitive issues
- ( ) e) None of the above

4 Select all that apply in order to determine the risk level of your application.

- ☐ a) Does the research involve participants who are particularly vulnerable or unable to give informed consent or in a dependent position?
- ☐ b) Will participants be asked to take part in the study without their consent or knowledge at the time or will deception of any sort be involved?
- ☐ c) Is there a risk that the research topic might lead to disclosures from the participant concerning their involvement in illegal activities or other activities that represent a threat to themselves or others?
- ☐ d) Could the study induce psychological stress or anxiety, or produce humiliation or cause harm or negative consequences beyond the risks encountered in a participant's usual everyday life?
- ☐ e) Does the study involve imaging techniques such as MRI scans or ultrasound?
- ☐ f) Does the study involve sources of non-ionising radiation (e.g. lasers)?
- ☑ g) Does the study involve physically intrusive procedures, use of bodily materials, or DNA/RNA analysis? (including collection of human tissue)

You should only select the statement below if you have not selected any of the above. Your application will be invalid if you select the below statement in addition to any of the above.

- ☐ I have answered no to all questions in the risk checklist above and I believe that my research is low risk

Based on your answers to the above filter questions your research has been categorised as High Risk and upon submission will be subject to review at the next relevant Research Ethics Subcommittee meeting. You can now access an overview of the available sections of the application by selecting the navigate tile in the action panel on the left. Alternatively you can proceed through each section of the application by selecting the next tile.

Meeting dates and submission deadlines can be found here

Section A: General Information

A Applicant Details

Title	First Name	Surname
Dr	Charlotte	Mills

Department Name: Diabetes & Nutritional Sciences Division

Address: 4.103, FWB, 150 Stamford St

City: London

County: UK

Postcode: SE1 9NH

Telephone:

KCL Email:

A2 Applicant Status

Staff

A3 Applicant Role

Other

A4 What is your role in the project?

Postdoctoral Research Associate

A5 Is King's College London the research sponsor? (•) Yes ( ) No

If no, please explain why ethical approval is being sought through KCL and not the sponsoring Institution.

N/A

A6 Who is the Principal Investigator?

Title	First Name	Surname
Dr	Sarah	Berry

Organisation: Diabetes & Nutritional Sciences Division

Address: 4.09, FWB, 150 Stamford st

Postcode: SE1 9NH

Telephone:

Email:

A7 Faculty/Institute/School

Please refer to the information icon if you are unsure of your Faculty/Institute/School.

Life Sciences and Medicine

A9 Job Title

Senior Lecturer in Nutritional Sciences

A13 Other Investigators/Collaborators

Title	First Name	Surname
Dr	Wendy	Hall

Organisation: Diabetes & Nutritional Sciences Division

Address: 4.108, FWB, 150 Stamford st

Postcode: SE1 9NH

Telephone:

Email:

What is the role of this investigator?

Co-PI

Section B: Project Information

B1 Project Title

The INTER-CARDIO study: The impact of commonly consumed fat on cadiovascular health; a randomised controlled trial

B2 Proposed start date 05/03/2018

B3 Expected completion date 31/12/2018

B4 Is this a funded project?

- (•) Yes
- ( ) No

How is the project being funded?

Externally funded

Please state your Funder Reference Number:
If you are unaware of your Grant Award Reference or Contract's Funder Reference please contact your Award Management Division campus team leader who will provide the relevant information.

BB/N020987/1

B5 What are the aims and objectives of the project?

Provide the academic/scientific justification of the project as well as detailing and explaining the principal research question, objectives and hypotheses to be tested.

Please Note: Applications to the BDM and PNM RESC should include a full list of references/citations to back up the academic/scientific justification of the project.

Background: Dietary fat consists mainly of triacylglycerol molecules (TAG), onto which 3 fatty acids can be attached at one of three positions i.e. position 1, 2 or 3. Dietary fat is one of the major modifiable risk factors implicated in the causation of cardiovascular disease (CVD), and the effects of differences in the properties of dietary fat, such as fatty acid chain length, degree of unsaturation and geometry of double bonds (cis or trans) are well documented. However, until recently, little attention has been given to the position of fatty acids in TAG molecule. The position of fatty acids in the TAG molecule (1, 2 and 3) determines the physical properties of the fat (such as melting point), and this in turn affects its absorption from the gut, metabolism in the blood and distribution into tissues. Following a high fat meal, there is a short-term change in blood concentrations of TAG, which is termed postprandial lipaemia. It is known that an elevated postprandial lipaemia (Patsch et al 1992) is associated with increased risk of CVD, via effects on lipids (Karpe 1997), inflammation (Vogel et al 1997), vascular function (Berry et al 2008) and blood clotting activity (Miller 1998). Any factors affecting rates of absorption and digestion will influence the magnitude and duration of the postprandial response, and therefore CVD risk.

In vegetable fats and oils (such as palm oil and cocoa butter), saturated fatty acids (SFA) are found predominantly

in the external positions (positions 1 and 3) and unsaturated fatty acids in the middle position (position 2) of the TAG. In animal fats (such as lard) however, the middle position of the molecule contains a higher proportion of SFA. It is suspected that these differences in the positional compositions of the plant and animal fats could be responsible for the differences in their effects on blood lipids and CVD risk (Kritchevsky 1995).

The process of interesterification, which is commonly used by the food industry, changes the position of fatty acids in the TAG. For example, when palm oil is interesterified to produce a harder fat that can be used by the food industry, there is an increase in the proportion of SFAs (specifically palmitic acid) in the middle position of the TAG molecule. Interesterified palm oil thus has more palmitic acid in position 2 of TAG than un-interesterified palm oil. Although this process of interesterification is becoming widely used by the food industry, the effect of these positional changes in the TAG on CVD risk factors is uncertain.

Previous research has shown that the positional composition of TAG influences rates of TAG absorption and therefore the level of postprandial lipaemia (reviewed by Berry 2009). Most research to date by our group (Hall et al. 2014; Filippou et al 2014a & b; Sanders et al. 2011 & 2003; Berry et al 2008, 2007a & b) and others (Zampelas et al 1994, Yli-Jokipii et al 2001) has largely focused on the postprandial effect of interesterification of palm oil or shea butter. However these findings are not relevant from a public health perspective, as straight palm oil is not commercially used by the food industry for interesterification. Instead the palm oil fractions; palm stearin and palm kernel are interesterified to create a 'hardstock' blend which is then blended with rapeseed oil. This blend of interesterified 'hardstock' and rapeseed oil are now used in most commercially available spreads in the UK (e.g. Flora, Clover, Utterly Butterly). Despite the wide use of this interesterified fat blend, only one pilot study and one full study carried out by our group (Hall, et al 2016 and unpublished, respectively) have investigated the acute effects of these nutritionally relevant fats (interesterified palm stearin and palm kernel oil) on cardio-metabolic risk factors and there is no direct comparison with application appropriate alternative fats (e.g. butter).

Aim: The current study therefore aims to investigate the acute effects of commonly consumed interesterified fat ('spread') versus an application appropriate fat (butter) on postprandial lipaemia and cardiometabolic health in a full study.

Hypothesis: Interesterified blends of a palm kernel and palm stearin fat with rapeseed oil, will have differing postprandial lipid metabolism and absorption and cardiometabolic outcomes compared to an application appropriate alternative (butter).

Expected value: The study will provide novel information on the acute effects of commercially relevant applications of interesterified fats on postprandial lipaemia. This data will be used to assess the atherogenic potential of the lipoprotein profile over the course of the study with physical outcomes also to be assessed.

References

Berry SE. Triacylglycerol structure and interesterification of palmitic and stearic acid-rich fats: an overview and implications for cardiovascular disease. Nutr Res Rev. 2009 Jun;22(1):3-17.
Berry SE, Tucker S, Banerji R, Jiang B, Chowienczyk PJ, Charles SM, Sanders TA. Impaired postprandial endothelial function depends on the type of fat consumed by healthy men. J Nutr. 2008 Oct;138(10):1910-4.
Berry SE, Miller GJ, Sanders TA. The solid fat content of stearic acid-rich fats determines their postprandial effects. Am J Clin Nutr. 2007a Jun;85(6).
Berry SE, Woodward R, Yeoh C, Miller GJ, Sanders TA. Effect of interesterification of palmitic acid-rich triacylglycerol on postprandial lipid and factor VII response. Lipids. 2007b Apr;42(4):315-23.
Filippou A, Teng KT, Berry SE, Sanders TA. Palmitic acid in the sn-2 position of dietary triacylglycerols does not affect insulin secretion or glucose homeostasis in healthy men and women. Eur J Clin Nutr. 2014a Sep;68(9):1036-41.
Filippou A, Berry SE, Baumgartner S, Mensink RP, Sanders TA. Palmitic acid in the sn-2 position decreases glucose-dependent insulinotropic polypeptide secretion in healthy adults. Eur J Clin Nutr. 2014b May;68(5):549-54.
Hall WL, Brito MF, Huang J, Wood LV, Filippou A, Sanders TA, Berry SE. An interesterified palm olein test meal decreases early-phase postprandial lipemia compared to palm olein: a randomized controlled trial. Lipids. 2014 Sep;49(9):895-904.
Hall WL, Iqbal, S, Li H, Gray R, Berry SEE. Modulatin of postprandial lipaemia by a single meal containing commonly consumed interesterified palimitic acid rich fat blen compared to a non-interesterifed equivalent. European Journal of Nutrition 2016 Aug: 1-9 [Epub ahead of print]
Jenkins DJ, Kendall CW, Josse AR, Salvatore S, Brighenti F, Augustin LS, Ellis PR, Vidgen E, Rao AV. Almonds decrease postprandial glycemia, insulinemia, and oxidative damage in healthy individuals. J Nutr. 2006 Dec;136(12):2987-92.
Karpe F. Postprandial lipid metabolism in relation to coronary heart disease. Proc Nutr Soc 1997;56:671-8.
Kritchevsky D. Fatty acids, triglyceride structure, and lipid metabolism. J.Nutr.Biochem. 6, 172-178. 1995.

Miller GJ. Postprandial lipaemia and haemostatic factors. Atherosclerosis 1998;141 Suppl 1:S47-S51.
Patsch JR, Miesenbock G, Hopferwieser T et al. Relation of triglyceride metabolism and coronary artery disease. Studies in the postprandial state. Arterioscler Thromb 1992;12:1336-45.
Sanders TA, Filippou A, Berry SE, Baumgartner S, Mensink RP. Palmitic acid in the sn-2 position of triacylglycerols acutely influences postprandial lipid metabolism. Am J Clin Nutr. 2011 Dec;94(6):1433-41
Sanders TA, Berry SE, Miller GJ. Influence of triacylglycerol structure on the postprandial response of factor VII to stearic acid-rich fats. Am J Clin Nutr. 2003 Apr;77(4):777-82.
Vogel RA, Corretti MC, Plotnick GD. Effect of a single high-fat meal on endothelial function in healthy subjects. Am J Cardiol 1997;79:350-4.Yli-Jokipii, K., et al. "Effects of palm oil and transesterified palm oil on chylomicron and VLDL triacylglycerol structures and postprandial lipid response." J.Lipid Res. 42.10 (2001): 1618-25.
Zampelas, A., et al. "The effect of triacylglycerol fatty acid positional distribution on postprandial plasma metabolite and hormone responses in normal adult men." Br.J.Nutr. 71.3 (1994): 401-10.
Zock, P. L., et al. "Positional distribution of fatty acids in dietary triglycerides: effects on fasting blood lipoprotein concentrations in humans." Am.J.Clin.Nutr. 61.1 (1995): 48-55.

B6 Where will the research be conducted? i.e in a facility within the college, in a private organisation, in a public place etc

Metabolic Research Unit in Nutritional Sciences Department, Franklin Wilkins Building, King's College London.

B7 If outside of the UK, please state the country/countries in which data collection is expected to occur.

N/A

B8 Selection of methodology from list: (select each that applies)

- [x] Questionnaires
- [ ] Semi-structured interviews
- [ ] Unstructured Interviews
- [ ] Focus Groups
- [ ] Observation
- [ ] Clinical Procedures or Interventions
- [x] Non-clinical Procedures or Interventions
- [x] Randomised Controlled Trial
- [ ] Oral history
- [ ] Secondary analysis of pre-existing data from human participants
- [ ] Audio/video recording or photography in a public place
- [ ] Audio/video recording or photography in a private place
- [x] Administration of food substances
- [ ] Behavioural/Cognitive Testing
- [ ] Other

If you are using any standardised methods for any of the above selected methodologies, please provide an overview of any standardised documentation to be used. Please provide full names and references where appropriate.
Please note you are not required to submit any standardised forms as supporting documents.

The study will follow a standardised protocol which has been used in previous studies by our group as published; Hall (2016), Hall (2014), Sanders (2011), Sanders (2003), Flippou (2014), Berry (2007a, 2007b and 2008) as well as recently approved project RESCMR - 16/17 - 4397.

B9 Give a summary of the project methodology.

A randomised controlled, double blind, crossover study will be conducted (n= 40). Participants will received one of three test meals (a muffin and a milkshake) in a random order containing 50 g test fat, 15 g protein and 85g carbohydrate (3.13 MJ) test fats will be produced by ADM Ltd (composition and structure will be confirmed in our lab). At least seven day wash-out period between study days will be required between study days. Participants will be asked to avoid exercise and alcohol for 24 h before the study visit and will be given a standardised low fat meal to have for dinner the nights before the study visit.
Participants will arrive on each study visit at approximately 07:30 h, following a 10 h overnight fast. A cannula will then be inserted into their vein and a baseline fasting blood sample will be taken. The test meal will then be consumed by the participant, within 15 minutes. Further samples will be collected at regular intervals following the test meal to determine parameters as described below.
A second meal challenge as a muffin providing 35 g of rapeseed oil (~15% protein, 50% carbohydrate, 35% fat) will be given at 5 hours to replicate a realistic meal pattern.
A total of 180 mL (approximately 36 teaspoons) blood will be collected on each study day so that a total of 540 mL blood will be collected over the whole 3 visits. Volunteers will be advised to sip water regularly throughout the visit, we will supply 150 mL for them every hour. At the end of the study participants will be provided with a complementary meal or snack.
The primary outcome of the study is flow mediated dilation (FMD), a non invasive measure for assessing nitric-oxide dependent endothelial function by ultrasound imaging of the brachial artery before and after hyperaemia will also be performed at 0, 4.5 and 7.5 hours.
Other outcomes include, plasma triacylglycerol (a measure of cardiovascular risk), plasma fatty acid composition, lipoprotein sizes and particle numbers which will be measured hourly. NOx, 8-isoprostane F2a and IL-6 will be measured at 0, 4, 6, 8 hours. Neutraphil NADPH oxidase at 0, 4, 6 hours, and extracellular vesicles at 0, 2 and 4 hour. Non esterifed fatty acids, glucose and insulin will be measured at 0.5, 1, 2, 3, 4, 5, 5.5, 6, 7, 8 hours.
Statistical methods: Anonymised data will be entered onto an Excel spreadsheet database. The data will be analysed using linear mixed modelling.

If the summary of your methodology would be supported by a flowchart please attach this here (an editable flowchart can be found via the link in the guidance icon)

Type	Name	File Name	Date	Version	Size
Other	Study flow chart_INTER-CARDIO V1 03 Jan 2018	Study flow chart_INTER-CARDIO V1 03 Jan 2018.pptx	26/01/2018 12:00:00 AM	1	63.5 KB

B10 I confirm that the researcher who will be administering all tests and/or procedures is competent in the methods.

- (•) Yes
- ( ) No

Section C(I): Participants

C1 Detail your projected number of participants and provide justification for this sample size.

Based on work by our group (Purcell et al, 2014), we estimate that a sample size of 20 males and 20 females to detect a (clinically relevant) difference (for males and females separately) between means of 2.16% difference in FMD to be detected (90% power, 0.05 alpha, SD 2.9). We will allow for a 20% drop out rate and therefore will recruit 50 participants.

C2a What are the Inclusion Criteria? Where appropriate explain how you will screen your participants. (the selection criteria should be clearly defined for multiple participant groups)

- Males and females aged 35-75 years
- Healthy (free of diagnosed diseases listed in exclusion criteria)
- Able to understand the information sheet and willing to comply with study protocol
- Able to give informed consent

C2b What are the Exclusion Criteria? Where appropriate explain how you will screen your participants. (the selection criteria should be clearly defined for multiple participant groups)

- Medical history of myocardial infarction, angina, thrombosis, stroke, cancer, liver or bowel disease or diabetes
- Body mass index < 20 kg/m2 or > 35 kg/m2
- Plasma cholesterol ≥7.5 mmol/L
- Plasma triacylglycerol > 3 mmol/L
- Plasma glucose > 7 mmol/L
- Blood pressure ≥140/90 mmHg
- Current use of antihypertensive or lipid lowering medications
- Alcohol intake exceeding a moderate intake (> 28 units per week)
- Current cigarette smoker (or quit withint the last 6 months)
- ≥ 20% 10-year risk of CVD as calculated using a risk calculator

C3 What are the upper and lower age limits? Provide justification for these where appropriate.

Aged between 35 and 75 years old. At this age/ period of life, metabolic changes occur and risk of cardiovascular disease increases. This will enable a more sensitive measure of metabolic differences due to interesterification to be detected.

C4 How will potential participants be identified and approached?

Subjects will be recruited from the student and staff population of Kings College London (KCL), via the KCL recruitment newsletter. We will also recruit via the use of, social media (Facebook, Twitter etc...), posters and newspaper advertisements. Individuals responding to advertisements will initially be sent the patient information sheet and a health questionnaire to complete to assess whether the study is suitable for them or not. Eligible respondents will then be invited to attend a screening session at KCL (Metabolic Research Unit, Franklin Wilkins Building) during which volunteers will be asked to complete a consent form have a fasting blood sample taken (17 mL, 3.5 teaspoons; for measurement of glucose and lipid concentrations, liver function tests and full blood counts) and a brief medical examination (to measure blood pressure, waist circumference, weight, height, and body composition (% body fat)). Subjects accepted onto the study according to the inclusion and exclusion criteria will complete a 3-day diet diary for assessment of habitual nutrient intake. Volunteers who do not meet the study inclusion criteria will be contacted by email or letter and informed that they will not be asked to take part on this occasion, explaining the reason why.

C5 If any participants are under 16 will you seek additional consent from parents or carers? ○ Yes ○ No ◉ N/A

C6 Please specify any incentives being offered and a justification for their use.

Participants will receive £150 in compensation for the time dedicated to the study. If the participant is withdrawn from the study after completion of at least 1 study visit only due to events beyond their control (e.g. illness, no longer eligible, unable to tolerate study procedures) they will receive a pro rata honorarium. No honorarium will be given for attendance at the screening visit, but they will receive breakfast and a full health screening including blood pressure, body composition, full blood count, full lipid profile, liver function and glucose tests. Participants will be able to claim the cost of reasonable travel expenses (maximum £10 per visit).

Informed Consent

C7 Will informed consent be sought? (•) Yes ( ) No

C7a How will this be sought? Who will take consent and how will it be recorded?
Note: Justification must be provided for not gaining written consent

Interested volunteers will be sent an information sheet by post or email. If they are eligible according to the initial email screening questionnaire they will be invited to attend a screening session at KCL. At the screening visit it will be the responsibility of the qualified researcher to obtain written (signed and dated by the participant and researcher) informed consent from each individual participating in the study after adequate explanation of the aims, methods, objectives and potential hazards of the study. The researcher will also explain to the subjects that they are completely free to refuse to enter the study or to withdraw from it at any time and volunteers will have the opportunity to ask questions. The participant will be provided with a copy of their signed and dated consent form and any other written information which they should be instructed to retain. Screening results will only be given to the volunteer's GP if results are outside the normal range and the volunteer wishes it. In this case the volunteer will be supplied with a letter to pass on to their GP.

C7b How long will participants be given to decide if they wish to participate?

Potential participants will be able to discuss the study and read the information leaflet for at least 24 hours prior to a screening appointment, booked at the participant's convenience. They will be asked to sign the consent form at the screening visit. They will be informed over the phone or via email, prior to the screening visit, that they are free to withdraw from the study at any point and they are not obliged to give a reason.

C8 Could your past or present relationship with potential participants give rise to a perceived pressure to participate? If so, what steps will you take to mitigate this issue?

N/A

C9 Detail the process by which participants may withdraw from the research both during the research and after it has been completed. A final withdrawal date should also be provided, after which participants may no longer withdraw their data from the study.

Withdrawal from the study will be dealt with face-to-face or over the telephone if possible (otherwise by email or letter) so that the participant has the opportunity to give their reasons for withdrawal and express their wishes concerning any data that has been collected and any further contact. If they withdraw following screening but before attending the first study visit, their data will be not be used in the study. If they withdraw once the study has started their data will be used in the final report unless the participant requests withdrawal of their data. Participants will be informed that their data cannot be withdrawn once the study has been published in a journal or compiled in a report, and that any data that is collected after the point of randomisation will be anonymised. Should a subject decide to withdraw all efforts will be made to complete and report the observations as thoroughly as possible. If a participant does withdraw, an explanation of why the subject is withdrawing from the study will be recorded. If the reason for removal of a subject from the study is an adverse event or an abnormal laboratory test result, the principal specific event or test will also be recorded on the personal file. Any adverse events ongoing at the final visit which are considered in any way related to the study treatment or study regime will be followed up until resolved, the condition stabilises or is otherwise explained.
Subject Replacement: Subjects will not be replaced on the study in the event of subject withdrawal unless the study has not yet commenced or is in its very early stages. In order to avoid loss of statistical power through subject withdrawal, an adequate sample size will be conserved by initially recruiting 20% more subjects than required (n=50 (25 men, 25 women), to complete 40 subjects (20 men, 20 women)).

Section D: High Risk Research

D1g Risk Identified: The study involves physically intrusive procedures, use of bodily material or DNA/RNA analysis? Please fully explain how the risk will be mitigated.

Venepuncture can cause brief discomfort and there is a risk of bruising. Efforts will be made to minimise this risk by appropriate training of researchers. Cannulation, blood draws and saline flushing of the cannula will be carried out by a trained, experienced phlebotomist. Flow mediated dilatation is non invasive, but can cause brief discomfort, again, efforts will be made to minimise this risk by appropriate training of researchers.

D2 What are the potential risks and burdens to the participant?

Venepuncture can cause brief discomfort and there is a risk of bruising. Blood pressure and flow mediated dilatation measurement may cause bruising through cuff inflation and may temporarily cause minor discomfort. Efforts will be made to minimise this risk by appropriate training of research staff and students. All participants will be questioned about any potential food allergies before the dietary interventions so the risk of allergic reaction is minimised. It is possible that participants may be inconvenienced or may have to change their lifestyle by having to attend for screening and study visits and being contacted by the researcher. To minimise this effect, the requirements of the study will be extensively explained both orally and in writing in order to pre-select a sample of participants who would not find this inconvenient. This is a necessary element of a dietary intervention study and we do not anticipate that participants will be adversely affected by this as they will have already given informed consent once they have understood what will be asked of them. Should participants find the study inconvenient then they will be free to withdraw from the study without needing to explain their reason for doing so.

D3 What are the potential benefits to the participant?

Participants will be screened for clinical biochemistry indicators (full blood count, liver function) of general health and cardiovascular risk factors (fasting lipids, glucose, blood pressure, waist circumference and BMI). Any results deemed to be of clinical significance will be reported to the participant. A letter to their GP will be provided to the participant so that they may then make the choice of whether to pass this on to their GP or not. Participants will be given honoraria of £150 on completion of the study and will be reimbursed for their travel costs (train, tube or bus fares) where necessary. They will also receive breakfast at the screening visit, a low fat meal the night before each visit and a meal at the end of the 3 study visits. They may in future benefit from the outcome of this research study in that our understanding of dietary prevention of cardiovascular disease will be improved and will be provided with a copy of the final report on request.

D4 If you have guaranteed participant anonymity in the final report, confirm how this will be ensured.

Volunteers will be assigned a subject code. Following screening and acceptance onto the study, all further records, databases and reports will refer to each subject by their code, not by their name. All records, databases and reports will be kept in a safe place and only the researchers and principle investigators will have access to them.

Section E(I): Data Handling, Protection And Storage

E1 Will the identification of potential participants involve the review of identifiable personal information?

- ○ Yes
- ◉ No

E2 Who will have access to participants personal data?

The study researchers and principle investigators will have access to the anonymised data.

E3 How will you ensure the confidentiality of personal data?

Volunteers will be assigned a subject code. Following screening and acceptance onto the study, all further records, databases and reports will refer to each subject by their code, not by their name. All records, databases and reports will be kept in a safe place and only the researcher and supervisor will have access to them. Medical results will not be disclosed to any other party than the participant. The research records will be held securely at King's College London, according to the Data Protection Act 1988, and in accordance with the College Guidelines. The project researchers will have access to the anonymised data. Participants' personal details will be kept securely in the researchers office (Room 4.103, Department of Nutritional Sciences, Franklin−Wilkins Building, King's College London, 150 Stamford Street, SE1 9NH). Personal data stored in filing cabinets, cupboards and/or rooms will be kept in a locked room when not in use. Personal data held within computers, will be password protected and stored on encrypted memory sticks or restricted server access when not in use. Access to such data will be granted only to appropriate members of the research team. On completion of the study, the research project's paper records will be stored in a secure environment (filing cabinet in a locked office) that enables continued access to the required records by the project researchers and authorised members of the department.

E4 I confirm that all processing of personal information related to the study will be in full compliance with the UK Data Protection Act 1998 (DPA) (including the Data Protection Principles) — (•) Yes ( ) No ( ) N/A

E5 Will any of the following activities be undertaken?: (select each that applies)

E5a Use of personal details such as address, phone number, email etc. — (•) Yes ( ) No

E5b Sharing personal data with external sources. — ( ) Yes (•) No

E5c Publication of quotes attributed directly to participants. — ( ) Yes (•) No

E5d Publication of data from which participants could be identified. — ( ) Yes (•) No

E5e Use of audio and/or visual recording devices. — ( ) Yes (•) No

E5f Electronic storage of personal data (encrypted, password protected). — (•) Yes ( ) No

E6 Where will data be stored during and after the study?

Participants' personal details will be kept securely in the researchers' office (Room 4.103, Department of Nutritional Sciences, Franklin−Wilkins Building, King's College London, 150 Stamford Street, SE1 9NH). Personal data stored in filing cabinets, cupboards and/or rooms will be kept in a locked room when not in use. Personal data held within computers, will be password protected and stored on encrypted memory sticks or restricted server access when not in use. Access to such data will be granted only to appropriate members of the research team. On completion of the study, the research project's paper records will be stored in a secure environment (filing cabinet in a locked office) that enables continued access to the required records by the researcher team and authorised members of the department.

E7 Who will have ownership and responsibility for data storage during and after the study?

The principle investigators will take responsibility for the data storage during and after the study.

E8 How long will personal data be stored for after the study is completed?

Personal contact details, consent forms and other administrative records will be stored for no longer than 12 months and will be stored in paper form in a locked office in the Franklin-Wilkins Building (room 4.103, 4th floor).

E9 How long will research data be stored for after the study is completed?

Anonymised data from the study will be stored for at least 10 years as they can be used for purposes such as calculation of statistical power for future studies or for teaching purposes.

E10a Will data be archived for further use? — ( ) Yes (•) No

E11 How will results be disseminated?

- [x] Internal report (thesis)
- [x] Journals
- [x] Conference
- [x] Other

If other, please specify

Public engagement events.

Section G: Human Tissue

G1 Does the study involve the use or collection of bodily materials or tissue from a human being? (•) Yes ( ) No

G1a Where biological samples are to be taken, I confirm that the amount taken or the size of the sample is minimised to avoid the collection of excess material

- (•) Yes
- ( ) No
- ( ) N/A

G1b Is the tissue used in this study defined as "relevant material" as defined by the Human Tissue Act 2004?

- ( ) Yes
- (•) No

G1c Does the study involve the collection of new, relevant material? (•) Yes ( ) No

G1d Detail how this material falls under the review remit of CREC.

The blood samples collected will be processed to render them acellular on the day of collection and then frozen to store before analysis. Analysis will take place within 6 months of the end of the study and then the remaining plasma sample will be disposed of. Therefore whole blood and infranatant of whole blood (containing red cells, platelets and white cells) will be disposed of on the day of the study.

G1e Explain how the consent arrangements for your study meet the requirements for "appropriate consent" as per the HTA "Code of Practice on consent."

The participants will be healthy competent adults and therefore consent will be in writing.

G2 Does the study involve DNA or RNA analysis of any kind? ( ) Yes (•) No

G3 Are substances or products to be administered? (•) Yes ( ) No

G3a Are substances to be administered which are classified as medicinal products? ( ) Yes (•) No

G3b Are substances to be administered which are not classified as medicinal products? (•) Yes ( ) No

If yes detail the following: Name of the substance(s), Amounts to be administered, Number of times substance will be administered, How the product will be obtained and/or stored, prepared (if appropriate) and dispensed/distributed and Route of administration

Food substances will be administered as part of this study: the test fats will be provided by ADM Ltd and will be fit for human consumption. They will be from a blends of fats already commercially produced by ADM for use in the food industry. The test fats will be made into muffins in our research kitchen and will be served with a milkshake made from Nesquik powder (banana or strawberry flavour). The test meals will contain 50 g test fat (3.55MJ, 850 kcal, 15 g protein, 80 g carbohydrate) and the second standard meal will contain 35 g fat (35% fat, 15% protein and 50% carbohydrate). The first meal will be given after 10 hours of fasting, the second meal will be given 5 hours after the first.

G4 Does the study involve only moderately invasive/intrusive procedures? ( ) Yes (•) No

G5 Does the study involve other invasive/intrusive procedures? (•) Yes ( ) No

G5a Please provide further details of the procedure

Invasive procedures include venepuncture at screening to extract 17 mL for analysis of lipids, full blood count, liver function and glucose concentrations, and cannulation of the antecubital vein on study days for analysis of study outcomes in blood plasma (180 mL per day, 540 mL in total).

Tissue Sites

G6 Name the location or sites where the human tissue will be handled, analysed and stored.

Organisation	King's College London
Address	FWB, 150 Stamford St
City	London
County	Greater London
Postcode	SE1 9EH
Telephone	
Email	

G7 Give details of the investigators experience or training which qualifies them to conduct the required procedures.

Dr Charlotte Mills (BSc PhD RNutr), Dr Sarah Berry (BSc MSc PhD RNutr) and Dr Wendy Hall (BA MSc PhD RNutr) are experienced researchers with considerable expertise in running randomised controlled dietary intervention studies involving all the procedures to be used in the current study in the field of diet and CVD risk. Dr Sarah Berry has supervised 4 PhD students who conduct postprandial dietary intervention studies/clinical trials and approximately 30 project students in carrying out dietary intervention trials. The research team running the study will be fully trained in all procedures. Phlebotomists will be used to carry out cannulation on study days and to carry out phlebotomy at the screening sessions. Research division technicians will also be available to supervise daily tasks such as sample handling and use of the Metabolic Research Unit kitchen. Dr Mills, Dr Berry and Dr Hall have a food hygiene training and will make sure that appropriate hygienic practices are followed when foodstuffs are prepared and provided to participants.

G8 Provide details of the human tissue licence, where applicable.

N/A

Section H: Insurance, Risks and Ethical Issues

H1 I confirm that if my study involves any of the Risk Assessment criteria outlined in the information icon guidance, I will ensure that a Risk Assessment form is completed and signed by my Department before data collection commences.

(•) Yes ( ) No ( ) N/A

H2 I confirm that I have read the exclusion criteria for the College's Clinical Trials and Research Projects Involving Human Subjects Insurance Policy, detailed in the guidance icon, and that:

- (•) a) This project meets the inclusion criteria of the policy
- ( ) b) This project falls under the exclusion criteria and I have gained approval from the Finance Department, as instructed in the guidance icon
- ( ) c) This project falls under the exclusion criteria but approval has not been granted by the Finance Department

H3 I confirm that my travel insurance arrangements are as follows:

- ( ) a) I will secure College travel insurance (see guidance icon for further details)
- ( ) b) I will secure personal travel insurance
- (•) c) I do not require travel insurance as I will conduct the research in my country of legal residence
- ( ) d) I will not secure travel insurance

H4 I confirm that if Disclosure & Barring Service clearance is required for my study, this will be obtained prior to the commencement of data collection.

(•) Yes ( ) No ( ) N/A

H5 I confirm that the No Fault Compensation Scheme will be offered to all UK based participants.

(•) Yes ( ) No ( ) N/A

H6 Give the details of any other review body approvals or permissions obtained (including gatekeepers, other Ethics Committees, peer review, R&D permission).

The application to BBSRC for the grant, which gave details of this study, was approved by five peer reviewers.

H7 Give details of any other ethical issues which have not been addressed elsewhere in the application and explain how you will mitigate these risks.

N/A

Section I: Supporting Documents

I1 Participant Information Sheet

Type	Name	File Name	Date	Version	Size
Participant Information Sheet	Participant Information Sheet_INTERCARDIO_V1	Participant Information Sheet_INTERCARDIO_V1.docx	26/01/2018 12:00:00 AM	1	64.1 KB

Consent form (if applicable)

I2 Consent form

Type	Name	File Name	Date	Version	Size
Consent Form	Consent_INTER CARDIO V1_current	Consent_INTER CARDIO V1_current.docx	26/01/2018 12:00:00 AM	1	40.1 KB

Questionnaires/Surveys (if applicable)

I4 Questionnaires/Surveys

Type	Name	File Name	Date	Version	Size
Questionnaires	Screening questionnaire_INTER CARDIO V1_current	Screening questionnaire_INTER CARDIO V1_current.docx	26/01/2018 12:00:00 AM	1	30.7 KB

Indicative questions, topic guides etc (if applicable)

I5 Indicative questions, topic guides etc

Evidence of any other approvals or permissions (includes gatekeeper, R&D, other ethical approvals) (if

applicable)

I6 Evidence of any other approvals or permissions (includes gatekeeper, R&D, other ethical approvals)

Approach letters to gatekeeper organisations (if applicable)

I7 Approach letters to gatekeeper organisations

Advertisement document (email, poster, flyer etc) (if applicable)

I8 Advertisement document (email, poster, flyer etc)

Type	Name	File Name	Date	Version	Size
Advertisement Document	KCL circular advert_INTER CARDIO V1_current	KCL circular advert_INTER CARDIO V1_current.docx	26/01/2018 12:00:00 AM	1	14.4 KB
Advertisement Document	Poster advert_INTER CARDIO V1_current	Poster advert_INTER CARDIO V1_current.pptx	26/01/2018 12:00:00 AM	1	1.2 MB

Cover Letter (for amendments and modifications) (if applicable)

I9 Cover Letter (for amendments and modifications)

Other (if applicable)

I10 Other

Researcher/Applicant

J1 Researcher/Applicant Signature

I undertake to abide by accepted ethical principles and appropriate code(s) of practice in carrying out this study. The information supplied above is to the best of my knowledge accurate. I have read the Application Guidelines and clearly understand my obligations and the rights of participants, particularly as regards obtaining valid consent. I understand that I must not commence research with human participants until I have received full approval from the ethics committee.

Please note that in order to authorise your application you must sign off using your KCL email address i.e. joe.bloggs@kcl.ac.uk and your KCL password.

Signed: This form was signed by Charlotte Mills on 26/01/2018 19:38

KING'S College LONDON

PARTICIPANT INFORMATION SHEET

Ethics reference: Ethics ref: RESCMR - xxx

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET
INTER-CARDIO: THE ACUTE EFFECTS OF COMMONLY CONSUMED FATS ON CARDIOVASCULAR HEALTH: A RANDOMISED CONTROLLED TRIAL.

The INTER-CARDIO study
We would like to invite you to participate in this original research project undertaken as part of a research council (BBSRC) funded project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of this study?
This research project is a short term dietary intervention study to investigate how commonly consumed fats are metabolised and their impact cardiovascular health. Following ingestion of a fat rich meal there is an increase in fat in the blood which is believed to affect processes involved in heart disease. Dietary fat has a defined molecular structure, which the food industry can modify via a process called 'interesterification', in order to make liquid fats more solid. These fats are found in most of the spreads, bakery and confectionary products that we consume but their affects on fat metabolism and therefore processes involved in heart disease risk is unknown.

Why have I been chosen?
You have been contacted as you have expressed an interest in our research. In order to participate in this study you need to be able to say 'yes' to the following:

- I am a male or female aged between 35-75 years old
- I do not smoke and have not recently given up smoking (within the last 6 months)
- I have never had a heart attack, stroke, angina, thrombosis, liver or kidney diseases, diabetes, chronic gastrointestinal disorder or cancer
- I do not take medication to lower blood fats (e.g. statins, fibrates) or to stabilise blood glucose (e.g. acarbose, metformin or sulfonylureas) or blood pressure
- I do not have a history of excess alcohol intake or substance abuse
- I do not have food intolerances, allergies or hypersensitivity
- I am not already participating in a clinical trial
- I am prepared to follow dietary instructions before and during the study
- I have not recently donated blood (within the last 3 months)
- I have not recently lost more than 3 kg/7 lb (in the last 2 months)

What will happen to me if I take part?
If you would like to participate you would first need to complete a screening questionnaire with us over the telephone or via email (approx. 5 mins), after which potentially eligible volunteers will be invited to attend a clinic screening appointment (approx. 45 mins) in the Metabolic Research Unit on the 4th Floor, Corridor A, Franklin-Wilkins Building, 150 Stamford Street, SE1 9NH.

Summary of screening visit:
1) You should avoid eating or drinking anything, except water, from after 20.00 h the previous night.

2) The visit will last approximately 45 min.

3) We will explain all the details of the study and answer any questions you have. If you are still happy to take part in the study, you will be asked to sign a consent form.

4) We shall ask you questions about your medical history, your food habits and measure your weight, height, percentage body fat, blood pressure and waist and hip circumference.

5) We will need to take a small venous blood sample to extract capillary blood (17 mL, about 3.5 teaspoons) to check that your blood biochemistry is normal.

6) Then you will be provided with breakfast.

The results of the screening blood test will be provided within 2 weeks. If any abnormal results are found we will offer to provide you with a letter for your GP. If, after screening, you are discovered to be unsuitable for the study your data will be destroyed.

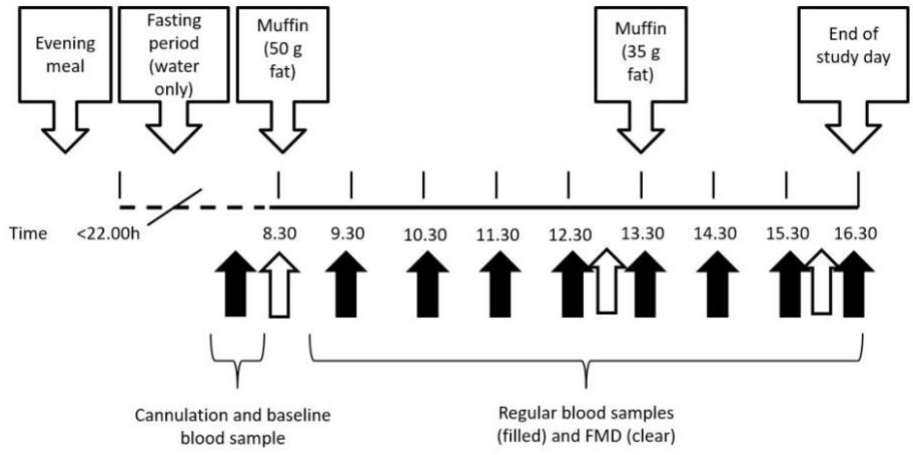
If you are eligible for the study you will attend the Metabolic Research Unit at the Franklin-Wilkins Building for three full day study visits. Prior to the first visit you will be given a 3-day diet diary in which we would like you to record everything that you eat and drink for 3 days (two week days and one weekend before the next visit). At the start of the study visit, you will consume the test meal (muffin and a milkshake containing 50g of the test fat. A cannula will be inserted into your arm and blood samples will be made at frequent time intervals during this visit.
We will be measuring the function of the artery in your arm (brachial artery), using a non-invasive, ultrasound technique, called flow mediated dilatation (FMD) when you arrive at 4.5 and 7.5 hours after your first muffin.
You will be given another muffin to consume after 5 hours. This visit takes approximately 9 hours, including time to consume dinner afterwards. There will be at least 1 week between each of the three visits.

Summary of study day visit:

1) Following screening, if your results comply with the study inclusion criteria you will be invited to attend the Metabolic Research Unit in the Franklin-Wilkins Building on 3 further occasions at least 7 days apart; each of these visits will take approximately 9 h each.
2) We will ask you to avoid tea, coffee, fatty foods, drinking alcohol, and any strenuous exercise the day prior to each visit.

3) We shall also ask you not to consume anything, except water, from after 20.00 h the night before. We will provide you with a standardised low fat meal for the night before the visit.
4) You will be asked to report to the Metabolic Research Unit in the Diabetes & Nutritional Sciences Division between 07:30 and 08.30 h, in a fasted state (i.e. without having consumed any food or drink from after 20.00 h the previous night, apart from water). **Make sure you drink some water on the morning of the study to avoid dehydration**.
5) At each of the 3 visits, a small flexible tube called a cannula will be inserted in a vein in your arm and a sample of blood will be taken (at baseline 20 mL, or 3½ tsp). Baseline flow mediated dilatation (FMD) will be measured.
6) You will be given the test meal to consume within 15 min. The test meal on all study days will consist of a muffin and a milkshake.
7) Following the test meal we would ask you to stay in the Metabolic Research Unit but you are free to work/ read/ use your laptop for the remainder of the study day in between measurements.
8) 0.5 hours following commencing eating the test meal you will have a second blood sample taken (20 mL 3½ tsp) and further blood samples will be taken at regular intervals up until and including 8 h after the meal. In total you will have 180 mL/ 30 tsp blood taken on each study day, and up to 557 mL / ~90 tsp blood taken over the course of the study, including the screening visit.
9) We will also measure FMD 4.5 and 7.5 hours after your first muffin.
10) 5 hours into the study we will provide you with another muffin.
11) Following the final blood sample and after the cannula has been removed, you will be provided with a meal.

An example of the overall study is shown in a diagram below (times may vary):



How will this benefit me?

You will have a free health check at screening, including liver function tests, full blood count, blood lipid profile and glucose levels, blood pressure measurements and body composition measurements. Should you wish to find out the results of this study you are welcome to contact the study team (using the details below) for a copy of the final report once the study is finished.

Will my participation be kept confidential?
Any information collected about you during this research will be kept strictly confidential. Your GP will not be told that you are taking part in the study, nor will they receive any results from the study, unless you instruct us to provide a letter for you to pass on to them. Subject confidentiality and anonymity will be observed throughout the study by use of subject codes in place of names, and the storage of subject details in a secure place. Only the investigators have access to this data.

What will happen to my study results?
Your anonymised data will be shared with other researchers. We hope to publish the results of the whole study in a scientific journal. You will not be identified in any publication. We will be happy to discuss the overall results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish. The results may also be used in student project reports (or thesis), presented at conferences and disseminated via public engagement activities. The data will remain fully anonymised and you will not be able to be identified in any of these occasions.

Who is organising and funding the study?
The study is funded and organised by the Biotechnology and Biological Science Research Council (BBSRC) via a Diet and Health Research Industry Club (DRINC) grant. In recognition of your time commitment, you will be paid an honorarium of £150 on completion of the study (£50 for each completed study visit exclusive of the screening visit). Any reasonable travel expenses will be refunded for the screening and study visits. The study is being run by the Division of Diabetes and Nutritional Sciences at King's College London,

Do I have to take part?
It is up to you to decide whether to take part or not. If you do decide to take part you will be asked to sign a consent form. You can withdraw from the study at any time by informing one of the researchers and you are not obliged to give a reason. You can also withdraw your data from the study if you wish at any time until the final research is prepared for submission for publication (estimated 31st December 2018). A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.
If you decide to take part, please let us know if you have been involved in any other study in the last year.

Thank you for your interest.

For further information, please contact: The INTER-CARDIO study team on 020 7848 4345; intermet@kcl.ac.uk; Diabetes and Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150 Stamford Street, London SE1 9NH.

If this study has harmed you in any way or if you wish to make a complaint about the conduct of the study you can contact King's College London using the details below for further advice and information.

Participant information sheet V1_ Jan 2018

Dr Sarah Berry

Date received	Initials	LJMU REC Ref

Application for Ethical Approval

No research must be started without full, unconditional ethical approval. There are a number of routes for obtaining ethical approval depending on the potential participants and type of study involved – please complete the checklists below to determine which is the most appropriate route for your research study.

A. Pedagogic Research (ROUTE FOR STAFF ONLY)

To find out if your study can be conducted under the University's Code of Practice for Pedagogic Research please answer the questions below.			
1.	Is the proposed study being undertaken by a member of LJMU staff?	Yes	
2.	Is the purpose of the study to evaluate the effectiveness of LJMU teaching and learning practices by identifying areas for improvement, piloting changes and improvements to current practices or helping students identify and work on areas for improvement in their own study practices?		No
3.	Will the study be explained to staff and students and their informed consent obtained?	Yes	
4.	Will participants have the right to refuse to participate and to withdraw from the study?	Yes	
5.	Will the findings from the study be used **solely** for internal purposes? *e.g. there is no intention to publish or disseminate the findings in journal articles or external presentations*		No
If you have answered **Yes to all Qs1-4** your study may be eligible for consideration under the University's Code of Practice for Pedagogic Research. You should **not** complete this application form but seek further guidance at http://www2.ljmu.ac.uk/RGSO/114123.htm or by contacting Dave Harriss			
If you have answered **No to any of Qs1-4** you should complete the checklists below to determine which route you should use to apply for ethical approval of your study.			

B. National Research Ethics Service (NRES)

To find out if your study requires ethical approval through NRES answer the questions below. Does you study:			
1.	Involve access to NHS patients or their data, or involve participants identified from, or because of, their past or present use of NHS services?		No
2.	Include adults who lack capacity to consent as research participants?		No
3.	Involve the collection and/or use of human tissue as defined by the Human Tissue Act 2004? **	Yes	
If you have answered **Yes to any of Qs1-3** you should **not** complete this application form. You must seek approval for your study through the NHS National Research Ethics Service (NRES). For further information and details of how to apply to NRES can be found at http://www.nres.nhs.uk/ *Please note that once ethical approval has been received from NRES a completed LJMU Research Governance Proforma must be submitted to LJMU REC with written evidence of full, unconditional ethical approval from NRES prior to commencing their research.*			
If you have answered **No to Qs1-3** complete the checklist below to determine whether your application is eligible for proportionate review or if a full review by the University's REC is required.			
** *Studies involving the use of human tissue from healthy volunteers which are taking place within the University's Research Institute for Sports and Exercise Sciences (RISES) can apply for approval through the University REC (for further information contact Dave Harriss:*			

C. Full versus Proportionate Review

Does the proposed study:			
1.	Expose participants to high levels of risk, or levels of risks beyond those which the participant is likely to experience whilst participating in their everyday activities? These risks may be psychological, physical, social, economic, cause legal harm or devaluate a person's self-worth. *e.g. untrained volunteers exposed to high levels of physical exertion; participants purposefully exposed to stressful situations; research where participants are persuaded to reveal information which they would not otherwise disclose in the course of everyday life.*		No
2.	Involve the administration of drugs, medicines or nutritional supplements as part of the research design?	Yes	
3.	Include adults who may be classed as vulnerable? *e.g. adults with learning disabilities or mental illness; drug/substance users; young offenders; prisoners/probationers; those in a dependent relationship with the researcher*		No
4.	Include children or young adults (below 18) where parental consent will not be sought?		No
5.	Involve the discussion or disclosure of topics which participants might find sensitive or distressing? *e.g. sexual activity; criminal activity; drug use; mental health; previous traumatic experiences; illness; bereavement*		No
6.	Use questionnaires which focus on highly sensitive areas? *e.g. illegal activity; criminal activity; disclosure and analysis of findings based on sensitive personal information as defined by Data Protection Act e.g. racial or ethnic origin; political opinions; religious beliefs; trade union membership; physical or mental health; sexual life*		No
7.	Incorporate interviews or focus groups which involve the discussion of highly sensitive areas? *e.g. illegal activity; criminal activity; disclosure and analysis of findings based on sensitive personal information as defined by Data Protection Act e.g. racial or ethnic origin; political opinions; religious beliefs; trade union membership; physical or mental health; sexual life*		No
8.	For research accessing and analysing existing datasets. Will the dataset include information which would allow the identification of individual participants?	No	
9.	Involve deliberately misleading participants in any way?		No
10.	Involve recruiting participants who have not been provided with a participant information sheet and asked to sign a consent form? *Please note that for questionnaire based studies a consent form is generally not request as consent is implied by the completion of the questionnaire. Applicants conducting **questionnaire-only** studies should answer NO*		No
11.	Involve the collection and/or use of human tissue from healthy volunteers? *Under these circumstances human tissue is as defined by the Human Tissue Act 2004 - "Any, and all, constituent part/s of the human body formed by cells." Research studies involving the use of plasma or serum are not covered by the HTA.*	Yes	
12.	Involve high levels of risks to the researcher? *e.g. lone working at night; interviewing in your own or participants homes, observation in potentially volatile or sensitive situations*		No

If you have answered **No to all Qs1-12** your study is eligible for proportionate review. You should complete the following application form and submit it electronically with any supporting documentation e.g. participant information sheets, recruitment letters, consent forms to **EthicsPR@ljmu.ac.uk** . Your application will be reviewed by a sub-committee of the University REC and you will be informed of the outcome within 2 weeks. Please note that if the allocated reviewer finds that your application has been wrongly submitted for proportionate review you will be notified and your application will be forwarded for consideration at the next University REC.
If you have answered **Yes to any of Qs1-12** your study is not eligible for proportionate review and will be considered at the next meeting of the University REC. You should complete the following application form and submit it electronically with any supporting documentation e.g. participant information sheets, recruitment letters, consent forms to **researchethics@ljmu.ac.uk** . *Please note that applications involving the use of human tissue originating from the School of Sports and Exercise Science should complete the Research Ethics Application Form for Studies Involving the Use of Human Tissue available at http://ljmu.ac.uk/RGSO/93044.htm* Guidance on completing the LJMU REC application form can be found at http://ljmu.ac.uk/RGSO/93044.htm ***Please note that following submission of your application to the relevant email address a signed copy of the application's signature page only must be sent to the Research Ethics Administrator, Research and Innovation Services, 4th Floor Kingsway House, Hatton Garden.*** Visit http://ljmu.ac.uk/RGSO/93126.htm for REC submission and meeting dates.

Where teaching practices involve invasive (psychological or physiological) procedures on students or others staff should refer to the guidance provided at http://ljmu.ac.uk/RGSO/93087.htm regarding the development of departmental/faculty codes of practice.

Research Mode

Undergraduate – specify course

Postgraduate

- [ ] MRes
- [ ] MPhil
- [ ] PhD
- [ ] Prof Doc e.g. EdD or DBA
- [ ] Other taught Masters programme – specify course

- [ ] Postdoctoral
- [x] Staff project
- [ ] Other – please specify

Has this application previously been submitted to the University REC for review? – **No**

If yes please state the original REC Ref Number

SECTION A – THE APPLICANT

A1. Title of the Research

The effect of oily fish and fish oil supplementation on markers of cardiovascular health and exercise performance

A2. Principal Investigator (PI) *(Note that the in the case of postgraduate or undergraduate research the student is designated the PI. For research undertaken by staff inclusive of postdoctoral researchers and research assistants the staff member conducting the research is designated the PI.)*

Title	*Dr*	Forename	*Graeme*	Surname	*Close*
Post	Reader in Applied Physiology and Sports Nutrition				
School / Faculty	Sport and Exercise Sciences				
Email				Telephone	

Relevant experience / Qualifications

- PhD Sport Nutrition
- Trained in blood taking and first aid
- 75 research papers and £750,000 competitive grant funding
- Supervised 7 PhD students and currently another 9 students
- Accredited with Sport Nutrition Register (SENr), British Association of Sport and Exercise Sciences (BASES) and United Kingdom Strength and Conditioning Association (UKSCA).

A3. Co-applicants *(including student supervisors)*

Co-applicant 1 / Academic Supervisor 1*(where the application is being submitted by a student, either undergraduate or postgraduate, details of their main dissertation supervisor must be included. The form must be submitted with a letter or email from their named supervisor indicating that they have read the application and are willing to supervisor the student undertaking the proposed study –* ***STUDENT APPLICATIONS WILL NOT BE REVIEWED UNTIL NOTIFICATION OF REVIEW BY THE NAMED SUPERVISOR IS RECEIVED***

Title	*Mr*	Forename	*Jamie*	Surname	*Pugh*

Post: PhD Candidate

Department / School / Faculty: Sport and Exercise Sciences

Email: | Telephone:

Relevant experience / Qualifications

Postgraduate Certificate Exercise & Nutrition Science
BSc Sport & Exercise Science

Title	*Dr*	Forename	*Ellen*	Surname	*Dawson*

Post: Lecturer

Department / School / Faculty: Sport and Exercise Sciences

Email: | Telephone:

Relevant experience / Qualifications

Dr. Ellen Dawson is an experienced researcher who is familiar with exercise physiology and cardiovascular research. Dr Dawson has been a member of staff at LJMU since 2007 and an active member of the cardiovascular group. Her current research is focused on vascular function in health and disease. This includes adaptation of arteries to acute injury, or improved vascular function with exercise training. The aim of the research is to understand the mechanisms underlying vascular function (both dysfunction and improved function) and its associated links with cardiovascular disease and health. Dr Dawson has experience with the cardiovascular methods being used in the proposed study.

Where there are more than 2 co-applicants please append an additional page to your application containing the relevant details

SECTION B – PROJECT DETAILS

B1. Proposed Date for Commencement of Participant Recruitment *(Please enter the date when you propose to start recruiting participants – note that no recruitment can take place without full, unconditional ethical approval)*

Start Date	**15/08/2016**

B2. Scientific Justification. State the background and why this is an important area for research *(Note this must be completed in language comprehensible to a lay person. Do not simply refer to the protocol. Maximum length – 1 side of A4)*

Evidence from observational studies, prospective cohort studies and randomized clinical intervention studies indicate that moderate doses of long-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) significantly decrease risk of fatal coronary heart disease (CHD). [1]

The exact mechanisms through which LC n-3 PUFA has an effect on CHD are not well established but may include a decrease in fasting and postprandial triacylglycerol levels, a decrease in arrhythmias, modulation of platelet aggregation and decreased synthesis of pro-inflammatory agents and improved vascular function. In addition, there is strong positive correlation between patients with cardiovascular disease and low mood/depression. Omega-3 highly unsaturated fatty acids (HUFAs) were recently reported in a meta-analytic review published by the the British Journal of Psychiatry to have an effect size of .061 in reducing clinical symptoms of depression.

The UK recommendation is that people eat at least two portions (with a portion being 140 g) of fish per week, one of which should be oily fish [2]

Despite recommendations, and sufficient availability, the majority of the UK population does not consume enough fish, particularly oily fish, and should be encouraged to increase consumption [3]

A relatively new efficacy biomarker of fish intake is the Omega-3 Index. This Index is defined as the total levels of eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) as a percentage of the total fatty acid levels in erythrocytes. This index is not only a biomarker of intake, but now also emerging as a risk factor for fatal and non-fatal cardiovascular events. A standardised analysis of fatty acid patterns correctly classifies persons to either low, intermediate or high risk.

An optimal target level of the Omega-3 Index is 8%, and an undesirable level is less than 4%, with 4–8% being an intermediate-risk zone. [4]

Consumption of fish provides energy, protein and a range of other important nutrients, including the

[1] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2757681/
[2] https://www.gov.uk/government/publications/sacn-advice-on-fish-consumption
[3] https://www.food.gov.uk/sites/default/files/ndns-scotland-executive-summary.pdf
[4] https://www.abdn.ac.uk/rowett/documents/fish_final_june_2012.pdf

long- chain n-3 polyunsaturated fatty acids (LC n3 PUFA) and micronutrients (including various vitamins and minerals). A portion of 140 g of fish provides about 50–60% of the daily protein requirements for an adult. Fish is usually low in saturated fats, carbohydrates and cholesterol [5]

B3. Give a summary of the purpose, design and methodology of the planned research
(Note this must be completed in language comprehensible to a lay person. Do not simply refer to the protocol. Maximum length – 1 side of A4)

Volunteers will be asked to attend 2 testing and 1 screening sessions at the University where bloods will be taken, urine samples collected, and functional cognition measures and the cardiovascular measures carried out, as well as a submaximal exercise test.

Volunteers will be asked to eat 2 portions of fish per week and take a supplement daily for 12 weeks. Participants will be randomized into one of three groups;
Group 1: placebo supplement + oily fish
Group 2: fish oil supplement + white fish
Group 3: placebo supplement + white fish

Volunteers will be asked to discontinue any intervention should they start to feel adverse reactions and instructed to seek medical advice.

At the end of 12 weeks participants will return to the University for a repeat session of testing (bloods, urine, functional measures).

B4. State the principal research question

Can you confer the same biological changes from regular consumption of oily fish from omega-3 supplementation?

B5a. Give details of the proposed intervention(s) or procedure(s) and the groups of people involved (*including psychological or physical interventions, interviews, observations or questionnaires)*

[5] Fish as a dietary source of healthy long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) and vitamin D : A Review of Current Literature June 2012 Baukje de Roos Alan Sneddon Helen Macdonald

Procedure or Intervention	Participants	Number of participants	No. of procedures per participant	Avg. Time to complete
E.g. Interview	*E.g. LJMU staff/students, general public, service users, professionals*	*25*	*1*	*1 hour*
1. Supplementation	LJMU staff/students, general public	*90*	*1*	*12 weeks*
2. Exercise test	LJMU staff/students, general public	*90*	*2*	*30 min*
3. Height measurement	As above	*90*	*3*	*5 min*
4. Weight measurement	As above	*90*	*3*	*5 min*
5. Waist to hip measurement	As above	*90*	*3*	*5 min*
6. Intima-medial thickness	As above	*90*	*2*	*5 min*
7. Arterial stiffness	As above	*90*	*2*	*30 min*
8. Flow mediated dilation	As above	*90*	*2*	*30 min*
9. Overnight fast	As above	*90*	*3*	*8 hours*
10. Hamilton Depression Rating Scale	As above	*90*	*2*	*5 min*
11. Becks Depression Inventory	As above	*90*	*2*	*5 min*
12. Profile of Mood States	As above	*90*	*2*	*5 min*
13. Depression, Anxiety and Stress scales	As above	*90*	*2*	*5 min*
14. Test of Everyday Attention	As above	*90*	*2*	*5 min*
15. Venous blood sample	As above	*90*	*3*	*5 min*
16. Urine sample	As above	*90*	*2*	*2 min*

To include additional interventions place your mouse cursor in the last cell of the final column and press the tab button on your keyboard. A new row will be created for the above table.

B5b. Where questionnaires are to be used have these previously been validated?

[X] **Yes** [ ] **No** [ ] **Not Applicable**

*If yes, state by whom and when. If no, you **must** append copies of the proposed questionnaire (s) to this application.*

1. Hamilton Depression Rating Scale (Hedlung & Vieweg, 1970)
2. Becks Depression Inventory (Beck, 1961)
3. Profile of Mood States (McNair, Lorr, & Droppleman, 1981)
4. Depression, Anxiety and Stress scales (Martin et al., 1998)

5. Test of Everyday Attention (Shelly et al., 2001)

B5c. Where interviews or focus groups (structured or semi-structured) are proposed you must append an outline of the questions you are going to ask your participants. Please confirm that you have attached an outline of your interview / focus group questions.

☐ **Yes** ☒ **Not Applicable**

B6. How will the findings of the research be disseminated? *(e.g. thesis, dissertation, peer-reviewed articles, conference presentations, reports)*

Peer reviewed articles and conference presentation. *BBC "Trust Me I'm a Doctor" programme* *BBC website (bbc.co.uk/trustme)*

SECTION C – THE PARTICIPANTS

C1. How will the participants been selected, approached and recruited? If participants are to be approached by letter/email please append a copy of the letter/email. Please include details on how much time participants will have to decide if they want to take part. *(where different groups of participants have been identified in section B5a above provide details on how each group will be selected, approached and recruited.)*

Recruitment will include putting call outs through the media (eg BBC local and national radio, websites and online forums, posters, social media, local groups and societies and flyers in the street). This process will be aided by the BBC *The BBC will be responsible for recruiting participants. Recruitment will be through the media (eg BBC local and national radio, websites and online forums, posters, social media, local groups and societies and flyers in the street). Interested participants will contact the BBC study team via email (trustme@bbc.co.uk) or through an online form (available at bbc.co.uk/trustme). After contacting the BBC study team, individuals will be sent the Participant Information Sheet and / or telephone call with a member of BBC staff experienced with recruitment for this sort of experiment. They will also be sent the Consent Form to read. If following this process participants are still interested in the study they will be invited to a screening session.*

C2. How was the number of participants decided? *(e.g. was a sample size calculation performed)*

Previous proteomic research has shown that approximately 25 people per group are needed for statistical analysis. Therefore, to account for drop-outs, 30 has been chosen.

C3a. Will any of the participants come from any of the following groups? (Please tick all that apply)
Please note that the Mental Capacity Act 2005 requires that all research involving participation of any adult who lacks the capacity to consent through learning difficulties, brain injury or mental health problems be reviewed by an ethics committee operating under the National Research Ethics Service (NRES). For further information please see
http://www.ljmu.ac.uk/RGSO/101579.htm

- [ ] Children under 16
- [ ] Adults with learning disabilities
- [ ] Adults with mental illness (if yes please specify type of illness below)

- [ ] Drug / Substance users
- [ ] Young offenders
- [ ] Those with a dependant relationship with the investigator *e.g. your employees or students*
- [ ] Other vulnerable groups please specify

Justify their inclusion

C3b. If you are proposing to undertake a research study involving interaction with children or vulnerable adults do you have current, valid clearance from the UK Disclosure and Barring Service (DBS)?

[ ] **Yes** [ ] **No** [X] **Not Applicable**

C4a. What are the inclusion criteria? *(Please include information on how you will ensure that your participants will be informed of your inclusion criteria and how you will ensure that any specific inclusion criteria are met)*

Participants included are required to be;
- > 40 years old
- BMI > 20
- + CVD scoring tool see table 1 below

TABLE 1
Scoring tool for recruitment[1]

	1 point		2 points	
CVD risk factors	Men	Women	Men	Women
Total cholesterol (mmol/L)[2]	5.18–6.21	5.18–6.21	6.22–7.99	6.22–7.99
HDL cholesterol (mmol/L)[2]	0.91–1.16	1.17–1.29	<0.90	<1.16
Glucose (mmol/L)[2,3]	6.00–6.99	6.00–6.99	NA	NA
BMI (kg/m^2)[4]	25.0–29.9	25.0–29.9	30.0–39.9	30.0–39.9
Waist circumference (cm)[4]	>94	>80	>102	>88
Systolic blood pressure (mm Hg)	130–139	130–139	140–159	140–159
Diastolic blood pressure (mm Hg)[5]	NA	NA	90–99	90–99
First-degree relative diagnosed with MI or T2D (age of diagnosis) (y)[6]	NA	NA	<55 in male relatives; <65 in female relatives	<55 in male relatives; <65 y in female relatives

[1] Scoring tool was based on the Framingham risk score system (13) and adapted from Chong et al (14) to include a score for a family history of MI or T2D (15). Participants required ≥2 points to have an RR ≥1.5 of developing CVD, which could be achieved through a combination of CVD risk factors. CVD, cardiovascular disease; MI, myocardial infarction; NA, not applicable; T2D, type 2 diabetes.

It will be ascertained during screening sessions as to whether participants fit the inclusion criteria. Relevant measurements will be made during screening session (see appended Screening Questionnaire)

Participants will be excluded from taking part in the study if they do not fit the inclusion criteria above.

C4b. What are the exclusion criteria? *(Please include information on how you will ensure that your participants will be informed of your exclusion criteria and how you will ensure that any specific exclusion criteria are met)*

Participants will be excluded from taking part in the study if they do not fit the inclusion criteria above.

- Infection, immune disorder including HIV, autoimmune disease, or fever of unknown origin
- Unstable medical conditions requiring immediate intervention
- Unstable or rapidly progressive neurological diseases
- History hemorrhagic or ischemic stroke within the last 3 months
- Allergy, hypersensitivity, or intolerance to fish, fish oils or omega-3 fats
- Any known food allergies
- Pregnancy or breastfeeding (by urine pregnancy test; self-report)

C5. Will any payments/rewards or out of pocket expenses be made to participants?

[X] **Yes** [ ] **No**

If yes what or how much? **Travel expenses will be reimbursed.**

SECTION D – CONSENT

D1. Will informed consent be obtained from (please tick all that apply)

X	**The research participants?**
	The research participants' carers or guardians?
	Gatekeepers to the research participants? *(i.e. school authorities, treatment service providers)*

D2. Will a signed record of consent be obtained? *Please note that where the study involves the administration of a questionnaire or survey a signed record of consent is not required for completion of the questionnaire as long as it is made clear in the information sheet that completion of the questionnaire is voluntary. Under these circumstances return of the completed questionnaire is taken as implied consent.*

If implied consent is to be assumed by return of questionnaires, the following statement (or similar) must be used:

"I have read the information sheet provided and I am happy to participate. I understand that by completing and returning this questionnaire I am consenting to be part of this research study and for my data to be used as described in the information sheet provided"

Participation in any other interventions within the same study e.g. interviews, focus groups must be supported by obtaining appropriate written consent.

X Yes **No** **Implied consent for questionnaire** **Verbal consent**

Where the study involves the use of more than one intervention for example interviews and a questionnaire please the space below to detail the method of consent to be used for each intervention eg
Questionnaire – implied consent
Interview – written consent
Telephone interview – verbal consent

PLEASE APPEND COPIES OF ANY PROPOSED CONSENT FORMS TO THIS APPLICATION

D3. All participants must be provided with written information detailing the purpose, procedures, risks and benefits of participating. An approved template for the participant information sheet can be found at http://ljmu.ac.uk/RGSO/93717.htm. Please check the box below to confirm that a participant information sheet has been appended to this application. X

APPLICATIONS SUBMITTED WITHOUT A PARTICIPANT INFORMATION SHEET WILL NOT BE REVIEWED.

D4a. Will participants be able to withhold consent (refuse to take part)?

X Yes **No**

If no please explain why not

D4b. Will participants be able to withdraw from the study whilst it is ongoing (after they have consented to take part)?

X Yes **No**

If no please explain why not

D4c. Will participants be able to withdraw from the study after data collection has ended (will it be possible to identify and remove an individual's data once it has been collected or has been collected anonymously)?

[X] **Yes**	[ ] **No**

If no please explain why not

THE ABILITY OF PARTICIPANTS TO REFUSE TO TAKE PART OR TO WITHDRAW FROM A STUDY MUST BE MADE CLEAR IN THE WRITTEN INFORMATION PROVIDED TO PARTICIPANTS

SECTION E - RISKS AND BENEFITS

E1. Where will the intervention(s) take place? *Please note that where research is to be conducted in participants' homes or other non-public places applicants must be aware of appropriate lone working policies / practices and complete a full risk assessment.*
Applicants should also be aware of potential embarrassment or distress for participants in asking them to discuss personal or sensitive topics in public places.

[X]	**LJMU premises**	[ ]	**NHS or other external organisations**	[ ]	**Public places**	[X]	**Participant homes or other non-public places**

E2. Will individual or group interviews/questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting or is it possible that criminal or other disclosures requiring action could take place during the study? (e.g. during interviews or focus groups)

[X] **Yes** [ ] **No** [ ] **Not Applicable**
If yes give details of procedures in place to deal with these issues. Information given to participants should make it clear under what circumstances action may be taken. Where interviews or questionnaires discuss sensitive or distressing topics signposting to relevant support organisations must be included in the associated participant information sheet.

Participants will be required to complete a number of short questionnaires in regards to various cognitive functions. These questionnaires are all previously validated and are commonly used in clinical practice. The questionnaires will be administered by a clinical researcher in Nutritional Neurosciences (Dr Rachel Gow) who will also be available for any questions before the study, via email, and will be available in person for any questions or concerns that participants may have during screening and experimental sessions. These are all self-report and only take a few minutes each to complete: 1. Hamilton Depression Rating Scale 2. Becks Depression Inventory 3. Profile of Mood States 4. Depression, Anxiety and Stress scales 5. Test of Everyday Attention

E3. Explain any potential benefits for individual participants of the study. Where there are no benefits to individual participants provide brief details of the potential broader benefits of the study for example to society or to future service users.

Participants will find out their own their blood lipid profile, omega-3 index as well as their current fitness level.

E4. Describe in detail any potential adverse effects, risks or hazards (mild, moderate, high or severe) of involvement in the research for the RESEARCHERS. *Explain any risk management procedures which will be put in place e.g. lone working procedures, counselling, peer support.*

The researchers will be subject to a some potential risks, relating to transmission of pathogen via blood. To reduce the risk those taking and handling blood will be fully trained and PPE will be adhered to at all times where indicated.

SECTION F – DATA ACCESS AND STORAGE

F1.Personal Data Management

Will the study involve the collection and storage of personal, identifiable or sensitive information from participants? Please note that signed consent forms constitute personal data. *(e.g. names, addresses, telephone numbers, date of birth, full postcode, medical records, academic records)*

[X] **Yes** [ ] **No**

If yes please provide details of what personal information will be collected and stored

Name **Age** **Sex** **Address** **Phone number** **Health status**

*Applicants should note that personal identifiable information or sensitive information relating to participants **must not** be transferred in or out of the EEA without the explicit consent of participants. Such information must be handled with great care and only used in the way described in the written information you give your participants.*

*You **must** store any hard copies of personal date (e.g. printed data sheets, signed consent forms) in locked cupboards or filing cabinets and any electronic data containing personal information **must** be stored securely on LJMU password protected computers.*

*Personal data **must not** be stored on USB drives or other portable media or stored on home or personal computers.*

Where the use of verbatim quotes is proposed in future publications or presentations or it is intended that information is gathered using audio/visual recording devices explicit consent for this must be sought from participants.

F2. Will you share personal, identifiable data with other organisations outside of LJMU or with people outside of your research team? *(e.g. supervisor, co-applicants)*

[X] **Yes** [ ] **No** | [ ] **Not Applicable**

If yes please provide further details

Data will be shared with the BBC. Files will be sent using a secure password encryption and stored in locked cupboards or password protected computers.

F3. For how long will any personal, identifiable data collected during the study be stored?

5 years

SECTION G – USE OF HUMAN TISSUE SAMPLES OR OTHER HUMAN BIOLOGICAL MATERIAL

Where human tissue is stored for the purpose of research The Human Tissue Act 2004 requires that either an appropriate license issued from the Human Tissue Authority is in place or that the research receives ethical approval from an appropriate Research Ethics Authority, namely a REC within the National Research Ethics Service (NRES). However where such cellular material is collected for a research purpose but is processed on the same day to leave it acellular and any residual cellular material is immediately discarded, this does not constitute storage and is not licensable. Therefore any research using human tissue where the tissue is rendered acellular immediately and the cellular component discarded can be ethically reviewed by the University REC.

Please tick the appropriate box

This study is being conducted under the Research Institute for Sports and Exercise Science (RISES) HTA licence [x]

This study is being undertaken outside of RISES but all cellular material will be processed and discarded within 24 hours [ ]

G1. What types of human tissue or other biological material will be included in the study?

Blood and urine

G2. Who will collect the samples?

PhD students and LJMU staff members will collect samples, only if they have phlebotomy experience and have undergone HTA training. All sample collection will be overseen by Jamie Pugh and Dr Graeme Close.

G3. Will informed consent be obtained from the donors for the use of the samples:

- In this research study?	**Yes**
- In future research?	**Yes**

G4. Will the samples be stored:

– **Fully anonymised?** **No**
(link to donor broken)

– **Psuedo-anonymised form?**
(linked to donor but not identifiable to researcher) **Yes**

If Yes who will have access to the code and personal information about the donor?
Only the direct research team will have access to the link

– **Identifiable to the researcher?** **No**

If Yes please justify

G5. What types of tests or analysis will be carried out on the samples?

Proteomic analysis will be conducted on the urine samples – University of Glasgow

Blood samples will be used to assess;
omega-3 index,– University of Southampton
blood lipid profiles, inflammation, eicosanoid metabolism, blood microparticles – University of Reading

G6. Will the research involve the analysis of human DNA in the samples?

No

G7. Is it possible that the research could produce findings of clinical significance for individuals? ***(including relatives of donors)***

Yes / No

If yes will arrangements be made to notify the individuals concerned?

Should participants have cholesterol levels higher than 6.22 mmol/L they will be advised to visit their GP.

G8. Give details of where the samples will be stored, who will have access and the custodial arrangements.

Samples will be stored in HTA designated freezers in the life sciences building. The research team will have access to the samples.

G9. What will happen to the samples at the end of the research? *(delete options that do not apply)*

Destruction

Transfer to a research tissue bank licensed by the Human Tissue Authority

Storage at LJMU pending ethical approval for use in another project

Please provide any further relevant details e.g. if the samples are to be stored at LJMU for future research please indicate the location of storage.

Samples will be stored in HTA designated freezers in the life sciences building overseen by Monica Barclay

G10. Please confirm that you have undertaken relevant training in the requirements of the Human Tissue Act 2004 for the use of human tissue samples in research.

Yes, I have received training. Please insert date of training received.

Yes, Graeme Close and Jamie Pugh have received training. Please insert date of training received. 11/14

DECLARATION OF THE PRINCIPAL INVESTIGATOR

- The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
- I undertake to abide by the ethical principles underlying the Declaration of Helsinki and LJMU's REC regulations and guidelines together with the codes of practice laid down by any relevant professional or learned society.
- If the research is approved I undertake to adhere to the approved study procedures and any conditions set out by the REC in giving its favourable opinion.
- I undertake to seek an ethical opinion from LJMU REC before implementing substantial amendments to the approved study plan.
- If, in the course of the administering any approved intervention, there are any serious adverse events, I understand that I am responsible for immediately stopping the intervention and alerting LJMU REC.

- I am aware of my responsibility to comply with the requirements of the law and relevant guidelines relating to security and confidentiality of personal data.
- I understand that any records/data may be subject to inspection for audit purposes if required in the future.
- I understand that personal data about me as a researcher will be held by the University and this will be managed according to the principals of the Data Protection Act.
- I understand that the information contained in this application, any supporting documentation and all correspondence with LJMU REC relating to the application will be subject to the provisions of the Freedom of Information Act. The information may be disclosed in response to requests made under the Act except where statutory exemptions apply.
- I understand that all conditions apply to my co-applicants and other researchers involved in the study and that it is my responsibility that they abide by them.

X	**TICK TO CONFIRM THAT YOU HAVE READ AND AGREE TO THE DECLARATION ABOVE**

SUBMITTING YOUR APPLICATION FOR REVIEW

Once you have completed the above application form please submit it electronically to either EthicsPR@ljmu.ac.uk for proportionate review or to researchethics@ljmu.ac.uk for full review by the University REC. If possible please submit your application form and any additional supporting documentation as a single pdf file.

APPLICATIONS MUST BE SUBMITTED VIA AN LJMU EMAIL ACCOUNT AND FOR STUDENT APPLICATIONS SUPPORTED BY AN EMAIL / LETTER FROM THE MAIN SUPERVISOR CONFIRMING THAT THEY HAVE READ AND APPROVED THE STUDY / APPLICATION.

CHECKLIST OF DOCUMENTS SUBMITTED ELECTRONICALLY (Please note that applications submitted without the required supporting documents will not be reviewed).

X	Ethics Application Form (MANDATORY)
X	Protocol (MANDATORY) see note below
	Email / letter from supervisor
X	Copies of any recruitment/advertisement material e.g. letters, emails, posters etc.
X	Participant Information Sheet
	Carer Information Sheet
	Gatekeeper Information Sheet
X	Participant Consent Form
	Carer Consent Form
	Gatekeeper Consent Form
	Non-validated questionnaires
	List of interview questions
X	Risk Assessment Form
	Other please specify

Note

A research protocol is a document describing in detail how a research study is to be conducted in practice, including a brief introduction or background to the study, the proposed methodology and a plan for analysing the results. For the purposes of your application for ethical approval it is something which can be presented in a variety of formats dependent on its origin for example:

- *for postgraduate research students it may be the programme of work embedded within their programme registration form (RD9R)*
- *for studies which have obtained external funding it is often the description of what they propose doing which they submitted to the funder*
- *for other students it is the study proposal they have written and had assessed/approved by their supervisor.*

APPENDIX A: EXPERIMENTAL PROTOCOL

The effect of oily fish and fish oil supplementation on markers of cardiovascular health and exercise performance

Participants will be required to visit the laboratory on 3 occasions, once for initial screening and twice for experimental visits. There will be around one week between screening and the first experimental visit and then a supplementary period of 12 weeks between the first and second experimental visits, during which participants will complete an identical protocol.

Screening

During this session, participants' height, weight and blood pressure will be recorded. A blood sample will also be collected for analysis of the omega-3 index. Participants will also meet with the LJMU study team. During this meeting participants will have the chance to ask any questions they have. The LJMU readiness to exercise questionnaire will also be completed. If participants are still interested in the study they will be asked to provide written consent.

Informed written consent will also be sought from each participant by the BBC. Before consent is obtained it will be made clear to participants, through written and verbal means, that they are free to leave the experiment at any time during the process. If they don't wish to feature on television, but are happy to take part in the experiment, it will be made clear that although the BBC cannot guarantee they won't appear in the final film, they will do their best to ensure they are not 'featured'. Should an individual withdraw from the study at any time, all research data and personal data will be destroyed. It will also be made clear to volunteers that should they participate there is no guarantee that they will appear in the broadcasted television item.

During this screening sessions, measurements will be taken, by the research team, in line with the inclusion and exclusion criteria, outline previously. Should participants not fit the inclusion criteria they will be informed at this point by a member of the research team and excluded from the study at this point.

Experimental visits

Participants will complete the following assessments before returning to the laboratory 12 weeks later, where they will undergo the same experimental protocols, in the same order.

Experimental Techniques

Following a 10 minute rest period participants' body composition will be assessed, via height and weight measurement of waist to hip ratio. A fasting blood and urine sample will also be obtained.

Flow mediated dilation

Following 10 minutes of supine rest brachial artery endothelial function will be measured. For this a small blood pressure cuff will be placed around the forearm.. Brachial artery diameter and velocity will be measured throughout using pulsed Doppler and 2D ultrasound (We will use a 12-MHz ultrasound probe attached to the high resolution ultrasound machine (Tearson) with a 12MHz ultrasound probe. A one minute baseline recording will be made before the blood pressure cuff is inflated to 220 mmHg for 5 minutes. The cuff will then be deflated and the response monitored for a further 3 minutes.

Arterial stiffness

Systemic and central artery stiffness will then be measured using a Sphygmocor system. A blood pressure recording will be taken at the brachial artery and an ECG waveform obtained. A tonometer will then be applied with gentle pressure to the radial artery (wrist) to record the blood pressure waveform used to measure systemic arterial stiffness. Furthermore, the tonometer will be placed with gentle pressure at the femoral/carotid artery (thigh/neck) to measure aortic pulse wave velocity (a measure of arterial stiffness).

IMT

We will measure carotid artery intima-medial thickness (IMT) using ultrasound imaging. Resting IMT and diameter of the left carotid artery will be measured 2 cm proximal to the carotid bifurcation from 3 different angles. . We will use a 12-MHz ultrasound probe attached to the high resolution ultrasound machine (Tearson). Recording will be performed over a 20 second period and the whole procedure will take no more than 15 minutes.

Cognitive Assessment

Participants will be required to complete a number of short questionnaires in regards to various cognitive functions. These questionnaires are all previously validated and are commonly used in clinical practice. The questionnaires will be administered by a clinical researcher in Nutritional Neurosciences (Dr Rachel Gow) who will also be available for any questions or concerns that participants may have. These are al self-report and only take a few minutes each to complete:

1. Hamilton Depression Rating Scale
2. Becks Depression Inventory
3. Profile of Mood States
4. Depression, Anxiety and Stress scales
5. Test of Everyday Attention

Incremental cycling tests

Participants will undergo a submaximal incremental cycling test to determine maximal fat oxidation. The protocol will begin at 50 W for 4 min with subsequent stages increasing by 30 W every 4 min. Between each exercise stage, participants will rest on the ergometer without pedalling for 2 min, in which time, a capillary blood sample was taken from the finger and plasma lactate concentration analysed. The test will terminated when participants blood plasma lactate reaches a value $\geqslant$4 mmol L^{-1}. Expired gas analysis, to assess VO_2 uptake will be performed, as well as recordings of heart rate and rating of perceived exertion (RPE).

Blood sampling

A resting venous blood sample (20mL) will be taken in order to quantify omega-3 index, eicosanoid metabolism, blood microparticles, blood lipid profiles, and inflammatory cytokines.

Screening Questionnaire

Age	
Weight (kg)	
Height (cm)	
BMI	
Waist circumference	
Total cholesterol.	
HDL cholesterol	
Glucose	
Systolic Blood Pressure	
Diastolic Blood Pressure	
First degree relative diagnosed with MI or T2D?	

Total points score…….

* If you are unsure of your body mass index and how to calculate this please use the following website:
http://www.nhlbi.nih.gov/health/educational/lose_wt/BMI/bmicalc.htm

TABLE 1
Scoring tool for recruitment[1]

	1 point		2 points	
CVD risk factors	Men	Women	Men	Women
Total cholesterol (mmol/L)[2]	5.18–6.21	5.18–6.21	6.22–7.99	6.22–7.99
HDL cholesterol (mmol/L)[2]	0.91–1.16	1.17–1.29	<0.90	<1.16
Glucose (mmol/L)[2,3]	6.00–6.99	6.00–6.99	NA	NA
BMI (kg/m^2)[4]	25.0–29.9	25.0–29.9	30.0–39.9	30.0–39.9
Waist circumference (cm)[4]	>94	>80	>102	>88
Systolic blood pressure (mm Hg)	130–139	130–139	140–159	140–159
Diastolic blood pressure (mm Hg)[5]	NA	NA	90–99	90–99
First-degree relative diagnosed with MI or T2D (age of diagnosis) (y)[6]	NA	NA	<55 in male relatives; <65 in female relatives	<55 in male relatives; <65 y in female relatives

[1] Scoring tool was based on the Framingham risk score system (13) and adapted from Chong et al (14) to include a score for a family history of MI or T2D (15). Participants required ≥2 points to have an RR ≥1.5 of developing CVD, which could be achieved through a combination of CVD risk factors. CVD, cardiovascular disease; MI, myocardial infarction; NA, not applicable; T2D, type 2 diabetes.

LIVERPOOL JOHN MOORES UNIVERSITY PARTICIPANT INFORMATION SHEET

The effect of oily fish and fish oil supplementation on markers of cardiovascular health and exercise performance

Dr Graeme Close, Jamie Pugh School of Sport and Exercise Sciences.

You are being invited to take part in a research study. Before you decide it is important that you understand why the research is being done and what it involves. Please take time to read the following information. Ask us if there is anything that is not clear or if you would like more information. Take time to decide if you want to take part or not.

You MUST NOT take part in this study if:

- You are younger than 40 years
- You are a current smoker
- You are pregnant
- You suffer any of the following;
  - Infection, immune disorder including HIV, autoimmune disease, or fever of unknown origin
  - Unstable medical conditions requiring immediate intervention
  - Unstable or rapidly progressive neurological diseases
  - History hemorrhagic or ischemic stroke within the last 3 months
  - Allergy, hypersensitivity, or intolerance to fish, fish oils or omega-3 fats
  - Have any known food allergies
  - Pregnancy or breastfeeding (by urine pregnancy test; self-report)

LJMU and BBC collaboration

This study is a joint project between Liverpool John Moores University (LJMU), University of Southampton, University of Reading, University of Aberdeen, University of Glasgow, and the BBC (British Broadcasting Corporation). This collaboration will not affect your rights as a research participant in any way. Importantly, you will still have the right to withdraw from the process at any time, and have all information/data stored confidentially and only accessed by members of the research team.

As well as consenting to take part in the research study the BBC will also seek your informed consent. This will give the BBC permission to use footage/ images of you on TV programmes and/or websites. The proposed study will form part of the BBC's "Trust Me I'm A Dr" series. As such, the research process will be filmed. If you do not wish to feature on television, but are happy to take part in the experiment, the BBC cannot guarantee you will not appear in the final film; however they will do their best to ensure you are not 'featured'. It should also be clear that participation in this study is no guarantee you will appear in the broadcasted television item.

1. What is the purpose of the study?

Recent evidence has shown that moderate doses of long-chain n-3 polyunsaturated fatty acids (more commonly known as omega-3 fatty acids, or fish oils) reduce the risk of coronary heart disease (CHD).

In the UK, it is recommended that people eat at least two portions (with a portion being 140 g) of fish per week. Despite these recommendations, and sufficient availability, the majority of the UK population do not eat enough fish and should be encouraged to increase consumption. However iit is not known whether the benefits from eating fish comes from the omega-3 fatty alone, or in combination with other macro and micronutrients within the fish. To this end, we would like to investigate the health benefits of oily fish consumption, compared to omega-3 supplementation alone.

2. Do I have to take part?

"No. It is up to you to decide whether or not to take part. If you do you will be given this information sheet and asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw will not affect your rights/any future treatment/service you receive."

3. What will happen to me if I take part?

You will be required to visit the laboratory on 3 occasions, once for initial screening and twice for experimental visits. There will be around one week between screening and the first experimental visits and then a supplementary period of 12 weeks between the first and second experimental visits. During these 2 visits you will complete the same tests, as detailed below.

During the screening session, your height, weight and blood pressure, using a standard blood pressure cuff, will be recorded. A blood sample (1 mL – less than 1/2 teaspoon) will also be collected for analysis of the omega-3 index. We will use these measures to ensure that you fit within the criteria to take part in the study. You will also meet with the LJMU study team and have the chance to ask any questions you may have. You will be informed at this point whether you meet the criteria to participate in the study.

Around 1-2 weeks later you will return to the laboratory to complete the first experimental visit. You will complete the following assessments before returning to the laboratory 12 weeks later, where you will undergo the same experimental protocols, in the same order.

Experimental visits
To begin with during the experimental visits, we will first repeat the same measures of height, weight, and blood pressure as well as collect another urine and blood sample (20mL).

Cardiovascular Tests

1. Artery function will be measured painlessly using an ultrasound. This is similar technology to that used to scan pregnant women. We will scan an artery in your upper arm (the brachial artery) A blood pressure cuff will be placed around your lower arm. This cuff will then be inflated for 5 minutes and then released. The artery is scanned for a further 3 minutes after the cuff is released. The inflation of the cuff may cause a pins and needles sensation or slight numbness in the arm, but this goes as soon as the cuff is released. This will take about 15 minutes
2. Blood vessel stiffness will be measured non-invasively using a commercial system (SphygmoCor) at your wrist, neck and groin. A pen like probe is placed on the skin to measure the stiffness of your blood vessels. This will take about 30 min.

3. The intima-medial thickness (IMT) is the thickness of the artery wall. We will measure the carotid (side of neck) artery IMT using ultrasound imaging (same as above). Recording of this artery will be performed over a 20 second period from 3 different angles and the whole procedure will take no more than 15 minutes.

Cognitive Tests

You will be required to complete a number of short questionnaires in regards to various cognitive functions. These questionnaires are all previously validated and are commonly used in clinical practice. The questionnaires will be administered by a clinical researcher in Nutritional Neurosciences (Dr. Rachel Gow) who will also be available for any questions or concerns that you may have. These self-report and only take a few minutes each to complete:

1. Hamilton Depression Rating Scale
2. Becks Depression Inventory
3. Profile of Mood States
4. Depression, Anxiety and Stress scales
5. Test of Everyday Attention

Fitness Test

1. A submaximal fitness test on a stationary bicycle will be completed; the test will involve cycling through increasing intensities. Prior to the test, you will be fitted with a heart rate monitor, which is strapped comfortably around your chest. The test will comprise an initial four minute period of easy cycling, and the resistance of the ergometer will then increase every 4 minutes. Throughout the test, breath samples will be taken to measure expired air. To do this, a face mask will be fitted. Finger prick blood samples will also be taken during this test. You may feel some discomfort during this as a small lancet needle is used to pierce the skin. This produces a droplet of blood on the surface of the skin.

Supplementation

In the 12 weeks between the two experimental visits, you will be required to eat 2 portions of fish per week and take a supplement daily for 12 weeks. The fish will either be white fish (e.g. cod) or oily fish (e.g. salmon) and provided as part of a premade ready meal (supplied by a company called Soulmate Food). The supplement will either be an omega-3 supplement capsule or a placebo capsule (olive oil), although you will not know which until after completing the study. You will be provided will all of the capsules after the first experimental visit The amount of omega-3 contained in the daily supplement will around 1.5g. This amount is naturally found in around 100g of fresh tuna or herring. There is currently no Recommended Daily Allowance in the UK but this amount is typically used for research studies.

4. Are there any risks / benefits involved?

There are a number of discomforts and risks that you need to be aware of in your consideration of participating in this research process. These are:

Exercise

You will experience fatigue during the maximal aerobic capacity tests and moderate intensity exercise. This will be short lived and you should have fully recovered within hours of the process. However, during such vigorous exercise there is a very minimal risk of unforeseen heart failure. The risk is extremely small and the procedures are regularly conducted within the laboratory. In addition,

you will be screened prior to exercise to ensure this risk is as low as possible. Experimenters will be first aid and immediate life support trained.

Blood Sampling:
Blood samples will be taken on several occasions. You will feel a sharp pain when the needle is inserted, but this will be short-lived. The researchers are experienced in this technique so the pain experienced will be minimal. You may also develop a small bruise on your arm, which can be prevented by applying pressure on the arm (the researcher will remind/instruct you to do this, as is good practice). Finger prick blood samples will also be taken. You may experience some sensitivity where the blood sample is taken, but this will be short-lived and normally only last ~24 hours.

Are there any benefits?
The tests conducted during the study will provide you with information about your current activity level, fitness, body composition and other markers of health.

5. Will my taking part in the study be kept confidential?

All information/data will be stored confidentially and only accessed by members of the research team. The results of this study are expected to be published in a scientific journal and by the BBC, but names of participants will never be published. You will be notified if potentially clinically significant information results from the personal data collected from you, and be provided with guidance on seeking medical advice.

Whilst your personal information and results will remain confidential, it should be noted that as experimental visits at LJMU will be filmed and broadcast for a BBC programme. While you may request to not be featured on the show, it cannot be guaranteed that you will not be seen at all in the final footage.

What happens now?
If after reading this information sheet you still want to participate in the study you should re-contact the BBC, outlining your continued interest. You will then be directed to the study team and the protocol described within will commence. You will be asked to complete an informed consent form to confirm that you are happy to participate in this study. You will be asked to keep a copy of this information sheet and the signed consent forms. Finally, you will be invited to the Laboratories in Liverpool John Moores University to undergo the Screening visit.

If you any concerns regarding your involvement in this research, please discuss these with the researcher in the first instance. If you wish to make a complaint, please contact researchethics@ljmu.ac.uk and your communication will be re-directed to an independent person as appropriate.

This study has received ethical approval from LJMU's Research Ethics Committee

REC reference number:
Approval Date:

Lead Researcher
Dr Graeme Close:

LIVERPOOL JOHN MOORES UNIVERSITY
CONSENT FORM

Title of Project.

"The effect of oily fish and fish oil supplementation on markers of cardiovascular health and exercise performance".

Name of Researcher: Dr Graeme Close, Jamie Pugh

School: Liverpool John Moores University

1. I confirm that I have read and understand the information provided for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason and that this will not affect my legal rights. ☐
3. I understand that any personal information collected during the study will be anonymised and remain confidential ☐
4. I agree to take part in the above study ☐

Name of Participant	Date	Signature
Name of Researcher	Date	Signature

External Collaborators

Leila Finikarides – BBC "Trust Me I'm a Doctor"

Parveen Yaqoob – University of Reading
p.yaqoob@reading.ac.uk

Philip C. Calder – University of Southamptom

Dr Rachel Gow – The Institute for Food, Brain and Behaviour

APPENDIX H Risk assessment and HTA risk assessment.

Assessment Number……..

Risk Assessment

Building……TRB (Physiology labs)…… School/Service Department……..Sports Science……
Location……Byrom Street……..

Activity…… **The effect of oily fish and fish oil supplementation on markers of cardiovascular health and exercise performance**

Risk Assessment			
Building	Tom Reilly Building	Date of Risk Assessment	22.07.16
School/Service Department	Liverpool John Moores Sport Sciences Department	Assessment carried out by	Jamie Pugh
Location	Byrom Street, Liverpool	Signed	
Activity	Lab Testing	Persons consulted during the Risk Assessment	Dr Graeme Close
STEP 1 What are the Hazards? *Spot hazards by* • *Walking around the workplace* • *Speaking to employees* • *Checking manufacturers*	• Submaximal cycle ergometer test • Blood sampling • Flow-mediated dilation • Controlled diet (fish)		

instructions	• Urine collection • General lab hazards • Supplementation
STEP 2 Who might be harmed and how? *Identify groups of people.* *Staff and students are obvious, but please remember* • *Some staff/students have particular needs* • *People who may not be present all the time* • *Members of the public* • *How your work affects others if you share a workplace*	Blood sampling: Participants – infection, discomfort/ bruising, Phlebotomists - needle stick injury. Submaximal cycle ergometer test: Participants – Injury, falling off Flow-mediated dilation: Participants – discomfort, feeling faint, anxiety, Experimenters – cuffs not secured properly could injure participant / researcher when inflated. Controlled diet (Fish): Participants - food hygiene issues and food allergies Urine collection – Researchers through bacterial infection from urine. Surface contamination. General: Participants & LJMU staff/students - Trips, slips or falls in the laboratory e.g. loose cables, spilt water. Supplementation
STEP 3 (a) What are you already doing? *What is already in place to reduce the likelihood of harm, or to make any harm less serious*	Blood sampling • LJMU guidelines on health and safety for blood collection will be followed throughout. • Investigators are experienced in blood sampling methods and have undertaken phlebotomy training. Cycle ergometer • All subjects familiarised with use of the ergometer. • Subjects will perform warm up and stretches prior to exercise. • Participants will have no history of muscle injuries. • 2 investigators will be present at all tests. • Trained first aiders will be available at all tests. • University guidelines on emergency aid (ambulance) will be followed.

	Flow-mediated dilation • Double check the cuff is secured properly before inflation. • No known risk associated with the use of ultrasound. Controlled diet: • All food will be pre-packaged and supplied by soulmatefood.com. Foods are prepared by a catering company qualified under Food Safety Standards. • Food allergies will be assessed via the readiness to exercise questionnaire and re-confirmed during screening visits via verbal confirmation Urine collection Follow COSHH guidance. Ensure PPE guidance is followed. General • Make sure the lab is clean and tidy. Ensure all equipment is calibrated properly and participants are aware of their surroundings and protocols. • All work stations, equipment etc. will be thoroughly cleaned and all waste disposed of appropriately post research to ensure the lab maintains safe and clean. Supplementation: Supplements are needed to be stored in a cool, dry environment, which subjects will be informed of storage and consumption information will be provided on the packaging. The dose of 1 tablet a day has been shown to have no known side effects. As the supplement is deemed to be safe for consumption for children and adults they are not required to be stored in childproof containers.

STEP 3 (b) What further action is needed? *Compare what you are already doing with good practice. If there is a gap, please list what needs to be done.*	
STEP 4 How will you put the assessment into action? *Please remember to prioritise. Deal with the hazards that are high risk and have serious consequences first*	The assessment will be put into action using the actions above (step 3a). As no risk has serious consequences and all actions will be put into place.

STEP 5 REVIEW DATE: Risk assessment to be carried with and place in Risk assessment holders by the investigators when in both laboratory and biochemistry labs undertaking data collection......