Effects of Fish Oil-derived N-3 Polyunsaturated Fatty Acids on the Generation and Functions of Platelet-Derived Extracellular Vesicles

A thesis submitted in partial fulfilment for the Degree of Doctor of Philosophy

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Declaration

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

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Abstract

Background: Extracellular vesicles (EVs) are membrane-enclosed vesicles derived from a wide range of cell types and these structures are suggested to have roles in the development of cardiovascular diseases (CVDs), thereby being considered as emerging markers of CVD. Platelet-derived EVs (PDEVs) comprise the major EV population in the circulation and are an important contributor to the pathophysiology of CVDs through their procoagulant activities. N-3 polyunsaturated fatty acids (PUFAs) are abundant in oily fish and fish oil and reported to reduce CVD risk, but there has been little research to date examining the effects of n-3 PUFA on the generation and function of EVs, including those produced in vitro from platelets.

Objective: The aim of this project was to investigate the effects of fish oil-derived n-3 PUFA supplementation on conventional, thrombogenic and the 'emerging' cardiovascular risk markers, extracellular vesicles (EVs), in subjects with moderate risk of CVDs. The generation, composition and procoagulant activities of EVs derived from platelets were of particular interest in this thesis, as well as their relationship with conventional and thrombogenic risk markers.

Design: A total of 40 subjects aged 40-70 years with moderate risk for CVDs were recruited to a randomised, double-blind, placebo-controlled crossover intervention study. Subjects were supplemented with capsules containing either fish oil (1.8 g/d n-3 PUFA) or control oil (high-oleic safflower oil) for a period of 12 weeks. Effects of fish oil supplementation on thrombogenic risk markers, including coagulation, thrombin generation, platelet aggregation and fibrinolysis were investigated. Nanoparticle Tracking Analysis (NTA) and fluorescence flow cytometry were employed to analyse numbers of PDEVs. Total lipid fatty acid composition of PDEVs was analysed by gas chromatography (GS) and their coagulatory behaviour was assessed using a range of functional assays, including fibrin clot formation, thrombin generation, fibrinolysis and ex vivo thrombus formation.

Results: There was a strong relationship between numbers of circulating EVs and thrombogenic risk markers of CVDs. Supplementation with n-3 PUFAs resulted in significant modification of a range of CVD risk markers (both conventional and thrombogenic), modulation of the fatty acid composition of in vitro-generated PDEVs from unstimulated and stimulated platelets, alteration of the surface expression of PS in the case of PDEVs derived from unstimulated platelets, a reduction in the ability of PDEVs generated in vitro from unstimulated and stimulated platelets to support fibrin clot formation and thrombin generation and an accompanying improvement in fibrinolysis.

Conclusion: Circulating EVs are associated with thrombogenic activity, implicating them in increasing risk of CVDs. Dietary n-3 PUFA supplementation altered the fatty acid composition of, and the surface expression of PS by, in vitro-generated PDEVs and these PDEVs were less procoagulant, which indicates potential anti-coagulatory properties of n-3 PUFAs mediated through modification of PDEVs.

Table of Contents

Abbreviations

AA arachidonic acid

ADP adenosine diphosphate

AHA American Heart Association

ALA α-linolenic acid

BMI body mass index

BP blood pressure

CAD coronary artery disease

COX-1 cyclooxygenase-1

CRP-XL collagen-related peptides-XL

CHD congenital heart defects

CVDs cardiovascular disease

DAG diacylglycerol

DBP diastolic blood pressure

DGLA dihomo-γ-linolenic acid

DHA docosahexaenoic acid

DPA docosapentaenoic acid

ECM extracellular matrix

EDEVs endothelial cell-derived extracellular vesicles

EM electron microscopy

EPA eicosapentaenoic acid

ESCRT endosomal sorting complex required for transport

ETA eicosatetraenoic acid

EVs extracellular vesicles

FAMEs fatty acid methyl esters

FCM flow cytometry

FETA FFQ EPIC tool for analysis

FFQ food frequency questionnaire

FSC forward scatter

GPIbα glycoprotein Ibα

GPVI glycoprotein VI

GS gas chromatography

HDL-C high density lipoprotein-cholesterol

IL-1 interleukin-1

IL-6 interleukin-6

ILVs intraluminal vesicles

IP_3 inositol triphosphate

ISEV International Society of Extracellular Vesicles

ISTH International Society of Thrombosis and Haemostasis

LDL-C low density lipoprotein-cholesterol

LEVs large total extracellular vesicles

MCP-1 monocyte chemotactic protein-1

MI myocardial infarction

MS mass spectrometry

MUFAs monounsaturated fatty acids

MVBs multivesicular bodies

N/A not applicable

NO nitric oxide

$ONOO^-$ peroxynitrite

NTA nanoparticle tracking analyser

PAI-1 plasminogen activator inhibitor type 1

PAR protease-activated receptors

PBS phosphate-buffered saline

PC phosphatidylcholine

PDEVs platelet-derived extracellular vesicles

PE phosphatidylethanolamine

PFP platelet-free plasma

PGH_2 prostaglandin H_2

PGI_2 prostacyclin

PL A_2 phospholipase A_2

PLC-γ phospholipase C-γ

PPP platelet-poor plasma

PRP platelet-rich plasma

PS phosphatidylserine

PUFAs polyunsaturated fatty acids

RCTs randomised control/clinical trials

ROS reactive oxygen species

SACN Scientific Advisory Committee on Nutrition

SBP systolic blood pressure

SD standard deviation

SDE serious adverse events

SEC size exclusion chromatography

SEM standard error of the mean

SEM scanning electron microscopy

SEVs small total extracellular vesicles

SFA saturated fatty acid

SSC side scatter

TAG triacylglycerol

TC total cholesterol

TEM transmission electron microscopy

TEVs total extracellular vesicles

TF tissue factor

TNFα tumour necrosis factor-alpha

TRPS tunable resistive pulse sensing

TXA_2 thromboxane A_2

tPA tissue plasminogen activator

TRAP-6 thrombin receptor activating peptide 6

VFP vesicle free plasma

VLDL very low-density lipoprotein

VSMCs vascular smooth muscle cells

vWF von Willebrand factor

WHO World Health Organisation

WP washed platelets

Accomplishments

Paper

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Scientific Reports, 10(1), 1-8.

Oral presentation

UK Society for Extracellular Vesicles Forum 2021

London, United Kingdom (virtual)

Tittle: Effects of n-3 PUFAs on the thrombogenicity of EVs generated in vitro from washed platelets (a 3-minute flash talk poster presentation)

Nutrition Society Summer Conference 2021

University of Reading, United Kingdom

Tittle: Association between circulating extracellular vesicles and thrombogenic risk markers for cardiovascular disease (a 15-minute presentation under Nutrition Society Theme Highlights 2021)

3rd Food and Nutritional Science Research Symposium 2020

University of Reading, United Kingdom

Tittle: Association between circulating extracellular vesicles and thrombogenic risk markers for cardiovascular disease

Food and Nutritional Science Research Seminar 2019

University of Reading, United Kingdom

Tittle: Effects of n-3 polyunsaturated fatty acids on the generation and functions of platelet-derived extracellular vesicles (HI-FIVE Study)

1st Food and Nutritional Science Research Symposium 2018

University of Reading, United Kingdom

Tittle: Effects of n-3 polyunsaturated fatty acids on platelet aggregation

Poster

International Society for Extracellular Vesicles Annual Conference 2020

Philadelphia, United State of America (virtual)

Tittle: Association between circulating extracellular vesicles and thrombogenic risk markers for cardiovascular disease

2nd Food and Nutritional Science Research Symposium 2019

University of Reading, United Kingdom

Tittle: Effects of n-3 polyunsaturated fatty acids on coagulation and fibrinolysis

Diet and Health Research Industry Club Dissemination Workshop 2019

University of Reading, United Kingdom

Tittle: Effects of n-3 polyunsaturated fatty acids on coagulation and fibrinolysis

Chapter 1 Introduction and literature review

1.1 Cardiovascular diseases

1.1.1 Introduction to cardiovascular diseases

Cardiovascular disease (CVD) is defined as a group of disorders of the heart and blood vessels and the term CVD refers individual conditions affecting the cardiovascular system, including coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism, heart attacks and strokes. According to the World Health Organisation (WHO), CVDs are a major cause of death throughout the world, accounting for an estimated 17.9 million deaths each year and of these deaths, and majority is due to heart attacks and strokes (WHO, 2021). CVD is known as a complex multifactorial disease, which various risk factors are related to the occurrence of CVDs (Mulle and Vaccarino, 2013). Atherosclerosis is the underlying cause of most CVDs and initiated by repeated damage of the vascular endothelium caused by several risk factors. Therefore, it is important to understand the pathophysiology of atherosclerosis to develop preventative strategies (Jansen et al., 2017).

This literature review will mainly focus on cardiovascular risk factors, its pathogenesis and impacts of fish oil-derived n-3 PUFAs on CVD risk factors.

1.1.2 Cardiovascular risk factors

It is well appreciated that multiple risk factors contribute to the development of CVDs, including physiological factors, behavioural factors and environmental risk factors and these risk factors are commonly used to assess CVD risk (Tzoulaki et al., 2016). Some of these risk factors are modifiable by lifestyle changes, such as high levels of plasma cholesterol levels and blood pressure, vascular dysfunction, inflammation and hypercoagulation, while others cannot be modifiable (non-modifiable risk factors), such as sex, genotype, age and ethnicity (Stanner et al., 2018). Changing risk factors of CVDs can be a strong preventative strategy against CVD

(or cardiovascular events) since WHO (2021) reported that 75% of premature CVDs can be prevented by modifying these risk factors.

Epidemiological studies have identified some risk factors as conventional risk factors, including blood pressure, triacylglycerol (TAG), total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), high fasting plasma glucose and high body mass index (BMI) and reported their strong relationship with the development of CVD (Joseph et al., 2017, Vilahur et al., 2014, Alonso et al., 2021). According to the INTERHEART study, conventional risk factors accounted for over 75% of CVD (Yusuf et al., 2004). In addition to the conventional CVD risk factors, there are a number of risk markers, such as those influencing thrombosis, a condition predisposing to the development of CVDs (Raskob et al., 2014). Thrombogenic risk markers can include hypercoagulation (enhanced fibrin clot/thrombus formation), altered clot fibrinolysis (Kotze et al., 2014, Woodman et al., 2003), increased platelet function (Renga and Scavizzi, 2017, Adili et al., 2018), and increased thrombin generation (Al Dieri et al., 2012, Loeffen et al., 2015, Carcaillon et al., 2011, Lutsey et al., 2009) and they have been suggested to contribute to atherosclerosis and abnormal thrombosis (or blood clotting) which involve the pathophysiology of CVDs, and therefore, higher risk of future CVD (Stanner et al., 2018).

1.1.3 Pathogenesis of CVD: atherosclerosis and thrombosis

Atherosclerosis is described as a chronic inflammatory disease of the vascular wall, with impaired vascular haemostasis and the development of plaque (Schafer and Bauersachs, 2008), initiated at the early stage of life and progressing asymptomatically throughout adulthood until clinical evidence presents (Mujica et al., 2010). The development of atherosclerotic lesions or plaques is a complicated process driven by circulating factors and different cell types in the vessel wall, which cause the accumulation of lipids in the sub-endothelium and continuous inflammation. It is commonly identified by the presence of endothelial dysfunction, leucocyte infiltration and platelet activation (Douglas and Channon, 2014).

Healthy arteries include three layers: the tunica intima, media, and adventitia as presented in **Figure 1.1A**. A single layer of endothelial cells comprises the tunica intima, which is the base membrane of the luminal side of blood vessels, and the tunica media consists of collagen with fibroblasts and vascular smooth muscle cells (VSMCs) (Siracuse and Chaikof, 2012). The vascular endothelium covers the entire vascular system and is considered as a dynamic organ (Sandoo et al., 2010), which plays significant roles in vascular homeostasis by regulating blood flow and vascular tone (Sandoo et al., 2010, Rajendran et al., 2013). The endothelium has a role in regulating permeability between the bloodstream and vascular wall, growth of medial smooth muscle cells, leukocyte and platelet adhesion and activation, thrombosis and fibrinolytic properties (Baker et al., 2018). This is achieved by the balanced secretion of vasodilators, such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium derived hyperpolarizing factor, and vasoconstrictors, such as thromboxane (TXA_2) and endothelin-1 from the endothelium (Vélez and García, 2015). Among these vasoactive factors, NO is the most important product of the endothelium as it inhibits proliferation of vascular smooth muscle cells, activation and aggregation of platelet and cell adhesion and migration (Douglas and Channon, 2014). However, an impairment in the endothelium due to shear stress and chronic inflammation results in imbalanced release of endothelial vasoactive mediators (Sandoo et al., 2010), leading to endothelial dysfunction (early sign of atherosclerosis) (Hadi et al., 2005). Endothelial dysfunction is generally characterised by increased endothelium-derived contracting factors, inflammation, proliferation, oxidation, and thrombotic activity and decreased vasodilation. The oxidation is linked to excessive production of reactive oxygen species, in particular superoxide, which tend to react with NO to form peroxynitrite ($ONOO^-$). The formation of $ONOO^-$ not only impairs the bioavailability of NO in the blood vessel wall, but also impairs the function of proteins and lipids (Douglas and Channon, 2014, Rajendran et al., 2013).

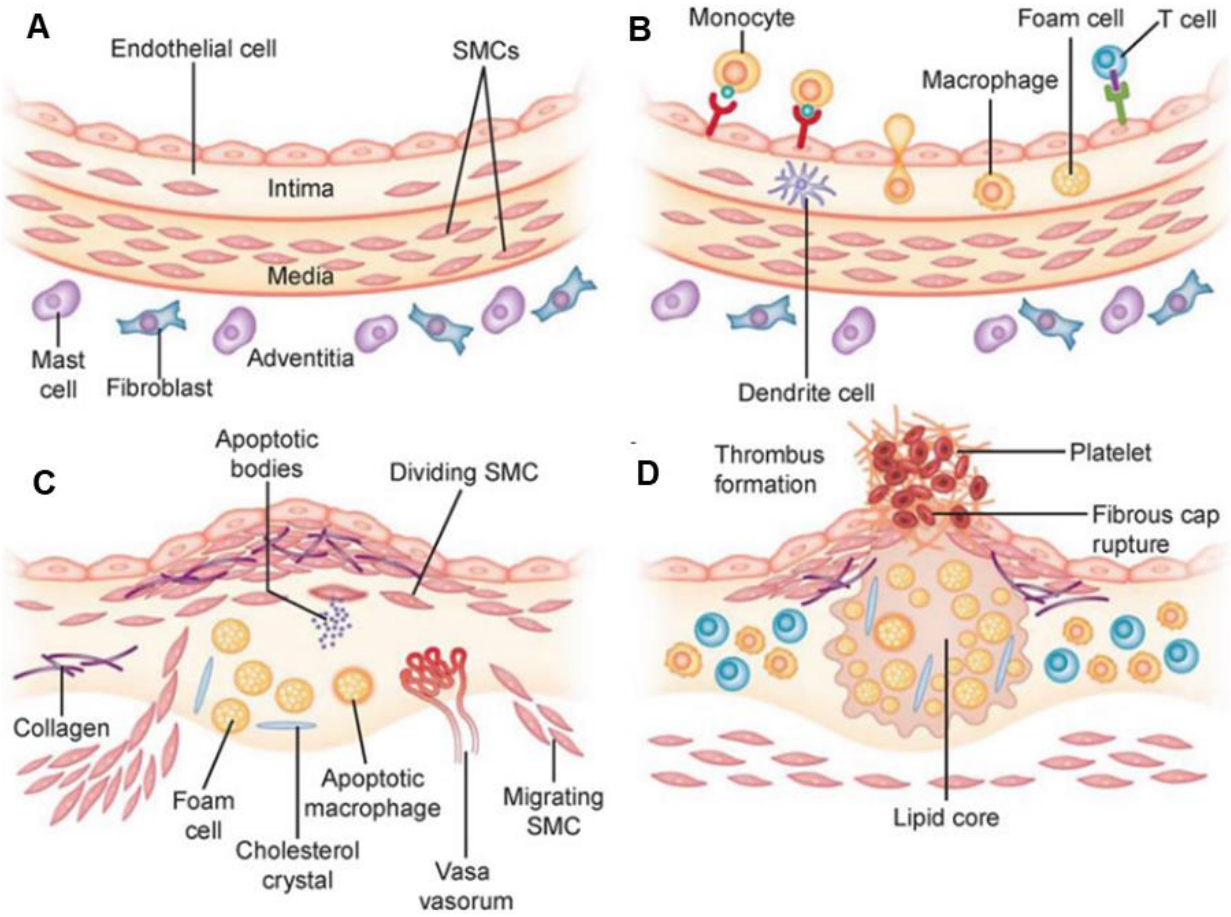


Figure 1.1. Development of an atherosclerotic plaque. (**A**) Normal arteries include three layers: the tunica intima, media, and adventitia. (**B**) the adhesion of lipids, monocytes and platelets to the damaged endothelium initiate the process of atherosclerosis. (**C**) atherosclerotic plaque is formed by accumulation of foam cell and smooth muscle cells, and the release of extracellular matrix proteins such as collagen and elastin. (**D**) platelet aggregation and thrombus formation as a result of plaque rupture (Siracuse and Chaikof, 2012).

The development of an atherosclerotic plaque is usually initiated by the exposure of deleterious lipids, in particular low-density lipoprotein cholesterol (LDL-C), to the underlying extracellular matrix (ECM), which is translated to endothelial dysfunction. When LDL-C is oxidized by reactive oxygen species (ROS), it stimulates endothelial cells to produce cellular adhesion molecules, chemotactic proteins and growth factors, and to limit the production of NO (Siracuse and Chaikof, 2012). These molecules favour the initiation of activation and adhesion of blood monocytes to the endothelium, which consequently facilitate the migration of the monocytes into intima, followed by differentiation to macrophages and formation of foam cells after uptake of oxidized LDL-C (Siracuse and Chaikof, 2012, Baker et al., 2018).

Further interaction of foam cells with T-cells potentiates the secretion of inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNFα), resulting in increased endothelial permeability, which leads to the recruitment of more LDL-C and monocytes to the endothelium (**Figure 1.1B**). This inflammatory response also induces the migration and proliferation of VSMCs from the media to the early lesion, which consequently synthesises ECM proteins, such as collagen and elastin, to form a strong fibrous cap, which covers and stabilises plaque by isolating the lipid core from circulating blood (Baker et al., 2018, Douglas and Channon, 2014). The earliest stage of plaque development is described as a fatty streak (**Figure 1.1C**), while the final stages involve plaque cap rupture, which allows blood coagulation components to interact with the prothrombotic plaque interior, promoting platelet aggregation, the coagulation cascade and thrombus formation, thereby resulting in the condition as (athero)thrombosis (**Figure 1.1D**) (Douglas and Channon, 2014, Libby et al., 2011).

Upon the damage to endothelial lining of a blood vessel or plaque rapture, the sequence of events may be triggered by exposure of tissue factor and collagen on the vessel wall, which activates two major clotting pathways (the extrinsic and intrinsic pathway of coagulation), leading to thrombin generation and fibrin clot formation, as presented in **Figure 1.2** (Stanner et al., 2018). The extrinsic pathway is initiated by active tissue factor, which initially forms a complex with clotting protein factor VII/VIIa, known as tissue factor-factor VIIa complex, which in turn, activates factor X into its active form (factor Xa) (Smith et al., 2015). In parallel, the coagulation cascade is triggered by negatively charged collagen on the damaged endothelium, resulting in sequential activation of factor XII, factor XI, and factor IX (the intrinsic pathway of coagulation). Activated factor IX with its cofactor, factor VIII form an enzyme complex, which in turn, activates factor X into factor Xa (Palta et al., 2014). Activated factor X in conjunction with its cofactor factor Va, tissue phospholipids, platelet phospholipids and calcium forms the prothrombinase complex, which stimulates the cleavage of prothrombin (factor II) to thrombin (factor IIa) (Stanner et al., 2018). Thrombin as the final coagulation

protein catalyses the cleavage circulating fibrinogen to fibrin monomers and activates factor XIII, which has the ability to crosslink fibrin polymers incorporated in the platelet plug (stable clot). Additionally, thrombin is a potent activator of platelets through protease-activated receptors (PAR) (Borissoff et al., 2009).

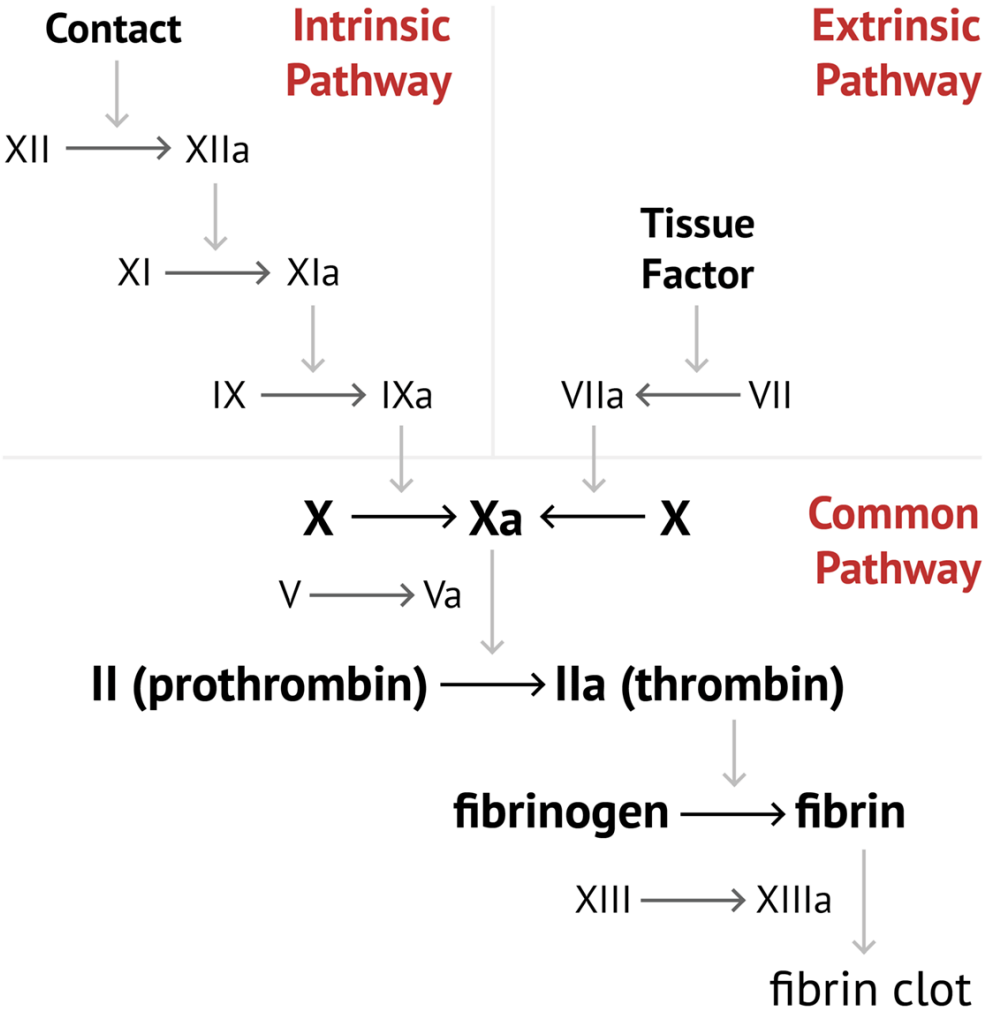


Figure 1.2. The coagulation cascade.

1.1.4 Platelets and their roles in haemostasis, thrombosis and CVD

Platelets are anuclear cells derived from megakaryocytes in the bone marrow with a half-life of 10 days(Jayachandran et al., 2011). Although they lack a nucleus, platelets have a unique

cytoskeleton (alpha and dense granules) and highly complex membrane structure including membrane glycoproteins. In normal physiological conditions, blood platelets are implicated in haemostasis and thrombosis, particularly in the prevention of excess blood loss and tissue healing. However, they also contribute to pathological conditions, such as inflammation, atherosclerosis and (athero)thrombosis and their enhanced activity is associated with cardiovascular diseases since activated platelets secrete substances from their storage granules, which can mediate pathophysiological processes (Mackay et al., 2012, van der Meijden and Heemskerk, 2018).

Normal vascular endothelium regularly inhibits abnormal platelet activation through the release of PGI_2 and NO (Siracuse and Chaikof, 2012). In the early stages of atherosclerosis, platelet adhesion and activation are initiated rapidly by subendothelial matrix proteins, such as collagen, von Willebrand factor (vWF) and fibronectin, which interact with specific platelet receptors. Platelets adhere to the endothelium via the interaction of subendothelial vWF with glycoprotein Ibα (GPIbα) component of the platelet GPIb-V-IX complex and collagen with platelet glycoprotein VI (GPVI) (Jennings, 2009, Kaplan and Jackson, 2011, van der Meijden and Heemskerk, 2018), leading to platelet granule secretion (alpha granules containing fibrinogen, vWF and factor V; dense granule, containing ADP), the formation of TXA_2 and the activation of fibrinogen receptor (αIIbβ3) (Vélez and García, 2015). This process results in the engagement of more vWF and fibrinogen, leading to the recruitment of additional platelets and the formation of a platelet plug. Adherent platelets, thereafter, interact with circulating leukocytes via P-selectin, which induces recruitment of leukocytes to the vessel well (Renga and Scavizzi, 2017). Moreover, activated platelets release coagulation factors, expose phosphatidylserine (PS) on the membrane and promote thrombin and fibrin formation (Swieringa et al., 2018). As platelets are central to the aetiology of atherosclerosis, it is important to understand how they are activated, what the consequences of the activation are and how this can be modified.

Platelet activation is initialized by soluble agonists, released from vascular cells, erythrocytes or leukocytes at the sites of vascular injury. Regardless of the agonist, this process involves shape change, aggregation, and liberation of arachidonic acid, which in turn leads to the production of prostaglandins and lipoxygenase products (Willoughby et al., 2002, Kaplan and Jackson, 2011). The agonists bind to their specific receptors on the platelet surface; thrombin and thrombin receptor activating peptide-6 (TRAP-6) trigger platelet activation through PAR (De Candia, 2012); adenine diphosphate (ADP) is engaged with the purinergic P_2Y_1 and P_2Y_{12} receptors; collagen and collagen-related peptides-XL *(*CRP-XL) are specific for GPVI (Jennings, 2009); epinephrine stimulates platelets through the α2-adrenergic receptor in the presence of other platelet agonists (Zhou and Schmaier, 2005). GPVI is associated with the Fc receptor and FcRγ stimulates platelet activation through phospholipase C-γ (PLC-γ), while the PAR, P2Y and α2-adrenergic receptor coupled to G-protein receptors (GPCR) activate platelets through PLC-β. Both signals result in the production of two messengers: diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG mediates calcium influx in platelets, whereas IP_3 releases calcium from intracellular stores (Rumbaut and Thiagarajan, 2010). Even though agonists have specific signalling cascades, they all liberate calcium from intracellular stores, which is critical in the process of platelet activation. This triggers the subsequent discharge of the content of platelet granules, including ADP and P-selectin into the lumen of the open canalicular system, resulting in platelet shape changes due to invagination of the membrane (Shin et al., 2017, Bachmair et al., 2014). Phospholipase A_2 (PLA_2) liberates arachidonic acid from the platelet membrane and liberated or external arachidonic acid is a precursor of prostaglandin H_2 (PGH_2) synthesis, driven by cyclooxygenase-1 (COX-1). PGH_2 is converted into TXA_2 by thromboxane synthase (Bachmair et al., 2014), which is another agonist of platelets (Rumbaut and Thiagarajan, 2010). This process ends with the activation of the GPIIb/IIIa receptor (out-side-in signalling), which induces platelet-platelet attachment and stabilisation of the platelet aggregate through fibrinogen binding and thrombus formation. Apart from the physical contribution to the

formation of plaque, platelets release proinflammatory substances, such as P-selectin, CD40 and RANTES (Bachmair et al., 2014), as well as procoagulant platelet-derived extracellular vesicles, which contribute to atherosclerotic plaque formation in the vessel wall, with the release of further growth factors, lipid products and cytokines (van der Meijden and Heemskerk, 2018).

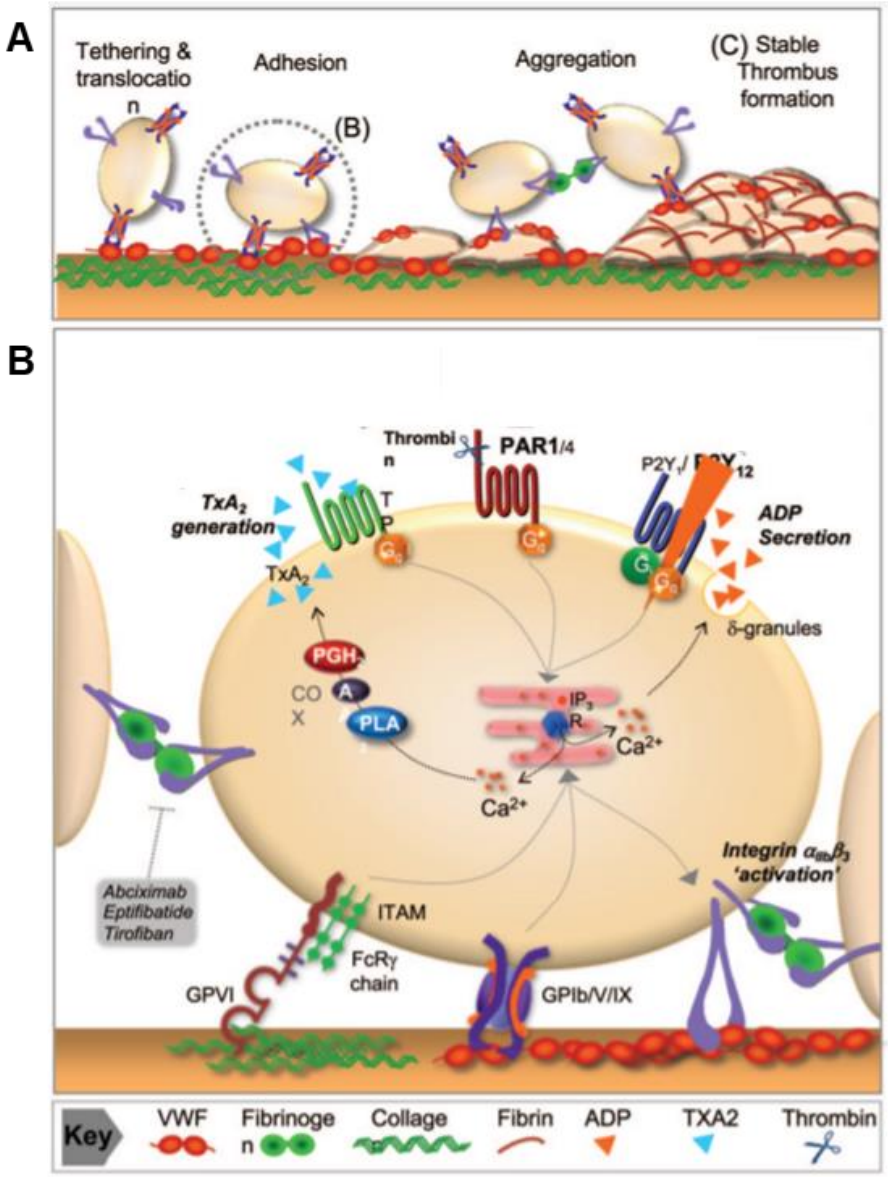


Figure 1.3 Platelets and their roles in thrombosis. (**A**) Platelets are recruited from circulating blood though the interaction of platelet GPIb-V-IX and vWF following disruption of the endothelium. (**B**) The mechanism for platelet adhesion and aggregation (Kaplan and Jackson, 2011).

1.2 Emerging risk markers; extracellular vesicles

1.2.1 Introduction to extracellular vesicles

Extracellular vesicles (EVs) are a group of heterogenic membrane-enclosed vesicles released by many different cell types in most eukaryotes in response to cellular stress and activation (Anouar and Daskalopoulou, 2018, Coumans et al., 2017). EVs were first observed by Chargaff and West (1946) and described as 'procoagulant platelet-derived particles' (Chargaff and West, 1946). They were later identified by Wolf (1967) as 'platelet-dust' (Wolf, 1967). Although these structures were initially considered as cell debris without biological activity, it is now well established that EVs are mediators of cell-to-cell communication (Boulanger et al., 2017). EVs carry bioactive cargo, such as proteins, lipids and nucleic acids (Boulanger et al., 2017, van Niel et al., 2018, Yáñez-Mó et al., 2015), and have important emerging roles in both physiological and pathological conditions involving coagulation and inflammation (Van der Pol et al., 2016). Therefore, interest in their potential use as novel biomarkers has grown considerably (Anouar and Daskalopoulou, 2018), resulting in numerous publications demonstrating the existence of EVs and their contribution to health and diseases.

1.2.2 Classification and biogenesis of EVs

EVs comprise a heterogeneous population with respect to cellular origin, size, density, numbers, content, membrane composition and mechanism of formation (Xu et al., 2016, Anouar and Daskalopoulou, 2018), and this heterogeneity makes the classification of EVs complex. Currently, there are at least three categories of EVs based on their size and biogenesis: exosomes, microvesicles, and apoptotic bodies, as presented in **Figure 1.4** (Anouar and Daskalopoulou, 2018). Exosomes (30-150 nm) are small EVs, created within the endosomal network and released following the fusion of multivesicular bodies (MVBs) with the plasma membrane (van Niel et al., 2018). Microvesicles (50nm to >1μm) are formed from the cell surface by outward budding and fission of the plasma membrane in response to activation (van Niel et al., 2018) and they contain cytoplasmic content on their surface (Kang et al.,

2017). Apoptotic bodies (1-5 μm) consist of vesicles resulting from cellular apoptosis (Kang et al., 2017). Distinguishing these EV classes is difficult due to their overlapping size and the lack of standardised methods (Brisson et al., 2017, Lee et al., 2012, Aatonen et al., 2014). The term EVs was introduced by the International Society of Extracellular Vesicles (ISEV) (Théry et al., 2018) to refer to all subtypes and this term will be used as such throughout this thesis, unless otherwise indicated.

The underlying mechanism for the biogenesis of EVs is still not completely certain because their generation may vary depending on the cell of origin (van Niel et al., 2018). The liberation of exosomes involves the formation of intraluminal vesicles (ILVs) in the endosomal lumen by the inward budding of endosomal membrane to form MVBs (van Niel et al., 2018). This process of MVB formation, ILV budding and protein cargo sorting into ILVs, is effected by membrane trafficking pathways, such as the endosomal sorting complex required for transport (ESCRT) system and followed by transfer of MVBs through microtubules regulated by cholesterol (Record et al., 2018). Once they reach the plasma membrane, the exosomes are secreted into the extracellular environment by fusion with the plasma membrane (Record et al., 2018). Another pathway implicates the movement of MVBs through the perinuclear area of the cell and fusion with lysosomes (Ailawadi et al., 2015). By contrast, the biogenesis of microvesicles involves direct budding from the plasma membrane through reorganisation of membrane phospholipids and remodelling of the cytoskeleton (van Niel et al., 2018). Cell activation or apoptosis triggers the disruption of asymmetric phospholipid membrane, followed by elevation of intracellular calcium, regulated by calcium-dependent enzymatic machinery, which involves amino-phospholipid translocases (flippases and floppases), scramblases and calpain (van Niel et al., 2018, Anouar and Daskalopoulou, 2018) and results in the externalisation of the negatively charged phospholipids, mainly PS (Zaldivia et al., 2017). These changes lead to reorganisation of the actin cytoskeleton and the physical folding of the membrane, thereby releasing apoptotic bodies into the circulation (van Niel et al., 2018) by the outward blebbing of the apoptotic cell membrane (Kang et al., 2017).

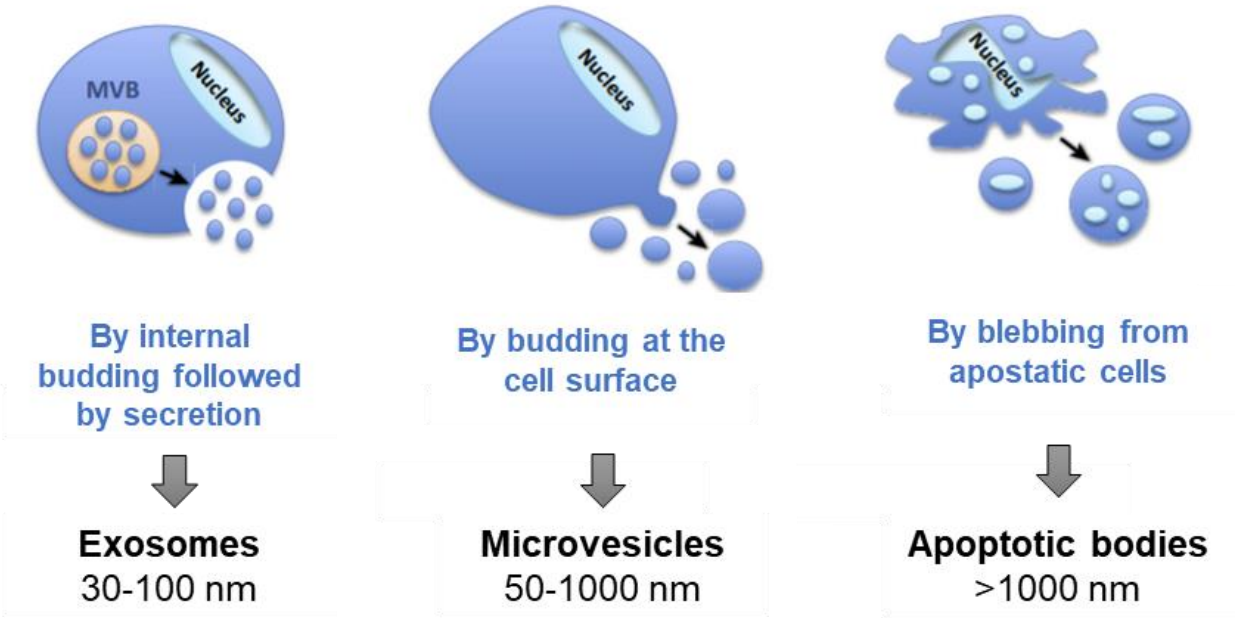


Figure 1.4. Main EV classes based on their size and biogenesis. Exosomes are small EVs, generated by inward endosomal budding and released following the fusion of multivesicular bodies (MVBs) to the extracellular space. Microvesicles are formed by outward budding of the cell membrane surface. Apoptotic cells are produced from death cells as a result of their apoptosis (Kanada et al., 2016).

1.2.3 Platelet derived extracellular vesicles

Platelets make a major contribution to the EV population in the circulation (Aatonen et al., 2014) comprising between 60–90% of circulating EVs in healthy subjects (Brisson et al., 2017). Platelet derived extracellular vesicles (PDEVs) can be distinguished from vesicles released from other sources by expression of GPIbα, integrins αIIbβ3 (CD61) and P-selectin (CD62P), which are platelet-specific markers (Kushwaha et al., 2018). There are thought to be several triggers for release of EVs from platelets, including platelet activation by soluble agonists, shear stress and glycoprotein (GP) IIb/IIIa outside-in signalling. Following activation or shear stress, intracellular calcium concentration in platelets is increased, leading to the activation of scramblase (bidirectional phospholipid transporter), remodelling of the cytoskeleton and loss of phospholipid asymmetry of the plasma membrane, which consequently results in the formation of EVs (Zaldivia et al., 2017, Melki et al., 2017). In contrast, unstimulated platelets have been shown to form EVs through destabilisation of the

actin cytoskeleton by GPIIb/IIIa signalling (Cauwenberghs et al., 2006). Heijnen and colleagues reported that activated platelets release a heterogeneous population of both microvesicles and exosomes, depending on the formation mechanism (Heijnen et al., 1999). Several selective markers have been used to identify platelet-derived exosomes and microvesicles (**Table 1.1**). For example, platelet exosomes have been reported to selectively express CD63, CD81, CD9, Hps70 and TSG101 (Heijnen et al., 1999, Ambrose et al., 2018), whereas platelet microvesicles selectively express annexin V, factor V, prothrombin αIIbβ3, GPIbα and P-selectin (Heijnen et al., 1999, Burnouf et al., 2014), suggesting that microvesicles share a similar surface phenotype with their parent platelets. However, both subpopulations have been shown to carry and transfer cellular signals, indicating their potential roles in cellular communication (Dovizio et al., 2015). In fact, distinguishing these subtypes of PDEVs is hampered by the lack of reliable separation and characterisation techniques (Brisson et al., 2017). Aatonen *et al* (2014) reported challenges in the separation of platelet exosomes and microvesicles based on size and density (Aatonen et al., 2014).

Bioactive molecules carried by PDEVs are diverse and altered depending on environmental conditions, but similar to EVs from other cell types, they comprise proteins, lipids, and nucleic acids (Melki et al., 2017) and include adhesion receptors, coagulation factors, transcription factors, growth factors, active enzymes, cytokines and chemokines, and their receptors (Gasecka et al., 2019). PDEVs present a lipid bilayer membrane, which is enriched in free cholesterol and phospholipids (Gasecka et al., 2019). Although their phospholipid composition is originated from 'parent' platelets, the content may be different from that of platelets as the selective sorting of molecules into PDEVs may be regulated by the specific agonists or microenvironment (Cointe et al., 2017). In fact, the lipid bilayer of PDEVs has been shown to contain a higher cholesterol content than platelets, perhaps indicating an enrichment of lipid rafts (Biró et al., 2005). The diversity in the composition of PDEVs may account for a wide range of biological roles.

Table 1.1. Characterisation of PDEVs

	Platelet exosomes	Platelet microvesicles	Apoptotic bodies
Size	30-100 nm	100-1000 nm	0.5-5 μm
Origin	Multivesicular bodies	Plasma membrane	Cellular fragments
Production mechanism	ESCRT	Budding from microdomains	Apoptosis
Specific surface markers	CD9, CD63, CD81, Alix, TSG101, HPS10, MHC I-II	CD42b, CD31 and CD41/61	PS
Platelet specific proteins	CD31, CD41, CD42a and PF4	GPs, tissue factors, P-selectin, PECAM-1 and factors V factor V and prothrombin	Histones, fragmented DNA

Table adapted and modified from (Zaldivia et al., 2017, Tao et al., 2017, Cointe et al., 2017).

1.2.4 Isolation and characterisation of PDEVs

EVs can be isolated from most cell types and biological fluids, such as saliva, urine, nasal and bronchial lavage fluid, amniotic fluid, breast milk, plasma, serum and seminal fluid (Yáñez-Mó et al., 2015). There are multiple techniques currently available to isolate EVs, but isolation may be challenging due to their small size and different structure, as well as a lack of universal standardised protocols. Differential centrifugation and size exclusion chromatography (SEC) are the most common techniques for isolation, and flow cytometry (FCM) and nanoparticle tracking analysis (NTA) for characterisation of EVs (Kang et al., 2017). There is particular interest in analysis of PDEVs since they comprise the majority of circulating EVs (Aatonen et al., 2014).

Diverse methodologies have been employed to isolate PDEVs from blood, but the International Society of Thrombosis and Haemostasis (ISTH) guidance emphasises (1) the use of a large needle size (21- gauge needle minimum), (2) discard of the first few millilitres of

collected blood, (3) collection of blood into an appropriate tube containing appropriate anticoagulant (citrate) and gentle mixing, (4) limited agitation and horizontal transportation of tubes, (5) processing of collected blood within a maximum of 2 hours, and (6) appropriate centrifugation to remove all platelets (Cointe et al., 2017, Coumans et al., 2017). PDEVs can be isolated from platelet poor plasma (PPP), obtained by centrifugation of whole blood or they can be derived from washed platelets (WP) following stimulation and then purification of PDEVs in vitro. In this method, WPs are stimulated with platelet agonists, such as TRAP-6, thrombin or collagen to trigger the formation of EVs, which can be isolated using different techniques, including differential centrifugation, density gradient and gel filtration or size exclusion chromatography (Kailashiya, 2018). As an alternative, platelets in platelet-rich plasma (PRP) can be activated with agonists and the PDEVs thus generated can be isolated by the removal of platelets and other cell debris by SEC to obtain a PDEV sample (Gasecka et al., 2019).

Several techniques are available for the characterisation of PDEVs, with sensitivity and accuracy being crucial. One of the most widely employed methods for the characterisation of PDEVs is FCM, which allows enumeration and detection of PDEV surface markers, such as PS and the platelet markers, CD41 and CD62P (Kang et al., 2017). A combination of annexin V, which has a high affinity for PS, with platelet markers, such as CD61, CD42b, CD41 or CD62P, are the most commonly used markers to identify PDEVs (Cointe et al., 2017). However, conventional FCM is largely unable to characterise EVs smaller than 200 nm and is not able to determine the size distribution of PDEVs (Arraud et al., 2016). NTA is able to characterise PDEVs ranging from 30-1000 nm in diameter and tunable resistive pulse sensing (TRPS) allow detection of individual EVs ranging in size from 80-1000 nm (Cointe et al., 2017, Gasecka et al., 2019). Neither NTA nor TRPS are able to phenotype EVs, nor to distinguish EVs from lipoproteins or protein aggregates (Gasecka et al., 2019). PDEVs can be visualised by electron microscopy (EM), including cryo-EM, transmission-EM (TEM) and scanning-EM

(SEM), which have all been used for the high-resolution visualisation of PDEVs (Brisson et al., 2017, Arraud et al., 2014).

The biological functions of PDEVs in the circulation have been attributed to their bioactive cargo, highlighting the importance of proteomics, and lipidomics approaches in their analysis (Kailashiya, 2018, Coumans et al., 2017). However, the most convincing evidence for procoagulant and fibrinolytic activity of PDEVs comes from functional assays, including those assessing fibrin clotting, thrombin generation and fibrinolysis (Cointe et al., 2017, Coumans et al., 2017).

It is important to note that while there are several techniques which provide a limited amount of information about the number, size, characteristics, composition and functions of PDEVs, there is no single technique which offers full characterisation. Therefore, an appropriate combination of different techniques is important in achieving fuller characterisation. According to the MISEV 2018 guidelines (Théry et al., 2018), the combination of high-resolution imaging of isolated EVs with other techniques to assess size and concentration, such as NTA and flow cytometry, is recommended for the characterisation of pre-purified EVs.

1.2.5 Role of PDEVs in CVDs

Over the past few years, particular attention has been drawn to role of PDEVs in CVDs as there is a large body of evidence suggesting that elevated levels of circulating PDEVs are associated with increased risk or incidence of cardiovascular events (Badimon et al., 2016, Melki et al., 2017, Berezin and Berezin, 2019). Increased numbers of PDEV have been reported in atherosclerosis (Fortin et al., 2016, Namba et al., 2007), acute coronary syndrome (ACS) (van der Zee et al., 2006), carotid intima-media thickness (IMT) (Csongrádi et al., 2011), acute-phase atherosclerotic cerebral infarction (Kuriyama et al., 2010), extended damage following myocardial infarction (MI) (Hartopo et al., 2016) and arterial thrombosis (Namba et al., 2007, Gasecka et al., 2017). EVs released from platelets have also been implicated in diabetes mellitus (Cohen et al., 2002), hypertension (Preston et al., 2003), hyperlipidaemia,

hypercholesterolaemia (Suades et al., 2013), metabolic syndrome (Helal et al., 2011) and obesity (Murakami et al., 2007). The contribution of PDEVs to CVD could involve a role in haemostatic and thrombotic process, inflammation, cell survival and apoptosis, vascular dysfunction, angiogenesis (Aatonen et al., 2012, Badimon et al., 2018, Zaldivia et al., 2017), thrombin generation (Van Der Meijden et al., 2012), thrombus formation and platelet aggregation (Suades et al., 2012a).

1.2.6 Role of PDEVs in haemostasis and thrombosis

Although the primary role of platelets is in coagulation and clotting, EVs released from those platelets have been suggested to have 50~100-fold higher procoagulant capability than even activated platelets (Sinauridze et al., 2007). PS-exposing EVs are particularly procoagulant and pro-thrombotic (Heemskerk et al., 2013), promoting the rate and extent of platelet activation, thrombin generation and thrombus formation (Nomura and Shimizu, 2015, Tripisciano et al., 2017b, Aatonen et al., 2014) and participating in coagulation and thrombosis (Zarà et al., 2019, Boulanger et al., 2017).

In resting platelets, the plasma membrane presents phosphatidylcholine (PC) in the outer leaflet, while the negatively charged PS and phosphatidylethanolamine (PE), which have procoagulant activity, are localised in the inner leaflet. However, when platelets are activated, PS and PE are flipped to the outer leaflet prior to EV shedding, leaving PDEVs with exposed PS on the outer leaflet (Brisson et al., 2017). PS-exposing PDEVs provide a catalytic phospholipid surface for the recruitment of blood coagulation factors and promote the formation of the tenase (factors IXa, VIIIa, and X) and prothrombinase (factors Xa, Va, and prothrombin) complexes, thereby supporting further activation of coagulation cascade to convert fibrinogen to fibrin (Cointe et al., 2017, Voukalis et al., 2019). It has been demonstrated that PDEVs not only propagate coagulation by the expression of PS but also support thrombin generation independently of tissue factor in a factor XII-dependent manner (Van Der Meijden et al., 2012). Moreover, the expression of tissue factor on the PDEV surface has been proposed to contribute to their procoagulant activity (Biro et al., 2003), since tissue factor binds

to factor VII to activate factor X and consequently trigger the extrinsic (tissue factor-dependent) pathways of coagulation (Zwicker et al., 2011). Procoagulant ability of PDEVs has also been attributed to the expression of integrins αIIbβ3 (CD61) and P-selectin (CD62P) and factor X on their membrane (Sinauridze et al., 2007, Kushwaha et al., 2018, Zubairova et al., 2015a), which promote fibrin clot formation by directly attaching to fibrin fibers and by increasing rapid thrombin generation (Zubairova et al., 2015a). The fact that PDEVs in the circulation may vary in their size, content and mechanisms leading to their formation has implications with respect to their procoagulatory behaviour. Recent studies have suggested that smaller EVs may be more procoagulant than larger EVs (De Paoli et al., 2018, Tripisciano et al., 2017b). However, it has also been suggested that despite being larger, platelet-derived microvesicles may be considerably more procoagulant compared with than platelet-derived exosomes due to greater expression of PS (Heijnen et al., 1999). In addition, procoagulant activity differs depending on the activator (Aatonen et al., 2012).

Several clinical trials have demonstrated increased numbers of PDEVs in thrombotic disorders (Chen et al., 2015, van der Zee et al., 2006, Huisse et al., 2007, Giannopoulos et al., 2014). Suades and co-workers reported that blood enriched with PDEVs induced platelet and fibrin deposition in atherosclerotic arteries in an ex vivo study. Similarly, PDEVs enhanced platelet aggregation and platelet adhesion to collagen as well as shortening the time for clotting in this study (Suades et al., 2012b). The ability of PDEVs to support thrombin generation has been demonstrated by several studies (Tripisciano et al., 2017b, Van Der Meijden et al., 2012, Aleman et al., 2011). Van Der Meijden *et at* (2012) revealed that these vesicles lead to thrombin generation in a factor XII-driven contact activation manner and fail to trigger coagulation in factor XII-deficient plasma (Van Der Meijden et al., 2012). It has been also suggested that EVs derived from platelets could, in turn, evoke platelet activation through the mechanism by which pre-treated PDEVs with PLA_2 deliver the concentrated arachidonic acid to platelets (Barry et al., 1997). An in vitro study reported that the ability of PDEVs to rapidly bind to monocytes via P-selectin results in the delivery of platelet adhesion receptor GPIbα to

monocytes and this promotes further interaction of GPIbα+monocytes with additional ligands, such as vWF, to be recruited on activated endothelial cells (Chimen et al., 2020). Therefore, PDEVs may contribute to coagulation and thrombosis in a variety of ways through their bioactive cargo and cell surface molecules.

1.3 Fish oil and n-3 polyunsaturated fatty acids

1.3.1 Introduction to n-3 polyunsaturated fatty acids

N-3 polyunsaturated fatty acids (PUFAs) are classified as unsaturated fatty acids which comprise hydrocarbon chains containing double bonds, with a carboxyl group at one end and a methyl group at the other and they are identified by having the first double bond in the third position from methyl end group. The main n-3 PUFAs are alpha-linolenic acid (ALA) (18:3 n-3) derived from plants such as flaxseed, rapeseed, soybean and walnut, eicosapentaenoic acid (EPA) (20:5 n-3) and docosahexaenoic acid (DHA) (22:6 n-3) derived from oily fish or fish oil. ALA is an essential fatty acid as it cannot be synthesised by humans and must be obtained through the diet; although it can be converted to EPA and DHA in a multistep elongation and desaturation reaction, as shown in **Figure 1.5,** this conversion is not sufficient to meet recommended requirement (Grundt and Nilsen, 2008). It is reviewed that 8-20% of ALA is partially converted to EPA, while the conversion rate of ALA to DHA is even lower (0.5-9%) (Stark et al., 2008). N-3 PUFAs have several roles in membrane structure and function, tissue metabolism, and genetic regulation (Grundt and Nilsen, 2008). The Scientific Advisory Committee on Nutrition (SACN) has recommended consumption of at least two portions of fish per week, at least one of which should be oily, to meet the 0.45 g/d requirement of n-3 PUFAs (SACN, 2004). For patients with hypertriglyceridemia, the American Heart Association (AHA) recommends supplementation with 2–4 g/d of fish oil (Skulas-Ray et al., 2019).

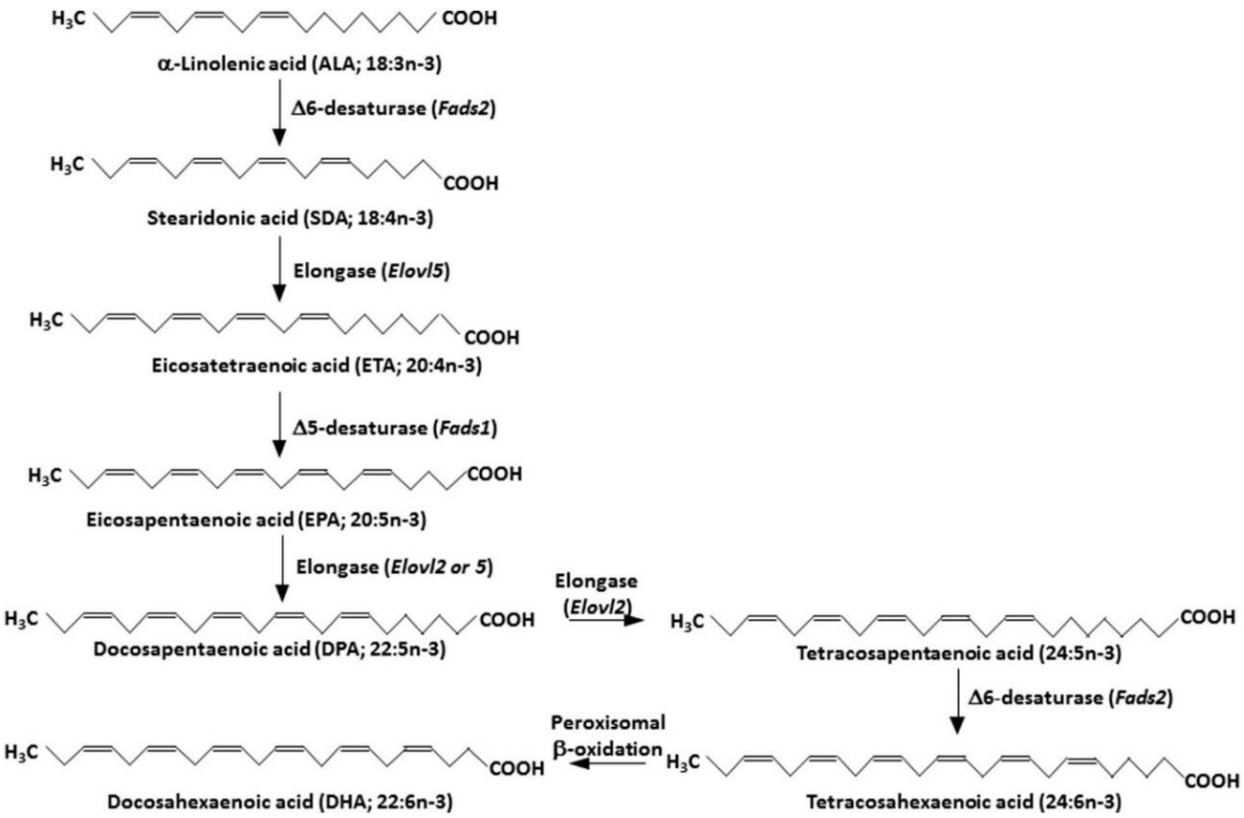


Figure 1.5. Pathway of conversion of ALA to EPA and DHA (Baker et al., 2016).

1.3.2 Effects of n-3 PUFAs on CVDs

Over the past few decades, there has been particular interest in the potential beneficial effects of n-3 PUFAs on CVDs as these fatty acids are suggested to have a role in the prevention of CVDs, with much of the research on modulation of risk factors that contribute to the development of CVDs (Calder, 2014, Maki and Dicklin, 2018, Calder, 2018). Early epidemiological studies reported reduced coronary mortality in Alaska natives (Newman et al., 1993), Greenland Eskimos (Dyerberg and Bang, 1979) and Japanese residing in fishing villages (Hirai, 1980), owing to high oily fish consumption. A link between consumption of n-3 PUFAs and lowered CVD morbidity and mortality was supported by further epidemiological studies and randomised control trials (Bucher et al., 2002, Studer et al., 2005, Calder, 2004, Zheng et al., 2012), which was in agreement with a global burden study, where low consumption of seafood n-3 PUFA was one of the leading dietary risk factor, accounting for

more than 2% of global cardiovascular mortality (Afshin et al., 2019). Additionally, Harris *et al* (2021) reported an association between higher circulating levels of marine n-3 PUFA and a lower risk of CVD mortality in their meta-analysis (Harris et al., 2021). Moreover, several meta-analyses and systematic reviews of multiple human intervention studies support evidence that fish oil supplementation decreases the risk of myocardial infarction (Casula et al., 2013, Hu et al., 2019), coronary disease (Chowdhury et al., 2014), sudden cardiac death, and nonfatal cardiovascular events (Marik and Varon, 2009, Hu et al., 2019). However, several other meta-analyses published do not support an association between n-3 PUFA supplementation and risk of all-cause death, cardiac mortality, sudden death, myocardial infarction, stroke (Rizos et al., 2012), coronary heart disease or any major vascular events (Aung et al., 2018) and a recent systematic review including 79 randomised trials concluded that n-3 PUFA supplementation had little or no impact on risk of cardiovascular events, coronary heart deaths, coronary heart disease events, stroke, or heart irregularities (Abdelhamid et al., 2018). More recently, the STRENGTH Study, including 13,078 subjects with high risk for CVD reported that n-3 PUFA supplementation, compared with corn oil did not affect major adverse cardiovascular events (Nicholls et al., 2020), which raises important questions regarding the widely-cited beneficial effects of n-3 PUFAs in CVDs. The lack of support for beneficial effects of n-3 PUFAs in recent meta-analyses has been attributed to widespread use of statins since 2000 and significant advances in therapeutic interventions in CVDs, both of which could make it more difficult to demonstrate a clear benefit of a small effect of n-3 PUFAs (Khoukaz and Fay, 2021).

Despite some uncertainties regarding the effects of n-3 PUFAs with respect to mortality from CVDs, the effect of n-3 PUFAs on CVD risk factors, such as plasma TAG concentration is well-established and approved by the European Food Safety Authority (EFSA) (EFSA Panel on Dietetic Products and Allergies, 2010) and the United State Food and Drug Administration (US FDA). In fact, a significant decrease in plasma TAG concentration is considered a hallmark of fish oil supplementation (EFSA Panel on Dietetic Products and Allergies, 2010).

However, the impact of n-3 PUFAs on other CVD risk factors is not so clear: beneficial effects on blood pressure and inflammation, haemostasis, vascular function, plaque stability have been reported in some cases (Cawood et al., 2010, Calder, 2018, Golanski et al., 2021, AbuMweis et al., 2018, Baker et al., 2018), while in others, there were no effects on endothelial dysfunction, inflammation and haemostasis (Shaikh et al., 2014, Jeansen et al., 2018).

1.3.3 Effects of n-3 PUFAs on platelet function, coagulation and fibrinolysis

Trials investigating the potential effects of dietary long chain n-3 PUFAs (EPA and DHA) on platelet function have been of interest since an increase in platelet activity is associated with a higher risk of CVD (Phang et al., 2012b) and anti-platelet effects of n-3 PUFAs have been reported by a number of studies. A meta-analysis by Gao *et al* (2013) analysed 15 RCTs and indicated that supplementation with <1.83 g/d of n-3 PUFAs significantly inhibited ADP-induced platelet aggregation in subjects with CVDs, but there was no effect in healthy subjects (Gao et al., 2013). A systematic review which examined 22 placebo-controlled trials in healthy subjects and seven placebo-controlled trials in patients with CVD concluded that n-3 PUFA supplementation reduced ADP- and collagen-induced platelet aggregation, with effects being more clear in healthy subjects (Bachmair et al., 2014). This inhibitory effect of n-3 PUFAs has been attributed to the incorporation of these fatty acids into platelet phospholipid membranes, thereby altering their role in haemostasis (Golanski et al., 2021). The incorporation of n-3 PUFAs into the platelet plasma membrane results in replacement of n-6 PUFAs, particularly AA, which is a precursor for the synthesis of pro-aggregatory eicosanoids (Adili et al., 2018). Replacement of the precursor with EPA results in the production of eicosanoids with lower biological potency and a less procoagulatory profile (Golanski et al., 2021). However, not all studies demonstrate that n-3 PUFA reduce platelet aggregation (Mackay et al., 2012, Poreba et al., 2017, Veljović et al., 2013, Bagge et al., 2018) and this may be partly due to the heterogeneity of studies using different methods.

Studies have investigated the impact of n-3 PUFAs on coagulation, fibrinolysis, and thrombin generation, as summarised in **Table 3.1**. The beneficial effects of n-3 PUFAs on coagulatory

and fibrinolytic activities were reported in both health (Vanschoonbeek et al., 2004, Din et al., 2013a) and various diseases, including CVDs (McEwen et al., 2015), stable CHD (Gajos et al., 2011), type-2 diabetes (Vanschoonbeek et al., 2007, Kabir et al., 2007). McEwen *et al* (2015) also showed the anticoagulant and antithrombogenic effect of fish oil in both health and CVD. However, a major limitation is that some studies were uncontrolled, and a broad range of methodologies were performed to assess the relevant parameters, as discussed in **Chapter 3**. Notably, the results of these studies are contradictory, and some recent studies failed to demonstrate the favourable effects of n-3 PUFA supplementation in healthy subjects (Bagge et al., 2018) and patients (Poreba et al., 2017). The discrepancy of literature might be explained by the common use of cholesterol lowering medication (statins), which has antithrombotic effects (Poreba et al., 2017, Golanski et al., 2021).

1.3.4 Effects of n-3 PUFAs on EVs

As an emerging risk marker for CVDs there is interest in the potential for dietary modulation of EV numbers and function by n-3 PUFAs. Ten studies have been conducted in both healthy subjects, patients with various CVD-related diagnoses and in one case, subjects at moderate risk of CVDs as presented in **Table 5.1**.

Nomura and colleagues conducted four intervention studies investigating the effects of n-3 PUFAs on EVs in hyperlipidaemic patients (non-diabetic and diabetic) (Nomura et al., 2003, Nomura et al., 2009a), hyperlipidaemic patients with type2 diabetes (Nomura et al., 2009a) and arteriosclerosis with type2 diabetes supplementation (Nomura et al., 2018). Supplementation with 1.8 g/d of EPA for 4 weeks decreased the numbers of GPIX+PDEVs and CD14+MDEVs in hyperlipidaemic patients with diabetes, but had no effect in the non-diabetic subjects (Nomura et al., 2003). Similarly, a follow-up study reported a reduction in the numbers of EDEVs (annexin v+/CD51+) after six months of EPA supplementation in only hyperlipidaemic patients with diabetes exclusively (Nomura et al., 2009a). However, these two studies were lacking control subjects and did not exclude confounding factors, such as age and BMI, despite the fact that one study identified significant differences in age and BMI

between the diabetic and non-diabetic groups at baseline (Nomura et al., 2009a). Later, their two reports elegantly demonstrated a favourable effect of either only n-3 PUFA supplementation (1.8 g/d of EPA) or a combination of n-3 PUFA with cholesterol lowering medication (2 mg/d of pitavastatin) on numbers of PDEVs in hyperlipidemic subjects with diabetes (Nomura et al., 2009a) and arteriosclerosis obliterans patients with type2 diabetes (Nomura et al., 2018). In a separate study, intervention with 5.2 g/d of n-3 PUFA (n=21) for 12 weeks significantly reduced the numbers of CD61+PDEV and CD14+MDEVs, but not CD62+EDEVs or tissue factor+EVs in post-MI patients after intervention (Del Turco et al., 2008). The lack of effect may be a result of the relatively small sample size.

In healthy subjects, there are four studies reporting the impact of n-3 PUFAs on EVs, including two on PDEVs (Englyst et al., 2007, Phang et al., 2012a), one on circulating TEVs (Phang et al., 2016) and the other on EDEVs (Marin et al., 2011). In 35 healthy young men, n-3 PUFA supplementation at a dose of 6 g/d for 12 weeks elevated the numbers of PDEVs (Englyst et al., 2007). Marin *et al* (2011) randomised twenty elderly healthy subjects to three diets: a Mediterranean diet enriched in monounsaturated fatty acids (MUFAs) with olive oil, a saturated fatty acid (SFA) diet and a low-fat, high-carbohydrate diet enriched in n-3 PUFAs. In this dietary intervention trial, 4 weeks of a n-3 PUFA enriched diet decreased numbers of total EVs, activated and apoptotic EDEVs compared with the SFA-rich diet (Marin et al., 2011). Phang *et al* (2012) conducted an acute study to compare the differential effect of EPA and DHA on circulating PDEVs in healthy subjects, who received a single dose of EPA-rich (2 g EPA and 0.4 g DHA), DHA-rich (0.4 g EPA and 2 g DHA) or placebo oil for 24 hours within a one-week washout period between each intervention. They reported reduced activity of PDEVs, but not the generation of CD41+ PDEVs after EPA-rich oil exclusively and a gender-dependent effect of n-3 PUFA, showing this beneficial effect in only males (Phang et al., 2012a). However, this study was investigating an acute effect of n-3 PUFAs, and it was relatively small. Although Phang and colleagues carried out a chronic study (4-weeks supplementation) to investigate the impact of the same intervention on total EVs (CD36+EVs),

n-3 PUFA supplementation did not alter the numbers of total EVs (CD36+ EVs) (Phang et al., 2016). To our knowledge, only Wu *et al* (2014) examined the effect of fish oil supplementation on EVs in subject with moderate risk for CVDs and revealed that 8-week supplementation with fish oil at a dose of 1.8 g/d decreased the numbers of EDEVs, while no effect was observed on PDEVs (Wu et al., 2014), suggesting that the beneficial effect may depend on the EV subpopulation.

Overall, favourable effects tend to be reported in subjects with disease and are less likely to be observed in healthy subjects and smaller studies. There is considerable variation in published studies with regard to the study design, sample size of subjects, subject clinical characteristics, doses of n-3 PUFAs or the duration of intervention, which may contribute to variation in results. This could also be a result of the heterogeneity in methods for collection, isolation and quantification of EVs and techniques used to characterise them. In addition to the limited information regarding the effect of n-3 PUFAs on PDEV numbers, there is no information on the generation and functions of PDEVs generated in an in vitro setting and further intervention studies to explore this gap in knowledge are warranted.

1.4 Aims of the thesis

Therefore, this project aimed to investigate the effects of fish oil-derived n-3 PUFA supplementation on conventional, thrombogenic and the 'emerging' cardiovascular risk markers, extracellular vesicles (EVs), in subjects with moderate risk of CVDs, determined by the QRISK2 scoring system. Subjects with moderate risk for CVD was chosen in this project since they are not entirely healthy but free of diagnosed diseases so studying an ''at risk'' group such as ours would be particularly valuable in terms of disease prevention and also this is arguably more biological responsive to intervention.

1. To explore the effect of fish oil-derived n-3 PUFAs on conventional CVD markers and thrombogenic markers, including platelet aggregation, coagulation, fibrinolysis, and thrombin generation in subjects with moderate risk for CVDs (**Chapter 3**).

2. To investigate whether higher numbers of circulating EVs are associated with increased CVD and thrombogenic risk markers in subjects with moderate risk for CVD (**Chapter 4**).

3. To examine whether fish oil-derived n-3 PUFAs alter the number, size, fatty acid composition and surface PS exposure of PDEVs generated in vitro by stimulated and unstimulated platelets (**Chapter 5**).

4. To investigate the influence of fish oil-derived n-3 PUFAs on the coagulatory behaviour of PDEVs generated in vitro by stimulated and unstimulated platelets by examining their influence on fibrin clotting, thrombin generation, fibrinolysis and ex vivo thrombus formation (**Chapter 6**).

1.5 Hypotheses

It hypothesised that higher numbers of circulating EVs will be associated with increased CVD and thrombogenic risk markers in subjects with moderate risk for CVD and that supplementation with n-3 PUFAs will (i) decrease conventional CVD and thrombogenic markers and (ii) reduce the generation and the procoagulatory capacity of PDEVs generated in vitro by stimulated and unstimulated platelets.

Chapter 2 Materials and Methods

2.1 Introduction

The HI-FIVE (Human Investigation of the effects of FIsh oil on extracellular Vesicles) study, funded by the BBSRC Diet and Health Research Industry Club (BBSRC DRINC), investigated the effects of fish oil supplementation on the number, generation and function of extracellular vesicles (EVs) in subjects at moderate risk for cardiovascular diseases (CVDs). Additionally, the study assessed the effect of fish oil supplementation on conventional CVD markers and thrombogenic markers, including platelet aggregation, fibrin coagulation, fibrinolysis, and thrombin generation. This chapter will describe the objectives, study design, recruitment protocols and methodology for analysis of samples.

2.2 Objectives of the HI-FIVE study

The main objective of the HI-FIVE study was to investigate the effects of fish oil supplementation on the number, generation and behaviour of EVs in subjects with moderate risk of CVDs. The objectives of the study were divided into two strands in this project and this thesis focused on strand 2, while strand 1 was carried out by others in the research group, as indicated in **Figure 2.1.** The aim of strand 1 was to investigate the effect of fish oil-derived n-3 PUFA supplementation on the number and phenotype of total circulating EVs, and their fatty acid composition, while the aim to of strand 2 was to investigate the effect of fish oil-derived n-3 PUFA supplementation on the generation of EVs by platelets collected from subjects, as well as their fatty acid composition and coagulatory behaviour (clot formation, thrombin generation, fibrinolysis and ex vivo thrombus formation). Therefore, the scope of this project was:

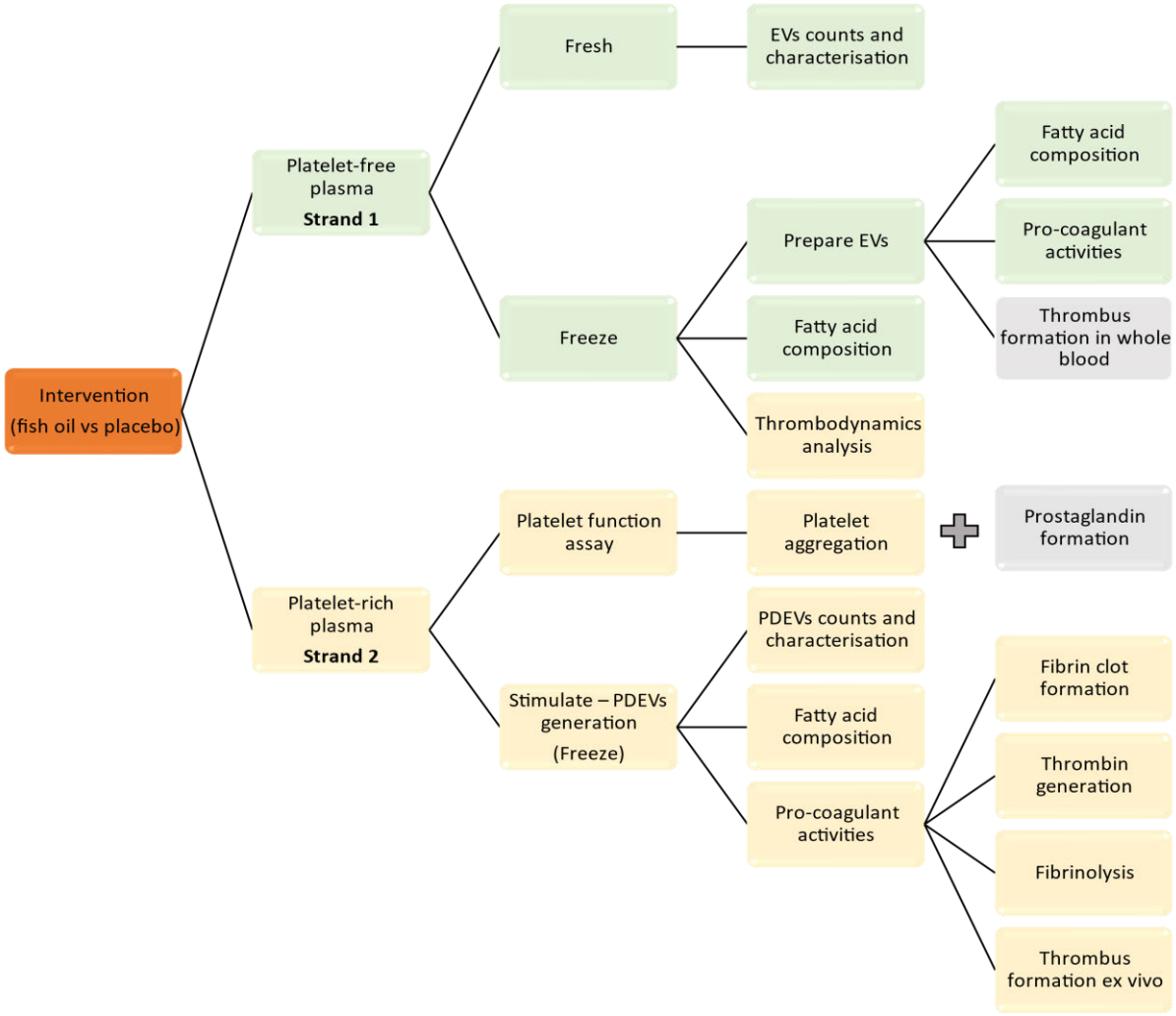


Figure 2.1. A schematic overview of HI-FIVE Study objectives. The scope of the current project is shown in yellow.

- To assess the effects of fish oil-derived n-3 PUFA supplementation on platelet function, fibrin clot properties (thrombodynamics) and thrombin generation, since it is important to understand this in the context of EVs generated from platelets. The hypothesis is that supplementation with n-3 PUFA will have beneficial effects on platelet aggregation, fibrin clot properties and thrombin generation.

- To investigate the influence of fish oil-derived n-3 PUFA on the generation of platelet-derived EVs (PDEVs) produced in vitro from unstimulated and stimulated platelets. The hypothesis is that supplementation with n-3 PUFA will alter the production and/or size of PDEVs generated in vitro.

- To evaluate the coagulatory behaviour (fibrin clot formation, thrombin generation, fibrinolysis and ex vivo thrombus formation) of PDEVs generated from unstimulated and stimulated platelets. The hypothesis is that supplementation with fish oil-derived n-3 PUFA will alter the behaviour of PDEVs.

2.3 HI-FIVE Study design

The study was a randomised, double-blind, placebo-controlled crossover intervention, conducted in the Hugh Sinclair Unit of Human Nutrition, University of Reading, in accordance with guidelines detailed in the Declaration of Helsinki and approved by the University of Reading Research Ethic Committee (reference: UREC 17/18). The study was registered at clinicaltrials.gov as NCT03203512.

A total of 40 eligible subjects aged between 40 and 70 years with moderate CVD risk (see criteria below) were randomly allocated to supplementation with capsules containing either fish oil or high-oleic safflower oil (control) in the first 12-week treatment period, followed by 12-week wash-out period. After a washout, subjects then crossed over to the other intervention for a further 12 weeks. Random assignment of subjects for intervention order ("1" and "2") was performed with an online software (https://www.randomizer.org/). The study capsules were blinded by a researcher not involved in the study and the code was not revealed until all statistical analyses had been completed.

The study comprised four intervention visits, which took place at the beginning and end of each 12-week intervention period (weeks 0, 12, 24 and 36) (**Figure 2.2**). After the first visit, subjects were asked to take 6 capsules per day of either fish oil (Wiley's Finest Easy Swallow Minis, Canada), providing a total daily intake of 1.8 g n-3 PUFA, providing 1080 mg EPA and 810 mg DHA, or high-oleic safflower oil (Wiley's Finest, Canada), providing 740 mg oleic acid plus 120 mg linoleic acid per day for 12 weeks (e.g. equivalent to 85-90 g of uncooked Atlantic Salmon) (US Department of Agriculture, 2019). Subjects were advised to take capsules with breakfast, lunch and dinner (2 at each meal). Weeks 12-24 represented a washout period

before subjects crossed over to the other intervention product until week 36. Subjects were provided with a daily checklist to support compliance.

Table 2.1. The fatty acid compositions of capsules used in the study

	Fish oil (wt %)	High-oleic sufflower oil (wt %)
Palmitic acid (16:0)	0.002	5.3
Stearic acid (18:0)	0.007	2.0
Oleic acid (18:1,n-9)	0.7	77.0
Linoleic acid (18:2,n-6)	0.2	13.4
ALA (18:3,n-3)	0.2	0.1
DGLA (20:3,n-6)	0.1	0.0
Arachidonic acid (20:4,n-6)	0.9	0.0
ETA (20:4,n-3)	4.7	0.01
EPA (20:5,n-3)	48.2	0.03
DPA (22:5,n-3)	2.8	0.0
DHA (22:6,n-3)	35.5	0.0

Data are mean ± SEM and are expressed as the percentages of the weight of each individual fatty acid relative to the total weight of all fatty acids (wt %) in either fish oil or control oil capsules; AA, arachidonic acid; ALA, α-linolenic acid; DGLA; dihomo-γ-linolenic acid, ETA; eicosatetraenoic acid, EPA, eicosapentaenoic acid; DPA; docosapentaenoic acid, DHA; docosahexaenoic acid.

Each visit lasted approximately 30 minutes. Before each study visit, subjects were asked to abstain from alcohol and strenuous exercise during the 24 hours prior to the study day. On the visit day, subjects were asked to come to the nutrition unit in an unfed state (fasted, not eating or drinking anything but water from 8 pm the night before). Measurements of height were taken by a wall-mounted Stadiometer, body mass (kg) and composition by Tanita Body Composition Analyzer (BC0418 MA; Ill, USA). Systolic (SBP) and diastolic blood pressure (DBP) were determined by the averages of three physician-measured readings (Omron M2 Upper Arm Blood Pressure Monitor, OMRON Healthcare Europe BV, United Kingdom). A blood sample of approximately 100 ml was drawn from subjects by either a qualified research nurse or a qualified researcher. On each visit day, subjects returned completed food frequency questionnaires (**Appendix F**), which was used to determine the subject's habitual dietary

intakes and to confirm low consumption of oily fish (less than one portion per month). Further details about visit days can be found in the ethics application (**Appendix A**).

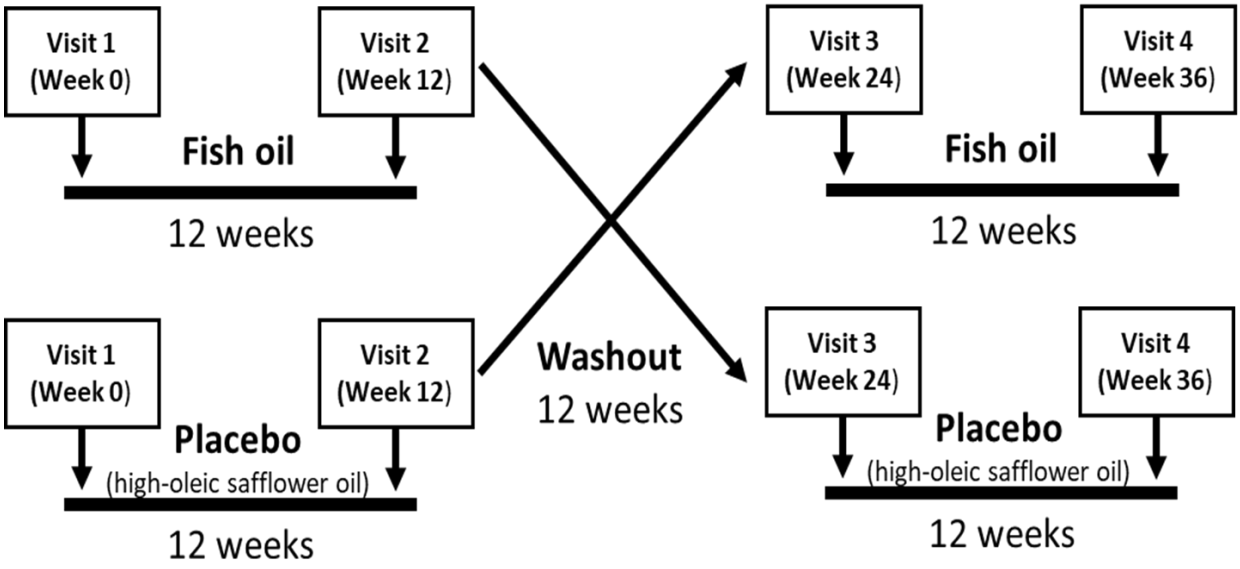


Figure 2.2. HI-FIVE Study design.

The duration of the intervention and washout periods was based on previous evidence that incorporation of n-3 PUFA in all cell types reaches a plateau after two to four weeks' intervention (Yaqoob et al., 2000, Hodson et al., 2018) and that a 12- week washout period is sufficient to avoid carryover effects (Walker et al., 2015).

High-oleic safflower oil was selected as the control oil because the daily dose of MUFA and n-6 PUFA it provided would not add significantly to the background dietary intake of these fatty acids. Also, compared to olive oil, safflower oil contains few polyphenols, which have been associated with bioactivity and potential effects on EVs.

2.4 Sample size

The sample size calculation was performed for the main endpoints: EV numbers, thrombus formation and platelet aggregation. A total of 34 subjects was required and we aimed to recruit 40 volunteers to allow for a 15% dropout. Based on previous study (Wu et al., 2014), a total of 34 subjects was a sufficient sample size to detect a 10% reduction in the number of EVs

following fish oil supplementation with a two-sided significance level of 5% and a power of 95%. This calculation is based on the assumption that the standard deviation is 2.4 (Wu et al. observed standard deviations between 1.38 and 2.4). Published data on thrombus formation suggest that 22 subjects are required to detect a 10% change in thrombus formation (Vaiyapuri et al., 2012) and 30 subjects would detect a significant effect of n-3 PUFA on platelet aggregation and PS exposure (Phang et al., 2012a).

2.5 Recruitment

The recruitment was achieved by using the Hugh Sinclair Unit of Human Nutrition volunteer database containing approximately 2000 volunteers (**Appendix G**), sending email advertisements to staff and students of the University of Reading and members of local community group (**Appendix H**), and distributing posters and leaflets (**Appendix I**) in public places, such as around the University campus and/or in community centres and shops. Potential volunteers were also recruited at public events (e.g. Pint of Science 2018) and through advertisement in local newspapers, magazines, social media (such as Facebook) and websites (**Appendix K**). The participant information sheet (**Appendix D**), which outlines the details of the study, and medical and lifestyle questionnaire (**Appendix E**) were provided to all interested volunteers and completed by telephone or email for initial screening. Then those indicating an interest in the study were invited to attend a screening visit at the Hugh Sinclair Unit of Human Nutrition and assessed for the eligibility based on exclusion criteria.

Exclusion criteria included:

- BMI: ＜18.5 kg/m^2
- Anaemia (haemoglobin concentration ＜12.5 g/L in men and＜11.5 g/L in women)
- Hyperlipidaemia (total cholesterol concentration ＞8 mmol/L)
- Diabetes (diagnosed or fasting glucose concentration ＞7 mmol/L) or other endocrine disorders
- Angina, stroke, or any vascular disease in the past 12 months

- Renal, gastrointestinal, respiratory, liver or bowel disease
- Inflammatory disease
- Take drug treatment for hypertension, hyperlipidaemia, inflammation, depression or thyropathy
- Take aspirin, ibuprofen or other nonsteroidal anti-inflammatory drugs (NSAIDs) > 4 times per month, or once in the week preceding the study
- Take any other anti-platelet or anti-coagulant drugs, like triflusal, clopidogrel and warfarin
- Having allergies
- Smoking (including e-cigarettes and nicotine products)
- Alcohol misuse or intakes ＞21 units/wk for men and ＞15 units/wk for women or have a history of alcohol misuse
- Regularly consume oily fish and/or dietary supplements
- Planning to start or on a weight reducing regimen
- Intense aerobic exercise (＞20 min, three times a week)
- Females who are pregnant, lactating, or if of reproductive age and not using a reliable form of contraception (including abstinence)
- Having participated in another clinical trial within the last three months.

2.6 Screening

Potential eligible subjects were invited to a screening visit in a fasted state (12 hours) in order to assess their further eligibility for the study using a medical and lifestyle questionnaire (**Appendix E**) administered via email or phone. During the screening visit, all the procedures were explained in detail and participants were offered the opportunity to ask questions. If they were willing to proceed, they gave their consent by completing the consent form (**Appendix C**), which was also signed by the researcher. For each subject, anthropometric measurements (weight, height and blood pressure) were measured as mentioned above (**Section 2.4**). An

overnight fasting blood sample of approximately 9 ml was taken into a serum-separating tube (Greiner Bio-One, Gloucestershire, United Kingdom) for the assessment of biochemical tests (blood count, lipid profile, glucose levels and markers of liver & kidney function, all of which were sent to Royal Berkshire Hospital Pathology Department for analysis) to identify subjects at moderate risk of CVDs. Serum-separating tubes were kept upright at room temperature for 30 minutes (and no more than 60 minutes) and then centrifuged at 1700 x g for 15 minutes at room temperature (Heraeus Labofuge 400R Centrifuge, Thermo Scientific, United Kingdom). The plasma (160µl) was collected and analyzed by iLab (iLab 600 Clinical Chemistry System, Diamond Diagnostics, United States) for triacylglycerol (TAG), TC, HDL-C, and glucose levels using standard reagent kits (Werfen Limited, Warrington, United Kingdom).

2.7 Selection of eligible subjects

Moderate CVD risk was determined by the QRISK2 scoring system, which was developed based on 2.3 million multi-ethnicity UK patients aged 35-74 Years with 140,000 cardiovascular events to estimate the 10-year risk of CVDs (CHD, stroke, and transient ischemic attack), as described by Hippisley-Cox *et al* (Hippisley-Cox et al., 2008). The online QRISK2 calculator (https://qrisk.org/2016/) provides a percentage risk of having a heart attack or stroke within the next 10 years, using traditional risk factors (age, systolic blood pressure, smoking status and ratio of total serum cholesterol to high-density lipoprotein cholesterol) together with body mass index, ethnicity, measures of deprivation, family history (cardiovascular disease in first degree relative under 60 years) and ethnic group (nine categories). Subjects with a score of 10%-20% were regarded as being at moderate risk and eligible for the study.

2.8 Participant flow diagram

Recruitment of 40 subjects (40-70y) with moderate risk of CVDs was completed in March 2019 after screening 58 subjects, as illustrated in the participant flow diagram (**Figure 2.3)**. The study was completed in November 2019, with 40 subjects successfully completing the trial, with no serious adverse events (SDE) and supplements well tolerated by all subjects.

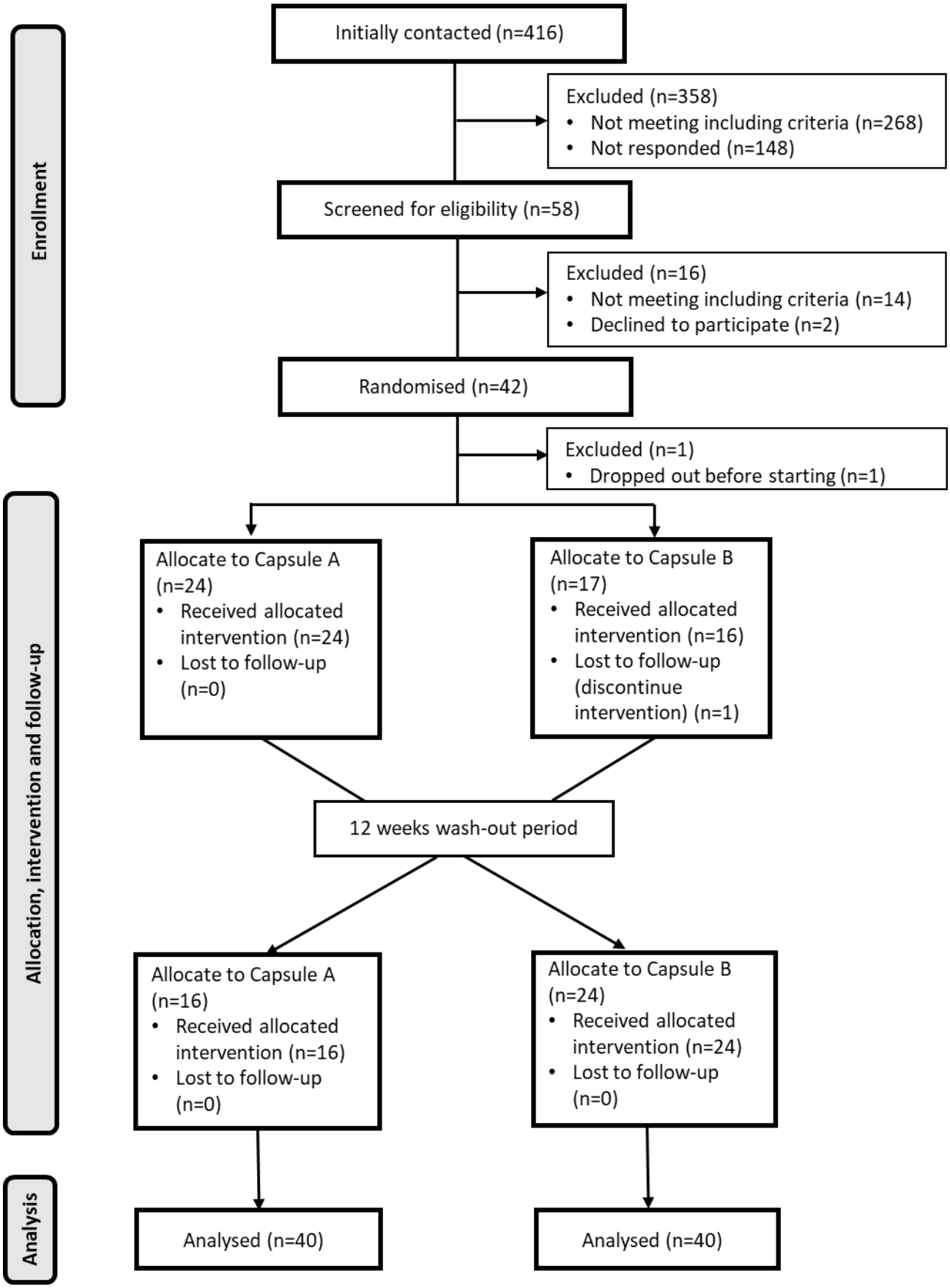


Figure 2.3. Participant flow. Capsule A, control oil supplement; Capsule B, fish oil supplement**.**

2.9 Compliance

Compliance was monitored by capsule counts and also by changes in plasma phospholipid fatty acid composition. Subjects were provided with capsules in excess of requirements, and remaining capsules at the end of the 12-week treatment period were checked. Compliance was > 98% throughout the trial, as indicated by capsule counts. Modification of the fatty acid composition of plasma phospholipids also reflected good compliance (**Chapter 3.2.1**).

2.10 Food frequency questionnaire analysis

Volunteers completed a short dietary questionnaire (**Appendix F**) to assess their habitual diet. Dietary assessment was conducted using FETA software (FFQ EPIC Tool for Analysis), which is a tool that provides energy, nutrient and food group intakes. Dietary intake was assessed both before and during both interventions and results suggested that subjects did not change their diets during the intervention (**Table 2.2**).

Table 2.2. Dietary intake of subjects before and during the intervention

	Fish Oil		**Control Oil**		***p - value***		
	Before	During	Before	During	Before-During Fish oil	Before-During Control oil	Before Fish oil-Before Control oil
Energy (kcal/d)	1606.5±90.6	1642.2±87.3	1642.0±84.8	1569.5±74.7	0.480	0.056	0.399
Carbohydrate (g/d)	195.2±12.4	196.7±12.1	196.2±11.8	187.7±10.2	0.812	0.112	0.881
Protein (g/d)	70.3±3.6	73.7±4.1	74.9±3.4	70.6±3.3	0.227	0.067	0.064
Fat (g/d)	60.0±3.8	61.3±3.5	64.9±3.5	60.5±3.3	0.588	0.067	0.055
n-3 PUFA (g/d)	1.1±0.1	1.1±0.1	1.2±0.1	1.1±0.1	0.989	0.060	0.131
Total PUFA (g/d)	11.6±0.9	12.0±0.9	12.1±0.7	11.1±0.7	0.511	0.054	0.382
Total MUFA (g/d)	21.1±1.6	21.8±1.3	22.7±1.3	22.1±1.1	0.472	0.068	0.117
Total SFA (g/d)	22.9±1.3	21.5±1.2	21.9±1.3	20.6±1.2	0.498	0.116	0.163
Alcohol (g/d)	8.6±1.3	8.0±1.1	8.1±1.2	8.1±1.2	0.615	0.969	0.406
Fibre (g/d)	18.8±1.1	18.9±1.1	18.7±1.1	17.6±0.9	0.881	0.063	0.849

Data are mean ± SEM. Dietary analysis was performed using a modified EPIC-Norfolk and FETA software. Differences in dietary intake were drawn using a paired t-test; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA; saturated fatty acid.

2.11 Blood collection and processing

Venous blood samples were collected from fasted subjects into vacutainer tubes (3.2%; Greiner Bio-One, UK) through a large diameter, 21-gauge needle mounted on a 19cm length of plastic tubing (Greiner Bio-One, UK). Prolonged use of a tourniquet was avoided to prevent platelet activation. Blood was processed within 30 minutes after transfer into polypropylene tubes. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 175 x g for 15 minutes, with no brake at room temperature (Allegra® 6 Series Centrifuge, Beckman Coulter®, CA, USA). Platelet-poor plasma (PPP) was obtained to use in the platelet aggregation assay by centrifugation of PRP at 13,000 x g at room temperature for 2 minutes and removal of the supernatant from the platelet pellet. To obtain platelet free plasma (PFP), blood was centrifuged at 1,500 x g at RT for 15 minutes and the upper two thirds were collected and centrifuged again at 13,000 x g for 2 minutes. The upper three quarters from each tube were collected and identified as PFP. Screening samples were frozen at -20 °C, while prepared PFP was aliquoted and stored at −80 °C for further analysis.

2.12 Measurement of platelet aggregation

Various methods have been developed to evaluate platelet function, including measurement of platelet aggregation (Bachmair et al., 2014). Although traditional light transmission aggregometry (LTA) is considered a ‘’gold standard’’ for platelet aggregation assessment (Chan et al., 2011), it requires specialist expertise (Lordkipanidzé et al., 2014) and only allows a limited volume of testing (Chan et al., 2018a), so it is not ideal for experiments which require a wide range of agonist concentrations. An alternative, 96-well high-throughput aggregometry technique, uses a 96-well plate format (Chan et al., 2018b) allows testing of a wide range of concentrations of different agonists, requiring only a small amount of sample (40 µl) compared with the LTA (200-250 µl). Therefore, to examine the influence of n-3 PUFA supplementation on platelet function, this high throughput assay for platelet aggregation was employed.

2.12.1 Preparation of 96-well platelet aggregometry

Clear half-area 96-well microplates (Greiner Bio-One, Stonehouse, Gloucestershire, UK) were prepared in advance as described by Chan and co-workers in 2011 (Chan et al., 2011). Briefly, plates were pre-coated with gelatine (Sigma-Aldrich, Dorser, UK) solution to avoid the surface activation of platelets before adding platelet agonists. The platelet agonists included (i) adenosine diphosphate (ADP, final concentration, 0.03-100 µM; Sigma-Aldrich, Dorser, UK), (ii) cross-linked collagen-related peptide (CRP-XL, 0.001-3 µg/ml; University of Cambridge, Cambridge, UK), (iii) epinephrine (EPI, 0.003-10 µM; Sigma-Aldrich, Dorser, UK), (iv) thrombin receptor-activating peptide-6 (TRAP-6, 0.015-50 µM; Bachem, St Helens, Merseyside, UK), which were prepared in 0.1% w/v ascorbic acid in distal water, (v) U46619 (0.01-30 µM; Enso Life Sciences, UK), which was prepared in 0.1% w/v PBS, (vi) arachidonic acid (0.3-1000 µM; Sigma-Aldrich, Dorser, UK), which was prepared in 0.1% w/v ethanol. Agonists (8 µl at each concentration) were added to pre-coated 96-well microplates and kept at -80 °C for 1 hour, following which they were transferred into a freeze-dryer and kept overnight at -20 °C. The plates were then removed from the freeze-dryer, vacuum-sealed and kept in the dark at room temperature for 8 weeks.

2.12.2 Platelet aggregation assay

PRP and PPP (prepared as described above) from each study visit were used in the platelet aggregation assay using pre-prepared 96-well microplates. PRP (40 µl) was then added into the appropriate wells of the microplate containing the agonists (ADP, CRP-XL, EPI, TRAP-6, U46619 and AA) and the plate was tapped gently to ensure that the PRP reached the bottom. The plate was shaken at 1,200 rpm at 37 °C for 5 minutes using a plate shaker (Thermo-shaker, Grant Instruments, UK). PRP or PPP (40 µl) were then added to the relevant control wells in plate (agonist-free control wells) which was tapped sharply to remove any aggregates from the centre of the wells. A plate-reader (Tecan Microplate Reader Spark, Switzerland) was used to determine absorbance at 405 nm. Absorbance data were converted to a percent

aggregation by reference to the absorbance of PRP and PPP as 0% and 100% aggregation controls, respectively. Dose-response curves in response to each agonist were obtained and curves were fitted by a four-parameter logistic non-linear regression using Prism (8.2; GraphPad Software, Inc., San Diego, CA)

2.13 Thrombodynamics analysis for fibrin clot formation and fibrinolysis

To understand how fibrin clot properties, such as coagulation and fibrinolysis, change as a consequence of supplementation with n-3 PUFAs, thrombodynamics analysis was performed using a thrombodynamics analyser and thrombodynamics kit (HemaCore, Moscow, Russia), which is a sensitive method to evaluate a tendency for hypercoagulability at the early stage and identify increased thrombogenic risk (Tuktamyshov and Zhdanov, 2015). The thrombodynamics analyser mimics the physiological processes associated with blood vessel wall damage in vivo by assessing the visualization of spatial clot growth in addition to detecting fibrinolysis as shown in **Figure 2.4**. In this assay, the formation of a fibrin clot is initiated by an immobilized activator in plasma to grow around the tissue factor (TF) bearing surface and fibrinolysis is then initiated in the presence of plasmin activator in plasma. This analysis provides information about both the activation and propagation phases of clot formation, including rate of clot growth and lag time.

For this analysis, there was a need to optimise the concentration of tPA in the assay and therefore concentrations ranging from 0.5-30 nM were tested, with the result that 4 nM of tPA was determined as the lowest concentration to begin consistent clot fibrinolysis and believed to be optimal to assess both fibrin clot formation and fibrinolysis parameters (**Figure 2.5**).

Coagulation and fibrinolysis were assessed in 120 µl of PFP (prepared as described above) thawed in a 37 °C water bath for 5 min following the manufacturer's instructions. Plasma was placed into a special plastic vial containing a lyophilised solution of protein-inhibitor and incubated for 15 minutes at 37 °C within the thermostat of the analyser, following which 4

nM of tissue-type plasminogen activator (tPA, final concentration) (Sigma-Aldrich, Dorset, UK) was added. This step was followed by addition of a lyophilized solution of calcium salt. The sample was immediately transferred into an optically transparent cuvette with two thin channels, which was placed into the 37°C temperature-controlled chamber of the instrument. Finally, an activating insert, the end edges of which were covered with immobilized tissue factor to activate clotting, was gently placed fully into the cuvette. An image of the fibrin clot was recorded over 60 min using a video microscopy system and the software facilitated quantification of both fibrin clot growth and lysis process.

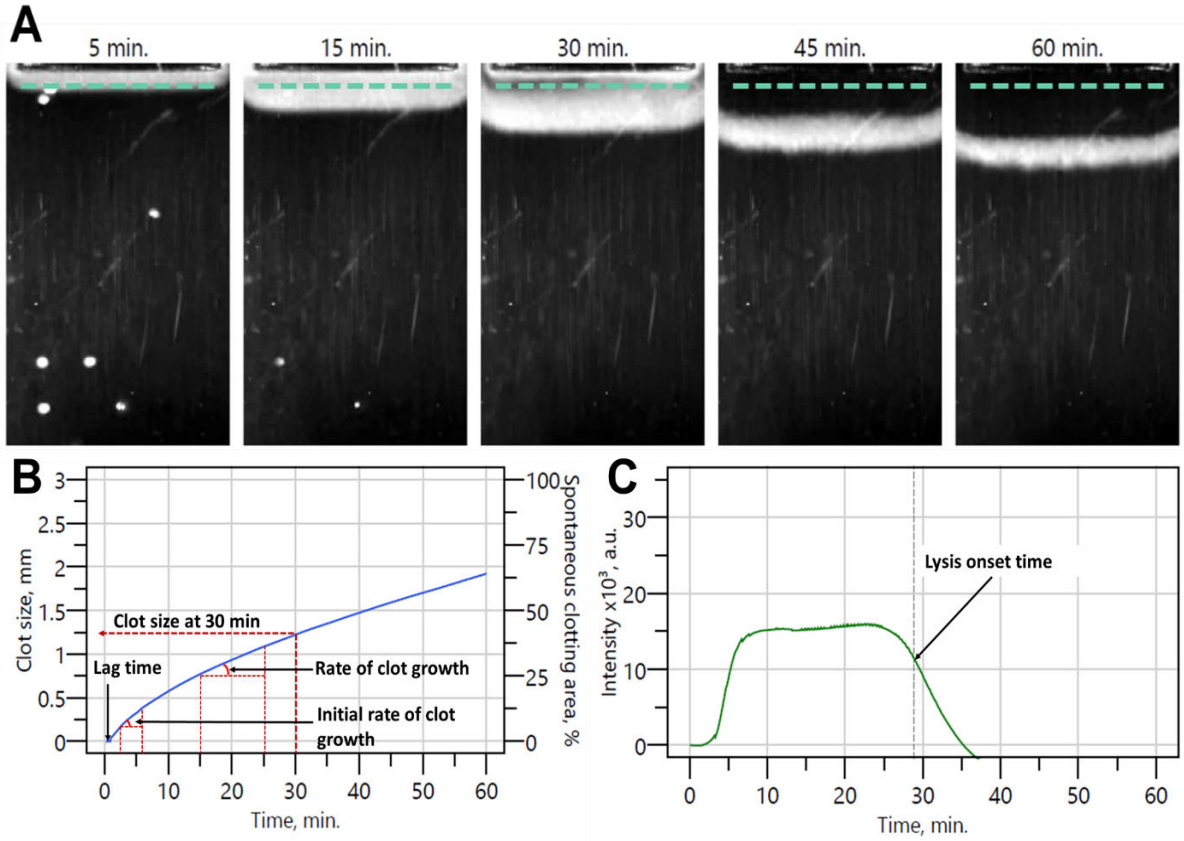


Figure 2.4. Clot formation is activated by introducing the TF bearing surface to PFP and fibrinolysis was assessed by addition of 4 nM tPA. (**A**) Representative images of the coagulation process and fibrinolysis were recorded over 60 min and all parameters were calculated by the thrombodynamics software (**B**) Plot of fibrin clot growth versus time was constructed to calculate coagulation-related parameters. Lag time was calculated as the time from the beginning of the measurement until the beginning of the clot growth when the first significant levels of fibrin can be detected. The rate of clot growth and the initial rate of clot growth, which indicate the propagation stage of blood coagulation were calculated on the interval 15-25 minutes and 2-6 minutes after the beginning of clot growth. The clot size was measured at the 30th minute of clot growth. Clot density was measured as amount of light scattering from a fibrin clot. (**C**) Plot of fibrin clot intensity versus time was constructed to calculate fibrinolysis-related parameters. Lysis onset time was calculated as the time, when the light scattering intensity (green line) in the clot reach to 30% reduction from the beginning

and the lysis progression was calculated as the linear rate of the light scattering intensity decrease as the percentage of the initial value in the following 5 min.

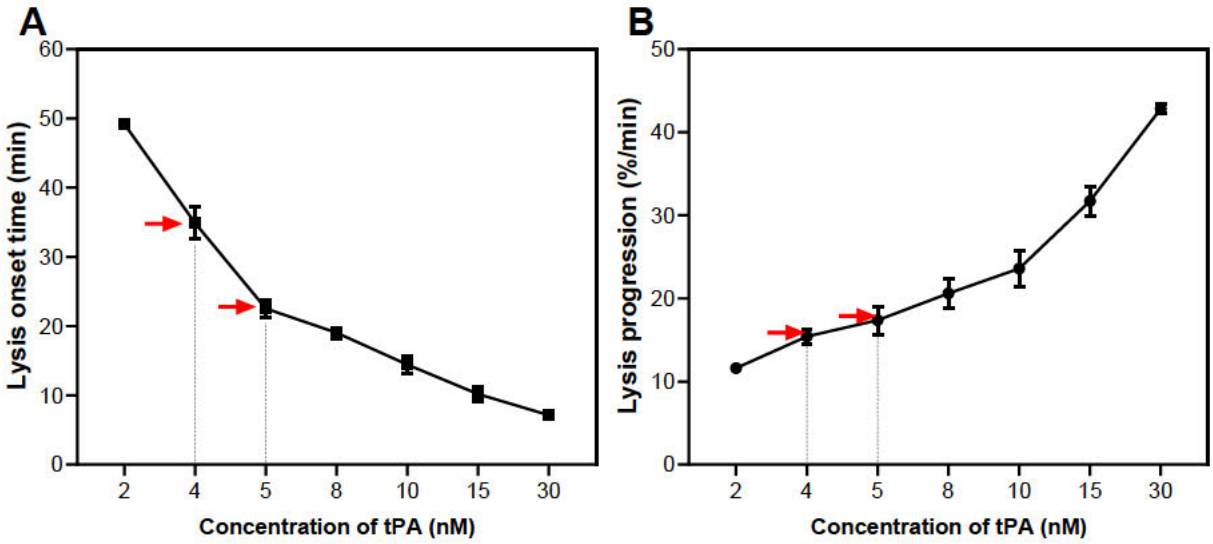


Figure 2.5. Effects of different concentrations of tPA on clot fibrinolysis. Data are mean ± SEM, (n=4). Dose-response curve of fibrinolysis parameters in the presence of increasing concentrations of tPA (0.5-30 nM) showed that **(A)** clot lysis onset time decreased, and **(B)** clot lysis progression increased within increasing concentrations of tPA in PFP; tPA, tissue plasminogen activator.

2.14 Isolation of EVs

2.14.1 Isolation of circulating EVs by size exclusion chromatography

Size exclusion columns (qEV, Izon Science) were used to isolate circulating EVs from PFP as recommended by the manufacturer. Size exclusion chromatography (SEC) separates EVs from other particles based on their size. In this gel filtration method, biological fluid passes through a porous matrix, where smaller particles (35 – 70 nm) enter into the pores in the matrix, thereby extending their elution time. Larger particles are eluted first since they cannot enter into the pores. In the present project, the column within operational temperature range (18-24°C) was equilibrated with 30 ml of PBS (Sigma-Aldrich, Dorset, UK), then 0.5 ml of PFP was loaded on top of luer-slip cap and allowed to enter the column. The top reservoir was then filled with PBS and 0.5 ml fractions were collected. Fractions were numbered in order of collection and the EV-rich fractions (7~9) were obtained.

2.14.2 Isolation of PDEVs

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

To isolate platelets, PRP was centrifuged at 1,000 x g, for 10 min, with low brake, at room temperature in the presence of prostacyclin (PGI_2, 1 µg/ml; Sigma-Aldrich, Dorser, UK), as described by Armstrong et al (Armstrong et al., 2017). The pellet was washed twice to obtain washed platelets in modified Tyrode's (MTH) buffer prepared by the supplementation of Tyrode`s HEPES stock (containing 134 mmol/l NaCl, 2.9 mmol/l KCl, 0.34 mmol/l Na_2HPO_4, 12 mmol/l $NaHCO_3$, 1 mmol/l MgCl2 and 20 mmol/l HEPES; Ph 7.4; filtered on 0.22 µm) with 0.1% of glucose (Sigma-Aldrich, Dorset, UK). Platelet concentration in the suspension was counted (Sysmex, USA) and adjusted to $3x10^8$ platelets/ml with MTH buffer. Washed platelets at 3 x 10^8 platelets/ml were supplemented with 2 mM of $CaCl_2$ (Sigma-Aldrich, Dorset, UK) then immediately stimulated with either 30 µM of TRAP-6 (stimulated platelets) or PBS (un-stimulated platelets). Stimulated and unstimulated platelets were incubated at 37°C for 2 hours in non-stirring conditions. Platelets were then removed by two sequential centrifugations at 1,200 x g for 10 minutes. The upper 90% of the supernatant, which contains the EVs, was collected and pelleted by centrifugation at 15,000 x g for 30 minutes at 4 °C. The supernatant was discarded and the pellet rich in PDEVs was suspended. Isolated PDEVs were pooled in a vial to ensure their homogeneity and then divided into aliquots of 30 µl each and stored at -80^{o}C until use.

2.15 Preparation of pooled vesicle free plasma

Venous blood samples were collected from three healthy, fasted subjects as described above (**Section 2.10**) to prepare pooled vesicle free plasma (VFP). Whole blood was first centrifuged twice at 2,500 x g for 15 min to remove blood cells and obtain pooled plasma. Vesicles were

removed from pooled plasma by ultracentrifugation at 20,000 x g for 1 hour at 4 °C, then the supernatant was collected, and the remaining pellets (EVs) discarded. The vesicle poor plasma was ultracentrifuged at 100,000 x g for 1 hour at 4 °C, followed by filtration on 0.1 µm (Merk Millipore, Billerica, MA) four times. This filtered supernatant was considered VFP and stored in 1 ml aliquots at -80 °C until use.

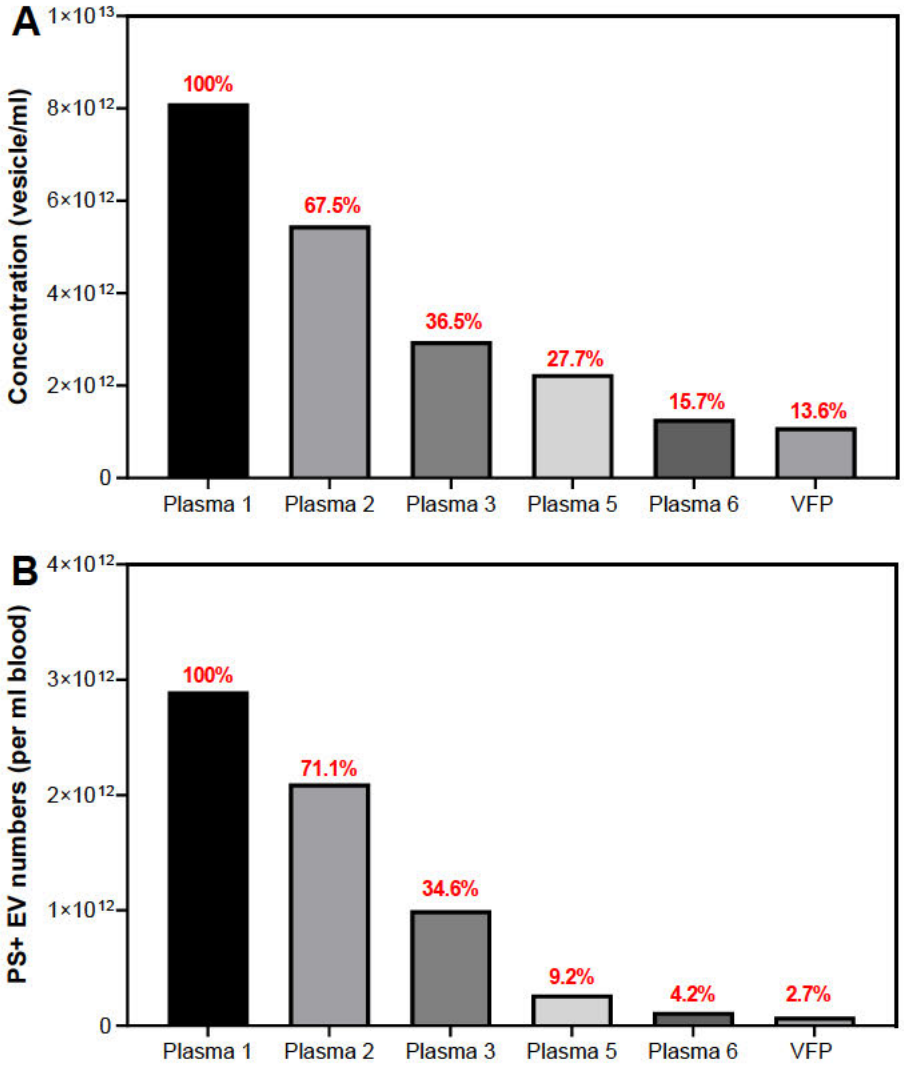


Figure 2.6. Removal of EVs during each stage of the preparation of VFP. (A) 84.4% of total EVs, as determined by NTA were removed from plasma and **(B)** 97.3% of PS+ EVs, as determined by FCM, were removed from plasma.

Removal of vesicles at each stage of preparation of VFP was verified by NTA and FCM (Annexin V staining), which showed that 86.4% of particles detectable by NTA were removed from plasma, with 97.3% removal of PS+ EVs from plasma, as shown in **Figures 2.6A and 2.6B, respectively.**

2.16 Characterisation and enumeration of PDEVs

2.16.1 Characterisation of PDEVs using nanoparticle tracking analysis

The size distribution and concentration of EVs derived from platelets were determined by Nanoparticle Tracking Analysis (NTA) using a NanoSight 300 (NS300; Malvern, Amesbury, UK). NTA is the most widely used technique with a light-scattering property under Brownian motion to analyse size distribution and concentration of particles (EVs in this case). This instrument is based on a video recording of light scattered particles in liquid suspension (Vestad et al., 2017). The NanoSight 300 is equipped with a 488 nm laser and an optical microscopy connected with a high sensitivity sCMOS camera. Its focused laser beam passes through the sample via a glass prism, illuminating vesicles and allowing their visualisation by recording five 1-minute videos of each sample (**Figure 2.7**). The NTA software (Nano 3.2 software) enables tracking of individual particles by dynamic Brownian motion and uses the solution temperature and viscosity to calculate the average EV concentration and the mean and mode EV sizes by the Stokes-Einstein equation. According to manufacturer, the NS300 is able to characterise the presence, size distribution and concentration of all types of nanoparticles from 10 nm to 1000 nm. However, a threshold of 70 nm diameter was set for analysis of circulating EVs, which were isolated through SEC. The isolation of EVs using SEC results in highly purified EVs, but some lipoprotein classes may co-isolate with them (Monguio-Tortajada et al., 2019); in order to achieve the best possible separation from lipoproteins, a threshold of 70 nm diameter was set. Since the majority of lipoproteins are smaller than this and the samples were always from fasted subjects, the EV preparations were essentially free of lipoprotein contamination. However, the threshold of 70 nm meant that EVs smaller than this could not be captured.

In this project, samples were diluted in filtered, sterile PBS (Sigma-Aldrich, Dorset, UK) to carry out the measurement in the recommended range (1-12 x 10^8 particle/ml) for NTA (Vestad et al., 2017) and manually injected into the NTA sample chamber using a 1 ml syringe and

syringe pump at room temperature. Five 1-minute videos of each diluted sample were captured at camera level 13 and frame rate of 25 per second and analysed by Nano 3.2 software.

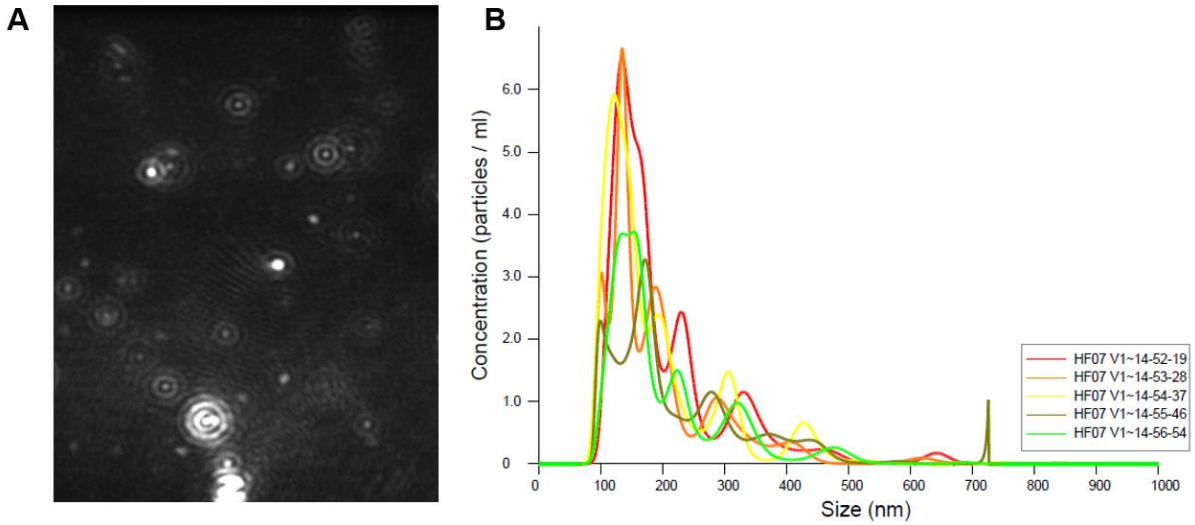


Figure 2.7. PDEV analysis by NTA. (A) Representative image displaying determination of the size and concentration by Brownian motion of in vitro-generated PDEVs. **(B)** Five videos recorded for 1-minute and the measurements of both size and concentration were based on the average of these videos.

2.16.2 Characterisation of PDEVs using flow cytometry

A flow cytometer (FCM; Canto II Flow Cytometer, BD Biosciences, UK), equipped with a blue (488 nm), a red (633 nm) and violet (405 nm) laser, was used to detect PS expression on PDEVs generated in vitro from platelets. FCM is the most commonly used method to characterise and phenotype PDEVs (Kang et al., 2017) by determining the concentration, cellular origin, pro-coagulant activity and surface marker expression of EVs. However, this technique is unable to provide an accurate result for the size distribution and it cannot reliably detect EVs smaller than 200 nm in diameter (Kailashiya, 2018) (Hartjes et al., 2019). In the case of characterisation of PDEV subpopulations within the total EV population, PDEVs can be distinguished by labelling platelet-specific surface protein labels, such as tetraspanins. Proper gating is essential for the analysis of EVs by flow cytometry to minimise contamination

by small platelets and cell fragments. Appropriately sized calibration beads are required to set the correct gates (Kailashiya, 2018).

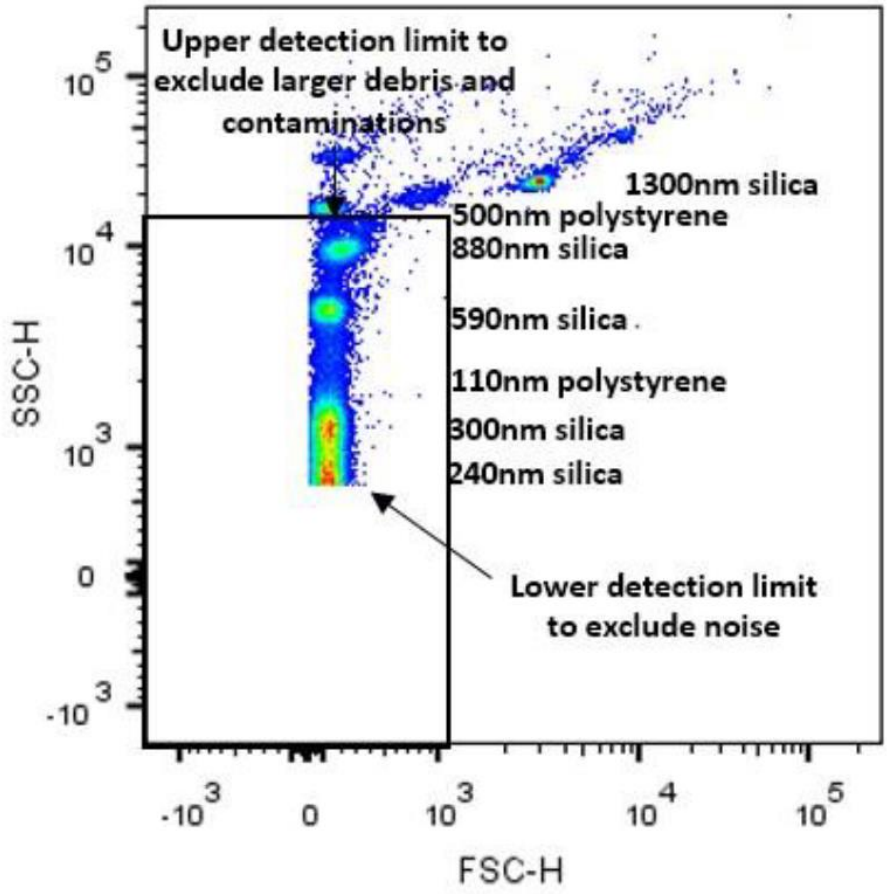


Figure 2.8. FACSCanto II flow cytometer set up as described by Ferreira *et al* (Ferreira et al., 2020). Forward scatter (FSC) vs side scatter (SSC) plot to show the EV analysis gate established by ApogeeMix beads. The gate included all particles of size 240nm-1µm, in which the lower detection limit was set by 240 nm silica beads to exclude noise, while the upper detection limit was set just above 880 nm silica beads to exclude larger debris and contaminations. SSC Voltage = 540, FSC Voltage = 500, signal triggered on SSC (Threshold 700).

Cytometer Setup and Tracking (CS&T) beads (BD Biosciences, Berkshire, UK) were used daily for quality control of the instrument's optics, electronics, and fluidics, and for adjusting fluorescence compensation. A set of rainbow calibration particles dyed with eight different fluorescent intensities (8 peaks) (BD Biosciences, Berkshire, UK) were also used periodically for routine calibration and performance tracking of the flow cytometer. Gating was informed by standard size calibration silica beads (ApogeeMix beads; Apogee Flow Systems, Hemel

Hempstead, UK) with the following sizes: 180 nm, 240 nm, 300 nm, 590 nm, 880 nm and 1300 nm. Additionally, 110 nm and 500 nm green, fluorescent latex beads were used to adjust for optimum side scatter (SSC) and forward scatter (FSC). The EV gate was set to include all particles ranging from 240 nm to 1 µm, in which the lower detection limit was established by 240 nm silica beads to exclude noise, while the upper detection limit was established just above the 880 nm silica beads to exclude platelets and large debris (**Figure 2.8**). The fluorescence signals used to measure each sample by setting the instrument to fluorescence mode and the signal was triggered on the detector correspondent to the fluorophore of the antibody of interest: CD-41 PE (λex. = 496 nm, λem. = 578 nm; Voltage 470, Threshold 350) and Annexin V APC (λex. = 650 nm, λem. = 660 nm; Voltage 642, Threshold 550). Triggering on a fluorescent offers higher sensitivity by removing interference from noise and those particles, which could be detected using light scattering triggering (Arraud et al., 2014).

All antibodies and reagents were filtered using 100 nm syringe filters (Sartorius, UK) and 100 nm centrifugal devices (Sartorius, UK) and analysed for 1 minute to evaluate the level of background particles that fell in the EV gate before each analysis day. To measure the PS expression of PDEVs, samples were assessed for their ability to bind cell marker-specific monoclonal antibody anti CD41 conjugated to Phycoerythrin (PE) (Biocytex, Marseille, France) to detect platelet-derived EVs and Annexin V conjugated to Allophycocyannin (APC) (ThermoFisher, UK), which binds externalized PS residues. For this double staining, frozen PDEVs were thawed, then 2 µl of each PDEV sample was incubated with both anti CD41 and Annexin V (3µl each) for at least 30 minutes in the dark at room temperature. The incubation was followed by dilution with 70 µl of Annexin V-binding buffer (10mM HEPES, 140mM NaCl, 2.5mM CaCl2, pH 7.4) and samples were analysed by FCM. Events were collected with low flow rate for 1 minute on the threshold corresponding to the labelled fluorophore avoiding an event rate higher than 5,000 events/second. For anti CD41, sample incubation with its isotype control had lower fluorescence than the antibody on its own; therefore the background was set on the fluorescence produced by the antibody only. EV positive events were calculated,

by subtracting the number of events obtained from the antibody mix on each experimental day from the absolute count of positive events of the labelled sample (**Figure 2.9**). Data was captured using FACSDiva Software version 6.1.3 and analysed using FlowJo version 10.

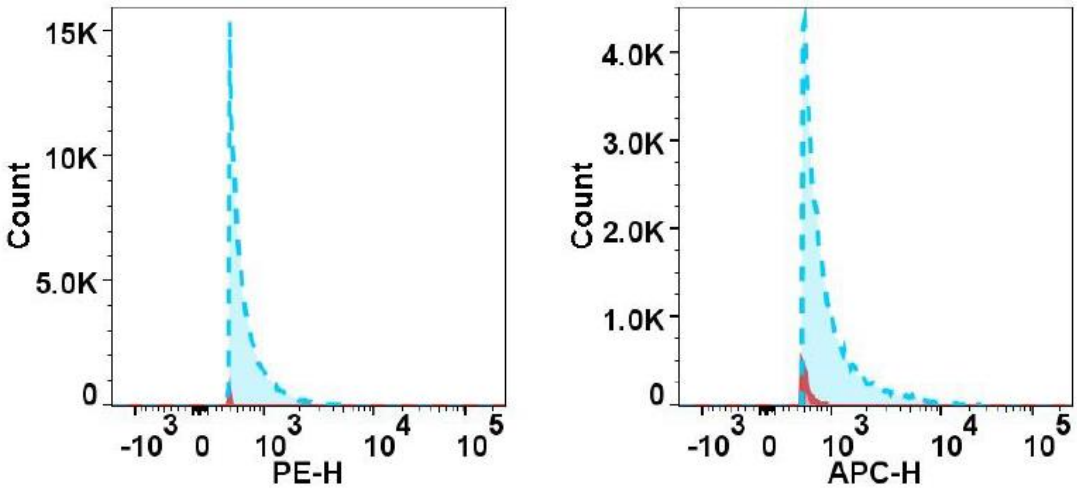


Figure 2.9. Representative flow cytometry histograms with signal triggered in fluorescence mode showing mean fluorescence intensity (MFI) of the antibody background (red) and PDEVs positive events (blue). PE = CD41, APC = Annexin V. The number of positive events was calculated subtracting the events collected in blue minus events collected in red.

2.16.3 Measurement of protein concentration of PDEVs using NanoDrop

The measurement of PDEV protein concentration was conducted by a NanoDrop-1000 spectrophotometer upon protein A280 measurements following the manufacturer's instruction (Thermo Scientific). Before starting to measurement, the instrument was cleaned with distilled water and blanked using 2 µl of nuclease20 free water. Sample (2 µl) was then loaded onto the pedestal and this was repeated three times to obtain the average protein concentration.

2.17 Fatty acid composition and lipid profile analysis

The fatty acid composition of PDEVs was analysed in collaboration with the University of Southampton as described by (Fisk et al., 2014). The volume of the PDEV sample was adjusted by addition of 0.9% NaCl to 800 µl. Total lipid was extracted from PDEVs using chloroform and methanol in a 2:1 (v/v) ratio in the presence of butylated hydroxytoluene (50 mg/l). Sodium chloride was added to aid the separation into aqueous and organic lipid

containing phases. The mixture was centrifuged at 1,000 x g for 10 min, low brake, at room temperature. The lower phase was collected into a new glass tube and dried under nitrogen at 40 °C. The lipid extracts were then dissolved in methanol containing 2% (v/v) sulphuric acid and incubated at 50°C for 2 h to generate fatty acid methyl esters (FAME). FAME were extracted using hexane and separated by gas chromatography using an Agilent 6890 series gas chromatograph equipped with flame ionisation detection (Agilent Technologies) and a BPX-70 fused silica capillary column (30m×0·25mm×25 μm; SGE Analytical Science. Quantification (with the use of the internal standards) was performed using ChemStation software (Agilent Technologies) and Microsoft Excel (Microsoft Corporation) by Helena Fisk. Results were reported as percent of total fatty acids.

Fatty acid composition of plasma phospholipids was also measured using gas chromatography. For this, a 400µl aliquot of frozen PFP was defrosted at room temperature using a roller mixer and centrifuged at 13,000 x g for 5 minutes at room temperature to remove denatured protein. 400µl of 0.9% NaCl was added to the PFP sample to make up 800µl in total, and 30µg of PC and 15µg of PE internal standards were then added for the quantitative analysis. Next, lipid extraction of plasma was performed as for PDEVs (see above). After drying with nitrogen, the lipid extract was dissolved in 1.0ml dry chloroform and transferred into chloroform-washed (2 x 1ml under vacuum) solid-phase extraction cartridges for separation of phospholipids. PC and PE were eluted by the addition of 2.0ml dry chloroform: methanol (60:40, v/v) and 2.0ml dry methanol respectively under vacuum. The PC and PE extracts were dried under nitrogen at 40°C. Before analysis by GC, the methyl esterification of plasma phospholipid extracts was performed as for PDEVs (see above).

For lipid profile analysis of plasma, PFP was defrosted at room temperature using a roller mixer, centrifuged at 500 x g for 5 min at room temperature and analysed for TC, TAG, HDL-C, LDL-C and TC/HDL-C ratio by a RANDOX clinical analyser (RANDOX Daytona+ Analyser, Randox Laboratories Ltd, United Kingdom).

2.18 Functional assays of PDEVs

2.18.1 Measurement of clot formation induced by PDEVs

The impact of PDEVs (before and after intervention) on fibrin clot formation in VFP was assessed in 96-well flat-bottom microplates (Greiner Bio-One, Stonehouse, Gloucestershire, UK) by a change in turbidity of the VFP at 405 nm (A405) every 30 seconds, for 1 hour at 37°C using a Flex Station 3 plate reader (Molecular Devices, United State). Prior to analysis, it was demonstrated that clot formation was significantly lower in VFP compared with PFP, indicating that the removal of EVs from plasma affected the ability to form fibrin clot (**Figure 2.10**). Therefore, pooled PFP (positive control) and VFP (negative control), were as benchmarks in this assay (for more details see **Section 2.15**). It was also necessary to optimise the concentration of in vitro-generated PDEVs (from stimulated and unstimulated platelets) in the assay and therefore protein concentrations of 2.5, 5 and 10 µg/ml of PDEVs were added to pooled VFP, with the result that addition of increasing amounts of PDEVs increased clot formation in a dose-dependent manner (**Figure 2.10**). A concentration of PDEVs of 5 µg protein/ml was selected as appropriate to evaluate the effect of PDEVs following the intervention on clot formation.

The assay was set up by diluting 30% of VFP in clot buffer (10 mM Tris [pH 7.4] and 0.01% Tween 20) and 5 µg/ml protein concentration of PDEVs (final concentration) was then added. Fibrin clot formation was initiated by addition of 5 mM $CaCl_2$ in a final volume of 100 µl of clotting mixture. Plates were read at 37 °C for 1 hour at 30 second intervals using a fluorescence plate reader (FlexStation 3, United State) at wavelength of 405 nm. Data were then analysed by an online application (https://drclongstaff.shinyapps.io/Clot_or_HaloCL/) to obtain relative parameters: time to 50% change, absorbance at 50% change, absorbance at peak, area under curve.

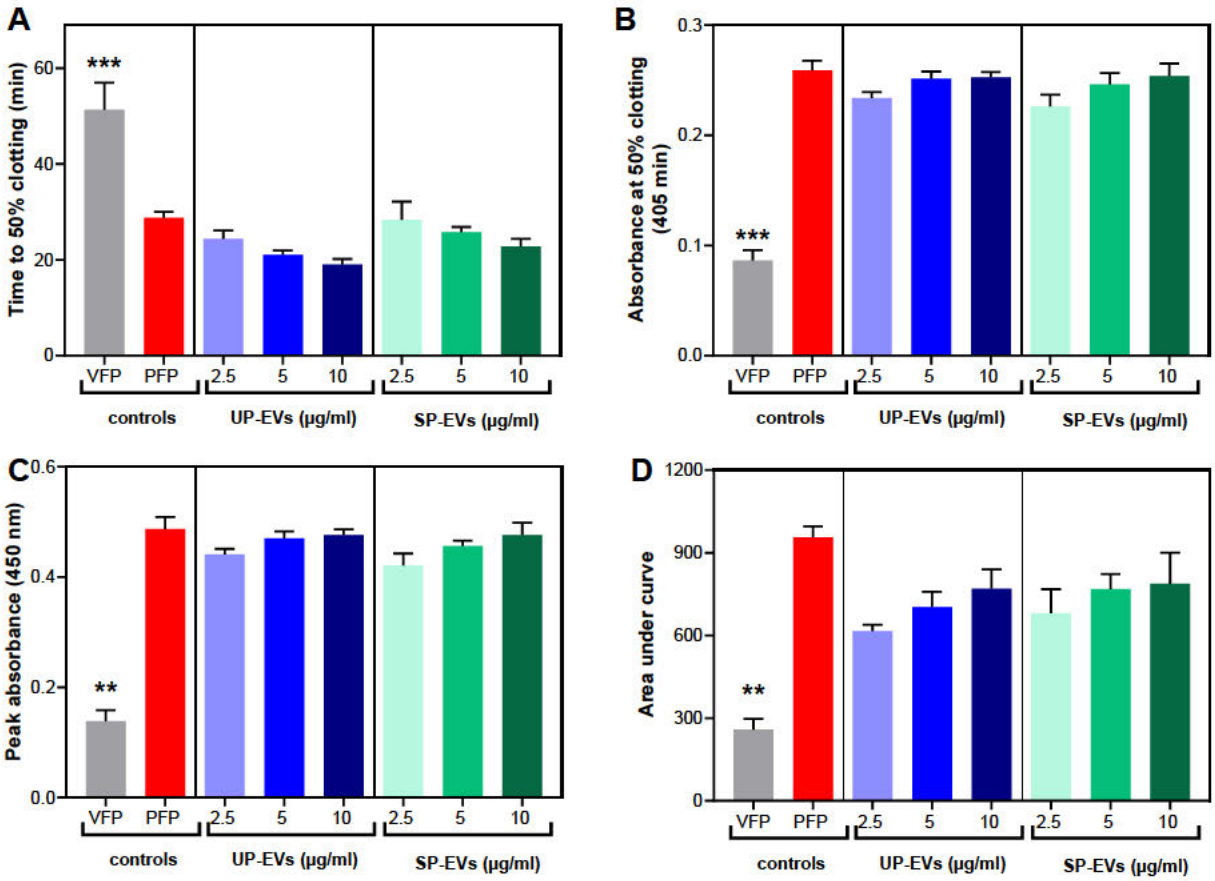


Figure 2.10. Effects of EVs generated in vitro from unstimulated or stimulated platelets on clot formation. Data are mean ± SEM, (n=4). Pooled VFP and pooled PFP were used for benchmarking purposes in the assessment of clot formation. Comparisons between pooled VFP and pooled PFP were drawn using two-tailed paired *t*-test difference shown at *p < 0.05, **p < 0.01 or ***p < 0.001. Pooled VFP induced less clot formation than pooled PFP. Dose-response effects of in vitro-generated PDEVs from unstimulated and stimulated platelets (2.5 - 10 µg protein/ml) were presented as **(A)** time to 50% change of clot, **(B)** absorbance at 50% change of clot, **(C)** peak absorbance of clot and **(D)** area under curve of clot; PFP, platelet-free plasma; SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles; VFP, vesicle-free plasma.

2.18.2 Measurement of thrombin generation

Thrombin formation was assessed using a commercially available, plate-based thrombin generation assay (Technothrombin TGA kit, Austria), which assesses a change in fluorescence as a result of cleavage of a fluorogenic substrate by thrombin over time upon activation of the clotting cascade by tissue factor. Two separate analyses were conducted: (i) determination of the effect of n-3 PUFA supplementation on thrombin generation in PFP from study samples relative to pooled VFP and (ii) determination of the effects of in vitro-generated

PDEVs derived from study samples on thrombin generation in pooled VFP. Prior to analysis, a thrombin calibration curve was constructed from dilutions of lyophilised Hepes-NaCl-buffer containing 0.5 % bovine serum albumin and ~ 1000 nM thrombin in buffer with BSA. A kinetic reading of a plate was initiated by additional of 50 µl of fluorogenic substrate solution containing fluorogenic substrate 1 mM Z-G-G-R-AMC and 15 mM $CaCl_2$. Calibration curves were recorded at 37 °C for 10 minutes with 30 seconds intervals using the plate reader (FlexStation 3, United State) at 360 nm for excitation and at 460 nm for emission.

The methods for both approaches were based on the use of pooled VFP as a negative control to allow assessment of thrombin generation specifically resulting from the presence of EVs. Preliminary, experiments demonstrated that thrombin generation in pooled PFP was approximately double that in pooled VFP (**Figure 2.11**), providing further support that the removal of EVs from plasma reduces thrombogenic activity (Tripisciano et al., 2017b, Zubairova et al., 2015b, Bidot et al., 2008).

For the first approach (i), 40 µl aliquots of either pre-thawed study sample PFP or pooled VFP or pooled PFP were added into the plate. For the second approach, PDEVs (10 µl of EV suspensions at 5 µg protein/ml, final concentration) produced from either unstimulated or stimulated platelets (UP-EVs and SP-EVs, respectively) from intervention samples, or PBS (negative control) were added to 30 µl VFP. The concentration of 5 µg protein/ml was determined as an appropriate concentration for this assay based on the data presented in **Figure 2.11**. This was followed by addition of a 10 µl suspension of phospholipid micelles containing recombinant human tissue factor (TF) in Tris-Hepes-NaCl buffer (RCL), which was provided in the kit. Formation of thrombin was initiated by additional of 50 µl of fluorogenic substrate solution containing fluorogenic substrate 1 mM Z-G-G-R-AMC and 15 mM $CaCl_2$. Plates were immediately read at 37 °C for 1 hour at 1 min intervals using a fluorescence plate reader (FlexStation 3, United State) at excitation and emission wavelengths of 360 and 460 nm, respectively. All samples were measured in duplicate. Fluorescent intensity was detected by TGA Evaluation Software to calculate thrombin generation in samples. Data were then

analysed by the TGA Evaluation Software manually to convert the unit of thrombin generation from RFU to nM and presented as three variables: lag time, time to the peak, the concentration of thrombin (nM), velocity-index and area under curve.

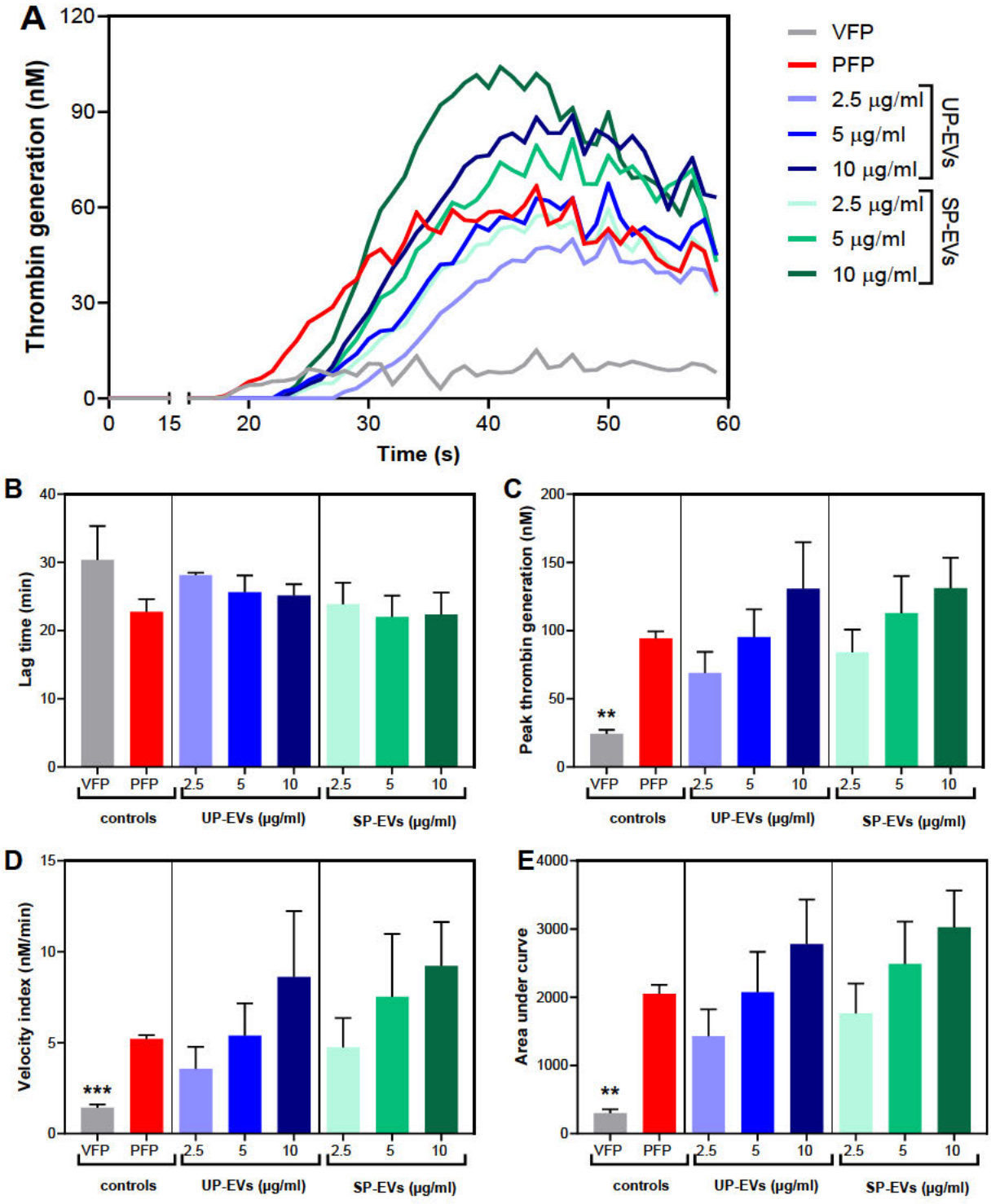


Figure 2.11. Effects of EVs generated in vitro from unstimulated or stimulated platelets on thrombin generation. Data are mean ± SEM, (n=4). Pooled VFP and pooled PFP were used for benchmarking purposes in the assessment of thrombin generation. **(A)** Thrombin generation induced by either VFP or PFP or in vitro-generated PDEVs at increasing

concentrations. Comparisons between pooled VFP and pooled PFP were drawn using two-tailed paired *t*-test difference shown at *p < 0.05, **p < 0.01 or ***p < 0.001. Pooled VFP induced less thrombin generation than pooled PFP. Dose-response effects of in vitro-generated PDEVs from unstimulated and stimulated platelets (2.5 - 10 µg protein/ml) were presented as **(B)** lag time, **(C)** peak thrombin generation concentration, **(D)** velocity index and **(E)** area under curve of thrombin generation; PFP, platelet-free plasma; SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles; VFP, vesicle-free plasma.

2.18.3 Measurement of fibrinolysis by PDEVs

The assessment of fibrinolysis induced by PDEVs added to VFP was performed in 96-well flat-bottom microplates (Greiner Bio-One, Stonehouse, Gloucestershire, UK) by a change in turbidity of VFP at 405 nm (A405) every 30 seconds, for 8 hours at 37°C using a Flex Station 3 plate reader (Molecular Devices, United State). The effect of EV removal from PFP on fibrinolysis was tested in preliminary experiments, demonstrating that fibrinolysis time was significantly reduced in the absence of EVs (**Figure 12**), suggesting that the potency of fibrinolysis was affected by the presence or absence of EV, in line with previous work (Zubairova et al., 2015b).

In this assay, pooled PFP (positive control) and VFP (negative control), served as benchmarks (for more details see **Section 2.15**). The assay for fibrinolysis of PDEVs generated in vitro was set up by diluting 30% of VFP in clot buffer (10 mM Tris [pH 7.4] and 0.01% Tween 20) in the presence of tPA (100 pM; Sigma-Aldrich, Dorset, UK) and 5 µg/ml protein concentration of PDEVs (final concentration), which was selected based on results shown in **Figure 12**. Fibrin clot formation to lysis was initiated by addition of 5 mM $CaCl_2$ in a final volume of 100 µl of clotting mixture. Plates were read at 37 °C for 8 hours at 30 second intervals using a fluorescence plate reader (FlexStation 3, United State) at wavelength of 405 nm. Data were then analysed by an online application (https://drclongstaff.shinyapps.io/Clot_or_HaloCL/) to obtain relative parameters: area under curve, time to 50% lysis from zero, absorbance at %50 lysis, time clotting to 50% lysis (time between 50% clotting and lysis), time peak to 50% lysis (time between the peak and 50% lysis).

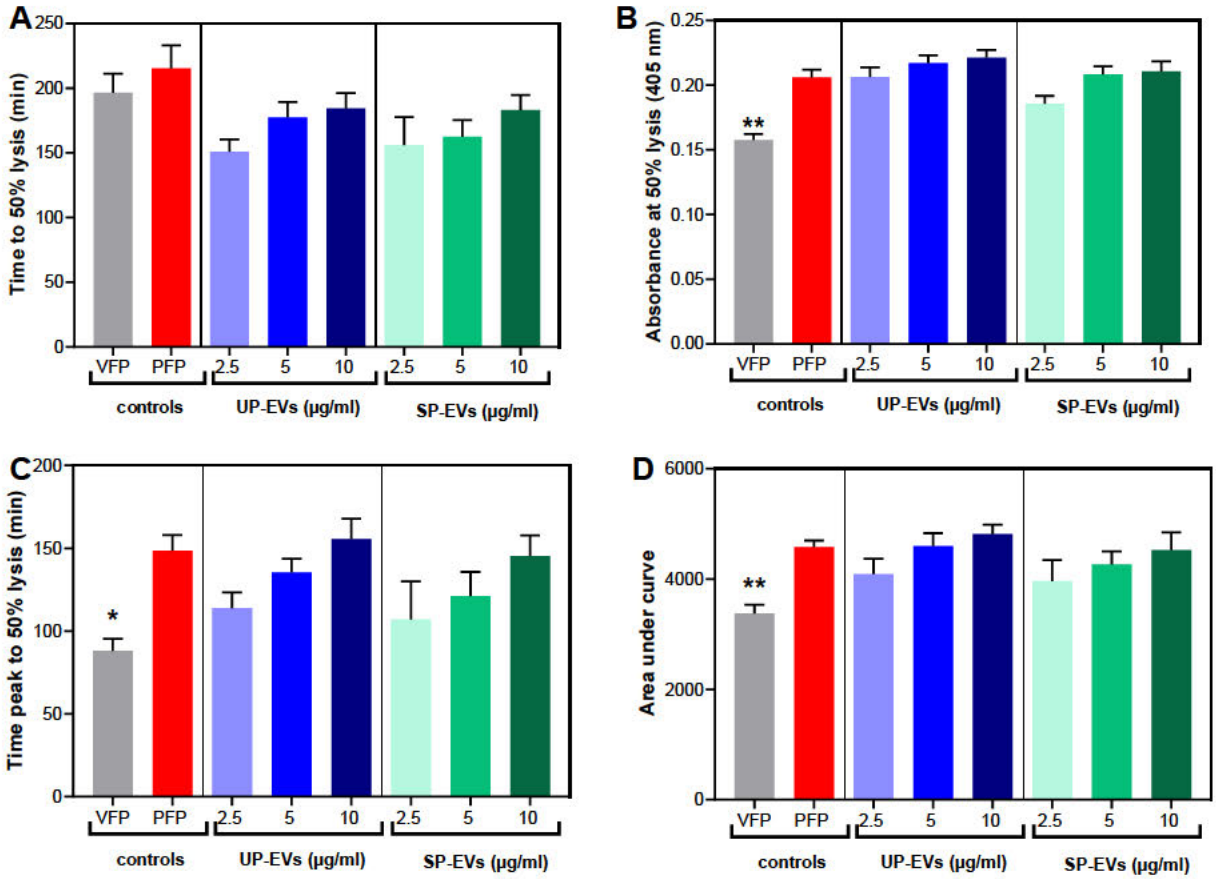


Figure 2.12. Effects of EVs generated in vitro from unstimulated or stimulated platelets on fibrinolysis. Data are mean ± SEM, (n=4). Pooled VFP and pooled PFP were used for benchmarking purposes in the assessment of fibrinolysis. Comparisons between pooled VFP and pooled PFP were drawn using two-tailed paired *t*-test difference shown at *p < 0.05 or **p < 0.01. Dose-response effects of in vitro-generated PDEVs from unstimulated and stimulated platelets (2.5 - 10 µg protein/ml) were presented as **(A)** time to 50% lysis, **(B)** absorbance at 50% lysis, **(C)** time peak to 50% lysis and **(D)** area under curve; PFP, platelet-free plasma; SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles; VFP, vesicle-free plasma.

2.18.4 Measurement of clot retraction by PDEVs

The assessment of clot retraction induced by PDEVs generated in vitro was performed using a thrombodynamics analyser (HemaCore, Moscow, Russia) in a protocol was modified by Tutwiler *et al* (2016) (Tutwiler et al., 2016). Briefly, 80 µl of PRP prepared from a healthy volunteer was incubated with either increasing concentration of PDEVs generated in vitro by unstimulated and stimulated platelets (protein concentrations of 2.5, 5 and 10 µg/ml, final concentration) or PBS (negative control) for 5 min at 37 °C within the thermostat of the

analyser. Samples were supplemented with 2 mM $CaCl_2$ (final concentration) and formation of a clot was initiated with 1 unit/mL thrombin (Sigma-Aldrich, Dorset, UK). As soon as thrombin was added, the mixture was transferred into an optically transparent, two channel-plastic cuvette that was coated with 4% Triton X-100 in PBS to avoid sticking of the clot to the cuvette chambers. The thrombodynamics analyser system allows tracking of the spontaneous contraction of the clot by accumulating digitized images based on the light-scattering properties of the clot every 15 s for 20 min. Data were manually analysed by thrombodynamics analyser software as recommended by the manufacturer, and results of control samples subtracted from PDEV values. Results were represented by percent of retraction (%), lag time (min), area under curve (%*min) and average velocity (%/min).

Addition of increasing concentrations of PDEVs, generated in vitro by unstimulated and stimulated platelets, decreased clot retraction in a dose-dependent manner, resulting in greater clot size with increasing concentrations of EVs (**Figure 2.13**). Unfortunately, the impact of PDEVs from the intervention study on clot retraction could not be assessed due to lack of sufficient sample, as this method was developed after the study had been completed. Nevertheless, the results of these preliminary investigations were highly consistent with the effects of PDEVs on clot formation, thrombin generation and fibrinolysis.

2.18.5 Measurement of ex vivo thrombus formation under flow

PDEVs prepared from the study samples were subjected to analysis of their effect on thrombus formation in whole blood under flow, a specialist technique conducted by Dr Jo Mitchell from the School of Biological Sciences, University of Reading. The assay was performed by adding either buffer (control) or 30 µl of PDEVs generated from TRAP-6 stimulated platelets (final protein concentration of EVs: 5 µl/ml) into whole blood in modified Tyrode's-HEPES buffer (134 mM NaCl, 2.9 mM KCL, 0.34 mM Na2HPO4, 20 mM HEPES, 1 mM MgCl2, pH 7.4). Cellix Vena8 Fluoro+ biochips with capillaries 0.01 cm high and 0.04 cm wide were coated with collagen (100 mg/ml) prior to thrombus formation studies. Whole citrated blood labelled with 3,3'-Dihexyloxacarbocyanine Iodide (DiOC6) was flowed for 10

minutes over the collagen coated capillary chambers to form platelet thrombi at an arterial shear rate of 1000s-1. Images were taken every 2-4 seconds using a Nikon A1R fluorescence confocal microscope at 20x magnification. Data were analysed using Fiji (Image J) by measuring the fluorescence intensity of the DiOC6 over time which corresponded to the increase in thrombus size.

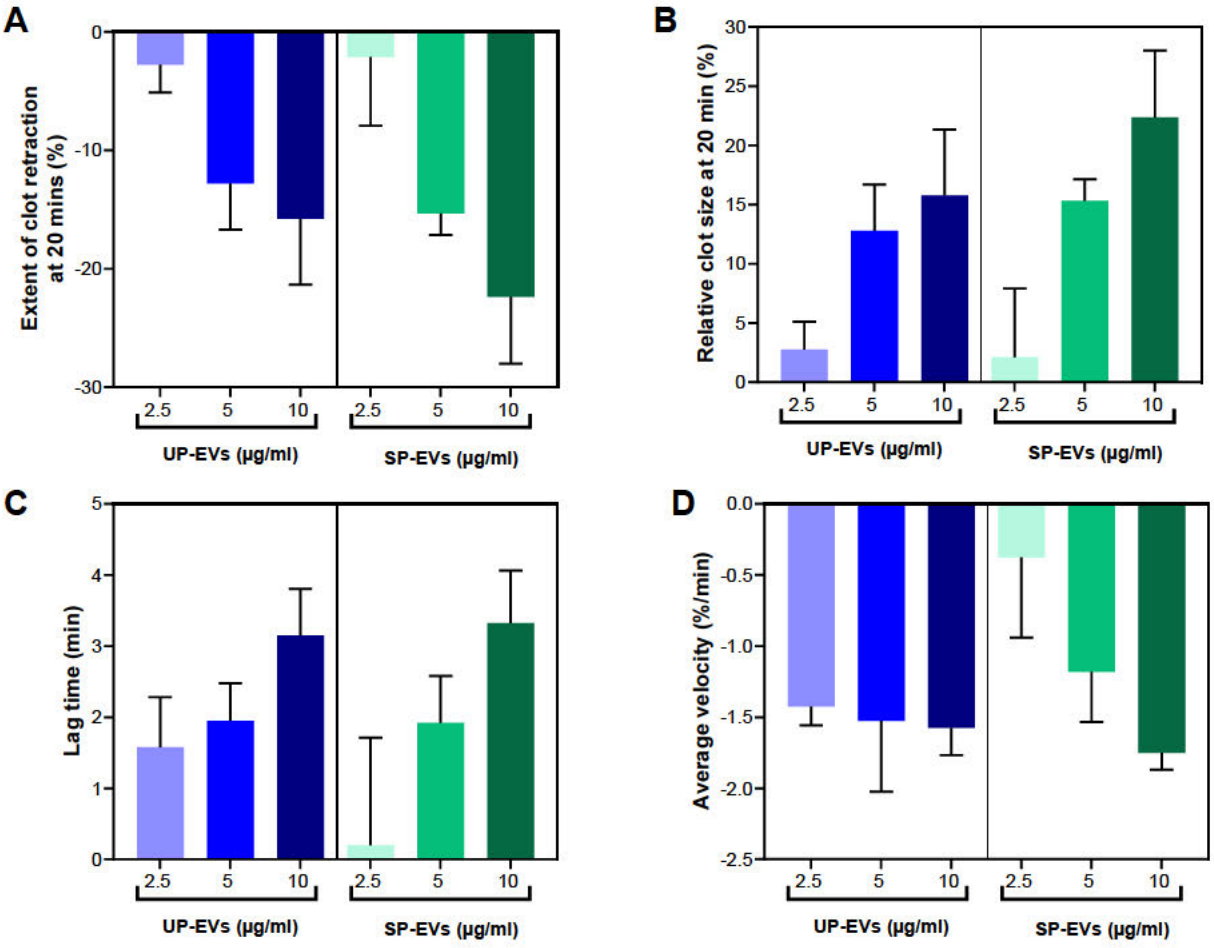


Figure 2.13. Effects of EVs generated in vitro from unstimulated or stimulated platelets on clot retraction. Data are mean ± SEM, (n=4). Pooled VFP and pooled PFP were used for benchmarking purposes in the assessment of clot retraction. The data represented the kinetics of clot contraction in PRP ($3x10^8$ platelets/ml) containing increasing concentrations of purified in-vitro generation EVs from platelets at a final concentration of 2.5, 5 and 10 µg/mL, subtracted from control (PRP containing only PBS). The final extent of clot contraction was optically tracked for 20 minutes, and the constructed curve was used to calculate **(A)** extent of clot retraction, **(B)** relative clot size, **(C)** lag time and **(D)** area under curve; SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles.

2.19 Statistical analysis

Two-tailed paired or unpaired student's *t-test* was performed to compare the means of two populations (**Chapter 3.2**, **Chapter 4.2.6, Chapter 5.2.1**). These statistical analyses were carried out using GraphPad Prism Software 9 (GraphPad, La Jolla, CA). Error bars denote SEM (standard error of the mean) and a p value of $p<0.05$ is considered statistically significant.

Dose-response curves of platelet aggregation for each baseline and intervention were constructed using a 4-parameter log-linear function in GraphPad Prism Software 9 (GraphPad, La Jolla, CA) (**Chapter 3.2.3**). These dose-response curves of platelet aggregation were used to calculate relative parameters including LogEC50, maximum response, minimum and hill slope values. Comparisons of these parameters after each intervention were drawn using 2-way ANOVA with the Turkey multiple comparisons test. These statistical analyses were carried out using GraphPad Prism Software 9. Error bars denote SEM (standard error of % aggregation values of mean) and a p value of $p<0.05$ is considered statistically significant.

Comparisons between PFP, VFP, after fish oil intervention and after control oil intervention were drawn using one-way ANOVA, followed by the Tukey multiple comparison test (**Chapter 3.2.6**). SPSS 24.0 software for Windows (SPSS, Inc., Chicago, IL) was used to perform this statistical analysis. Error bars denote SEM (standard error of the mean) and a p value of $p<0.05$ is considered statistically significant.

Comparisons after each intervention were drawn using General Linear Model (GLM). For this, GLM was fitted to analyse time course data for study test points in order to determine individual treatment effects with fixed factors of time (repeated measures) and treatment. SPSS 24.0 software for Windows (SPSS, Inc., Chicago, IL) was used to perform this statistical analysis. Error bars denote SEM (standard error of the mean) and a p value of $p<0.05$ is considered statistically significant.

For **Chapter 4**, normality of baseline values (V1) was tested using Shapiro–Wilk normality test. All variables with non-normal distribution were log-transformed to achieve normal distribution before correlation analysis. Pearson's correlation analysis was used to determine trend of correlations between each variable. Significant correlations of Pearson's test ($p < 0.05$) were then incorporated into the univariate linear regression analysis. To avoid multicollinearity in the multivariate analysis, variance inflation factor of <3 was set and a variance inflation factor of >3 was removed. Multivariate linear regression analysis was constructed using significant univariant determinants ($p < 0.05$) to identify the relationship of these determinants with thrombogenic risk factors. Stepwise regression analysis was then conducted as an additional analysis to determine which independent risk factors (conventional risk markers and EV parameters) influenced thrombogenic risk markers. In stepwise regression, thrombogenic risk markers (the rate of clot growth, clot size at 30 min, lag time for thrombin generation, peak thrombin concentration, velocity index and AUC) were selected as dependent variables, while only related independent variables, where $p < 0.05$ after an initial linear regression between the dependent, were selected as independent variables to avoid multicollinearity. This statistical analysis was performed with SPSS 24.0 software for Windows (SPSS, Inc., Chicago, IL).

Chapter 3. Effects of n-3 PUFAs on plasma fatty acid composition, CVD risk markers and coagulation-related parameters in subjects with moderate risk for CVD (HI-FIVE STUDY)

3.1 Introduction

Cardiovascular diseases (CVDs), including coronary artery diseases, ischaemic heart disease, stroke, hypertension, and peripheral arterial disease, are still a major cause of death throughout the world (Lozano et al., 2012). Atherosclerosis, the main underlying cause of cardiovascular events, is initiated by impaired vascular endothelium, leading to endothelial cell activation and apoptosis, which in turn results in endothelial dysfunction (Bachmair et al., 2014, Renga and Scavizzi, 2017). Multiple risk factors have been proposed to contribute to the development of atherosclerosis and CVD, and these risk factors are commonly used to assess cardiovascular risk (Tzoulaki et al., 2016). Evidence suggests that thrombosis (Kotzé et al., 2014), including hyper platelet aggregation (Renga and Scavizzi, 2017, Adili et al., 2018), hypercoagulability, impaired fibrinolysis (Kotze et al., 2014, Woodman et al., 2003), higher thrombin formation (Al Dieri et al., 2012, Loeffen et al., 2015), and altered conventional cardiovascular risk factors, including hypertension (Fuchs and Whelton, 2020), hypercholesterolemia and hypertriglyceridemia (Karalis, 2017), and obesity (Kachur et al., 2017, Bastien et al., 2014), are the most common determinants of atherosclerotic plaque formation and are therefore highly associated with CVD risk.

Fish oil is suggested to have a role in the prevention of CVD with much of the research on modulation of risk factors that contribute to the development of CVDs (Calder, 2014, Maki and Dicklin, 2018, Calder, 2018). An inverse relationship between the supplementation with n-3 PUFAs and CVD risks has been supported by a recent meta-analysis with several randomised clinical trials (RCTs) (Hu et al., 2019). Epidemiological studies report that consumption of fish oil reduces cardiovascular morbidity and mortality by lowering risk factors of CVD (Bucher et

al., 2002, Studer et al., 2005, Calder, 2004, Iso et al., 2006, Yamada et al., 2000). Moreover, some meta-analyses and systematic reviews of multiple human intervention studies also support evidence that fish oil supplementation decreases the risk of coronary disease (Chowdhury et al., 2014), sudden cardiac death, and nonfatal cardiovascular events (Marik and Varon, 2009) by reducing the development of atherosclerosis (Calder and Yaqoob, 2012). Although the exact underlying mechanism by which fish oil improves cardiac health is not clear, these cardioprotective effects could be associated with the high n-3 PUFA content in fish oil, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and their beneficial effects on conventional and thrombogenic risk markers of CVD. EPA and DHA can modify the composition of the plasma membrane of cells and platelets (Calder, 2014), thereby resulting in antiatherogenic and antithrombotic actions (Calder, 2018, Siscovick et al., 2017). However, not all meta-analyses have demonstrated favourable effects of n-3 PUFA consumption on all-cause death, cardiac mortality, sudden death, myocardial infarction, or stroke (Rizos et al., 2012, Kotwal et al., 2012, Aung et al., 2018). A recent systematic review including 79 randomised trials concluded that n-3 PUFA supplementation had little or no impact on the risk of cardiovascular events, coronary heart deaths, coronary heart disease events, stroke, or heart irregularities (Abdelhamid et al., 2018).

One of the proposed mechanisms for the protective role of n-3 PUFAs in CVDs is by lowering blood pressure. Several studies showed a reduction in blood pressure after treatment with fish oil, thereby decreasing CVD risk (Miller et al., 2014, Liu et al., 2011). The beneficial effect of n-3 PUFAs on other risk factors for CVD, such as plasma triacylglycerol (TAG), is well-established and has been approved by the European Food Safety Authority (EFSA) (EFSA Panel on Dietetic Products and Allergies, 2010) and the United State Food and Drug Administration (US FDA). Despite the positive findings, there are several studies that did not replicate the effects of n-3 PUFA on blood pressure (Campbell et al., 2013), plasma lipoproteins (Root et al., 2013, Abdelhamid et al., 2020) and platelet aggregation (Mackay et al., 2012, Poreba et al., 2017). Even though it is likely that treatment with n-3 PUFAs may

reduce those risk markers, thereby influencing CVD development, controversy regarding the beneficial effects of fish oil-derived n-3 PUFA on cardiovascular risk factors still remains.

Concerning thrombogenic risk markers, some trials point to beneficial effects of n-3 PUFAs on vascular health and function (Wu et al., 2012), endothelial function (Renga and Scavizzi, 2017), and thrombosis by favourable effects on platelet aggregation, hypercoagulation, fibrinolysis, and thrombin generation. Numerous studies have evaluated the effect of n-3 PUFAs on platelet aggregation using various methodologies over the last few decades. Although a meta-analysis (Gao et al., 2013) and a systematic review (Begtrup et al., 2017) showed a reduction in platelet aggregation in response to n-3 PUFAs, recent literature is contradictory (Poreba et al., 2017, Bagge et al., 2018, Kander et al., 2018). The heterogeneity in study design and techniques, including the use of various platelet agonists and their concentrations, makes it difficult to draw a clear conclusion.

A summary of studies investigating the effects of n-3 PUFAs on coagulation, fibrinolysis, and thrombin generation is presented in **Table 3.1**. Some trials demonstrated an improvement in coagulation-related parameters after n-3 PUFA supplementation, but this was not consistent, and they used a broad range of methodologies to assess coagulation (Nordøy et al., 2000, Vanschoonbeek et al., 2004, Vanschoonbeek et al., 2007, Gajos et al., 2011, Phang et al., 2014, McEwen et al., 2015). Four out of seven studies demonstrated a decrease in some aspects of coagulation and thrombin generation following n-3 PUFA supplementation and the effects on clot lysis were even less clear (**Table 3.1**) as four studies suggested that n-3 PUFAs enhance fibrinolysis (Din et al., 2013a, Gajos et al., 2011, Kabir et al., 2007, McEwen et al., 2015), but other four studies showed no effect (Nordøy et al., 2000, Woodman et al., 2003, Din et al., 2013b, Poreba et al., 2017).

Importantly, there have been no studies investigating the influence of n-3 PUFA supplementation on both coagulation and fibrinolysis in the same assay. The inconsistency in reported effects of n-3 PUFAs on thrombin generation may be due to the variety of techniques used to assess thrombin generation and this requires resolution.

In summary, there is a lack of clarity regarding the effects of n-3 PUFA on thrombogenic risk markers and this requires investigation in a well-designed study. The current randomized, double-blind, placebo-controlled, crossover trial aimed to investigate whether the effect of daily supplementation with 1.8 g/d of fish oil-derived n-3 PUFA altered conventional CVD markers and thrombogenic markers, including platelet aggregation, coagulation, fibrinolysis, and thrombin generation, in subjects with moderate risk for CVD. This chapter also serves as a foundation for examining the effects of PDEVs modified by n-3 PUFA on coagulation, fibrinolysis and thrombin generation, which are explored later in this thesis.

Table 3.1. Human intervention studies investigating the effect of n-3 PUFAs on coagulation, fibrinolysis and thrombin generation

Outcomes	Subjects	Study design	Dose of n-3 PUFA	Duration	Method	Results	Study info
Coagulation	41 patients with combined hyperlipemia	Double-blind parallel	3.36 g/d of n-3 PUFAs (+simvastatin) or placebo (corn oil)	5 weeks	Haemostatic Parameters	↓ activated factor VII	(Nordøy et al., 2000)
	25 healthy males	Clinical intervention	3.0 g/d of omega-3 PUFAs	4 weeks	Haemostatic Parameters	↓ Plasma fibrinogen level ↓ Factor V level	(Vanschoonbeek et al., 2004)
	57 overweight (Study 1) and 42 with type-2 diabetes (Study 2)	Clinical intervention	3.1 g/d of n-3 PUFAs or corn oil (Study 2: +antidiabetic drug)	4 weeks / 8 weeks	Haemostatic Parameters	Study 1: → Coagulation parameters Study 2: ↓ Prothrombin, factor V, factor VII, factor X, and of fibrinogen levels	(Vanschoonbeek et al., 2007)
	54 with stable CAD	Prospective, double-blind, RCT	1 g/d of n-3 PUFA (+anti-platelet drugs)	4 weeks	Fibrin clot properties	↑ Pores in the fibrin network	(Gajos et al., 2011)
	94 healthy	Double-blind, RCT	600 mg/d of EPA or DHA rich oil or sunola oil (placebo)	4 weeks	Haemostatic Parameters	Male: ↓ prothrombin, factor V and vWFAg levels with EPA	(Phang et al., 2014)
	40 healthy and 16 with CVD	Clinical intervention	640 mg/d of n-3 PUFAs (DHA 520 mg and EPA 120 mg)	4 weeks	Overall haemostatic potential assay	Healthy subjects: ↓ fibrin generation and ↑ delay to fibrin generation Subjects with CVD: ↓ overall haemostatic potential	(McEwen et al., 2015)
	74 with atherosclerosis and type2 diabetes	Prospective, double-blind, RCT, double-centre	2 g/d n-3 of PUFAs and placebo	3 months	Latex nephelometry	→ Plasma fibrinogen level	(Poreba et al., 2017)
	12 healthy subjects	Clinical intervention	2520 mg/d of n-3 PUFAs	10 days	Sonoclot Coagulation Analyser	→ Coagulation	(Bagge et al., 2018)

Table 3.1. Human intervention studies investigating the effect of n-3 PUFAs on coagulation, fibrinolysis and thrombin generation (continued)

Outcomes	Subjects	Study design	Dose of n-3 PUFA	Duration	Method	Results	Study info
Thrombin generation	25 healthy males	Clinical intervention	3.0 g/d of omega-3 PUFAs	4 weeks	Thrombogram method	↓ thrombin peak ↑ time to thrombin peak ↓ ETP	(Vanschoonbeek et al., 2004)
	57 overweight (Study 1) and 42 with type-2 diabetes (Study 2)	Clinical intervention	3.1 g/d of n-3 PUFAs or corn oil (Study 2: +antidiabetic drug)	4 weeks / 8 weeks	Thrombogram method	Study 1: ↑ time to thrombin peak Study 2: ↓ ETP	(Vanschoonbeek et al., 2007)
	54 with stable CAD	Prospective, double-blind, RCT	1 g/d of n-3 PUFA (+anti-platelet drugs)	4 weeks	CAT	↓ thrombin peak	(Gajos et al., 2011)
	42 adolescents	RCT	4 g/d of fish oil	8 weeks	TGA	→ thrombin generation	(Gidding et al., 2014)
	94 healthy	Double-blind, placebo-controlled, randomised	600 mg/d EPA or DHA rich oil or sunola oil (placebo)	4 weeks	CAT	→ thrombin generation	(Phang et al., 2014)
	40 healthy and 16 with CVD	Clinical intervention	640 mg/d of n-3 PUFAs (DHA 520 mg and EPA 120 mg)	4 weeks	CAT	Healthy subjects: ↓ thrombin peak Subjects with CVD: ↑ peak thrombin and lag time	(McEwen et al., 2015)
	74 with atherosclerosis and type2 diabetes	Prospective, double-blind, RCT, double-centre	2 g/d n-3 of PUFAs and placebo	3 months	CAT	→ thrombin generation	(Poreba et al., 2017)

Table 3.1. Human intervention studies investigating the effect of n-3 PUFAs on coagulation, fibrinolysis and thrombin generation (continued)

Outcomes	Subjects	Study design	Dose of n-3 PUFA	Duration	Method	Results	Study info
Fibrinolysis	41 patients with combined hyperlipemia	Double-blind parallel	3.36 g/d of n-3 PUFAs (+simvastatin) or placebo (corn oil)	5 weeks	Haemostatic Parameters	→ plasma plasminogen activator inhibitor-1 → tissue plasminogen activator	(Nordøy et al., 2000)
	59 treated-hypertensive Type 2 diabetic men and postmenopausal women	double-blind placebo-controlled trial of parallel design	4 g/day of EPA, DHA or olive oil (placebo)	6 weeks	Haemostatic Parameters	→ tissue-plasminogen activator → plasma plasminogen activator inhibitor-1	(Woodman et al., 2003)
	27 women with type-2 diabetes	Double-blind parallel, RCT	3 g/d of fish oil (1.8 g n-3 PUFAs) or (placebo)	2 months	Chromolize/PAI-1 kits	↓ plasma plasminogen activator inhibitor-1	(Kabir et al., 2007)
	54 with stable CAD	Prospective, double-blind, RCT	1 g/d of n-3 PUFAs (+anti-platelet drugs)	4 weeks	Plasmin-mediated fibrinolysis assay	↓ Lysis time ↑ Clot susceptibility to lysis	(Gajos et al., 2011)
	20 smoker male	Double-blind, RCT	2 g/d of n-3 PUFAs or olive oil placebo	6 weeks	ELISA	↑ tissue plasminogen activator	(Din et al., 2013a)
	20 male with a previous myocardial infarction	Double-blind, RCT	2 g/d of n-3 PUFAs or olive oil placebo	6 weeks	ELISA	→ plasma plasminogen activator inhibitor-1 → tissue plasminogen activator	(Din et al., 2013b)
	Forty healthy subjects and sixteen with CVD	Clinical intervention	640 mg/d of n-3 PUFAs	4 weeks	Overall haemostatic potential assay	Healthy subjects: ↑ overall fibrinolysis potential	(McEwen et al., 2015)
	Seventy-four patients with atherosclerosis and type 2 diabetes	Prospective, double-blind, double-centre, RCT	2 g/d of n-3 PUFAs or placebo	3 months	Plasmin-mediated fibrinolysis assay	→ Lysis time (*t50%*)	(Poreba et al., 2017)

3.2 Results

Baseline characteristics of 40 subjects (24 men and 16 women) collected before starting the study are shown in **Table 3.2**. The study population was mildly hypertensive and mildly hypercholesterolemic. There were some significant differences in baseline characteristics between male and female participants, in that female subjects were older and had significantly higher BMI, whereas blood glucose concentration and QRISK scores were significantly greater in males (**Table 3.2**).

Table 3.2. Baseline characteristics of the study participants at screening

Baseline characteristics	Combined n=40	Male n=24	Female n=16	p-value
Age (year)	64.5 ± 0.6	63.5 ± 0.9	66.1 ± 0.6	**0.028**
BMI (kg/m^2)	25.4 ± 0.5	25.8 ± 0.6	24.8 ± 0.6	0.303
Systolic BP (mm Hg)	134.0 ± 2.2	134.8 ± 3.0	132.8 ± 3.5	0.678
Diastolic BP (mm Hg)	78.8 ± 1.4	79.6 ± 1.4	77.6 ± 2.9	0.513
Total cholesterol (mmol/l)	6.0 ± 0.2	5.8 ± 0.2	6.4 ± 0.2	0.060
HDL-cholesterol (mmol/l)	1.6 ± 0.0	1.5 ± 0.1	1.6 ± 0.1	0.219
Cholesterol ratio	3.9 ± 0.1	3.9 ± 0.2	3.9 ± 0.2	0.771
TAG (mmol/l)	1.3 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	0.287
Glucose (mmol/l)	5.8 ± 0.1	6.0 ± 0.1	5.4 ± 0.1	**0.002**
Risk (%)	12.9 ± 0.5	14.1 ± 0.7	11.2 ± 0.5	**0.004**

Data are mean ± SEM, n = 24-16 / male-female. Comparisons between gender (male vs female) were drawn using two-tailed unpaired *t-test*, with differences shown at $p < 0.05$. There was a significant difference in age, glucose level and cardiovascular disease risk between male and female subjects; BMI, body mass index; BP, blood pressure; TAG, triacylglycerol.

3.2.1 Effects of n-3 PUFAs on plasma phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fatty acid composition

The fatty acid composition of the major fatty acids in plasma PC and PE before and after intervention is shown in Table 3. Supplementation with fish oil significantly increased the plasma PC content of EPA, DPA and DHA, while decreasing that of palmitic acid, palmitoleic acid, oleic acid and the n-6 PUFAs, linoleic acid, GLA, eicosadienoic acid, DGLA and arachidonic acid (**Table 3.3**). There was no significant impact of time and no treatment*time interaction.

In contrast to the PC results, intervention with fish oil resulted in an increase in the PE content of EPA and DPA, but not DHA, and there were decreases in DGLA and arachidonic acid only (**Table 3.3**).

3.2.2 Effects of n-3 PUFAs on CVD risk markers

Supplementation with fish oil, significantly lowered systolic blood pressure (SBP), diastolic blood pressure (DBP) and plasma TAG concentration, but increased plasma LDL-cholesterol concentration (**Table 3.4**). There was no significant effect of time and no treatment*time interaction with respect to any parameter, apart from SBP, where there were significant effects of time and a treatment*time interaction ($p < 0.05$ and $p < 0.05$, respectively; **Table 3.4**). Thus, for most of the parameters, there were main effects of treatment only. The significant interaction between treatment and time for SBP indicates that the effect of treatment was dependent on time, represented by a substantial decrease in SBP by fish oil compared with control oil at the end of the intervention (**Table 3.4**).

Table 3.3. Effects of n-3 PUFAs on PC and PE fatty acid composition

	PC					PE				
	Control Oil		Fish Oil		*P*-value	Control Oil		Fish Oil		p-value
	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	*treatment*	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	*treatment*
Myristic acid (14:0)	0.36±0.02	0.37±0.02	0.37±0.01	0.36±0.02	0.207	0.88±0.04	0.89±0.04	0.91±0.05	0.92±0.04	0.986
Palmitic acid (16:0)	30.79±0.18	30.59±0.17	30.77±0.19	30.06±0.20	**0.048**	22.60±0.30	22.00±0.26	22.60±0.31	21.97±0.34	0.855
Palmitoleic acid (16:1n-7)	0.78±0.03	0.74±0.03	0.76±0.03	0.66±0.03	**0.048**	1.47±0.10	1.35±0.10	1.37±0.10	1.20±0.10	0.713
Stearic acid (18:0)	13.86±0.15	14.02±0.17	13.97±0.17	14.01±0.18	0.576	14.08±0.26	13.85±0.24	14.27±0.25	13.72±0.20	0.422
Oleic acid (18:1n-9)	10.94±0.19	10.99±0.17	10.79±0.18	10.13±0.22	**0.004**	20.90±1.11	19.61±1.08	19.95±1.06	18.23±1.06	0.755
Vaccenic acid (18:1n-7)	1.35±0.03	1.33±0.03	1.34±0.03	1.29±0.02	0.573	1.33±0.05	1.27±0.06	1.27±0.05	1.17±0.05	0.643
Linoleic acid (18:2n-6)	22.22±0.37	22.52±0.31	22.33±0.38	22.23±0.44	**<0.001**	15.17±0.32	15.94±0.34	15.37±0.33	13.95±0.38	**<0.001**
GLA (18:3n-6)	0.08±0.00	0.09±0.00	0.09±0.00	0.06±0.00	**<0.001**	0.10±0.01	0.11±0.01	0.11±0.01	0.11±0.01	0.087
ALA (18:3n-3)	0.24±0.01	0.23±0.01	0.24±0.01	0.22±0.01	0.575	0.83±0.05	0.81±0.05	0.79±0.03	0.84±0.05	0.208
Arachidic acid (20:0)	0.14±0.01	0.13±0.01	0.13±0.01	0.12±0.01	0.697	0.17±0.01	0.16±0.01	0.16±0.01	0.15±0.01	0.723

Table 3.3. Effects of n-3 PUFAs on PC and PE fatty acid composition (continued)

	PC					PE				
	Control Oil		Fish Oil		*P*-value	Control Oil		Fish Oil		p-value
	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	*treatment*	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	*treatment*
Eicosenoic acid (20:1n-9)	0.17±0.01	0.18±0.01	0.18±0.01	0.17±0.01	0.188	0.31±0.02	0.32±0.02	0.35±0.02	0.30±0.02	0.232
Eicosadienoic acid (20:2n-6)	0.21±0.01	0.20±0.01	0.20±0.01	0.16±0.01	**<0.001**	0.32±0.02	0.29±0.02	0.31±0.02	0.31±0.02	0.195
DGLA (20:3n-6)	3.24±0.10	3.28±0.10	3.30±0.09	2.43±0.08	**<0.001**	1.64±0.10	1.73±0.11	1.78±0.11	1.31±0.08	**<0.001**
Arachidonic acid (20:4n-6)	9.47±0.27	9.57±0.28	9.70±0.26	7.85±0.21	**<0.001**	10.92±0.60	12.00±0.59	11.54±0.53	9.63±0.49	**0.001**
ETA (20:4n-3)	0.16±0.01	0.15±0.01	0.15±0.01	0.17±0.01	0.036	0.68±0.05	0.77±0.08	0.72±0.06	0.76±0.05	0.759
EPA (20:5n-3)	1.36±0.07	1.25±0.06	1.32±0.09	4.16±0.18	**<0.001**	1.49±0.13	1.47±0.10	1.45±0.13	5.07±0.30	**<0.001**
DPA (22:5n-3)	1.08±0.03	1.01±0.02	1.06±0.03	1.35±0.04	**<0.001**	1.43±0.07	1.49±0.07	1.49±0.07	1.50±0.06	0.670
DHA (22:6n-3)	3.54±0.14	3.34±0.11	3.29±0.15	5.57±0.14	**<0.001**	5.68±0.35	5.93±0.31	5.56±0.34	8.85±0.42	**<0.001**

Data are mean ± SEM (n = 40). Comparisons after each intervention were drawn using General Linear Model (GLM), with differences shown at $p < 0.05$; GLA, gamma linolenic acid; ALA, alpha-linolenic acid; DGLA, dihomo-γ-linolenic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

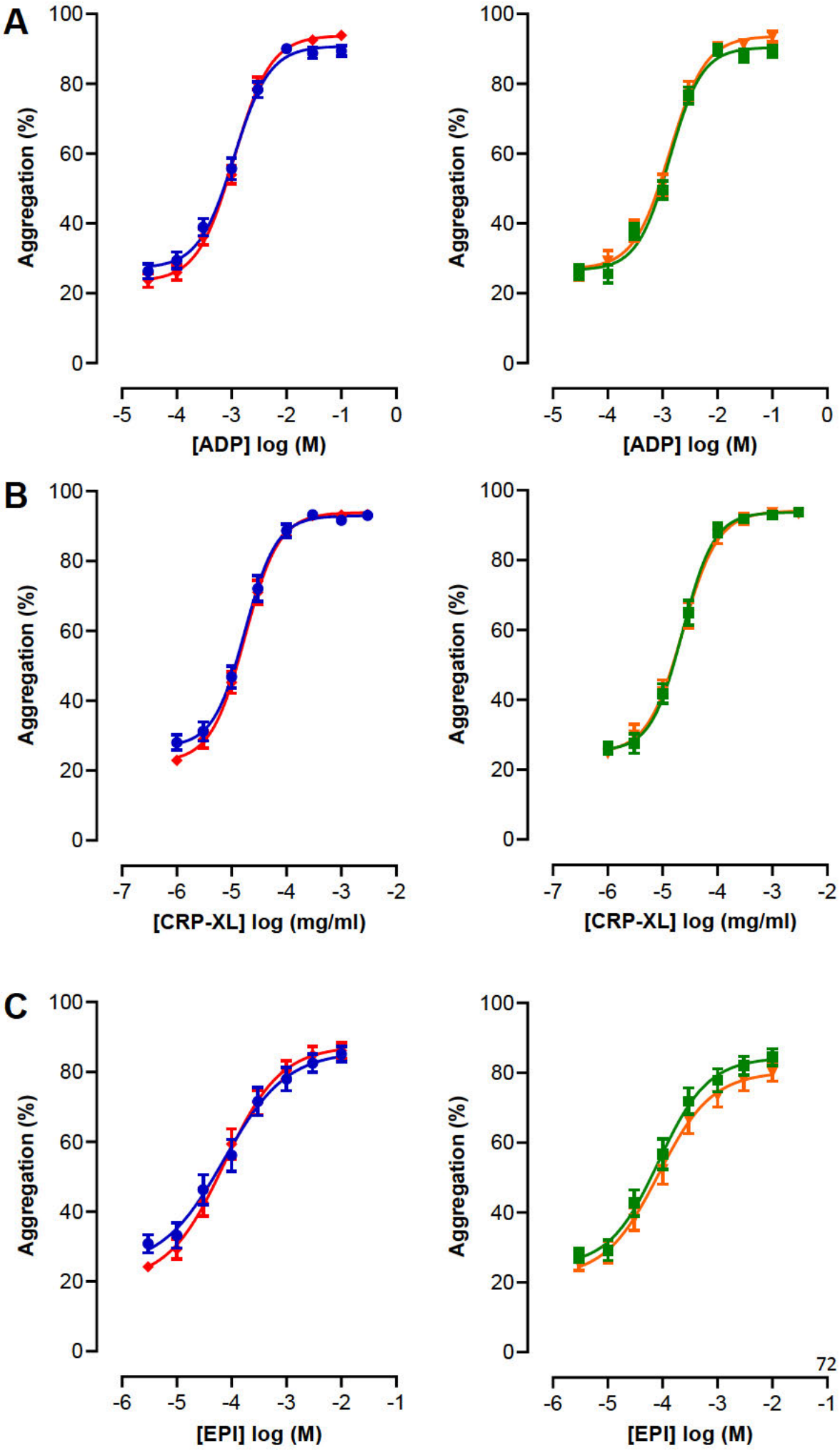
Table 3.4. Effects of n-3 PUFAs on CVD risk markers

	Control Oil		Fish Oil		*p*-value		
	Before	**After**	**Before**	**After**	**treatment**	**time**	**treatment x time**
Systolic BP (mm Hg)	131.85±2.19	135.23±2.14	134.03±2.18	127.38±2.19	**0.000**	**0.011**	**0.033**
Diastolic BP (mm Hg)	78.08±1.43	79.00±1.63	78.98±1.40	76.05±1.42	**0.002**	0.952	0.114
BMI (kg/m2)	25.47±0.46	25.52±0.47	25.57±0.48	25.62±0.48	0.675	0.076	0.675
TAG (mmol/l)	0.98±0.06	1.01±0.06	0.99±0.06	0.88±0.05	**0.016**	0.566	0.659
Total-cholesterol (mmol/l)	4.90±0.13	4.88±0.13	4.88±0.12	5.02±0.11	0.077	0.921	0.793
HDL-cholesterol (mmol/l)	1.38±0.05	1.42±0.05	1.39±0.05	1.45±0.05	0.379	0.938	0.341
LDL-cholesterol (mmol/l)	3.06±0.10	3.01±0.10	3.04±0.10	3.17±0.10	**0.014**	0.842	0.943

Data are mean ± SEM, (n = 40). Comparisons after each intervention were drawn using the General Linear Model (GLM), with differences shown at $p < 0.05$; BP, blood pressure; BMI, body mass index; TAG, triacylglycerol; n-3 PUFA, n-3 polyunsaturated fatty acid; SFA, saturated fatty acid.

3.2.3 Effects of n-3 PUFAs on platelet aggregation

The intervention had no effect on platelet aggregation in response to ADP, CRP-XL, epinephrine, TRAP-6 or U46619, as shown in **Figure 3.1**. There was also no effect on the sensitivity of platelets to each agonist, calculated as the log half maximal effective concentration (logEC50) by nonlinear regression analysis (**Figure 3.2**). Maximum aggregation value, minimum aggregation value and Hill slope were extracted from the dose-response curves for each agonist; there was no effect of the intervention (**Table 3.4)**.


A
Aggregation (%)
100
80
60
40
20
0
-5
-4
-3
-2
-1
0
[ADP] log (M)
B
[CRP-XL] log (mg/ml)
-7
-6
C
[EPI] log (M)
72

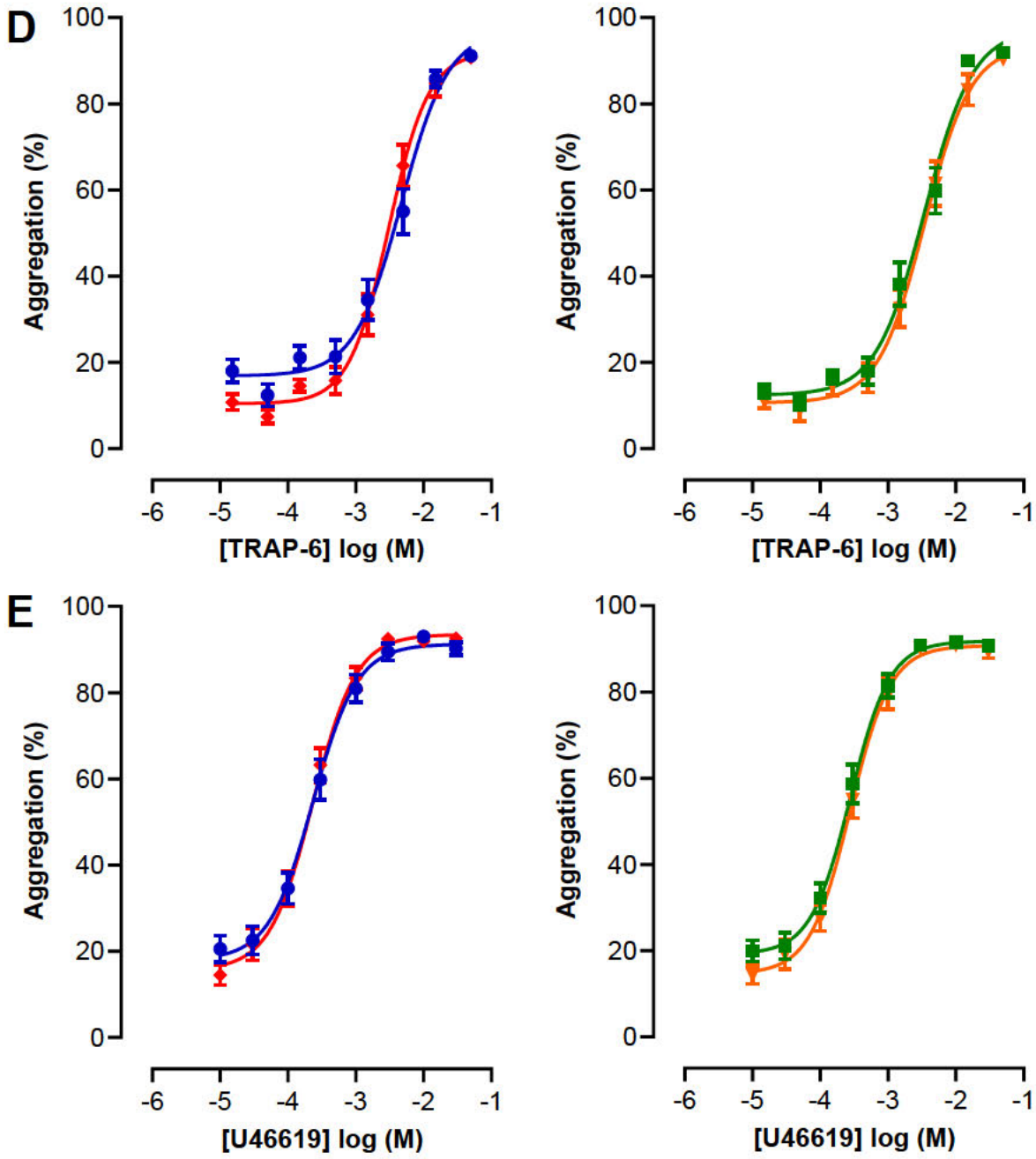


Figure 3.1. Effect of n-3 PUFA intervention on platelet aggregation in response to ADP, CRP-XL, epinephrine, TRAP-6 or U46619. Dose-response curves were constructed for ADP (A1-B2), CRP-XL (B1-C2), epinephrine (C1-D2), TRAP-6 (D1-E2) and U46619 (E1-F2) for each baseline and intervention. Each point represents the response of baseline for control oil (red diamond) or for fish oil (green square) against intervention for control oil (blue circle) or for fish oil (orange triangle). Data are shown as % aggregation values of mean ± SEM, (n=40). Comparisons after each intervention were drawn using 2-way ANOVA with the Turkey multiple comparisons test, with differences shown at $p < 0.05$. There was no significant effect of intervention on platelet aggregation in response to any platelet agonist (treatment effects: $p < 0.05$); ADP, adenosine diphosphate; TRAP-6, thrombin receptor activator peptide; CRP-XL, collagen-related peptides- cross-linking; EPI, epinephrine.

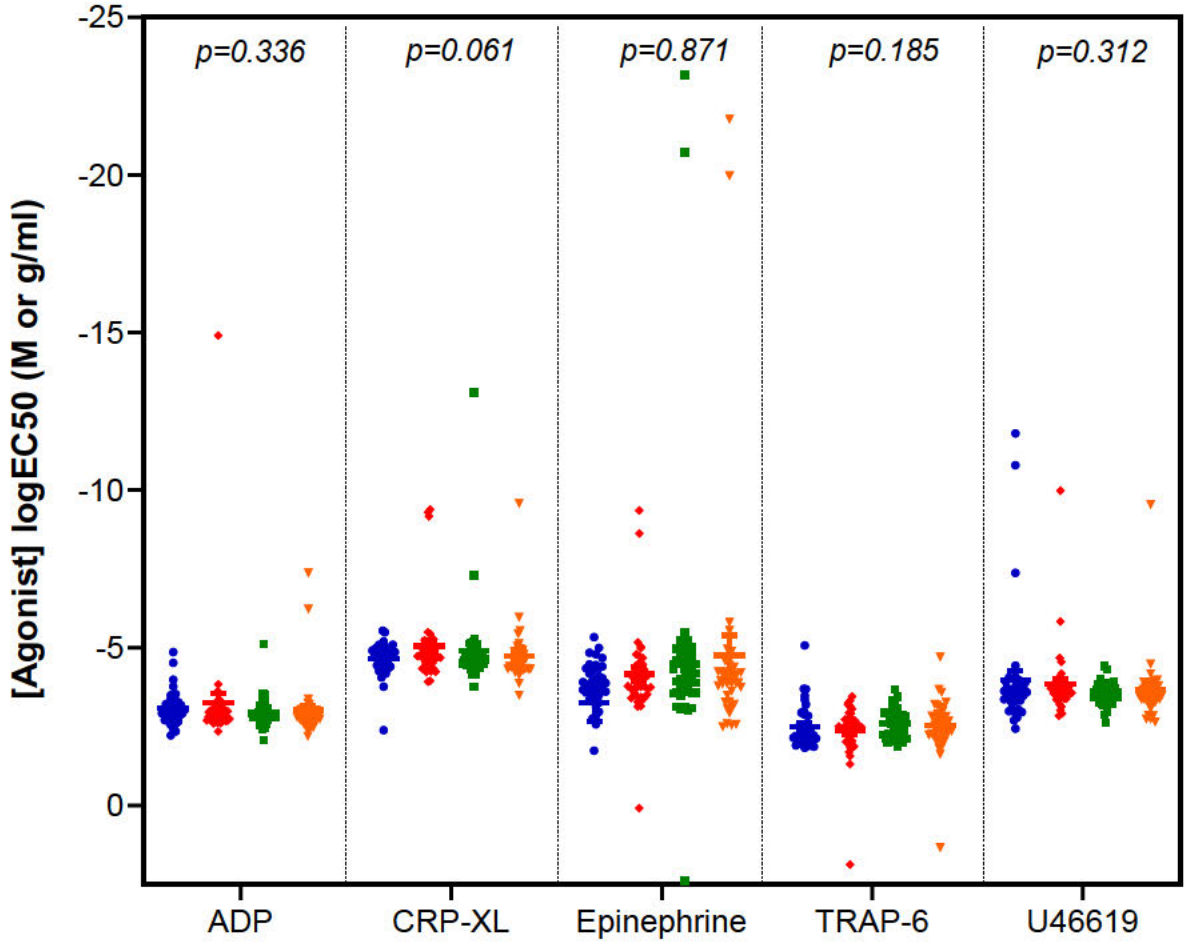


Figure 3.2. Effect of n-3 PUFA intervention on LogEC$_{50}$ values in response to ADP (0.03-100 µM), CRP-XL (0.001-3 µg/ml), epinephrine (0.003-10 µM), TRAP-6 (0.015-50 µM) and U46619 (0.01-30 µM). Scatter plot of LogEC50 (log concentration of agonist, M or mg/ml, giving a response half-way between maximum and minimum aggregation) values calculated from dose-response curves platelet responses to ADP, CRP-XL, epinephrine, TRAP-6 and U46619. Each point represents the response of baseline for control oil (red diamond) or for fish oil (green square) against intervention for control oil (blue circle) or for fish oil (orange triangle). Data are mean ± SEM, (n=40). Comparisons after each intervention were drawn using 2-way ANOVA with the Turkey multiple comparisons test, with differences shown at $p < 0.05$. There was no significant effect of intervention on LogEC50 of any platelet agonist (treatment effects: $p < 0.05$); ADP, adenosine diphosphate; TRAP-6, thrombin receptor activator peptide; CRP-XL, collagen-related peptides- cross-linking.

Table 3.4. Effect of n-3 PUFA intervention on additional platelet aggregation parameters

		Control Oil		Fish Oil		*p-value*
Agonists	**Parameters**	**Before**	**After**	**Before**	**After**	**treatment**
ADP	Maximum	26.77 ± 2.00	23.70 ± 1.68	26.35 ± 1.75	27.23 ± 2.07	*0.599*
	Minimum	90.64 ± 1.56	93.96 ± 1.35	90.30 ± 1.54	93.75 ± 1.85	*0.204*
	Hillslope	1.33 ± 0.18	1.31 ± 0.14	1.46 ± 0.20	1.34 ± 0.20	*0.935*
CRP-XL	Maximum	27.14 ± 2.34	22.09 ± 2.33	25.33 ± 2.06	24.70 ± 2.48	*0.515*
	Minimum	93.04 ± 1.39	93.83 ± 1.30	93.69 ± 1.39	94.07 ± 1.62	*0.960*
	Hillslope	1.51 ± 0.21	1.38 ± 0.17	1.50 ± 0.20	1.35 ± 0.19	*0.897*
Epinephrine	Maximum	26.38 ± 6.33	18.54 ±6.05	23.84 ± 4.83	22.56 ± 4.86	*0.823*
	Minimum	85.88 ± 3.92	87.20 ± 3.11	84.19 ± 2.89	80.39 ± 3.20	*0.575*
	Hillslope	0.81 ± 0.25	0.82 ± 0.19	0.94 ± 0.23	0.92 ± 0.24	*0.970*
TRAP-6	Maximum	16.89 ± 2.09	10.43 ± 1.81	12.46 ± 2.01	10.61 ± 2.05	*0.109*
	Minimum	96.97 ± 5.39	92.00 ± 3.29	97.58 ± 4.38	93.36 ± 4.26	*0.705*
	Hillslope	1.25 ± 0.24	1.47 ± 0.22	1.20 ± 0.19	1.27 ± 0.21	*0.815*
U46619	Maximum	19.30 ± 2.94	14.39 ± 2.94	18.92 ± 2.46	14.92 ± 2.49	*0.439*
	Minimum	91.73 ± 2.05	93.05 ± 1.83	91.64 ± 1.79	90.87 ± 1.86	*0.872*
	Hillslope	1.36 ± 0.23	1.33 ± 0.19	1.48 ± 0.23	1.46 ± 0.22	*0.948*

Data are mean ± SEM (n = 40). Maximum response, minimum and hill slope values were calculated from dose-response curves of platelet responses to ADP, CRP-XL, epinephrine, TRAP-6 and U46619. Comparisons after each intervention were drawn using 2-way ANOVA with the Turkey multiple comparisons test, with differences shown at $p < 0.05$. There was no significant effect of fish oil supplementation on any platelet aggregation related parameters; ADP, adenosine diphosphate; TRAP-6, thrombin receptor activator peptide; CRP-XL, collagen-related peptides- cross-linking.

3.3.4 Effects of n-3 PUFAs on fibrin clot coagulation

Clot formation and fibrinolysis were assessed using a thrombodynamics analyser (Hemacore, Russia). Intervention with fish oil significantly decreased the rate of clot growth (**Figure 3.3A**) and clot size at 30 min (**Figure 3.3E**). There was no significant effect of time and no treatment*time interaction. Although there was a trend for a decrease in the initial rate of clot growth (**Figure 3.3C**) and the stationary rate of clot growth (**Figure 3.3D**), and for an increase in lag time (**Figure 3.3B**) after fish oil supplementation, these were not statistically significant.

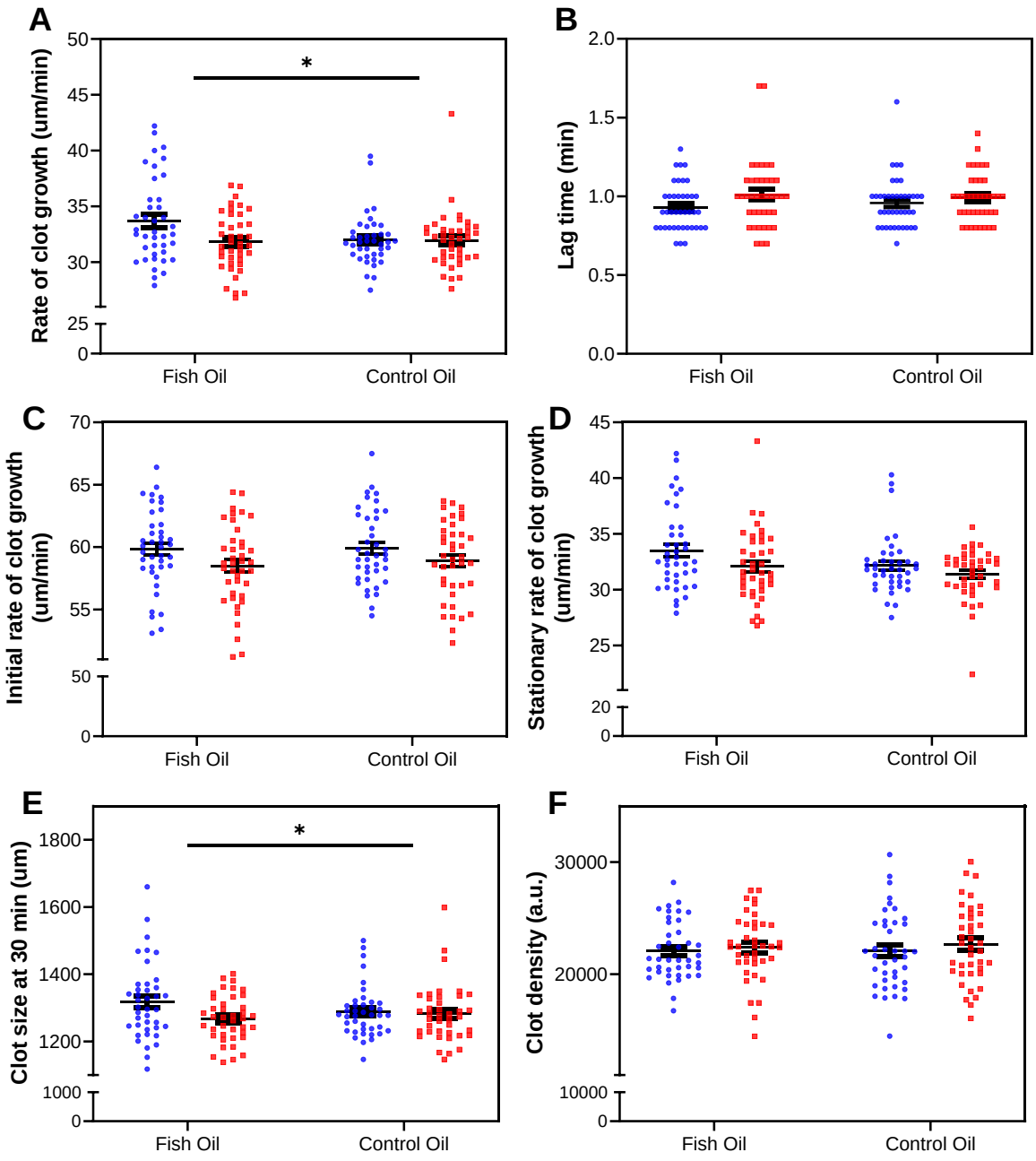


Figure 3.3. Effects of n-3 PUFA intervention on coagulation parameters in subjects at moderate risk for CVD. Each point represents the response of baseline (blue circle) either for control oil or baseline for fish oil against intervention (red circle) either for control oil or for fish oil. Data are mean ± SEM, (n=40). Comparisons after each intervention were drawn using General Linear Model (GLM), including pairwise comparison test with Bonferroni for treatment, period and treatment*time interaction, with differences shown at $p < 0.05$. There was a significant effect of fish oil on **(A)** rate of growth of PFP-derived clot and **(E)** clot size at 30 min (treatment effects: $p < 0.05$ and $p < 0.05$ respectively; general linear model). There was no significant effect of interventions on **(B)** lag time, **(C)** initial rate of clot growth, **(D)** stationary rate of clot growth and **(F)** clot density (treatment effects: $p < 0.05$; general linear model). **p<0.05*.

3.2.5 Effects of n-3 PUFAs on fibrinolysis

There was no effect of the intervention on fibrinolysis parameters, including lysis onset time or lysis progression, as shown in **Figure 3.4A** and **Figure 3.4B** respectively.

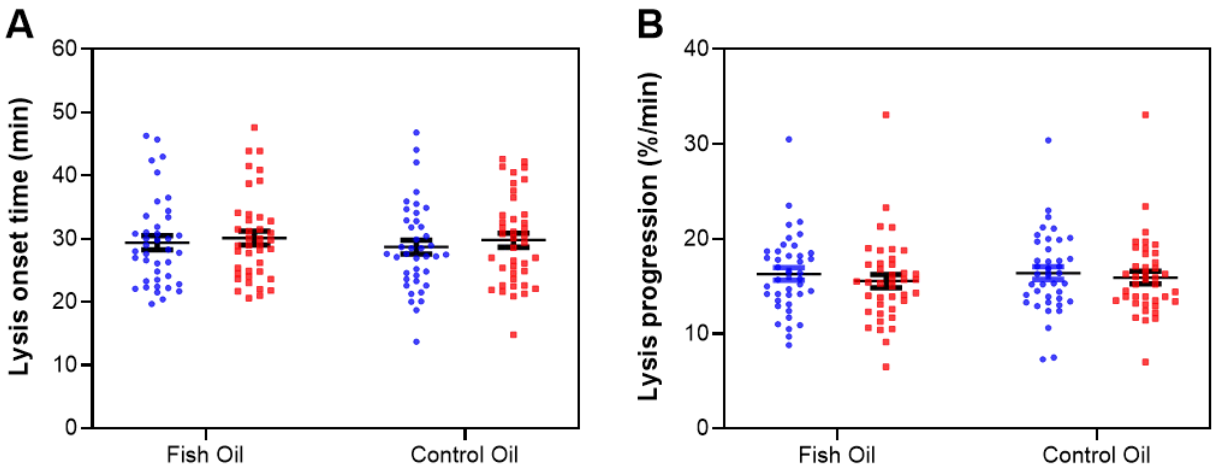


Figure 3.4. Effects of n-3 PUFA intervention on fibrinolysis parameters in subjects at moderate risk for CVD. Each point represents the response of baseline (blue circle) either for control oil or baseline for fish oil against intervention (red circle) either for control oil or for fish oil. Data are shown as mean ± SEM, (n=40). Comparisons after each intervention were drawn using General Linear Model (GLM), with differences shown at $p < 0.05$. There was no significant effect of fish oil on both **(A)** lysis onset time and **(B)** lysis progression (treatment effects: $p < 0.05$ and $p < 0.05$ respectively; general linear model). There was no significant effect of time and no treatment*time interaction (overall effects: $p > 0.05$; general linear model) for any fibrinolysis parameter (overall effects: $p > 0.05$; general linear model).

3.2.6 Effects of fish n-3 PUFAs on thrombin generation

Pooled VFP and pooled PFP from healthy subjects (n=3) were used for benchmarking purposes in the assessment of thrombin generation in PFP from subjects participating in the intervention study (**Figure 3.5).** Supplementation with fish oil resulted in prolonged lag time for thrombin generation, such that there was a main effect of treatment, but no time effect and no treatment*time interaction (**Figure 3.6A**). Peak thrombin generation, time to reach peak thrombin generation, velocity index and area under the curve were significantly reduced by fish oil (**Figures 3.6B to 3.6E**). For all parameters, there was a main effect of treatment, but no significant effect of time and no treatment*time interaction. The benchmarking data

demonstrates that thrombin generation in pooled PFP was approximately double that in VFP (**Figure 3.5**), and the absence of vesicles in VFP resulted in significantly prolonged lag time and time to reach peak thrombin generation **(Figures 3.6A and 3.6C)** as well as lower peak thrombin concentration, slope (velocity index) and area under curve **(Figures 3.6B, 3.6D and 3.6E)** compared to pooled PFP from the same subjects. Since the pooled PFP was from healthy subjects, comparison between this and PFP from subjects on the intervention study provides some insight into the effects of EVs from healthy subjects vs those with moderate CVD risk on thrombin generation parameters. In most cases, PFP from subjects with moderate CVD risk exhibited enhanced thrombin generation compared with that from healthy subjects, but fish oil reduced this to a level similar to that of healthy subjects (**Figure 3.6**).

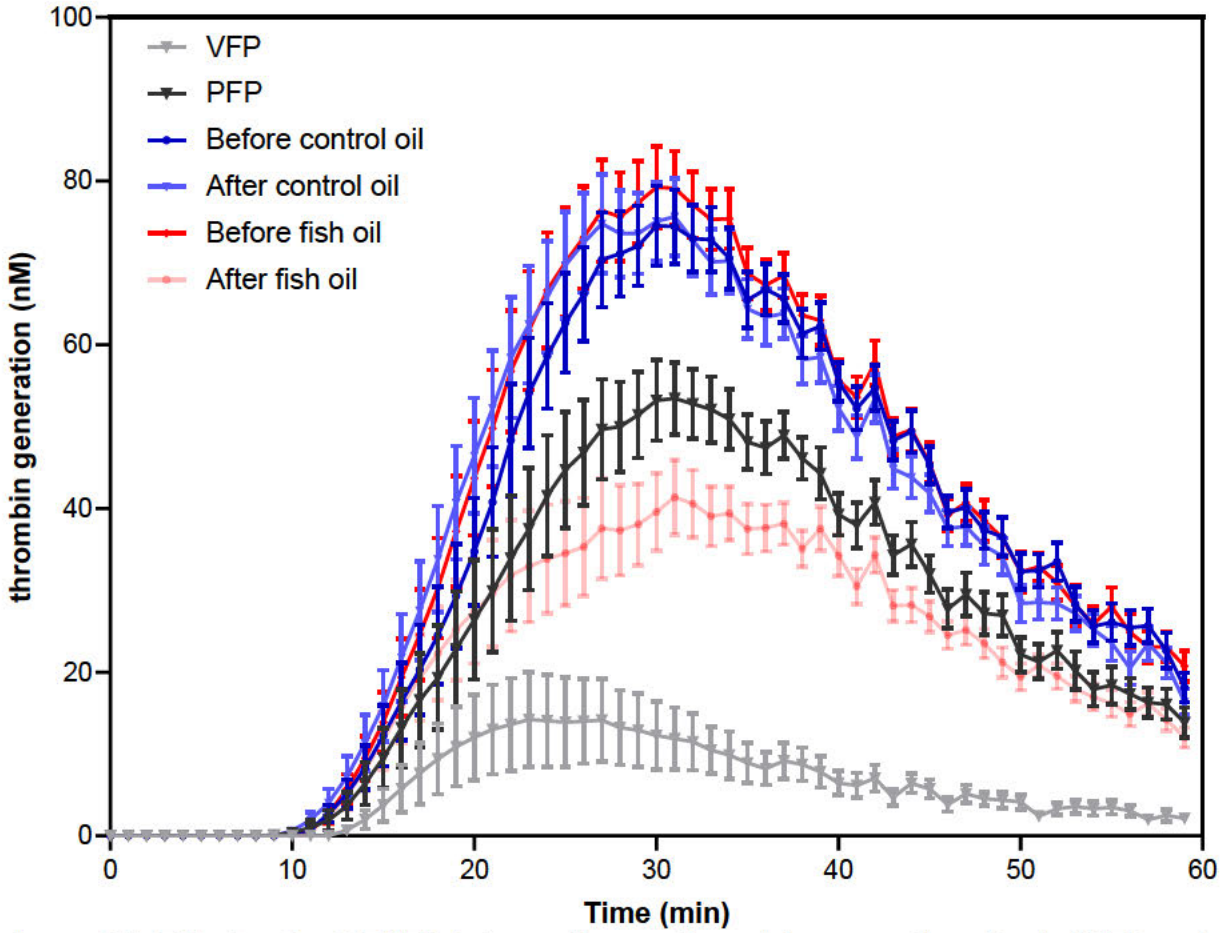


Figure 3.5. Effects of n-3 PUFA intervention on thrombin generation. Pooled VFP and pooled PFP were used for benchmarking purposes in the assessment of thrombin generation in PFP from subjects participating in the intervention study. Thrombin-dependent cleavage of a fluorogenic substrate was quantified over 60 minutes. Data are mean ± SEM, (n=3 for pooled VFP and PFP; n=40 for the intervention); VFP, vesicle-free plasma; PFP, platelet-free plasma.

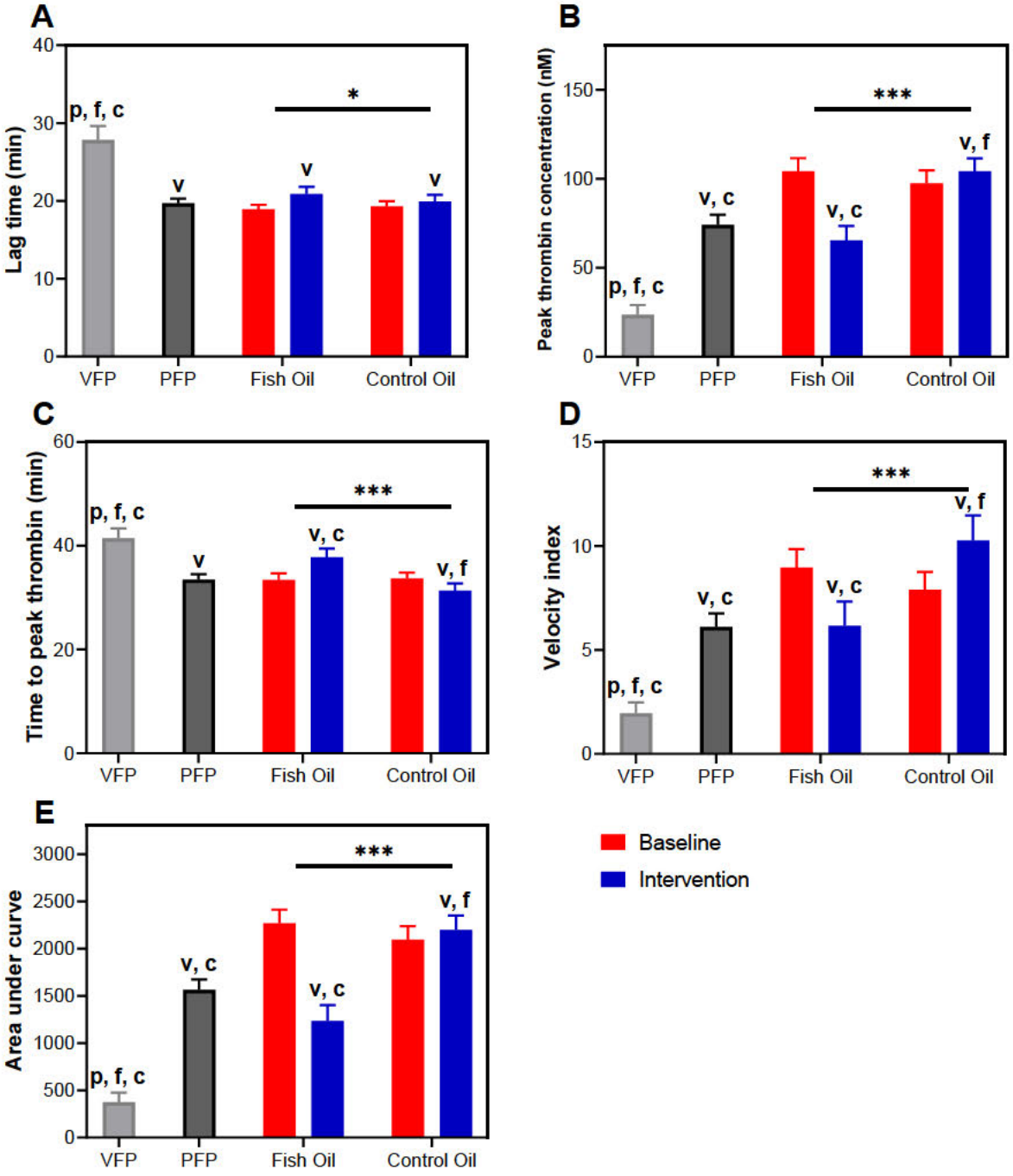


Figure 3.6. Effect of n-3 PUFA intervention on thrombin generation parameters. Data are mean ± SEM (n=40). Pooled VFP and pooled PFP were used for benchmarking purposes in the assessment of thrombin generation in PFP from subjects participating in the intervention study. Data were analysed using the General Linear Model (GLM), including pairwise comparison test with Bonferroni for treatment, period and treatment*time interaction with differences shown at $p < 0.05$. There was a significant effect of fish oil on **(A)** lag time for thrombin generation (treatment effect: $p < 0.05$; general linear model), **(B)** peak thrombin concentration, **(C)** time to reach thrombin, **(D)** velocity index and **(E)** area under curve (treatment effects: $p < 0.0001$; general linear model). Comparisons of the means between PFP, VFP, after fish oil intervention and after control oil intervention were drawn using one-way ANOVA, followed by the Tukey multiple comparison test, with differences shown as $p < 0.05$. The symbol of **p** denotes significantly different from PFP ($p < 0.05$); a symbol of **v** denotes significantly different from VFP ($p < 0.05$); a symbol of **f** denotes significantly different from fish oil post-intervention ($p < 0.05$) and a symbol of **c** denotes significantly different from after control oil post-intervention ($p < 0.05$). **p<0.05 and ***p<0.001*; VFP, vesicle-free plasma; PFP, platelet-free plasma.

3.3 Discussion

This randomised controlled crossover trial demonstrated that 12-weeks' supplementation with 1.8 g/d of n-3 PUFAs decreased blood pressure and plasma TAG concentration, increased LDL-cholesterol concentration, and decreased coagulation and EV-supported thrombin generation in subjects with moderate risk of CVD, although platelet aggregation and fibrinolysis remained unaffected.

Changes in intake of n-3 PUFAs can be rapidly reflected by the fatty acid composition of plasma phospholipids (Fisk et al., 2018, Visioli et al., 2004, Harris et al., 2007, Yaqoob et al., 2000), but incorporation of n-3 PUFAs tends to be negatively correlated with AA content in plasma phospholipid (Harris et al., 2007). Therefore, the fatty acid composition of plasma phospholipids is often used as a marker of compliance with fish oil-derived n-3 PUFA supplementation (Din et al., 2013b, Poreba et al., 2017, Kabir et al., 2007). In the current RCT, supplementation with 1.8 g/day of n-3 PUFA was reflected by a substantial increase in the n-3 PUFA content of both PC and PE compared to control oil, indicating good compliance. Fish oil supplementation resulted in an increase in the proportions of EPA, DHA and DPA in plasma PC, whereas in plasma PE, only EPA and DHA were elevated, for reasons which are unclear. In PC, the incorporation of n-3 PUFAs was at the expense of oleic acid and a range of n-6 PUFAs, whereas in PE, n-3 PUFA replaced only linoleic acid, DGLA and arachidonic acid. The incorporation of n-3 PUFAs into PC and PE by replacement of n-6 PUFA was reported by Mori *et al* (1987) (Mori et al., 1987), who investigated the effect of fish oil on the platelet PC and PE fractions. The incorporation of n-3 PUFAs by replacement of DGLA and arachidonic acid has also been demonstrated in plasma phospholipids (Yaqoob et al., 2000, Kew et al., 2004, Din et al., 2013a). However, data relating specifically to the influence of fish oil on the fatty acid composition of plasma PC and PE fractions is difficult to find. Replacement of oleic acid by n-3 PUFA is not reported in the literature, although it does appear to be a non-significant trend for decreased oleic acid in plasma phospholipid fatty acids after fish oil supplementation in at least two studies (Kew et al., 2004, Yaqoob et al., 2000). In the present

study, the significantly lower proportion of oleic acid in plasma PC following supplementation with fish oil may have been more evident because of the separate analysis of the PC and PE fractions.

In the present study, supplementation with n-3 PUFAs for 12 weeks reduced both SBP and DBP, although there was a time*treatment interaction for SBP only. The effect of n-3 PUFAs on blood pressure is broadly in agreement with published literature (AbuMweis et al., 2018) and a meta-analysis of 70 RCTs demonstrated a reduction in both systolic and diastolic blood pressure of 1.52 mm Hg and 0.99 mm Hg, respectively (Miller et al., 2014). The meta-analysis suggested that the effect of n-3 PUFA on diastolic blood pressure requires a dose of >2 g/day (Miller et al., 2014), although a separate systematic review failed to demonstrate dose-dependency (Colussi et al., 2017). The beneficial effect of fish oil on blood pressure has been explained by an improvement in endothelial function in response to reduced systemic vascular resistance (Mozaffarian, 2007) and the vasodilatory effects of eicosanoids, whose metabolism is altered by EPA and DHA (Abraham and Speth, 2019). Despite a lower dose than that employed by Miller *et al* (2014), the current trial provided confirmatory results for a reduction of SBP and DBP by 6.65 mm Hg and 2.93 mm Hg, respectively after n-3 PUFA.

A significant reduction in plasma TAG concentration is a hallmark of fish oil supplementation (EFSA Panel on Dietetic Products and Allergies, 2010, Abdelhamid et al., 2018, Eslick et al., 2009, Leslie et al., 2015) and the confirmatory results presented in this chapter therefore demonstrate the efficacy of the intervention and overall compliance of the subjects. The mechanism involves reduced production of hepatic very low density lipoprotein level and increased TAG clearance from the plasma (Mohebi-Nejad and Bikdeli, 2014). The current intervention also led to a small, but significant, increase in the concentration of LDL-cholesterol, which has been observed in other trials (Backes et al., 2016, Oelrich et al., 2013, Englyst et al., 2007), reported in a meta-analysis (AbuMweis et al., 2018) and suggested to be due to increased conversion of VLDL to LDL after fish oil supplementation (Barceló-Coblijn and Murphy, 2009). There was no effect of n-3 PUFA on either HDL-cholesterol or total-

cholesterol concentrations, which is in agreement with some reports (Cottin et al., 2016, Poreba et al., 2017, Root et al., 2013). Overall, therefore, the data supports the published effects of n-3 PUFAs on the plasma lipid profile.

Given the uncertainties about the effects of n-3 PUFAs on fibrin clot properties highlighted in the introduction to this chapter (**Table 3.1**), the current study investigated fibrin clot properties using a thrombodynamics analyser, which facilitates the measurement of both coagulation and fibrinolysis at the same time to better model physiological conditions. To the best of our knowledge, this is the first dietary intervention study to assess the effect of n-3 PUFAs on fibrin clot properties using the spatial clot growth assay, providing a real-time observation of clot growth and lysis. Supplementation with n-3 PUFAs decreased the propagation stage of the fibrin clot (rate of clot growth) and this was accompanied by a reduction in overall fibrin clot formation (clot size at 30 min). However, other coagulation parameters remained unchanged. Furthermore, there was no significant effect of n-3 PUFAs on fibrinolysis parameters, including lysis onset time and lysis progression. The study therefore demonstrated that n-3 PUFAs reduced clot formation but had no effect on clot lysis.

Although there are no similar studies with which to compare these results directly, earlier studies in various populations tend to agree with the current study. Of the confirmatory studies, there was no evidence regarding a dose-dependent response of n-3 PUFAs. Favourable effects of n-3 PUFA on coagulation were reported at doses ranging from 0.6 g to 3.36 g per day (compared with controls) as detailed in **Table 3.1**, but a contradictory result was reported by a recent RCT, where 2 g/d of n-3 PUFA supplementation did not affect coagulation (Poreba et al., 2017). The duration of studies reporting favourable effects of n-3 PUFAs on coagulation ranged from 4 weeks to 8 weeks, which suggests that 4 weeks of supplementation is sufficient. However, Poreba *et al* (2017) reported that 12 weeks of intervention at a dose of 2 g/d had no effect on coagulation. Although the majority of previous studies supported substantial reduction in coagulation following supplementation with fish oil, the lack of an unequivocal effect on coagulation could be due to a combination of the variation in doses of n-3 PUFAs

and the duration of intervention among previously published studies; thus the overall picture is not entirely clear.

Previous studies on fish oil and coagulation can also be evaluated from the perspective of whether they were conducted in healthy subjects or those with diagnosed disease. Only one study investigated the effect of fish oil on coagulation in both health and disease (CVD). However, this study was not only uncontrolled, but the sample size of subjects with CVD was very low (n=16) in comparison to the healthy group (n=40) (McEwen et al., 2015). Two further studies lacked a control treatment (Vanschoonbeek et al., 2004, Bagge et al., 2018), and were therefore largely disregarded, although they were in agreement with the current study. On the other hand, there were well-controlled studies using different methods of analysis, which reported findings consistent with the present study. For example, daily supplementation with 1 g/d n-3 PUFAs significantly reduced fibrin clot formation in subjects with stable CAD (Gajos et al., 2011). An improvement in activated factor II was reported in subjects with hyperlipemia after intervention with 3.36 g/d of n-3 PUFAs (Nordøy et al., 2000). Furthermore, fish oil had the ability to induce reductions in the level of factor II as well as the levels of factor V, fibrinogen, factor VII and factor X following an 8-week supplementation with high dose n-3 PUFAs (3.1 g/d) per day in subjects with diabetes mellitus (Vanschoonbeek et al., 2007). Although the current study does not report levels of coagulation factors, their contribution to the process of coagulation is relevant. Factor V plays an important role in clot formation as it leads to the conversion of prothrombin (factor II) to thrombin, which may have implications for potential mechanism of reduced coagulation by n-3 PUFAs (Tavares-Dias and Oliveira, 2009), but very little is known regarding the mechanism. In contrast with results of Vanschoonbeek *et al* (2007), intervention with 2 g/d of n-3 PUFAs for 12 weeks in subjects with atherosclerosis and diabetes mellitus had no effect on levels of fibrinogen (Poreba et al., 2017). This may be due to a more pronounced effect of fish oil on fibrinogen in individuals with a structural fibrinogen alpha-chain polymorphism (Vanschoonbeek et al., 2004). Some reports suggests that doses of n-3 PUFAs as low as 0.64 g/d are sufficient to reduce coagulation in healthy

subjects (McEwen et al., 2015); however, in one case this study was uncontrolled and therefore of lower quality and in another (controlled) study, the effect was only observed in males (Phang et al., 2014). Questions about effective dose and possible differences in response between sexes and between health vs non-healthy subjects therefore remain. The strength of the present study was to employ a relatively high dose of n-3 PUFAs in subjects with CVD risk (and therefore not healthy, but also free of diagnosed disease). Studying an 'at-risk' group such as this is particularly valuable in terms of disease prevention and is arguably more biologically responsive to intervention. In addition, the method used to assess the effect of n-3 PUFAs on coagulation in the present RCT aimed to imitate the damage of vessel wall with the detection of spatial fibrin clot growth differently from the existing methods (Koltsova et al., 2020). Therefore, the current RCT extended the exciting data as it is the first to evaluate the effect of n-3 PUFAs on coagulation using a sensitive method in subjects with moderate risk for CVD.

In the haemostatic system and coagulation cascade, thrombin plays a very important role as a potent cell-signalling mediator, suggesting involvement of thrombin generation in the development and progression of cardiovascular disease (Crawley et al., 2007). The current RCT demonstrated that subjects with moderate risk for CVD had significantly greater levels of peak thrombin and area under curve compared to healthy subjects, but this difference disappeared when subjects were supplemented with n-3 PUFAs. An elevated level of thrombin generation has been found to be associated with venous thromboembolism (Lutsey et al., 2009), acute ischemic stroke (Carcaillon et al., 2011) and conventional risk markers of CVD (e.g. obesity) (van Paridon et al., 2020), indicating that higher thrombin generation might be expected in subjects at risk for CVD. This is not always the case when healthy subjects are compared with CVD patients, but this may be because subjects with CVD are often being successfully treated with anti-platelet and cholesterol lowering medication (Kromhout et al., 2010). The assessment of thrombin generation in VFP provided insight into the impact of removal of EVs on thrombin generation. The current study provided clear evidence that the

presence of EVs in plasma enhanced tissue factor-stimulated thrombin generation, suggesting an important contribution of these vesicles to thrombin generation stimulated through the tissue factor pathway (Kaufmann et al., 2006, Bidot et al., 2008, Tripisciano et al., 2017a). EV-induced thrombin generation will be assessed in more detail in further chapters.

The present study demonstrated for the first time that supplementation with n-3 PUFAs reduced thrombin generation in PFP, as reflected by increased lag time and time to peak thrombin, decreased peak thrombin, velocity index and area under curve. This result is in accordance with several well-controlled studies, where fish oil resulted in moderate improvements in different aspects of thrombin generation in healthy subjects (McEwen et al., 2015) as well as patients with CAD (Gajos et al., 2011) and diabetes mellitus (Vanschoonbeek et al., 2007) and also with some uncontrolled and/or small studies (Vanschoonbeek et al., 2004) (McEwen et al., 2015). However, Phang *et al* (2014) reported that 0.6 g/d n-3 PUFAs for 4 weeks did not affect thrombin generation in healthy subjects, perhaps due to the combination of a low dose and short duration. There is a limited number of published studies examining the effects of higher doses of n-3 PUFAs on thrombin generation in healthy subjects (and no studies in subjects with mild/moderate risk of CVDs). The beneficial effect of fish oil may be more evident in the present study due to the subject profile; indeed, a higher level of plasma TAG (risk marker for CVD) is reported to be significantly associated with a greater degree of reduction in thrombin generation by fish oil (Vanschoonbeek et al., 2007). However, this was not the case in adolescents with hypertriglyceridemia supplemented with 4 g/d n-3 PUFA (Gidding et al., 2014), although the age of the subjects and the inadequate washout period of 4 weeks may have contributed to this.

According to the study of Vanschoonbeek *et al* (2007), a high dose of fish oil (3.1 g/d) resulted in moderate improvements in different aspects of thrombin generation in patients with diabetes mellitus (Vanschoonbeek et al., 2007). However, Poreba *et al* (2017) failed to report any change in thrombin generation in subjects with atherosclerosis and type 2 diabetes mellitus, although this study was conducted using a similar dose (2 g/d) of n-3 PUFAs and

was of a similar duration (12 weeks) to the present study. The discrepancy might be at least partly due to the fact that the patients were on lipid-lowering medication, which is known to influence thrombin generation (Ferroni et al., 2012). As indicated, a strength of the current study is that it investigates the effects of fish oil in an at-risk group in the absence of any prescribed medication, thereby focussing on potential prevention of future disease.

As described in the introduction to this chapter, the effects of n-3 PUFAs on platelet aggregation are unclear. Beneficial influences had been anticipated due to the fact that n-3 PUFAs, particularly EPA, are incorporated into platelet membrane phospholipids, replacing n-6 PUFAs, including arachidonic acid (AA) (Adili et al., 2018). This has been suggested to alter the synthesis of eicosanoids, such as thromboxane A_2 (TXA_2) , which is a potent pro-aggregatory eicosanoid, and to increase the release of anti-platelet prostaglandins (PGI_2), and thromboxanes (TXA_3), which contribute to a reduction in platelet aggregation (Wander and Patton, 1991). In the current study, platelet aggregation was assessed in response to eight concentrations of ADP, CRP-XL, epinephrine, TRAP-6, and U46619 in PRP from subjects supplemented with fish oil. However, in agreement with the most earlier studies, supplementation with n-3 PUFAs did not affect the extent of platelet aggregation in response to ADP (Harris et al., 2008, Veljović et al., 2013, Poreba et al., 2017), CRP-XL (McEwen et al., 2013), epinephrine (McEwen et al., 2013, Castaño et al., 2006), TRAP-6 (Bagge et al., 2018, Mackay et al., 2012), or U46619 (McEwen et al., 2013). It seems that a large body of evidence has been published to evaluate platelet functions in response to n-3 PUFAs, yet comparability is limited by the wide variety of protocols using different agonists and concentrations. In the current study, the use of a high-throughput platelet function assay with a wide range of concentrations of different agonists strongly indicates a lack of effect of n-3 PUFAs. On the other hand, in a recent systematic review by *Begtrup et al* (2017), fish oil was demonstrated to inhibit platelet aggregation in a healthy population. Similarly, a meta-analysis of 15 RCTs suggests inhibitory effects of n-3 PUFAs, particularly in the case of ADP-induced platelet aggregation, despite the significant heterogeneity in the results. In this meta-analysis,

however, the effects were only evident in subjects with poor-health status and in shorter-term studies (<8 weeks) (Gao et al., 2013). The lack of effect of n-3 PUFAs on ADP-induced platelet aggregation in the current study may therefore be due to an adaptive effect of longer-term supplementation (12 weeks), but this requires further evaluation. The dose of n-3 PUFA employed may also be important as higher doses are suggested to be more effective in inhibiting platelet aggregation (Tremoli et al., 1995). Although the literature is not consistent, most studies administering high doses of n-3 generally achieve significant reductions in platelet aggregation (Tomic-Smiljanic et al., 2019, Woodman et al., 2003, Baldassarre et al., 2006). Overall, the current study reinforces the fact that there is a lack of convincing evidence for beneficial effects of a moderate dose of fish oil on platelet aggregation, as assessed by currently applied methodologies.

In the current study, 1.8 g/day of n-3 PUFAs also failed to alter fibrinolysis, determined by measuring lysis onset time and lysis progression. This result is in agreement with a systematic review, where the lack of effect of supplementation with fish oil on fibrinolysis was reported (Begtrup et al., 2017). Furthermore, four out of eight studies using high doses of n-3 PUFAs (ranging from 2 g to 4 g daily) reported no effect of fish oil on fibrinolysis in hyperlipidemic (Nordøy et al., 2000), hypertensive and diabetic patients (Woodman et al., 2003), patients with previous myocardial infarction (Din et al., 2013b) or atherosclerosis and diabetes mellitus (Poreba et al., 2017). However, it is important to note that an earlier report by Din *et al* (2013) did demonstrate a increase in the level of tPA during supplementation with high dose n-3 PUFAs (2 g/d) in subjects who were healthy smokers. Also, three studies reported that lower doses of n-3 PUFA (ranging from 0.64 g to 1.8 g daily) increased fibrinolytic parameters in patients with diabetes mellitus (Kabir et al., 2007), CAD (Gajos et al., 2011) and in healthy subjects (McEwen et al., 2015). Hence, it is difficult to explain discrepancies in these findings, but it is possible that inconsistent outcomes could be attributed to different protocols used to evaluate fibrinolysis. The most commonly used method was the measurement of the levels of PAI-1 and tPA in plasma, which have important roles in the regulation of fibrinolysis as an

inhibitor and activator, respectively (Longstaff, 2018). Although reduced PAI-1 and increased tPA levels indicated improved fibrinolysis (Din et al., 2013a), and are thereby associated with improvement in the risk of CVDs, this does not necessarily equate with thrombogenic potential (Longstaff, 2018). On the other hand, the present study evaluated fibrinolysis using the spatial dynamics of the fibrinolytic system using a thrombodynamics analyser, which monitors dissolution of the fibrin clot following its formation. To our knowledge, this method has not previously been used to assess the effect of supplementation with n-3 PUFA on fibrinolysis. The main advantage of this assay might be sensitivity of the assay to changes in the fibrinolytic pathways (Koltsova et al., 2020).

3.4 Conclusion

This well-designed study provides support to the existing evidence regarding the beneficial effects of n-3 PUFAs on CVD risk markers and new evidence relating to thrombogenic risk markers in subjects with moderate risk for CVDs. It suggests that 12 weeks supplementation with a moderate dose of n-3 PUFAs decreased BP and TAG concentration in plasma, significantly reduced the formation of a fibrin clot and thrombin generation but did not influence clot breakdown or platelet function. These observations contribute to understanding of the mechanisms by which n-3 PUFAs have beneficial effects on cardiovascular health. Effects of n-3 PUFAs on thrombin generation might be due to alterations in the membrane composition of circulating procoagulant EVs in the plasma. Studies investigating the potential procoagulant effects of the circulating EVs and the influence of n-3 PUFAs on them are explored in subsequent chapters in this thesis.

Chapter 4. Relationship between circulating EV number and thrombogenic risk markers for CVD

4.1 Introduction

Thrombogenesis predisposes to cardiovascular diseases (CVD) (Raskob et al., 2014). Increased thrombogenic risk factors, characterised by hypercoagulation (enhanced clot formation), altered clot fibrinolysis (Kotze et al., 2014, Woodman et al., 2003), increased platelet function (Renga and Scavizzi, 2017, Adili et al., 2018), and increased thrombin generation (Al Dieri et al., 2012, Loeffen et al., 2015, Carcaillon et al., 2011, Lutsey et al., 2009) are suggested to contribute to endothelial dysfunction and abnormal angiogenesis, and therefore, higher risk of future CVD. **Chapter 3** (**Section 3.3.6**) demonstrated that subjects with moderate risk for CVD had significantly higher levels of thrombin generation compared to healthy subjects, indicating that increased level of thrombin generation in the circulation is associated with CVD.

Evidence for involvement of established risk factors in the development of atherothrombotic CVD (Mozaffarian et al., 2008) hypertension (Felmeden et al., 2003, von Känel et al., 2001, Junker et al., 1998), obesity (Yang and Christine De Staercke, 2012, van den Born et al., 2011, Allman-Farinelli, 2011), insulin resistance and hyperglycemia (Sambola et al., 2003b, Tripodi et al., 2011, Baalbaki and Bell, 2007) and hyperlipidaemia (Sambola et al., 2003b) associated with increased thrombogenesis, suggesting a link between these risk markers and thrombogenic tendency (Sambola et al., 2003a). Several studies also demonstrated that increased thrombogenic risk factors are associated with an adverse blood lipid profile (Kim et al., 2015, Morange et al., 2006), a high body mass index (BMI) (Morelli et al., 2017, Wannamethee et al., 2005, Morange et al., 2006) and high blood pressure (Elias et al., 2019, Wirtz et al., 2006). While conventional risk markers appear to be linked to thrombogenesis, there is little information about a link with circulating EVs, which could potentially play a role in thrombogenesis by virtue of their phospholipid surface and bioactive molecules, both on

their surface and in their internal cargo, which provide procoagulant capability (Zarà et al., 2019). Increased numbers of extracellular vesicles (EVs) have been reported in several thrombotic complications (Zwicker et al., 2009, Owens III and Mackman, 2011, Chirinos et al., 2005) and greater thrombogenic potential (Bucciarelli et al., 2012, Campello et al., 2016, Campello et al., 2018), including elevated clot formation (Silachev et al., 2019, Sinauridze et al., 2007) and increased thrombin generation (Tripodi et al., 2011, Ayers et al., 2014, Nielsen et al., 2018, Sinauridze et al., 2007)**. Chapter 3 (Section 3.3.6)** provided clear evidence that plasma EVs induced greater tissue factor-stimulated thrombin generation, suggesting an important contribution of EVs to this component of thrombogenic risk, in agreement with other studies (Kaufmann et al., 2006, Bidot et al., 2008, Tripisciano et al., 2017a, Pluchart et al., 2021, Zubairova et al., 2015b, Marchetti et al., 2014). However, there is little information about the relationship between circulating EVs numbers and thrombogenic risk markers.

Therefore, the aim of this research was to investigate whether higher numbers of EVs are associated with increased thrombogenic risk markers in subjects with moderate risk for CVD, and how these associations compare with those between conventional risk markers and thrombogenic risk using baseline data.

4.2 Results

4.2.1 Characteristics of the study population

Baseline characteristics of forty subjects with moderate risk for CVD are presented in **Table 4.1**. Some variables did not present as normal distributions, therefore common logarithmic transformation was applied for analysis. The median age was 65 y, and 24 out of 40 subjects were males. The mean and median values for SBP, DBP, TAG, HDL-C and glucose level were within normal reference ranges (SBP was within parameters for pre-hypertension and glucose concentration was within parameters for pre-diabetes).

Table 4.1. Characteristics of the Study Subjects

Sample Characteristic	All Subjects (n=40)
Age	64.5 (5.0)
BMI (kg/m^2)	25.4 ± 0.5
SBP (mmHg)	134.0 ± 2.2
DBP (mmHg)	78.8 ± 1.4
TC (mmol/l)	6.0 ± 0.2
TAG (mmol/l)	1.2 (0.9)
HDL-C (mmol/l)	1.6 (0.3)
TC/HDL ratio	3.9 ± 0.1
Glucose (mmol/l)	5.6 (0.8)
Risk (%)	10.9 (5.9)
TEVs (per ml blood) (NTA)	$3.6*10^{10}$ ($3.3*10^{10}$)
SEVs (per ml blood) (NTA)	$3.4*10^{10}$ ($3.1*10^{10}$)
LEVs (per ml blood) (NTA)	$1.3*10^{9}$ ($1.5*10^{9}$)
EVs Mean Size (nm)	98.0 ± 11.4
EVs Mode Size (nm)	73.7 ± 6.8
PS+EVs (per ml blood) (FCM)	$3.5*10^{7}$ ± $2.3*10^{6}$
PDEVs (per ml blood) (FCM)	$2.2*10^{7}$ ± $1.2*10^{6}$
EDEVs (per ml blood) (FCM)	$1.3*10^{6}$ ± $6.3*10^{5}$

Data which were normally distributed are shown as mean ± SEM and data which were not normally distributed are shown as median (inter-quartile range). BMI, body mass index; DBP, diastolic blood pressure; EDEVs, endothelial-derived extracellular vesicles; FCM, flow cytometry; HDL, high-density lipoprotein cholesterol; LEVs, large total extracellular vesicles (201nm-1000nm); NTA, nanoparticle tracking analysis; PDEVs, platelet-derived extracellular vesicles; PS+EVs, phosphatidylserine positive extracellular vesicles; SBP, systolic blood pressure; SEVs, small total extracellular vesicles (70-200nm); TAG, triacylglycerol; TC, total cholesterol; TEVs, total extracellular vesicles.

4.2.2 Association between conventional risk markers and fibrin clot properties, thrombin generation and platelet aggregation

Univariate regression analysis (**Table 4.3**) showed that higher DBP and plasma TAG concentration was associated with increased rate of clot growth, which relates to the propagation stage of coagulation (**Figure 4.1A and 4.1B**), although the initial rate of clot growth (the earliest stage of propagation of coagulation) was only associated inversely with plasma TC concentration (r=-0.330, p=0.038). Higher plasma TAG concentration was associated with increased clot size measured at 30 min, but there was a negative association with plasma HDL concentration (**Figure 1C and 1D**). Also, TC/HDL ratio was inversely associated with lysis onset time and positively associated with lysis progression (r=-0.410, p=0.009 and r=0.417, p=0.008, respectively).

Univariate regression analysis demonstrated that higher BMI and plasma TAG concentration were associated with increased thrombin generation, reflected by lag time for thrombin generation, peak thrombin concentration, velocity index and AUC (**Table 4.4** and **Figure 4.2A~4.2F**). DBP was positively associated with velocity index and negatively with time to peak thrombin generation (r=-0.341, p=0.031 and r=0.328, p=0.0039, respectively).

There was little association between conventional risk markers and platelet aggregation, apart from a positive association between SBP and U46619-induced platelet aggregation and an inverse association with plasma TC concentration (**Table 4.2**).

Table 4.2. Correlations between emerging (EV numbers, sizes and subpopulations) and conventional risk markers and thrombogenic risk factors

			Emerging risk markers								Conventional risk markers									
			NTA			FCM			NTA											
			TEVs (ml blood)	SEVs (ml blood)	LEVs (ml blood)	PS+ EVs (ml blood)	PDEVs (ml blood)	EDEVs (ml blood)	Mean size (nm)	Mode size (nm)	Age	BMI (kg/m^2)	SBP (mmHg)	DBP (mmHg)	TC (mmol/L)	TAG (mmol/L)	HDL (mmol/L)	TC/ HDL ratio	Glu (mmol/L)	Risk (%)
Clot formation	**Rate of clot growth (um/min)**	r	**.568****	**.581****	.179	.051	.083	**.319***	-.175	.159	-.160	.278	.086	**.353***	-.198	**.441****	-.284	.076	.150	.170
		p	**.000**	**.000**	.268	.754	.611	**.045**	.281	.326	.324	.082	.598	**.026**	.221	**.004**	.076	.640	.355	.295
	Lag time (min)	r	.022	.021	.047	.180	-.005	.097	-.046	-.018	-.142	.145	-.162	.016	.228	.244	.179	.054	-.060	-.165
		p	.895	.897	.776	.267	.974	.552	.780	.912	.381	.373	.318	.921	.157	.129	.269	.741	.714	.310
	Initial rate of clot growth (um/min)	r	.251	.261	-.037	-.019	-.074	.127	**-.330***	-.105	.054	.040	-.052	.015	**-.445****	.206	-.151	.220	.196	.074
		p	.118	.104	.821	.909	.652	.436	**.038**	.519	.743	.806	.752	.929	**.004**	.202	.353	.172	.225	.649
	Clot size at 30 min	r	**.480****	**.493****	.095	.085	.069	.291	-.235	.019	.046	.176	.000	.234	-.258	**.440****	**-.482****	.197	.152	.300
		p	**.002**	**.001**	.561	.601	.670	.068	.144	.906	.780	.277	.999	.147	.108	**.005**	**.002**	.223	.348	.060
	Clot density	r	.000	-.015	.208	.230	.025	-.005	.116	.064	.188	.166	.020	-.005	.207	.046	-.031	.199	-.146	.084
		p	.999	.928	.197	.154	.877	.976	.477	.697	.245	.306	.905	.075	.200	.776	.850	.219	.369	.604
Fibrinolysis	**Lysis onset time**	r	.171	.165	.168	.174	.058	**-.410****	-.146	-.128	.171	.033	.165	.165	.168	.174	.058	**-.410****	-.146	-.128
		p	.290	.308	.299	.284	.720	**.009**	.369	.432	.290	.814	.308	.309	.299	.284	.720	**.009**	.369	.432
	Lysis progression	r	-.115	-.099	-.274	-.146	-.036	**.417****	-.001	.074	-.115	-.149	-.099	-.282	-.274	-.146	-.036	**.417****	-.001	.074
		p	.480	.542	.087	.370	.827	**.008**	.995	.649	.480	.359	.542	.078	.087	.370	.827	**.008**	.995	.649

Table 4.2. Correlations between emerging (EV numbers, sizes and subpopulations) and conventional risk markers and thrombogenic risk factors (continue)

			Emerging risk markers								Conventional risk markers									
			NTA			FCM			NTA											
			TEVs (ml blood)	SEVs (ml blood)	LEVs (ml blood)	PS+ EVs (ml blood)	PDEVs (ml blood)	EDEVs (ml blood)	Mean size (nm)	Mode size (nm)	Age	BMI (kg/m²)	SBP (mmHg)	DBP (mmHg)	TC (mmol/L)	TAG (mmol/L)	HDL (mmol/L)	TC/ HDL ratio	Glu (mmol/L)	Risk (%)
Platelet aggregation	**ADP**	r	.156	.147	.205	.055	-.099	.056	-.029	-.076	-.026	.226	.100	.011	-.114	.203	-.186	.075	**-.359***	-.122
		p	.335	.364	.205	.877	.545	.731	.861	.641	.871	.160	.541	.948	.483	.209	.252	.646	**.023**	.454
	CRP-XL	r	.063	.058	.072	-.041	-.066	-.092	.290	.277	.082	.024	-.159	-.265	-.003	.105	-.082	.066	**-.488****	.073
		p	.700	.722	.657	.758	.686	.571	.069	.084	.613	.882	.328	.099	.986	.520	.617	.685	**.001**	.652
	EPI	r	.198	.188	.260	.130	.260	.254	.197	.173	-.199	.198	.206	.160	.085	.072	-.129	.181	-.026	.068
		p	.228	.251	.110	.404	.110	.118	.228	.291	.225	.227	.208	.330	.607	.664	.434	.271	.877	.680
	TRAP-6	r	.180	.172	.286	.209	-.025	.236	-.004	-.001	-.097	.181	.056	.212	-.002	.107	.024	-.012	.101	-.046
		p	.267	.289	.073	.208	.880	.143	.982	.994	.552	.264	.732	.190	.989	.511	.881	.941	.535	.776
	U46619	r	.095	.088	.148	-.006	-.065	.049	**-.338***	**-.382***	-.107	.145	**.394***	.211	**-.384**	-.007	-.050	-.268	.040	-.196
		p	.559	.587	.361	.820	.692	.763	**.033**	**.015**	.510	.372	**.012**	.191	**.014**	.965	.760	.095	.806	.226
Thrombin generation	**Lag time (min)**	r	**-.384***	**-.379***	-.288	-.113	.150	-.208	.040	-.161	-.051	**-.314***	.080	-.170	.255	**-.345***	.085	.146	.043	.011
		p	**.015**	**.016**	.072	.486	.356	.199	.806	.320	.756	**.048**	.626	.295	.113	**.029**	.602	.367	.791	.948
	Peak thrombin concentration (nM)	r	**.588****	**.590****	.301	.195	-.091	.118	-.011	.238	.134	**.433****	-.009	.249	-.074	**.405****	-.255	.132	-.037	.291
		p	**.000**	**.000**	.059	.228	.577	.468	.947	.139	.410	**.005**	.957	.122	.651	**.010**	.112	.417	.822	.069
	Time to peak thrombin (min)	r	**-.453***	**-.453***	-.311	-.075	.080	-.212	.110	-.079	-.017	-.241	-.019	**-.341***	.151	-.276	-.011	.127	-.065	-.011
		p	**.003**	**.003**	.051	.648	.624	.190	.498	.630	.917	.134	.909	**.031**	.351	.085	.944	.436	.691	.945
	Velocity index	r	**.577****	**.580****	**.320***	.159	-.059	.143	-.073	.152	.064	**.371***	.019	**.328***	-.065	**.352***	-.146	.062	.044	.200
		p	**.000**	**.000**	**.044**	.326	.717	.379	.653	.349	.693	**.018**	.908	**.039**	.688	**.026**	.369	.706	.787	.217
	AUC	r	**.563***	**.563***	**.320***	.180	-.113	.109	.013	.013	.121	**.399***	.006	.263	-.089	**.365***	-.265	.127	-.055	.246
		p	**.000**	**.000**	**.044**	.266	.488	.502	.935	.935	.485	**.011**	.971	.101	.583	**.021**	.099	.435	.734	.126

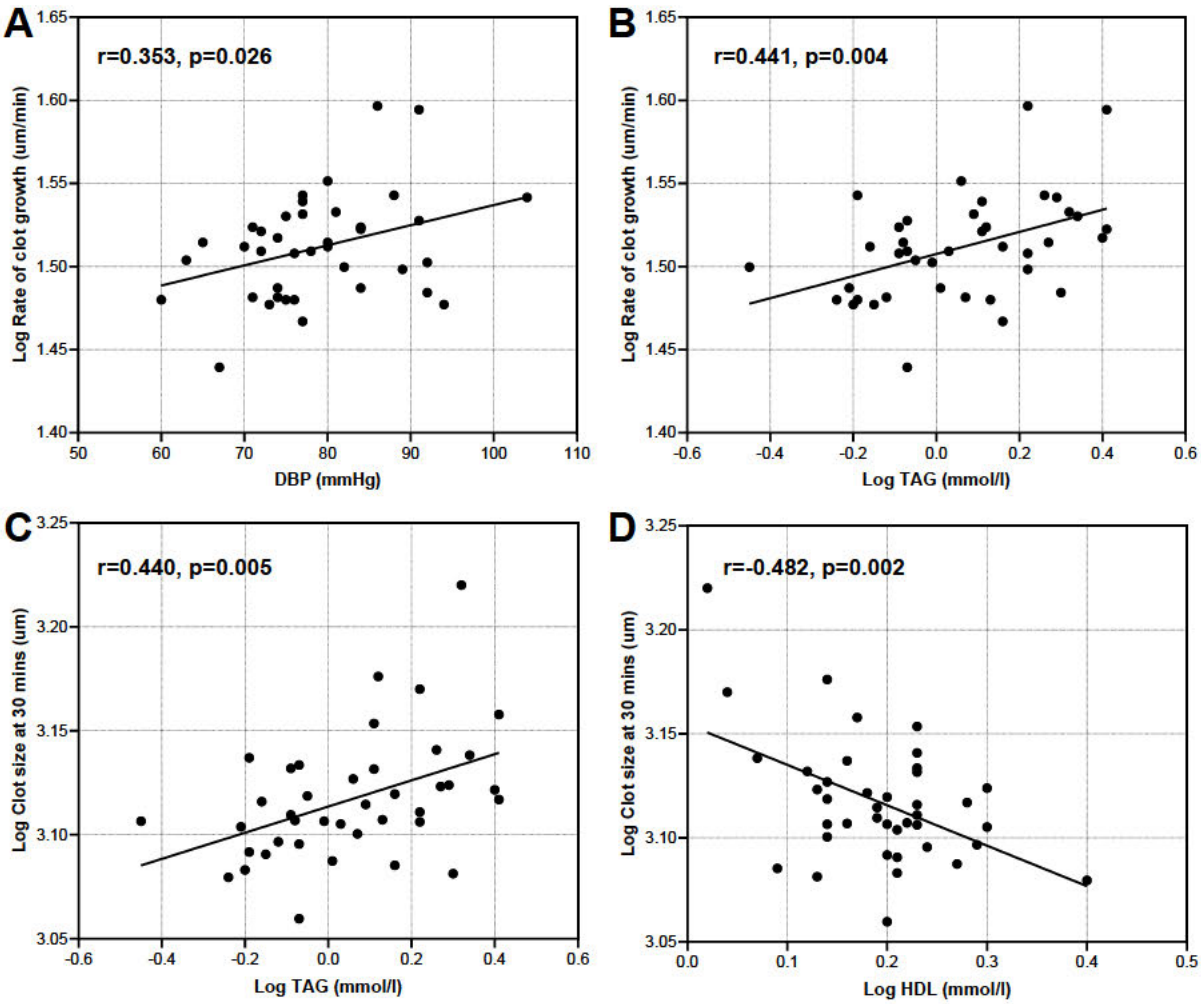


Figure 4.1. Correlations between conventional risk markers and fibrin clot properties. DBP, diastolic blood pressure; HDL, high-density lipoprotein cholesterol; TAG, triacylglycerol.

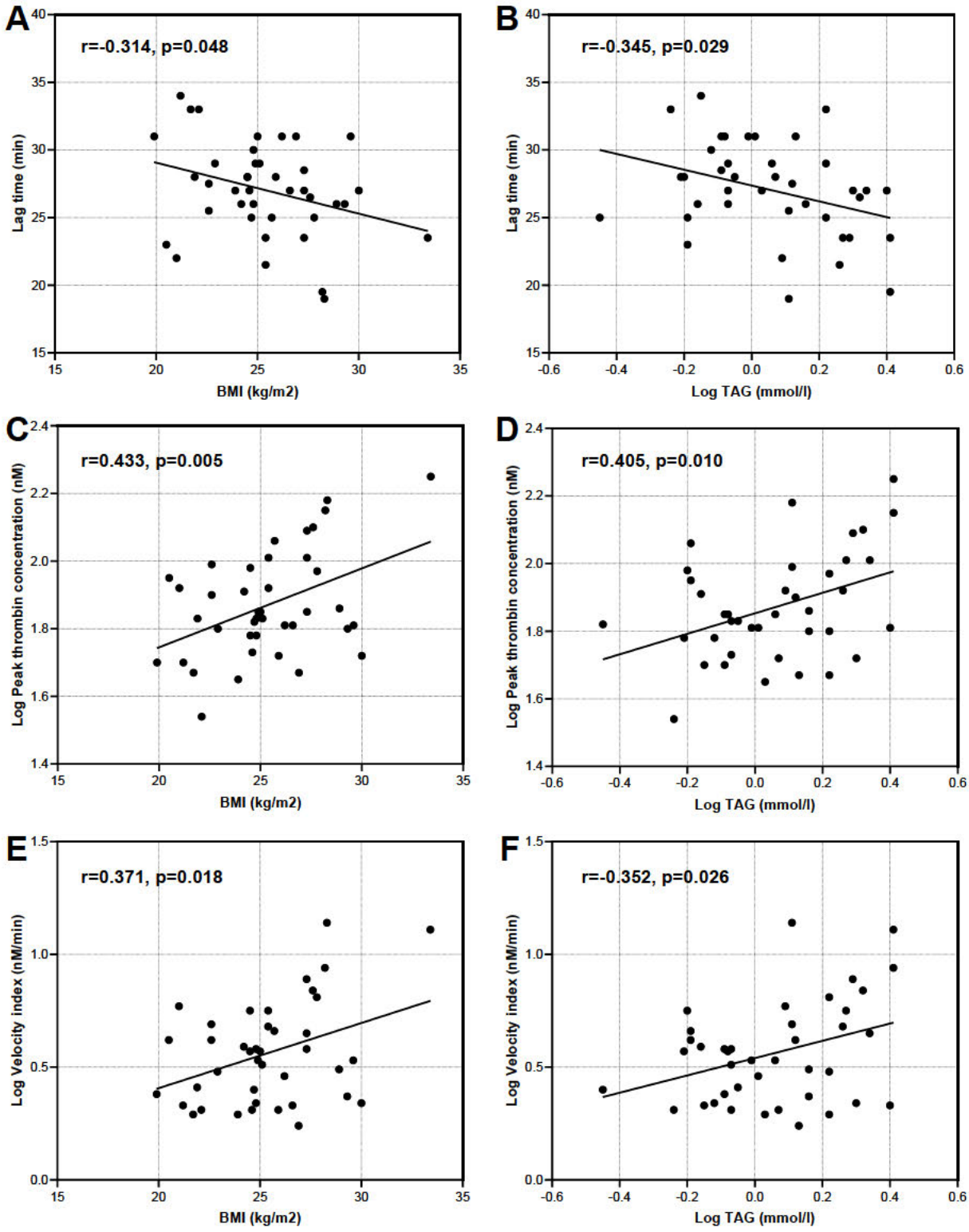


Figure 4.2. Correlations between conventional risk markers and thrombin generation. BMI, body mass index; TAG, triacylglycerol.

Table 4.3. Univariate and multivariate regression analysis for the association of clot formation-related risk markers with EV numbers and conventional risk markers

	Rate of clot growth (um/min)				Clot size (um)			
	Univariate analysis		Multivariate analysis R^2=0.403 *p=0.001*		Univariate analysis		Multivariate analysis R^2=0.477 *p<0.001*	
	B	***p***	**B**	***p***	**B**	***p***	**B**	***p***
Log TEVs (per ml blood)	0.059	**p<0.001**	**0.043**	**0.045**	0.047	**0.002**	**0.028**	**0.016**
Log SEVs (per ml blood)	0.059	**p<0.001**	-	-	0.048	**0.001**	-	-
EDEVs (ml blood)	0.041	**0.045**	0.028	0.130	-	**-**	-	-
DBP (mmHg)	0.001	**0.026**	0.000	0.912	-	**-**	-	-
TAG (mmol/l)	0.067	**0.004**	0.024	0.385	0.063	**0.021**	0.021	0.405
HDL (mmol/l)	-	-	-	-	-0.196	**0.002**	-0.148	**0.013**

In univariate analysis, TEV numbers, SEV numbers, EDEV numbers, DBP and TAG were significant factors for the rate of clot growth while TEV numbers, SEV numbers, TAG and HDL were significant factors for clot size at 30 min. Only TEV numbers, SEV numbers and HDL were still significantly correlated with clot size at 30 min when entered into multivariate model. DBP, diastolic blood pressure; EDEV, endothelial-derived extracellular vesicles; HDL-C, high-density lipoprotein cholesterol; SEVs, small total extracellular vesicles; TEVs, total extracellular vesicles.

Table 4.4. Univariate and multivariate regression analysis for the association of thrombin generation-related risk markers with EV numbers and conventional risk markers

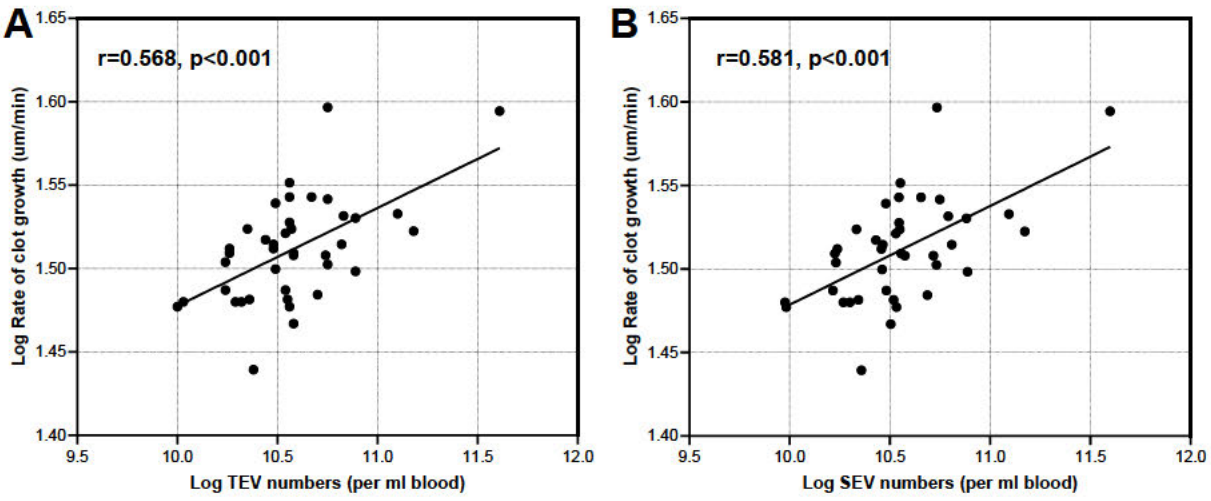
	Lag time (min)				Peak thrombin concentration (nM)				Velocity index (nM/min)				AUC			
	Univariate analysis		Multivariate analysis R^2=0.182 *p=0.125*		Univariate analysis		Multivariate analysis R^2=0.361 *p=0.003*		Univariate analysis		Multivariate analysis R^2=0.354 *p=0.018*		Univariate analysis		Multivariate analysis R^2=0.362 *p=0.007*	
	B	***p***	**B**	***p***	**B**	***p***	**B**	***p***	**B**	***p***	**B**	***p***	**B**	***p***	**B**	***p***
Log TEVs (per ml blood)	-4.428	**0.015**	-2.611	0.313	0.304	**0.000**	**0.263**	**0.013**	0.429	**0.000**	**0.432**	**0.011**	0.402	**0.000**	**0.372**	**0.013**
Log SEVs (per ml blood)	-4.303	**0.016**			0.300	**0.000**	-	-	0.425	**0.000**	-	-	0.395	**0.000**	-	-
Log LEVs (per ml blood)	-	**-**	-	-	-	-	-	-	0.223	**0.044**	-	-	0.214	**0.014**	-	-
BMI (kg/m^2)	-0.377	**0.048**	-0.126	0.590	0.023	**0.005**	0.006	0.485	0.029	**0.018**	0.003	0.809	0.030	**0.011**	0.007	0.583
DBP (mmHg)	-	**-**	-	-	-	**-**	-	-	0.008	**0.016**	0.000	0.951	-	**-**	-	-
TAG (mmol/l)	-5.800	**0.029**	-2.414	0.487	0.305	**0.010**	0.009	0.947	0.382	**0.026**	-0.058	0.774	0.379	**0.021**	-0.026	0.893

In univariate analysis, TEV numbers, SEV numbers was significant factors for lag time, peak thrombin concentration, velocity index and AUC and LEV numbers were significant factor for only velocity index and AUC, but none of these emerging risk markers was significantly correlated with thrombin generation-related parameters when entered into multivariate model. In univariate analysis, BMI and TAG were significant factors for all thrombin generation parameters and DBP was significant factors for velocity index; but none of these conventional risk markers was significantly correlated with thrombin generation-related parameters when entered into multivariate model. BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein cholesterol; SEVs, small total extracellular vesicles; LEVs, large total extracellular vesicles; TEVs, total extracellular vesicles.

4.2.3 Association between circulating EV numbers and fibrin clot properties

Higher numbers of TEVs and SEVs were associated with elevated clot formation, reflected by the rate of clot growth and clot size at 30 min, analysed by univariate regression analysis; **Table 4.3**. SEV numbers showed a higher degree of association than TEV numbers, but there was no relationship between EV size and these risk markers **(Figure 4.3A-4.3D)**. The mean size of EVs, detected by NTA was negatively correlated with only initial rate of clot growth **(Figure 4.3E)**. Higher EDEV numbers, measured by FCM, were associated with increased rate of clot growth **(Figure 4.3F)**, while numbers of PS+EVs and PDEVs were not associated with any clot formation-related risk markers (data not shown). EDEVs were also negatively associated with lysis onset time, but positively correlated with lysis progression **(Figure 4.3G and 4.3H)**.

When univariate determinants of the rate of clot growth and clot size at 30 min (except SEV numbers to avoid multicollinearity) were entered into a multivariate regression model, TEV numbers remained significantly associated with the rate of clot growth and clot size. Clot size was also associated with HDL concentration (**Table 4.3**). Finally, stepwise regression analysis suggested that circulating TEV numbers explained 32.3% of the variance for the rate of clot growth and 24.3% of the variance for clot size at 30 min (**Table 4.5**). An additional 13.1% of the variance in clot size at 30 min was predicted by HDL (**Table 4.5**).



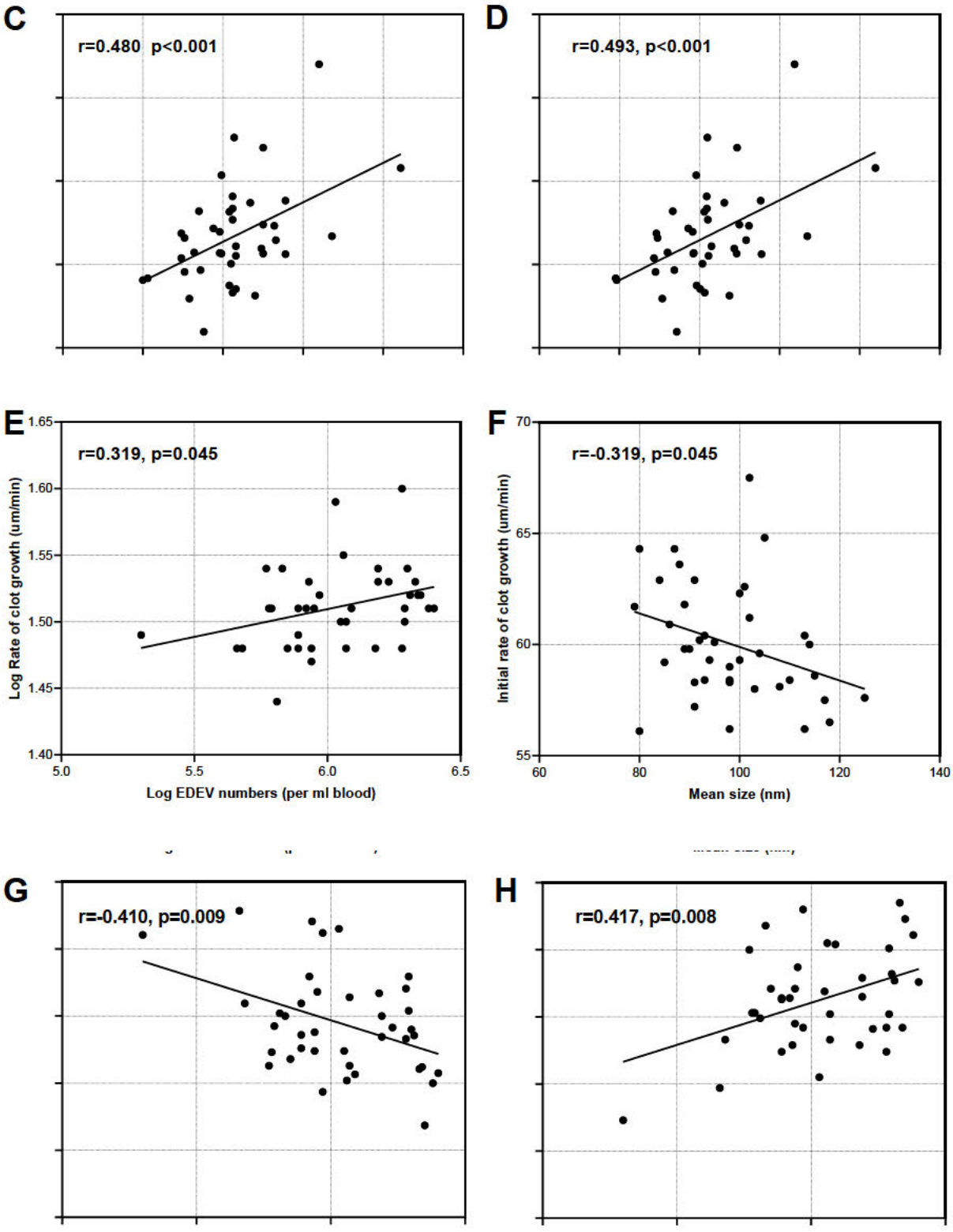


Figure 4.3. Correlations between EV numbers and fibrin clot properties. EDEVs, endothelial-derived extracellular vesicles; SEVs, small total extracellular vesicles; TEVs, total extracellular vesicles.

Table 4.5. Independent predictors of clot formation parameters determined by stepwise regression

		Model	B (95% CI)	SE (B)	β	*p*-value
Rate of clot growth	**1**	(Constant)	0.890 (0.594, 1.186)	0.146		<0.001
		TEV number R^2=0.323	0.059 (0.031, 0.087)	0.014	0.568	<0.001
Clot size at 30 min	**1**	(Constant)	2.611 (2.319, 2.904)	0.145		<0.001
		TEV number R^2=0.243	0.048 (0.020, 0.076)	0.014	0.493	0.001
	2	(Constant)	2.744 (2.458, 3.030)	0.141		<0.001
		TEV number	0.038 (0.012, 0.065)	0.013	0.393	0.006
		Log HDL R^2=0.376	-0.154 (-0.295, -0.043)	0.055	-0.378	0.008

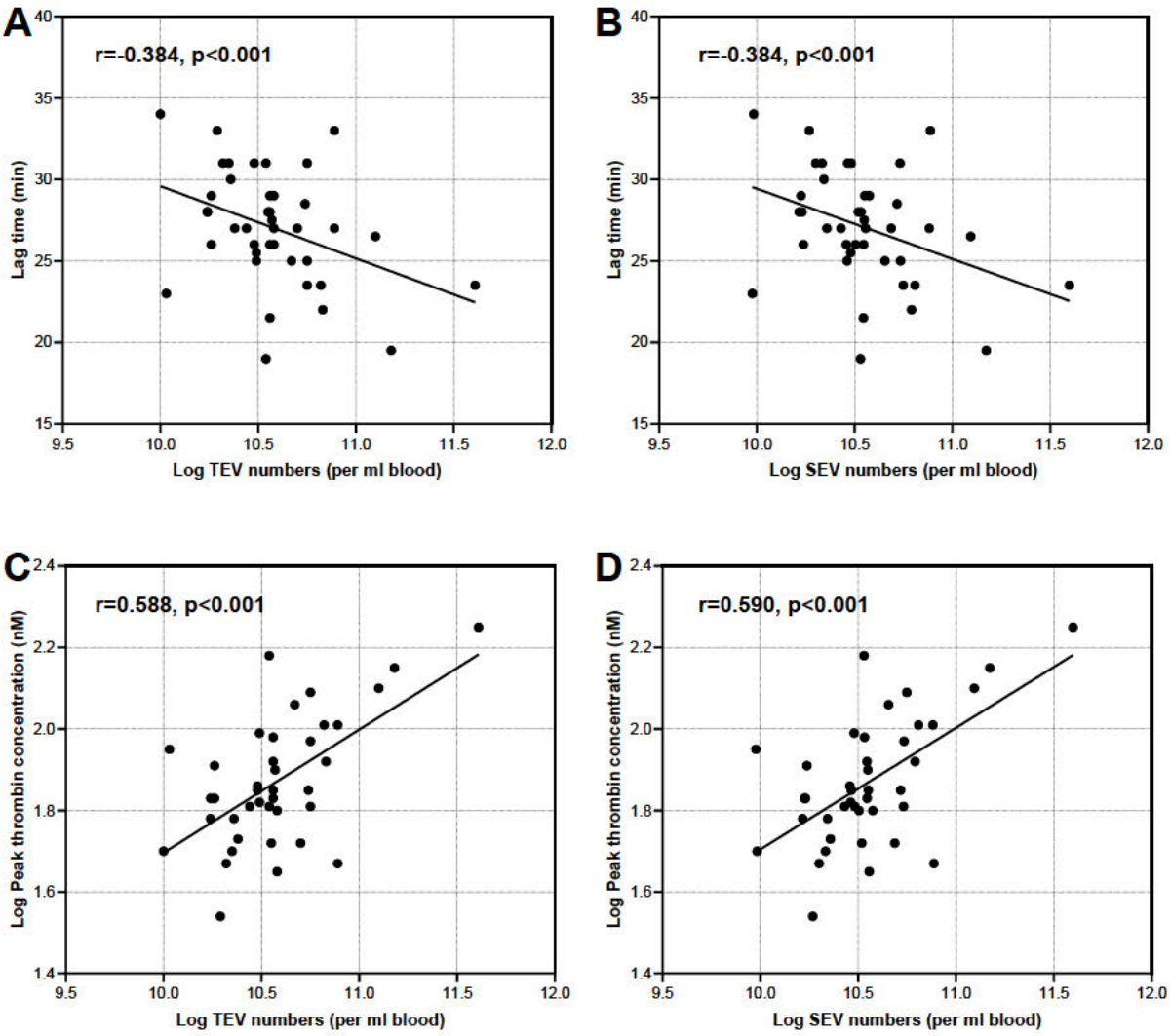
Unstandardized coefficients (B) indicates that as the independent variables (EV numbers and conventional risk markers) change by one unit, the dependent variable (clot formation parameters: rate of clot growth and clot size at 30 min) changes by B units. Regression coefficient (β) indicates that as the independent variables change by 1 SD, the dependent variable changes by β SD. For reference, 1-SD of log TEV numbers is 0.31/ log ml blood; 1-SD of log rate of clot growth is 0.03 um/min. CI, confidence interval; SD, standard deviation; SE, standard error; SEVs, small total extracellular vesicles; TEVs, total extracellular vesicles.

4.2.4 Association between circulating EV numbers and thrombin generation

Higher numbers of TEVs and SEVs were associated with increased thrombin generation, reflected by all parameters (lag time for thrombin generation, time to peak thrombin generation, peak thrombin concentration, velocity index, and AUC) analysed by univariate regression analysis (**Table 4.4, Figure 4.4)**. Velocity index and AUC were also positively correlated with numbers of LEVs (r=0.320, p=0.044), but to a lesser degree than TEV and SEV numbers and there was no relationship between EV size and thrombin generation. Numbers of PS+EVs, PDEVs and EDEVs, were not associated with any thrombin generation-related risk markers.

When univariate determinants of thrombin generation-related risk markers (except SEV numbers to avoid multicollinearity) were entered into a multivariate regression model, TEV

number remained significantly associated with peak thrombin concentration, velocity index, and AUC, but not lag time (**Table 4.4**). Stepwise regression analysis suggested that TEV numbers explained 14.7% of the variance for lag time, 34.6% of the variance for peak thrombin concentration, 33.3% of the variance for velocity index respectively and 31.7% of the variance for AUC, respectively (**Table 4.6**).



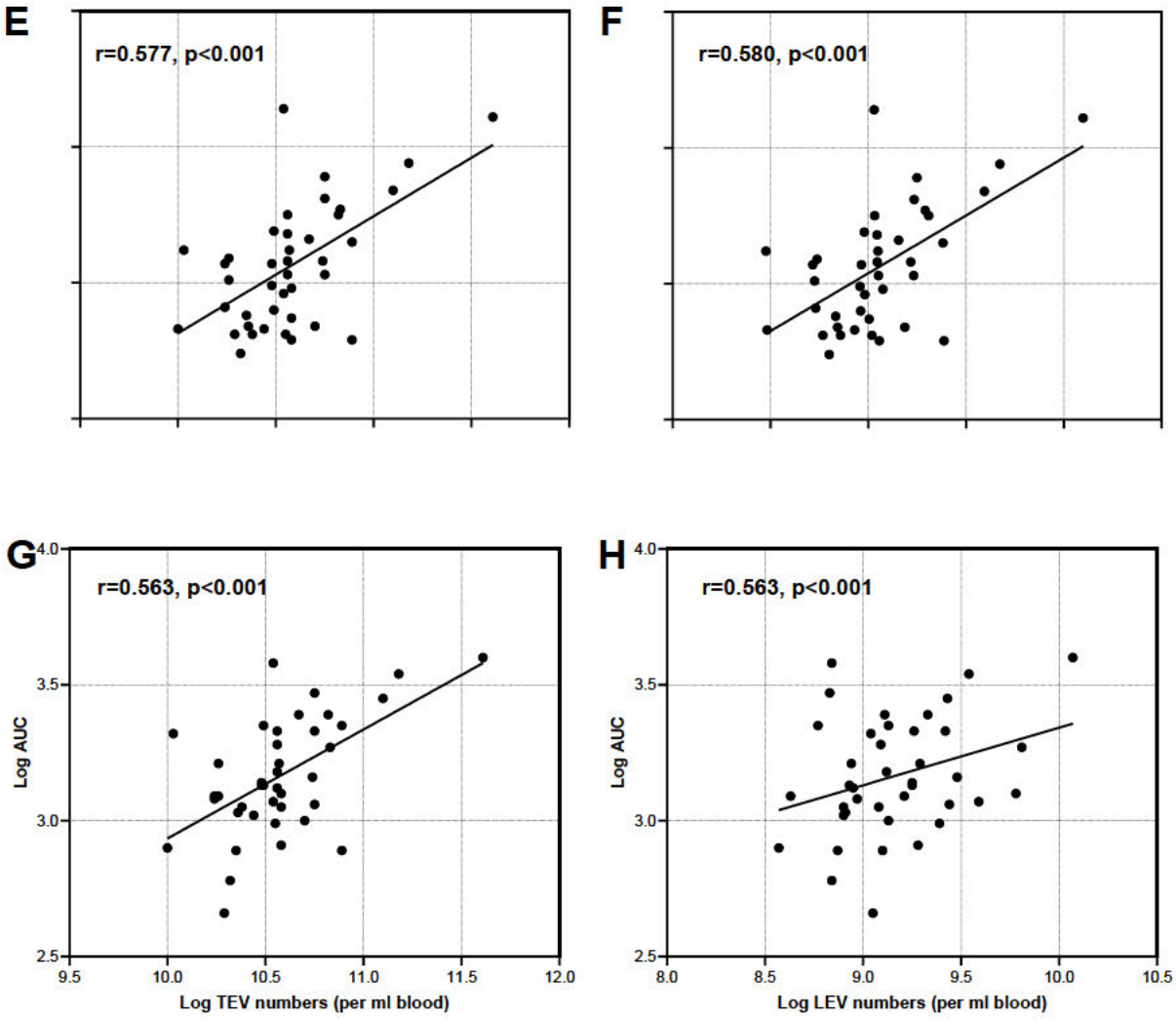


Figure 4.4. Correlations between EV numbers and thrombin generation. AUC, area under curve, LEVs, large extracellular vesicles; SEVs, small total extracellular vesicles; TEVs, total extracellular vesicles.

Table 4.6. Independent predictors of thrombin generation parameters determined by stepwise regression

		Model	B (95% CI)	SE (B)	β	*p*-value
Lag time	**1**	(Constant)	72.856 (35.995, 108.911)	18.293		<0.001
		TEV number R^2=0.147	-4.428 (-7.756, -0.850)	1.729	-0.384	0.015
Peak thrombin concentration	**1**	(Constant)	-1.347 (-2.800, 0.106)	0.718		0.068
		TEV number R^2=0.346	0.304 (0.167, 0.442)	0.068	0.588	<0.001
Velocity index	**1**	(Constant)	-3.979 (-6.090, -1.869)	1.043		<0.001

		TEV number R^2=0.333	0.429 (0.230, 0.629)	0.099	0.577	<0.001
AUC	**1**	(Constant)	-1.083 (-3.130, 0.963)	1.011		0.291
		TEV number R^2=0.317	0.402 (0.208, 0.595)	0.096	0.563	<0.001

Unstandardized coefficients (B) indicates that as the independent variables (EV numbers and conventional risk markers) change by one unit, the dependent variable (thrombin generation parameters: lag time for thrombin generation, peak thrombin concentration, velocity index and AUC) changes by B units. Regression coefficient (β) indicates that as the independent variables change by 1 SD, the dependent variable changes by β SD. For reference, 1-SD of log TEV numbers is 0.30/ log ml blood; 1-SD of log the level of peak thrombin concentration is 0.16/ log nM. AUC, area under curve; CI, confidence interval; SD, standard deviation; SE, standard error; SEVs, small total extracellular vesicles; TEVs, total extracellular vesicles.

4.2.5 Association between circulating EV numbers and platelet aggregation

There were no significant associations between numbers of circulating EVs and platelet aggregation induced by ADP, CRP-XL, epinephrine or TRAP-6, but size of circulating EVs, was negatively correlated with U46619-induced platelet aggregation (**Supplementary Table 4.1**).

4.2.6 Coagulation parameters are higher in subjects with circulating EV numbers above the median

Subject were divided into two groups based on EV numbers above or below the median. Subjects with high numbers of TEVs had a significantly higher rate of clot growth, stationary rate of clot, peak thrombin concentration and area under curve for thrombin generation than those with low numbers of TEVs (**Figure 4.5A~D**), but there were no differences in other thrombogenic risk markers between EV groups (data not shown). Those with higher numbers of SEVs had a higher rate of clot growth, stationary rate of clot growth, clot size at 30 minutes, peak thrombin concentration, time to peak thrombin, velocity index for thrombin generation and area under curve for thrombin generation compared to low EVs group (**Figure 4.6A~F)**.

Subjects in the high LEV group had significantly higher epinephrine-induced and TRAP-6-induced platelet aggregation than the low LEV group (**Figure 4.7A and 4.7B**), but there were no significant differences in other thrombogenic risk markers (data not shown). Subjects in the

high EDEV group had a significantly lower lysis onset time and significantly higher TRAP-6-induced platelet aggregation than the low EDEV group (**Figure 7C and 7D**). There were no other significant differences between the high and low EDEV groups (data not shown).

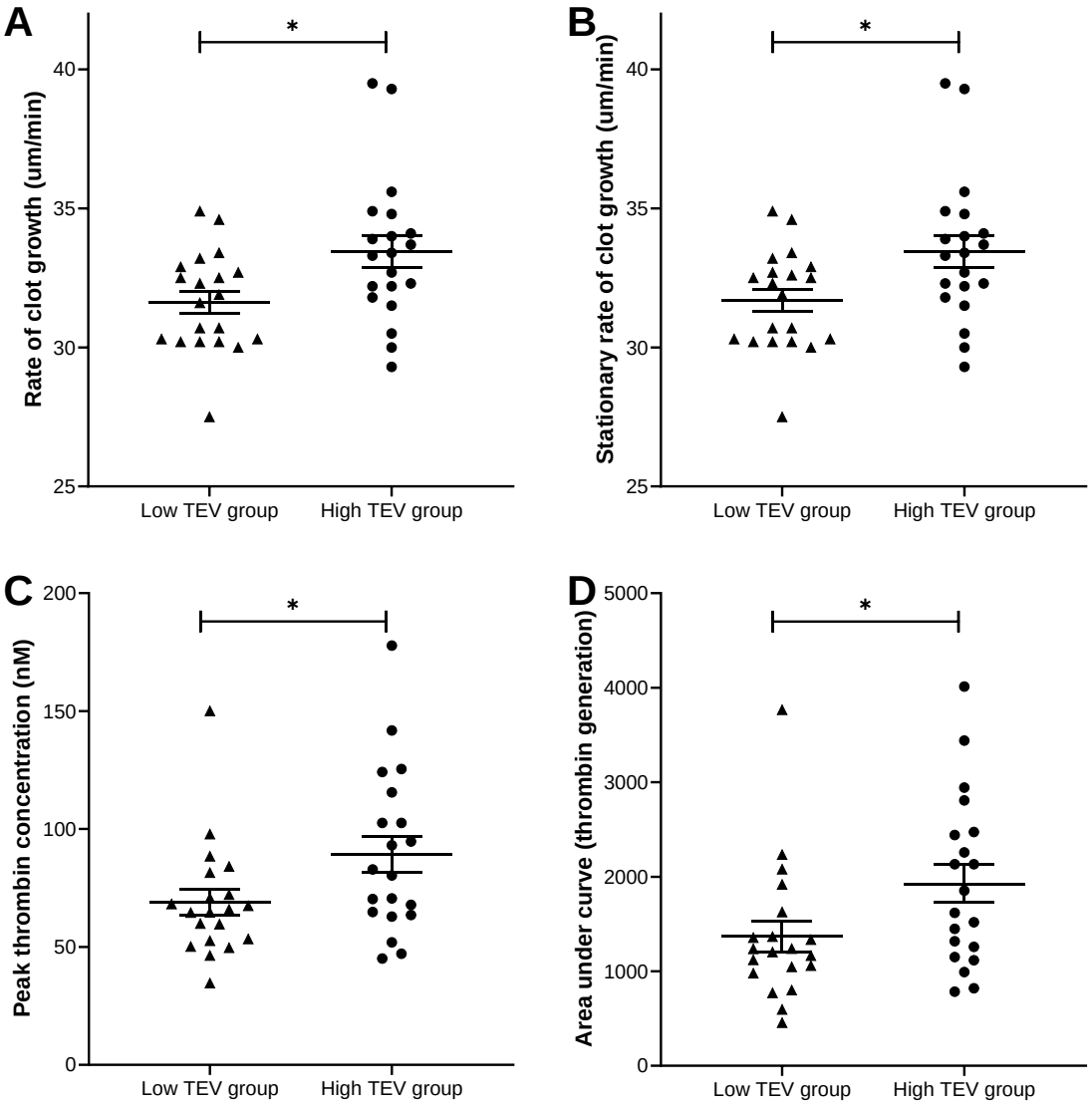


Figure 4.5. High numbers of TEVs are associated with increased clot formation and thrombin generation. Data are mean ± SEM (n=20 per group). Differences between the means of the low and high TEV groups were drawing using two-tailed unpaired t-tests, with differences shown at $p < 0.05$. There were statistically significant differences in **(A)** rate of clot growth, **(B)** stationary rate of clot growth, **(C)** peak thrombin concentration and **(D)** AUC for thrombin generation between the low and high TEV groups (categorized by the median of TEV numbers: $3.63*10^{10}$/ml blood). **p<0.05*. TEVs, total extracellular vesicles.

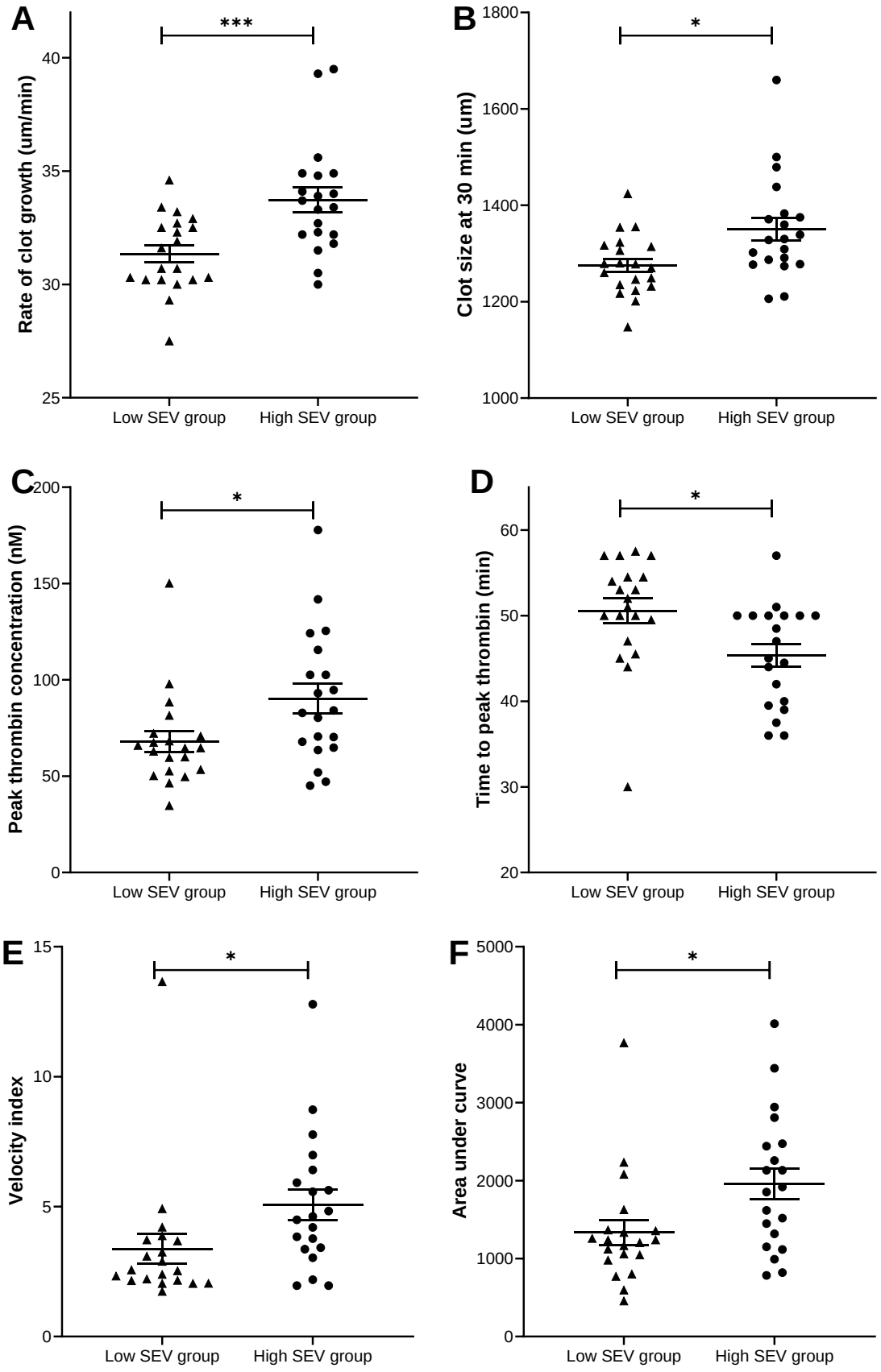


Figure 4.6. High numbers of SEVs are associated with increased clot formation and thrombin generation. Data are mean ± SEM (n=20 per group). Differences between the means of the low and high SEV groups were drawing using two-tailed unpaired t-tests, with

differences shown at p < 0.05. There were statistically significant differences in **(A)** rate of clot growth, **(B)** clot size at 30 minutes, **(C)** peak thrombin concentration, **(D)** time to peak thrombin, **(E)** velocity index and **(F)** AUC for thrombin generation between the low and high SEV groups (categorized by the median of SEV numbers: $3.40*10^{10}$/ml blood). ** p<0.05 and ***p<0.001*. SEVs, small total extracellular vesicles.

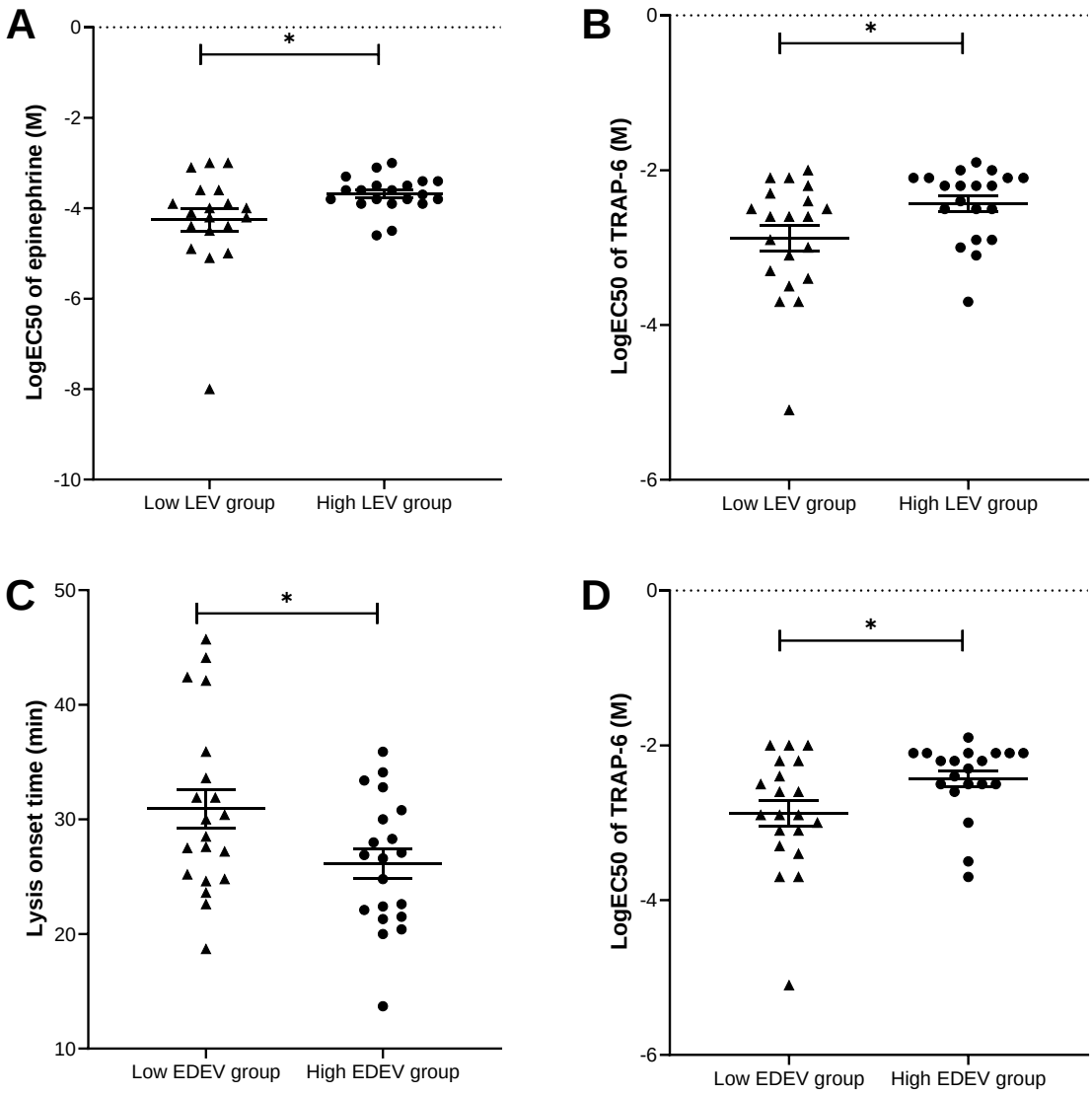


Figure 4.7. High numbers of LEVs and EDEVs are associated with increased platelet aggregation and high numbers of EDEVs are associated with increased fibrinolysis. Data are mean ± SEM (n=20 per group). Differences between the means of low and high LEVs and EDEVs groups were drawing using two-tailed unpaired t-test, with differences shown at p < 0.05. There were statistically significant differences in **(A)** epinephrine-induced platelet aggregation and **(B)** TRAP-6-induced platelet aggregation between the low and high LEV groups, and **(C)** lysis onset time and **(D)** TRAP-6-induced platelet aggregation between the low and high EDEV groups (categorized by the median of LEV and EDEV numbers: $1.29*10^{9}$/ml blood and 1.09*106/ml blood, respectively). * p<0.05. EDEV, endothelial cell-derived extracellular vesicles; LEVs, large total extracellular vesicles; TRAP-6, thrombin receptor activator peptide.

4.3 Discussion

To the best of our knowledge, this report is the first to investigate the relationship between numbers of circulating EVs and a range of parameters relating to blood coagulation pathways in subjects with moderate risk for CVD. There were positive associations between numbers of circulating TEVs and SEVs and coagulation parameters, including fibrin clot formation and thrombin generation, as well as a positive association between numbers of PS+ EDEVs and fibrin clot formation. In multivariate regression analysis, where SEV number was removed due to a high degree of collinearity, TEV numbers remained strongly associated with coagulation parameters. Numbers of circulating TEVs appear to be independent predictors of aspects of clot formation and thrombin generation. The addition of plasma HDL-cholesterol concentration improved the predictive ability for clot formation in stepwise regression analysis. Subjects with higher than median circulating EV numbers exhibited greater fibrin clot formation and thrombin generation than subjects with lower than median circulating EV numbers.

A hypercoagulable state has been observed in overweight and obesity (Campello et al., 2015, Kornblith et al., 2015), hyperlipidaemia (Nordøy et al., 2003, Kim et al., 2015, Ay et al., 2010), insulin resistance and hyperglycemia, hypertension (Lip, 2000, Carcaillon et al., 2011), suggesting that an increased tendency for coagulation might be associated with conventional risk markers of CVD, including BMI, blood pressure, blood lipids, and glucose level. Indeed, abnormalities in not only coagulation, but also fibrinolytic systems and platelets, have been demonstrated (Poli et al., 2000, Lee and Lip, 2003). The present study showed significant associations between conventional risk markers of CVD and parameters relating to thrombogenicity, including clot formation, thrombin generation, and platelet activation. For example, increased BMI was related to accelerated thrombin generation, in agreement with previous reports, which demonstrated a similar link in subjects with venous thromboembolism (Sonnevi et al., 2013), hypertension (Elias et al., 2019) and obesity (Campello et al., 2015). This suggests that weight loss might contribute to decreased pro-coagulatory activity, and indeed bariatric surgery in morbidly obese subjects resulted in a lowering of thrombin

generation (Ay et al., 2010). In addition, the present data revealed that higher DBP was associated with accelerated fibrin clot formation and thrombin generation, while higher SBP was related to increased platelet aggregation (U46619 induced), supporting the previous suggestion that increased hypertensive risk factors are associated with hypercoagulation (Wirtz et al., 2006), elevated thrombin generation (Elias et al., 2019), and platelet activation (Yang et al., 2016). The current study also demonstrated that plasma TAG concentration was positively associated with clot growth rate, clot size and thrombin generation, and plasma HDL concentration was inversely related to fibrin clot size, implying that blood lipid levels and hypercoagulation are connected in the development of CVDs. Although studies reported in the literature employed a wide variety of coagulation-related markers, the observations in the current study are aligned with them in as much as plasma TAG concentration being associated with coagulation factor II (Kim et al., 2015, Vanschoonbeek et al., 2007), factor VII (Kim et al., 2015, Nordøy et al., 2003, Vanschoonbeek et al., 2007), factor IX (Kim et al., 2015, Vanschoonbeek et al., 2007) and X (Kim et al., 2015), and thrombin generation (Ay et al., 2010, Vanschoonbeek et al., 2007) in health and disease (obesity, overweight, and hyperlipidaemia). Furthermore, plasma HDL-cholesterol concentration was inversely associated with clot size, suggesting that elevated HDL-cholesterol concentration may reduce the risk of thrombosis (Doggen et al., 2004). Despite good correlations between these conventional risk markers and thrombogenic risk factors, the only significant association which remained in multivariate regression analysis was that between HDL and clot size.

Emerging risk markers, including EVs, introduce new avenues for exploration in terms of the development of CVDs (Dickhout and Koenen, 2018) and there is already some evidence that elevated numbers of EVs are linked with thrombotic diseases (Mooberry and Key, 2016, Nomura and Shimizu, 2015). In the present study, numbers of TEVs and SEVs were associated with rate of clot growth and clot size at 30 min, suggesting that an increase in circulating EV numbers enhanced the propagation phase of the coagulation cascade and increased the size of the fibrin clot, and the association of TEVs with both clot formation

parameters was maintained in multivariate regression analysis, after removal of SEVs due to collinearity. SEV numbers actually demonstrated a stronger association with coagulation parameters than TEV numbers, suggesting that SEVs, which represent the size range of exosomes and microvesicles (71-200 nm), may play a greater role in fibrin clot formation than LEVs (200-1000 nm), although they do comprise the majority of the EV population (96%). Nevertheless Lawrie *et al* (2008) showed that the rate of fibrin clotting and thrombus formation were reduced after PS+EVs (>200 nm) were removed by the filtration (Lawrie et al., 2008). The current study, therefore, suggests greater procoagulant activity of smaller EVs, but future studies specifically designed to evaluate EVs within different size ranges are required to investigate this.

Stepwise regression analysis (including both conventional and emerging risk markers as independent variables), highlighted TEV number as an independent predictor of fibrin clot formation, and the addition of HDL-cholesterol concentration to TEV number improved predictability for clot size. Although this result did not infer a causal relationship between EV numbers and coagulation, several mechanisms for the role of EVs in coagulation have been proposed by previous studies (Hrachovinová et al., 2003, Tesselaar et al., 2007, Van Der Meijden et al., 2012). The negatively charged phospholipid surface of EVs, which contains phosphatidylserine (PS), (Sinauridze et al., 2007, Gilbert et al., 1991) promotes the formation of the tenase (factors IXa, VIIIa, and X) and prothrombinase (factors Xa, Va, and prothrombin) complexes (Spronk et al., 2014), thereby supporting further activation of coagulation cascade to convert fibrinogen to fibrin (Voukalis et al., 2019, Sinauridze et al., 2007). EVs may additionally (Tripisciano et al., 2017b) support the transformation of tissue factor to its active form via the exposure of PS (Tripisciano et al., 2017a, Voukalis et al., 2019). The findings in the present study indicate a potential contribution of circulating EVs to hypercoagulation in subjects with moderate risk for CVD.

In the present study, there was no relationship between PS+EV numbers or PS+ PDEVs and clot formation. Although Tripodi *et al* (2011) also reported no association between levels of

coagulation factors (factor II and factor VIII) and Annexin V+ EVs, in agreement with the current study (Tripodi et al., 2011), others demonstrated association of clotting time with numbers of Annexin V+ EVs (Ayers et al., 2014) and PDEVs, characterised by CD31+ CD41+ (Ayers et al., 2014) and Annexin V+ CV31+ CD41+ (Stagnara et al., 2012). Notably, different phenotyping and gating strategies to characterise EVs by FCM may have influenced this inconsistency in results. However, the lack of the association between numbers of EVs detected by FCM and coagulation-related parameters could be explained by the fact that smaller EVs (<200 nm) cannot be detected by FCM due to the detection limit (Aatonen et al., 2012), while NTA can detect small EVs, which form the majority of the EV population. EVs may also escape detection by FCM due to poor binding to Annexin V (Wang et al., 2016).

In contrast to PS+ EVs and PS+ PDEVs, numbers of PS+ EDEVs, detected as CD105+, were positively associated with the rate of clot growth. Increased EDEVs numbers have been reported in patients with acute coronary syndromes (Mallat et al., 2000, Bernal-Mizrachi et al., 2003) and acute ischemic stroke (Simak et al., 2006), and suggested to contribute to impaired endothelial function in vascular events (Markiewicz et al., 2013), but the present study revealed an association of PS+ EDEVs with fibrin clot formation for the first time, supporting their potential roles in coagulation (Diamant et al., 2004, Markiewicz et al., 2013, Voukalis et al., 2019). Although there are no published studies examining associations between EDEVs and coagulation-related risk factors, a few studies have investigated the relationship between EDEVs and endothelial dysfunction. EDEV numbers, identified as CD31+CD42- independently predicted impaired flow-mediated dilation (FMD) (Feng et al., 2010), arterial dysfunction (Feng et al., 2010, Wang et al., 2007, Wang et al., 2009) and arterial elasticity (Wang et al., 2007). The current study demonstrated that PS+ EDEV numbers were positively associated with fibrinolysis, and negatively associated with lysis onset time, perhaps suggesting a role for EDEVs in the breakdown of the fibrin clot. Furthermore, EVs derived from endothelial cells express plasminogen activators on their surface under pathological

conditions, which also supported the suggestion that they play a role in fibrinolytic activity (Lacroix and Dignat-George, 2012).

The pro-coagulatory ability of EVs is also manifested through enhancement of thrombin generation (Mooberry et al., 2016, Van Der Meijden et al., 2012, Sinauridze et al., 2007, Tripisciano et al., 2017b). The present study demonstrated that circulating EVs were associated with a range of thrombin generation parameters and stepwise regression analysis showed that numbers of circulating TEVs are independent predictors of thrombin generation, indicating that concentrations of circulating EVs might be a sensitive indicator of a prothrombotic state. It has been suggested that EVs support coagulation by enhancing thrombin generation through their externalised PS-rich catalytic surface (Tripisciano et al., 2017b). While this is supported by a study demonstrating that procoagulant EVs (PS+EVs), identified as Annexin V+ by FCM, were associated with thrombin generation (Ayers et al., 2014, Macey et al., 2011, Tripodi et al., 2011) the current study reported no such association between PS+EVs numbers and thrombin generation. One of these studies also demonstrated a significant association between numbers of PDEVs and peak thrombin concentration (Ayers et al., 2014), which was specific to PDEVs identified as CD31+/CD41+. Differences in phenotyping techniques to quantify PDEVs represent the most reasonable explanation for this discrepancy as the present study captured PS+ PDEVs by double anti-body labelling (CD41+/Annexin V+). This might limit the detection of PDEV numbers in the current study because not all PDEVs are enriched with PS on their surface to bind Annexin V (Connor et al., 2010). However, it would be expected that PS+ PDEVs would be more thrombogenic, and this does not appear to be the case based on the results of the current study compared with those of Ayers *et al* (2014). Also, the smaller sample size of the present study (40 versus 78) may have been a contributory factor to a difference in results.

The release of circulating EV numbers has been shown be dependent on the individual's health status and microenvironmental conditions at cellular level, for example pH and cell stress (Zaborowski et al., 2015). The relationship between EV numbers and thrombogenic risk

markers described in this chapter highlight a potential role for EVs in the progression of coagulation and thrombin generation, which may have implication with respect to cardiovascular events (Nozaki et al., 2009) stroke (Rosinska et al., 2019), coronary artery disease (Jansen et al., 2014) and acute coronary syndromes (Nijiati et al., 2017). Although the current study presents only limited phenotypic analysis of EV subtypes, it does demonstrate that subjects with higher than median numbers of EDEVs has greater platelet aggregation and fibrinolysis. Overall, increased numbers of EVs in the circulation were associated with increases in thrombogenic risk markers; this may have implications for risk of cardiovascular events, but causal links have not yet been established.

Potential limitations of the present study include a relatively small sample size for this type of analysis, as well as the fact that the study population comprised only subjects with moderate risk for CVD. As with any association study, it is unable to establish causal links between circulating EVs and thrombogenic risk markers and there is therefore a need for large prospective studies to verify the relationship between EVs and thrombogenic risk factors by confirming whether higher circulating EV numbers lead to increased thrombotic events.

4.4 Conclusion

In summary, this study demonstrated that higher circulating EV numbers were associated with enhanced thrombogenic risk factors in subjects with moderate risk for CVD. Circulating EV numbers independently predicted fibrin clot formation and thrombin generation, suggesting that circulating EVs may play a role in hypercoagulation.

Chapter 5 Effects of n-3 PUFAs on the generation and characteristics of EVs generated in vitro from washed platelets

5.1 Introduction

Extracellular vesicles (EVs) are membrane-enclosed vesicles derived by many different cell types (Coumans et al., 2017) and these structures have been pointed out to have emerging roles in both physiological and pathological conditions as they carry various cargoes, such as protein, lipids and nucleic acids one cell to another (Yáñez-Mó et al., 2015, Boulanger et al., 2017) (for more details see **Chapter 1**). Some studies reported increased levels of EVs in particularly cardiovascular events (Simak et al., 2006, Jansen et al., 2014, Walenta et al., 2012, Sinning et al., 2011, Horn et al., 2014) and others showed the positive association between elevated circulating EV numbers and cardiovascular risk factors, including smoking (Gordon et al., 2011), hypercholesterolemia (Suades et al., 2014), dyslipidaemia (Amabile et al., 2014), obesity (Esposito et al., 2006), hypertension (Preston et al., 2003), diabetes mellitus (Koga et al., 2005) and metabolic syndrome (Helal et al., 2011). Moreover, **Chapter 4** of this thesis demonstrated that circulating EV numbers were positively associated with thrombogenic risk markers, which may indicate an important roles of EVs in the development of cardiovascular disease (CVD) (Amabile et al., 2014).

Platelet-derived EVs (PDEVs) comprise the major EV population in the circulation, representing up to 60–90% of circulating EVs (Brisson et al., 2017). They are considered particularly important mediators, potentially contributing to the development and progression of CVD (Zaldivia et al., 2017) in similar ways to activated platelets (Willoughby et al., 2002), and potentially serving as biomarkers for cardiovascular health (Badimon et al., 2016). EVs release by activated platelets were first identified as playing a role in blood coagulation (Wolf, 1967) and their contribution to coagulation was attributed to exposure of negatively-charged phospholipids, including phosphatidylserine (PS), which then provide a catalytic surface for

coagulation factors to bind (for more details see **Chapter 1**). Indeed, EVs released from platelets have been found 50- to 100-fold more procoagulant than activated platelets due to an elevated density of PS, P-selectin and factor X (Sinauridze et al., 2007). Exposure of PS on the surface of PDEVs has been confirmed using flow cytometry (FCM) and electron microscopy (EM) (Brisson et al., 2017), and has been demonstrated to support prothrombotic and procoagulant activities (Tripisciano et al., 2017b). PDEVs are commonly detected using FCM via annexin V binding and NTA (Vajen et al., 2015, Kailashiya, 2018) (Arraud et al., 2016), but there is little information about the fatty acid profile of PDEVs, which is important, not only because they have been reported to carry pro-inflammatory lipids (Barry et al., 1999), but in the context of dietary lipid intervention, this may be modifiable.

Dietary and lifestyle interventions have been suggested to modify CVD risk factors (Zhang et al., 2017), and intervention with fish oil is no exception to this (Calder, 2014, Maki and Dicklin, 2018, Calder, 2018). However, there is, as yet, very little information about the impact of dietary interventions on EVs, which are emerging cellular markers of CVD, holding unique promise with respect to modulation by dietary fatty acid in particular. Intervention with a high-fat diet increased the number of circulating EVs (Heinrich et al., 2015, Ferreira et al., 2004), but to date, only ten studies have investigated the effects of n-3 PUFA supplementation on EVs and seven of them have reported data relating to PDEVs (see **Table 5.1**). This includes trials investigating chronic supplementation with n-3 PUFA in hyperlipidaemic patients (Nomura et al., 2003, Nomura et al., 2009a), patients post-myocardial infarction (Del Turco et al., 2008) and patients with arteriosclerosis (Nomura et al., 2018), each of which demonstrated a decrease in the number of circulating PDEVs, characterised by FCM or captured by ELISA. However, not all previous studies reported a reduction in PDEVs. One out of seven studies demonstrated an increase in the numbers of circulating PDEVs following a high dose of fish oil (Englyst et al., 2007). Moreover, a study by Wu *et al* (2014) showed no significant effect of n-3 PUFA supplementation for 8 weeks in subjects with moderate risk for CVD on numbers of PDEVs in plasma (Wu et al., 2014). Phang *et al* (2012) reported that acute supplementation

of healthy subjects with a single dose of n-3 PUFA did not alter the number of PS+ PDEVs (Phang et al., 2012a). As these two studies, one chronic and one acute, out of seven trials showed no effect, the inconsistency in reported impacts of n-3 PUFAs on circulating PDEVs may be due to the variety of techniques used to identify and characterise PDEVs. Importantly, there have been no previous studies examining the impact of supplementation with n-3 PUFAs on the ability of platelets to generate EV in vitro and the characteristics of those EVs which have been generated in vitro. The hypothesis is that modification of the fatty acid composition of the platelet membrane by n-3 PUFA, as has been demonstrated previously following intervention with fish oil (Din et al., 2008, Di Stasi et al., 2004), will alter the ability of platelets to generate EVs in vitro and will result in the generation of PDEVs which are enriched with n-3 PUFA in a similar way to circulating EVs.

The current randomized, double-blind, placebo-controlled, crossover trial therefore aimed to investigate whether daily supplementation of subjects at moderate risk of CVDs with 1.8 g/d of fish oil-derived n-3 PUFAs altered the number, size, fatty acid composition and surface PS exposure of PDEVs generated in vitro by stimulated and unstimulated platelets.

Table 5.1. Human intervention studies investigating the effect of n-3 PUFAs on numbers of EVs						
Study info	**Study design**	**Subjects**	**Supplementation/Diet**	**Duration**	**Methods and measured markers**	**Outcomes**
(Nomura et al., 2003)	Uncontrolled clinical intervention	41 hyperlipidemic (18 non-diabetic and 23 diabetic)	1.8 g/d of EPA	4 weeks	Isolation PDEV from washed platelets without any stimuli Flow cytometry **PDEVs**: labelled with GPIX+ **MDEVs**: labelled with PE-CD14+ Annexin V+ EVs	Increased levels of **PDEVs** and **MDEVs** in subjects with hyperlipidemic patients with type2 diabetes compared with control subjects ↓ number of **PDEVs** and **MDEVs** after EPA therapy in hyperlipidemic patients with type2 diabetes
(Englyst et al., 2007)	Uncontrolled clinical intervention	35 healthy men	6 g/d n-3 of PUFA	12 weeks	Isolation PDEV from platelet-rich plasma with stimuli (A23187) ELISA **PDEVs**: labelled with CD61	↑ numbers of **PDEVs** after the intervention
(Del Turco et al., 2008)	Randomised, parallel designed, controlled	46 patients with post-MI	5.2 g/d of n-3 PUFAs (4.3 g of EPA and DHA) or Olive oil (control oil)	12 weeks	Isolation EV pellet by centrifugation Flow cytometry **PS+EVs:** labelled with Annexin V **TF-exposing EVs**: labelled with **t**issue factor 4508CJ **PDEVs**: labelled with CD61+ **Monocyte-derived EVs**: labelled with CD14+ **EDEVs**: labelled with CD62E+	Increased level of **PS+EVs** in subjects with post MI compared with healthy subjects ↓ number of **PDEVs** and **monocyte-derived EVs** after n-3 PUFA supplementation → number of **TF-exposing EVs** and **EDEVs** ↓ EV activity after n-3 PUFAs
(Nomura et al., 2009a)	Randomised parallel designed, uncontrolled	191 hyperlipidemic patients with type2 diabetes	2 mg/d of pitavastatin 1.8 g/d of EPA 2 mg/d of pitavastatin and 1.8 g/d of EPA	6 months	ELISA **PDEVs**	↓ level of **PDEVs** after treatment with only EPA ↓ level of **PDEVs** after treatment with pitavastatin and EPA

(Nomura et al., 2009b)	Uncontrolled, clinical intervention	126 patients with hyperlipidemia (50 non-diabetic and 76 diabetic)	1.8 g/d of EPA	6 months	<u>Flow cytometry</u> **EDEVs**: labelled with PE-CD51+	Increased levels of **EDEVs** in subjects with hyperlipidemic patients with type2 diabetes ↓ number of **EDEVs** in diabetic patient group after the intervention → number of **EDEVs** in non-diabetic patient group after the intervention
(Marin et al., 2011)	Randomised crossover	20 healthy elderly subjects (age> 65 years)	A Mediterranean diet enriched with olive oil (24% en MUFA, <10% en SFA, 4% en PUFA, of which 0.4% was ALA); A SFA diet (12% en MUFA, 22% en SFA, 4% en PUFA, of which 0.4% was ALA); A low-fat, high-carbohydrate diet enriched with n-3 PUFAs (12% en MUFA, <10% en SFA, 8% en PUFA, of which 2% was ALA)	4 weeks	<u>Flow cytometry</u> **PS+ EVs in PFP:** labelled with Annexin V+ **Apoptotic EDEVs in PFP**: labelled with CD31+Annexin V+ **Activated EDEVs in PFP**: labelled with CD144+ CD62E	↓ number of **PS+EVs, apoptotic and activated EDEVs** after Mediterranean diet and a low-fat, high-carbohydrate diet Reduction after a Mediterranean diet was greater than a low-fat, high-carbohydrate diet
(Phang et al., 2012a)	Blinded, placebo-controlled intervention trial	30 healthy subjects (15M, 15F)	DHA rich oil= 0.4 g EPA and 2 g DHA or EPA rich oil= 2 g EPA and 0.4 g DHA or Placebo (sunola oil)	24 hours	<u>Flow cytometry</u> **PDEVs in PFP**: labelled with CD41+/Annexin V+	→ number of **PDEVs** after neither oil ↓ activity of **PDEVs** after EPA-rich oil ***Gender specific outcome:*** ↓ activity of **PDEVs** after EPA-rich oil in only males
(Wu et al., 2014)	Double-blind, placebo-controlled, crossover trial	84 subjects with moderate risk of CVD	1.5 g/d of LC n-3 PUFA Placebo (corn oil)	8 weeks	<u>Flow cytometry</u> **EDEVs in PFP**: labelled with CD31+CD42b2- **PDEVs in PFP**: labelled with CD31+CD42b+	↓ number of **EDEVs** → number of **PDEVs**

(Phang et al., 2016)	Double-blinded RCT	94 healthy subjects (43M, 51F)	DHA rich= 0.2 g EPA and 1 g DHA or EPA rich: 1 g EPA and 0.2g DHA or Placebo (sunola oil)	4 weeks	Flow cytometry **TEVs**: labelled with CD36+	→ number of **TEVs** regardless intervention
(Nomura et al., 2018)	Randomized, non-blinded, parallel-group	84 arteriosclerosis obliterans patients with type2 diabetes	2 mg/d of pitavastatin with 1.8 g/d of EPA or 300 mg/d sarpogrelate	12 months	ELISA **PDEVs**	↓ level of **PDEVs** after treatment with pitavastatin and EPA

ALA, alpha-linolenic acid; CVDs, cardiovascular diseases; DHA, docosahexaenoic acid; EDEV, endothelial-derived extracellular vesicles; en, energy; EPA, eicosapentaenoic acid; GPIX, glycoprotein IX; MDEVs, monocyte-derived extracellular vesicles; MI, myocardial infarction; MUFAs, monounsaturated fatty acids; n-3 PUFAs, n-3 polyunsaturated fatty acids; PDEVs, platelet-derived extracellular vesicles; PS+EVs, phosphatidylserine positive extracellular vesicles; SFAs, saturated fatty acids.

5.2 Results

5.2.1 Differences in size, number and PS expression of EVs generated in vitro from unstimulated and stimulated platelets

EV characteristics of PDEVs from both unstimulated and stimulated platelets were evaluated at baseline (V1). There were some significant differences in mode size, numbers and PS expression between the two types of PDEVs, in that greater number of EVs were generated in vitro from stimulated platelets and they were larger, although this was only when mode size was considered. However, PS expression was greater in EVs generated from unstimulated platelets (**Figure 5.1**).

To assess PDEV distribution, concentrations of PDEVs were grouped in 50 nm bin sizes (**Figure 5.2A and 5.2B**). EVs derived from unstimulated platelets displayed a significantly greater proportion of small PDEVs (101-150 nm) and a lower proportion of larger PDEVs (151-200 nm) compared with EVs derived from stimulated platelets ($p<0.001$; **Figure 5.2B**).

5.2.2 In vitro-generated PDEVs have a fatty acid composition profile which is different from that of platelets and circulating EVs

PDEVs generated in vitro from both stimulated and unstimulated platelets contained higher proportions of palmitic and stearic acid, and a higher proportion of ALA in comparison with platelets (data taken from the literature), but proportions of EPA, DPA and DHA were lower with respect to n-3 PUFA content. The total MUFA content of PDEVs produced in vitro was different from that of platelets, with an approximately 1.5-fold lower proportion of oleic acid. Proportions of linoleic acid and AA were approximately 4-fold lower and proportions of DGLA were approximately 3-fold higher than in platelets. (**Table 5.2**).

The fatty acid composition of circulating EVs was analysed by another PhD student on the HI-FIVE study and included for comparison in this chapter. PDEVs generated in vitro had higher proportions of palmitic acid, stearic acid, ALA, DGLA, arachidonic acid, EPA and DPA and lower proportions of oleic acid, linoleic acid, ETA and DHA than circulating EVs (**Table 5.2**).

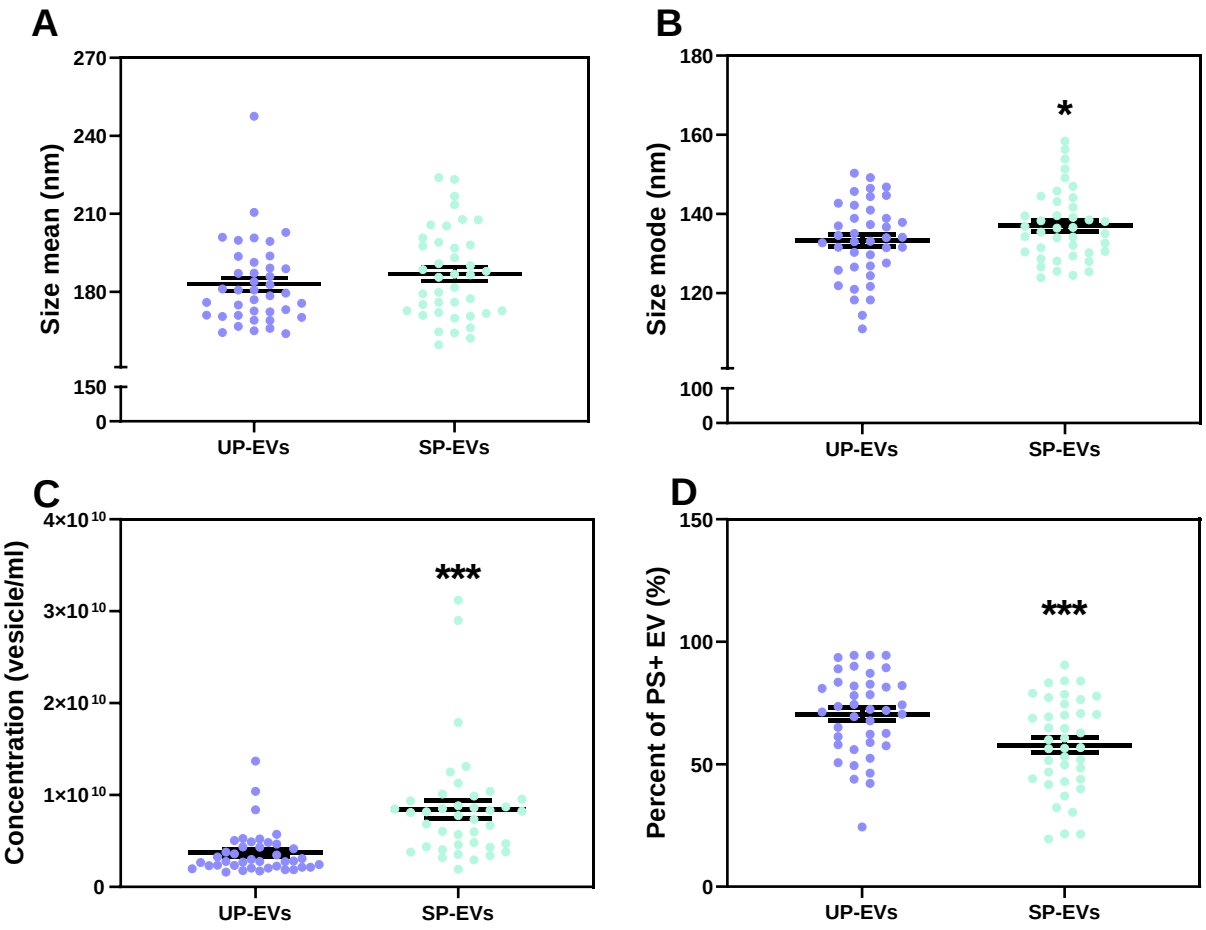


Figure 5.1. Differences in size (mean and mode), number and PS expression of EVs generated in vitro from unstimulated and stimulated platelets. Data are mean ± SEM, (n=40). Comparisons between EVs generated from unstimulated and stimulated platelets were drawn using two-tailed paired *t*-test difference shown at $p < 0.05$. There was a significant difference in **(B)** size mode, **(C)** numbers and **(D)** expression of PS. **p<0.05 and ***p<0.001*; SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles.

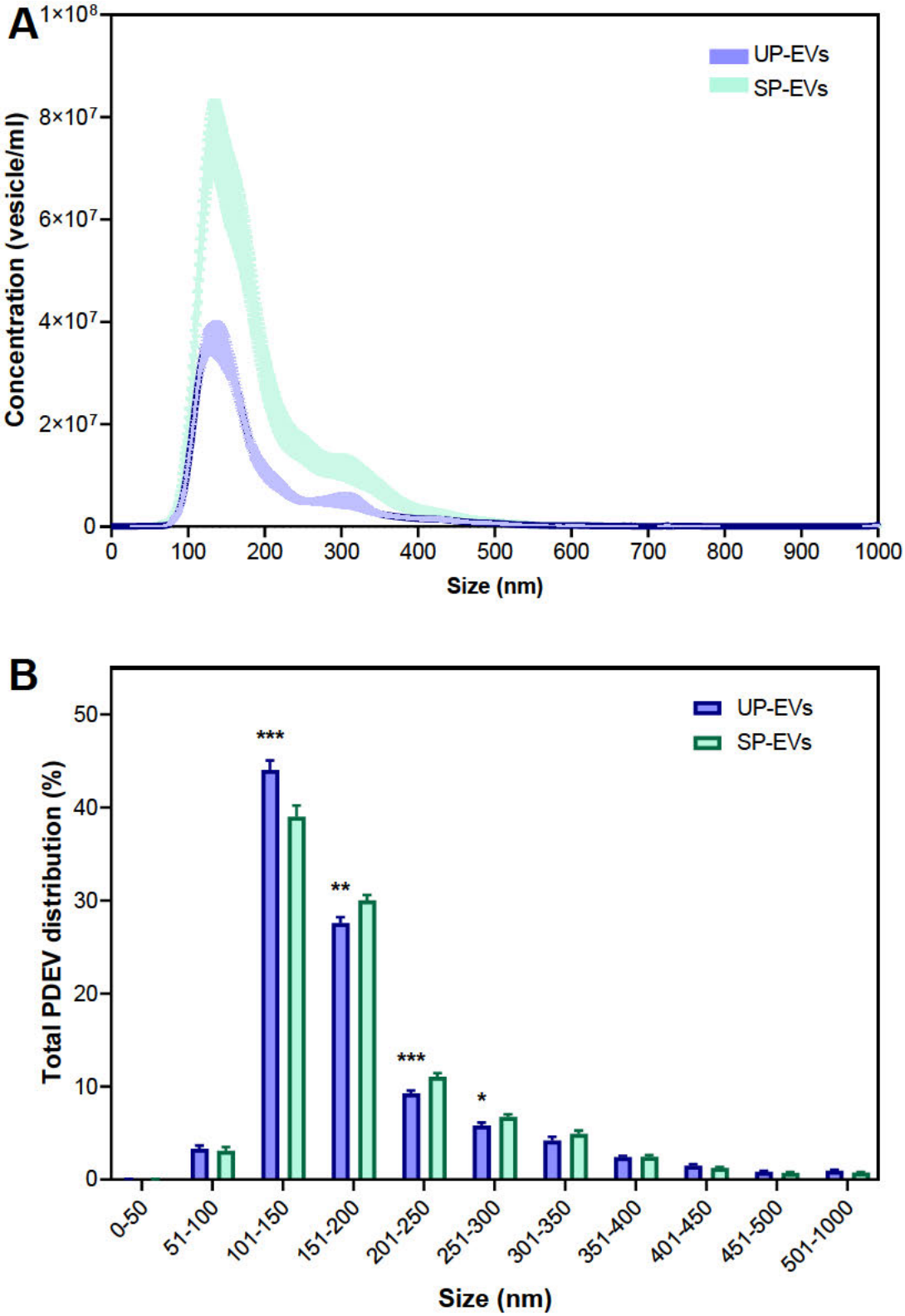


Figure 5.2. Size distribution of EVs generated in vitro from unstimulated and stimulated platelets. Data are mean ± SEM, (n=40). Comparisons between EVs generated from unstimulated and stimulated platelets were drawn using two-tailed paired *t*-test difference shown at $p < 0.05$. Size distribution of in vitro-generated EVs from **(A)** unstimulated or **(B)** stimulated platelets were presented in 50 nm size ranges. **(C)** There was a significant difference in the percentages of in vitro-generated PDEVs at the size range of 101–150 nm, 151–200 nm, 201–250 nm and 251–300 nm. **p<0.05, **p<0.01 and ***p<0.001*; UP-EVs, unstimulated platelet-derived extracellular vesicles.

Table 5.2. Fatty acid composition of circulating EVs, EVs generated in vitro from unstimulated and stimulated platelets, platelets and plasma total phospholipids

	UP-EVs	SP-EVs	Platelets[a]	Circulating EVs[b]	Plasma
Palmitic acid (16:0)	34.42±0.96	32.70±1.00	13.90±1.07	27.51±0.52	29.49±0.19
Stearic acid (18:0)	30.92±0.69	31.01±0.72	19.38±0.67	10.82±0.16	13.95±0.18
Oleic acid (18:1,n-9)	8.30±0.48	9.37±0.57	17.18±0.67	29.70±0.71	12.76±0.35
Linoleic acid (18:2,n-6)	2.51±1.19	2.83±1.26	9.31±0.35	16.99±0.5	21.00±0.33
ALA (18:3,n-3)	2.86±0.25	2.87±0.26	0.13±0.04	1.04±0.05	0.35±0.01
DGLA (20:3,n-6)	7.26±0.47	6.37±0.49	1.29±0.08	1.74±0.12	3.01±0.01
AA (20:4,n-6)	4.02±0.56	5.35±0.69	21.23±0.17	2.81±0.09	9.72±0.26
ETA (20:4,n-3)	0.41±0.07	0.45±0.07	N/A	1.40±0.17	0.24±0.17
EPA (20:5,n-3)	0.98±0.13	0.88±0.14	1.44±0.67	0.61±0.08	1.38±0.09
DPA (22:5,n-3)	0.59±0.06	0.63±0.07	1.09±0.08	0.38±0.03	1.12±0.03
DHA (22:6,n-3)	0.43±0.05	0.47±0.05	2.21±0.11	0.87±0.07	3.62±0.15
Total SFA	67.06	65.37	39.03	38.33	43.44
Total MUFA	12.01	12.95	20.19	35.53	16.12
Total n-3 PUFA	5.26	5.30	4.87	4.30	6.71
Total n-6 PUFA	15.66	16.38	35.91	21.54	33.73

Data are mean ± SEM and are expressed in percentages of the peak area of each individual fatty acid relative to the total of all the fatty acid peak areas (wt%). Circulating EVs from unstimulated and stimulated platelets and plasma only involved baseline data (visit 1) of their fatty acid composition in the current study, respectively. Data for the fatty acid composition of platelets[a] is estimated from healthy subjects (Rise et al., 2007). [b]Data for circulating EVs supplied by another PhD student for comparative purposes; AA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid; EVs, extracellular vesicles; N/A, not applicable.

5.2.3 Effect of n-3 PUFA supplementation on size and number of PDEVs generated in vitro by unstimulated or stimulated platelets

There was no effect of the intervention on the size of PDEVs generated in vitro, regardless of whether they were derived from unstimulated or stimulated platelets, as shown in **Figure 5.3**.

There was also no significant effect of the intervention on numbers of EVs generated in vitro by either unstimulated or stimulated platelets (**Figure 5.4A and 5.4B**).

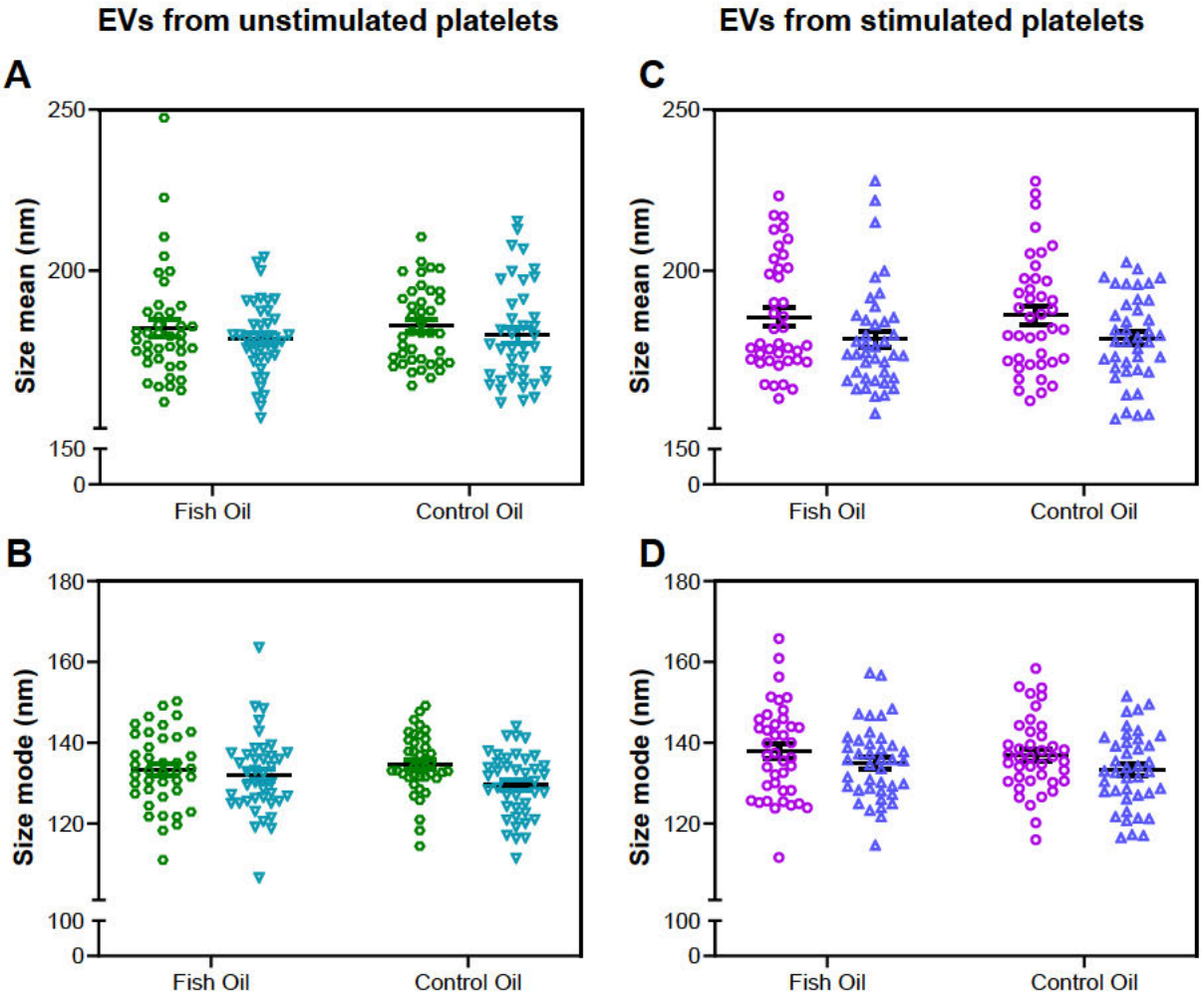


Figure 5.3. Effects of n-3 PUFA intervention in subjects at moderate risk for CVD on size of EVs generated in vitro from unstimulated or stimulated platelets. Each point represents the response of baseline (green circle for UP-EVs, EVs from unstimulated platelets; purple circle for SP-EVs, EVs from stimulated platelets) either for control oil or baseline for fish oil against intervention (turquois triangle for UP-EVs, blue triangle for SP-EVs) either for control oil or for fish oil. Data are mean ± SEM, (n=40). Comparisons after each intervention were drawn using General Linear Model (GLM), including pairwise comparison test with

Bonferroni for treatment, period and treatment*time interaction, with differences shown at $p < 0.05$. There was no significant effect of fish oil on **(A)** mean or **(B)** mode of UP-EVs and **(C)** mean or **(D)** mode of SP-EVs (treatment effects: $p > 0.05$; general linear model). There was no significant effect of time and no treatment*time interaction for parameters of both UP-EV and SP-EV sizes (overall effects: $p > 0.05$; general linear model); SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles.

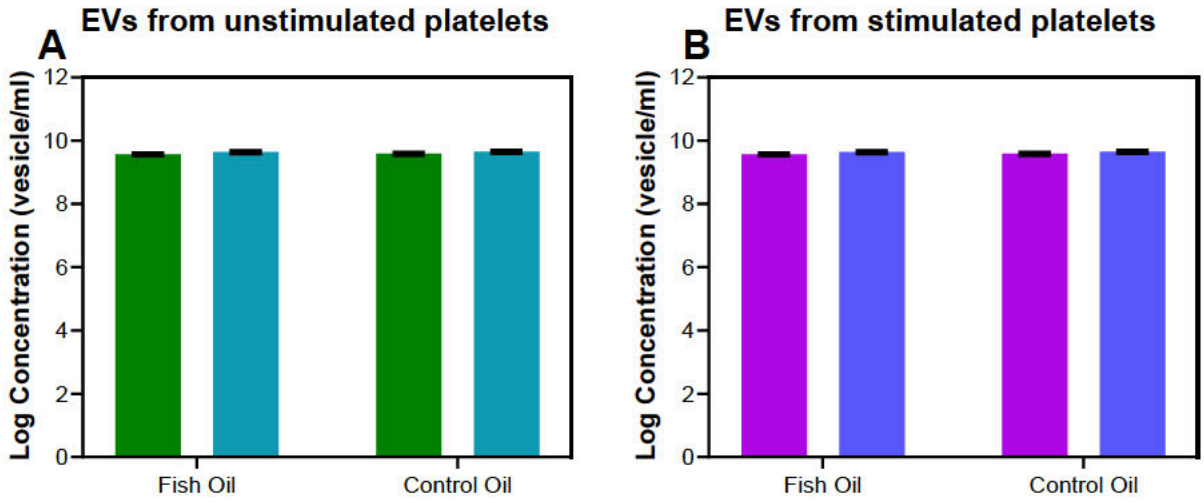


Figure 5.4. Effect of n-3 PUFA intervention in subjects at moderate risk for CVD on numbers of EVs generated in vitro from unstimulated or stimulated platelets. Each bar represents the response of baseline (green bar for UP-EVs, purple bar for SP-EVs) either for control oil or baseline for fish oil against intervention (turquois bar for UP-EVs, blue bar for SP-EVs) either for control oil or for fish oil. Data are mean ± SEM, (n=40). Comparisons after each intervention were drawn using General Linear Model (GLM), including pairwise comparison test with Bonferroni for treatment, period and treatment*time interaction, with differences shown at $p < 0.05$. There was no significant effect of fish oil on the number of either **(A)** UP-EVs or **(B)** SP-EVs (treatment effects: $p > 0.05$; general linear model). There was no significant effect of time and no treatment*time interaction for parameters of both UP-EV and SP-EV numbers (overall effects: $p > 0.05$; general linear model); SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles.

5.2.4 Effects of n-3 PUFAs on total lipid fatty acid composition of EVs generated in vitro from unstimulated or stimulated platelets and circulating EVs

The fatty acid composition of EVs derived from both stimulated and unstimulated platelets before and after intervention is shown in **Table 5.3**. Intervention with fish oil significantly increased the content of GLA, ALA, eicosenoic acid, EPA and total n-3 PUFA of PDEVs derived from stimulated platelets, while decreasing that of arachidonic acid, compared with the control group **(Table 5.3)**. There was no significant impact of time and no treatment*time interaction.

In contrast, for EVs derived from unstimulated platelets, supplementation with fish oil resulted in an increase in ETA, EPA and total n-3 PUFAs, with an accompanying decrease in arachidonic acid, but no effect on GLA, ALA or eicosenoic acid **(Table 5.3)**. There was no significant impact of time and no treatment*time interaction.

The effect of n-3 PUFA supplementation on the fatty acid composition of circulating EVs was assessed by another PhD student on the HI-FIVE study and included for comparison in this chapter (**Table 5.4**). Intervention with fish oil significantly increased proportions of EPA, DHA and DPA in circulating EVs, resulting in a substantial overall increase in total n-3 PUFA, and decreased the proportions of oleic acid, AA and total MUFA (**Table 5.4**). There was no significant impact of time and no treatment*time interaction.

Table 5.3. Effect of n-3 PUFA supplementation on the fatty acid composition of EVs derived from unstimulated or stimulated platelets

	EVs from unstimulated platelets					**EVs from stimulated platelets**				
	Fish Oil		Control Oil		p-value	Fish Oil		Control Oil		p-value
	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	*treatment*	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	*treatment*
Palmitic acid (16:0)	34.95±0.96	34.87±1.00	34.73±0.93	34.30±1.06	0.570	33.88±1.05	33.68±1.01	33.82±0.90	34.81±1.00	0.324
Stearic acid (18:0)	31.34±0.79	31.34±0.77	31.21±0.75	31.01±0.61	0.971	30.65±0.79	31.59±0.76	31.38±0.73	30.48±0.64	0.089
Oleic acid (18:1,n-9)	7.90±0.44	8.10±0.49	7.77±0.42	8.00±0.44	0.686	9.06±0.47	8.27±0.54	8.56±0.54	8.72±0.48	0.236
Linoleic acid (18:2,n-6)	2.60±0.19	2.48±0.18	2.54±0.15	2.65±0.19	0.433	2.80±0.16	2.57±0.19	2.57±0.16	2.73±0.18	0.196
GLA (18:3,n-6)	0.96±0.07	0.98±0.06	0.94±0.07	0.89±0.07	0.350	0.85±0.06	0.99±0.06	0.96±0.06	0.84±0.06	**0.006**
ALA (18:3,n-3)	2.76±0.24	2.83±0.24	2.89±0.24	2.75±0.25	0.236	2.73±0.24	2.93±0.27	2.94±0.16	2.76±0.25	**0.017**
Eicosenoic acid (20:1,n-9)	1.92±0.15	2.12±0.13	1.93±0.14	1.88±0.14	0.291	1.86±0.15	1.93±0.14	2.10±0.14	1.58±0.12	**0.011**
DGLA (20:3,n-6)	6.99±0.42	7.30±0.45	7.50±0.46	7.55±0.41	0.439	6.89±0.46	7.05±0.50	6.90±0.44	6.95±0.36	0.663
AA (20:4,n-6)	3.95±0.52	2.87±0.34	3.73±0.44	4.40±0.52	**0.021**	5.06±0.68	4.01±0.57	4.04±0.44	4.74±0.64	**0.011**

ETA (20:4,n-3)	0.39±0.07	0.55±0.07	0.42±0.07	0.42±0.07	**0.006**	0.44±0.07	0.56±0.03	0.41±0.06	0.48±0.08	0.167
EPA (20:5,n-3)	0.79±0.11	1.24±0.15	1.01±0.14	0.88±0.10	**0.018**	0.64±0.09	1.00±0.14	0.86±0.12	0.78±0.11	**0.042**
DPA (22:5,n-3)	0.62±0.07	0.64±0.06	0.57±0.06	0.63±0.07	0.676	0.65±0.07	0.65±0.07	0.59±0.07	0.57±0.06	0.689
DHA (22:6,n-3)	0.42±0.04	0.50±0.04	0.40±0.04	0.45±0.04	0.863	0.40±0.04	0.50±0.05	0.40±0.04	0.42±0.04	0.282
Total SFA	68.01±1.03	67.89±0.89	67.66±1.77	66.96±1.09	0.600	66.20±0.96	67.04±1.01	66.91±0.96	66.96±1.03	0.692
Total MUFA	11.58±0.47	11.72±0.50	11.34±0.66	11.44±0.45	0.761	12.42±0.45	11.72±0.53	12.37±0.55	11.90±0.46	0.759
Total n-3 PUFA	4.98±0.31	5.77±0.33	5.28±0.56	5.13±0.33	**0.010**	4.86±0.29	5.71±0.35	5.21±0.35	5.00±0.32	**0.003**
Total n-6 PUFA	15.44±0.60	14.62±0.51	15.72±1.19	16.47±0.60	0.069	16.52±0.68	15.54±0.58	15.50±0.56	16.15±0.63	0.114

Data are mean ± SEM (n = 40). Comparisons after each intervention were drawn using General Linear Model (GLM), with differences shown at $p < 0.05$; AA, arachidonic acid; ALA, alpha-linolenic acid; DGLA, dihomo-ɣ-linolenic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; GLA, gamma linolenic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles.

Table 5.4. Effect of n-3 PUFA supplementation on the fatty acid composition of circulating EVs (data supplied by another PhD student for comparative purposes)

	Circulating EVs				
	Fish Oil		Control Oil		p-value
	Before (wt%)	After (wt%)	Before (wt%)	After (wt%)	After (wt%)
Palmitic acid (16:0)	26.9±0.5	26.8±0.4	27.0±0.6	26.6±0.5	0.996
Stearic acid (18:0)	10.9±0.6	11.4±0.7	10.3±0.6	10.3±0.6	0.636
Oleic acid (18:1,n-9)	29.4±0.7	27.5±0.7	29.9±0.7	30.4±0.8	**0.011**
Linoleic acid (18:2,n-6)	17.1±0.5	17.0±0.6	17.7±0.5	17.6±0.5	0.807
ALA (18:3,n-3)	1.1±0.1	1.12±0.1	1.1±0.1	1.1±0.1	0.461
DGLA (20:3,n-6)	1.8±0.1	1.7±0.1	1.8±0.1	1.7±0.1	0.718
AA (20:4,n-6)	2.9±0.1	2.5±0.1	2.9±0.1	2.9±0.1	**<0.001**
ETA (20:4,n-3)	1.7±0.2	2.0±0.2	1.4±0.2	1.5±0.2	0.436
EPA (20:5,n-3)	0.7±0.1	1.6±0.1	0.6±0.1	0.5±0.1	**<0.001**
DPA (22:5,n-3)	0.4±0.03	0.6±0.03	0.4±0.02	0.4±0.03	**0.004**[#]
DHA (22:6,n-3)	0.9±0.1	1.9±0.1	0.9±0.1	0.9±0.1	**<0.001**
Total SFA	37.8±0.9	38.2±0.9	37.3±0.9	36.9±0.9	0.773
Total MUFA	34.3±0.8	32.1±0.8	34.9±0.8	35.1±0.9	**0.013**
Total n-3 PUFA	4.7±0.3	7.2±0.2	4.3±0.2	4.5±0.2	**<0.001**
Total n-6 PUFA	21.9±0.6	21.2±0.6	22.3±0.6	22.2±0.5	0.627

Data are mean ± SEM (n = 40). Comparisons after each intervention were drawn using General Linear Model (GLM), with differences shown at $p < 0.05$; ALA, alpha-linolenic acid; DGLA, dihomo-γ-linolenic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acid; PFP, platelet-free plasma; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

5.2.5 Effect of n-3 PUFA intervention on expression of PS by EVs generated in vitro from unstimulated or stimulated platelets

Expression of PS on the PDEV surface was assessed by flow cytometry. Intervention with fish oil significantly decreased the expression of PS by EVs generated from unstimulated platelets (**Figure 5.5A**). There was a similar trend for EVs generated from stimulated platelets, but this was not statistically significant (**Figure 5.5B**).

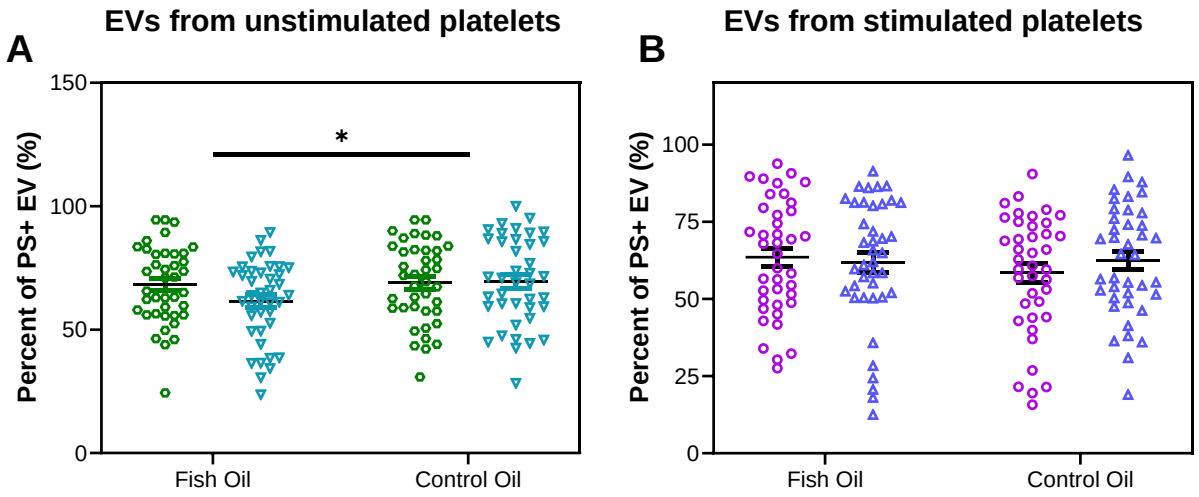


Figure 5.5. Effect of n-3 PUFA intervention in subjects at moderate risk for CVD on expression of PS by EVs generated in vitro from unstimulated or stimulated platelets. Each point represents the response of baseline (green circle for UP-EVs, purple circle for SP-EVs) either for control oil or baseline for fish oil against intervention (turquois triangle for UP-EVs, blue triangle for SP-EVs) either for control oil or for fish oil. Data are mean ± SEM, (n=40). Comparisons after each intervention were drawn using General Linear Model (GLM), including pairwise comparison test with Bonferroni for treatment, period and treatment*time interaction, with differences shown at $p < 0.05$. There was a significant effect of fish oil on **(A)** the percentage of PS+ UP-EVs (treatment effects: $p < 0.05$; general linear model). There was no significant effect of fish oil on **(B)** the percentage of PS+ SP-EVs (treatment effects: $p < 0.05$; general linear model). There was no significant effect of time and no treatment*time interaction for the percentage of both types of PS+ PDEVs (overall effects: $p > 0.05$; general linear model); PS+ PDEVs, PS-positive platelet derived extracellular vesicles; SP-EVs, stimulated platelet-derived extracellular vesicles. **p<0.05*; UP-EVs, unstimulated platelet-derived extracellular vesicles.

5.5 Discussion

This randomised controlled crossover trial demonstrated, for the first time, that n-3 PUFA supplementation had no effect on the number or size of EVs generated in vitro from stimulated or unstimulated platelets, but altered their fatty acid composition and, in the case of EVs derived from unstimulated platelets, decreased their surface expression of PS.

In the current study, intervention with n-3 PUFAs did not alter the mean size of EVs derived from either stimulated or unstimulated platelets. In vitro-generated PDEVs were smaller than 500 nm, with the majority ranging from 100-250, consistent with earlier studies (De Paoli et al., 2018, Kushwaha et al., 2018) and there were differences in size distribution depending on whether the PDEVs were generated from stimulated or unstimulated platelets, as reported previously (Aatonen et al., 2014, Aatonen et al., 2012), such that PDEVs generated from unstimulated platelets tended to be smaller than those generated from stimulated platelets, which may indicate preferential release of smaller EVs via the exosomal pathway by unstimulated platelets. Although platelets release EVs over a wide range of sizes upon agonist activation (Heijnen et al., 1999), it has been suggested that they predominantly shed larger EVs (>100 nm) from outward budding from their plasma membrane than smaller EVs (<100 nm), which might be classified as exosomes (Aatonen et al., 2014), consistent with data in the current study. Shedding of EVs following agonist stimulation of platelets involves Ca^{2}+ influx and activation of the Ca^{2}+-dependent protease calpain (Crespin et al., 2009), while the mechanisms for EV generation by unstimulated platelets are postulated to involve glycoprotein (GP) IIb/IIIa signalling (Cauwenberghs et al., 2006). However, there is no indication as to whether these two distinct pathways affect the size of EVs. It has been suggested that smaller EVs may be more procoagulant than larger EVs (De Paoli et al., 2018, Tripisciano et al., 2017b), although size is not a key determinant of the coagulatory activity of PDEVs (Ferreira et al., 2020), and coagulatory behaviour of PDEVs is examined in the next chapter (**Chapter 6**).

The present study demonstrated, for the first time, that fish oil supplementation had no impact on the ability of washed platelets to generate EVs in vitro (according to NTA data), regardless of whether the platelets were stimulated or not. This in vitro data does not necessarily extrapolate to mean that fish oil does not alter the numbers of circulating PDEVs. Indeed, another PhD student on the project demonstrated that supplementation with n-3 PUFA significantly reduced the numbers of total circulating EVs, the majority of which were PDEVs, as analysed by NTA. They also demonstrated that n-3 PUFA decreased the number of circulating PS+ PDEVs, which comprised 63 percent of all circulating EV population at baseline. It should be noted, however, that the latter were identified by flow cytometry, which is only able to detect PDEVs with a diameter >200 nm, and therefore only a small subpopulation of the total PDEVs (Aatonen et al., 2014). In the current study, size distribution according to NTA showed that 25-28% of in vitro-generated PDEVs were larger than 200 nm. However, a previous study suggested that a limitation of NTA of PDEVs is that larger particles may mask smaller ones when at high concentration in an EV suspension, which could result in underestimation of PDEV numbers (Aatonen et al., 2012). Also, NTA is unable to distinguish protein aggregated from vesicles (Aatonen et al., 2017). Therefore, a combination of techniques, including FCM and NTA, can provide better insight, although it is still challenging to report an accurate number of circulating PDEVs across their entire size distribution.

Despite the fact that there is a lack of comparable data for characterisation of in vitro-generated PDEVs following an intervention study, the results presented in this chapter can be compared with data reported by another PhD student on the HI-FIVE study and with previously published data. Seven previous studies reported effects of n-3 PUFA on numbers of circulating PDEVs, four using FCM (Nomura et al., 2003, Del Turco et al., 2008, Phang et al., 2012a, Wu et al., 2014) and three using ELISA (Nomura et al., 2009a, Nomura et al., 2018, Englyst et al., 2007). Five of these studies involved patients with various disease status, such as hyperlipidaemia, type 2 diabetes and post-MI, with only one study investigating healthy subjects. Although there is variation in methodology and subject group, most of the previous

studies (four out of five) indicated that n-3 PUFA decrease numbers of circulating PDEVs, in agreement with the outcome of the HI-FIVE study. Of the studies reporting significant effects of n-3 PUFA on numbers of circulating PDEVs, there appeared to be no relationship with dose (**Table 5.1)**, but effects in general tend to be reported in subjects with disease, such as hyperlipidemia (Nomura et al., 2003, Nomura et al., 2009a), post-myocardial infarction (Del Turco et al., 2008), type 2 diabetes (Nomura et al., 2009a) and arteriosclerosis accompanied by type 2 diabetes (Nomura et al., 2018), while studies in healthy subjects (Phang et al., 2012b) or those with moderate risk of CVD (Wu et al., 2014) tend to report no effect.

As indicated earlier, another PhD student on the HI-FIVE project demonstrated a significant decrease in numbers of circulating total EVs and of PS+ PDEV after supplementation with 1.8 g/day of n-3 PUFAs. This result is in line with a study conducted by Nomura *at al* (2003), where supplementation with 1.8 g/day of EPA significantly reduced the number of GPIX+ PDEVs in hyperlipidemic patients with type 2 diabetes, although this uncontrolled study was conducted in a small sample size of 23 subjects and captured different types of PDEVs using different phenotyping techniques (not PS+) (Nomura et al., 2003). Later, two reports by Nomura and colleagues confirmed a favourable effect of either only n-3 PUFA supplementation (1.8 g/d of EPA) or a combination of n-3 PUFA with cholesterol lowering medication (2 mg/d of pitavastatin) on numbers of PDEVs in hyperlipidemic subjects with diabetes (Nomura et al., 2009a) and arteriosclerosis obliterans patients with type2 diabetes (Nomura et al., 2018), this time conducting PDEV analysis by ELISA (Nomura et al., 2018). In a separate study, a reduction in CD61+ PDEV numbers was demonstrated in post-MI patients after intervention with 5.2 g/d of n-3 PUFA (n=21); however, this relatively small study represented a different phenotype of PDEVs (CD61+) (Del Turco et al., 2008).

While previous studies reported consistent PDEV-lowering effects of n-3 PUFAs in disease, studies in healthy subjects are less so. For example, in healthy men, a particularly high dose of 6 g/d of n-3 PUFAs for 12 weeks resulted in an unexpected increase in the number of CD61+ PDEVs (Englyst et al., 2007). Authors attributed this to a 53% increase in LDL-C

concentration and a 7% increase in platelet aggregation in response to collagen, although there was no specific evidence for a causal link for either parameter (Englyst et al., 2007). One study reported no effect of a single 2 g dose of either EPA or DHA on numbers of PS+ PDEVs (analysed using similar techniques to those described in this chapter) in healthy subjects 24 hours after supplementation (Phang et al., 2012b). The lack of effect may be attributed to it being an acute intervention in healthy population, which could indicate that n-3 PUFAs need to be incorporated into platelet membrane phospholipids in order to subsequently alter numbers of circulating PDEVs in vivo. Di Stasi *et al* (2004) reported that 2 g/d of n-3 PUFA increased total n-3 PUFA content of platelets after 1 week (Di Stasi et al., 2004).

A study in subjects at moderate risk for CVD, similar to the current study and conducted in our research laboratory, reported no effect of 1.5 g/d of n-3 PUFA supplementation on CD42+ PDEVs (Wu et al., 2014). However, the phenotyping analysis of PDEVs in this and two other studies (Nomura et al., 2003, Del Turco et al., 2008) was later found to be flawed because polystyrene calibration beads were used for setting gates in the FCM, and these were later discovered to result in greater light scattering compared to similar-sized biological EVs (Van der Pol et al., 2016). It is therefore likely that EV size has routinely been underestimated when employing FCM because the population gated as EVs is likely to have additionally included some small platelets and apoptotic bodies. This highlights how the use of different protocols and calibration techniques to characterise and phenotype PDEVs prior to the recent standardisation and guidance has resulted in inconsistency in some of the reported effects of fish oil on PDEV numbers.

There is almost no relevant data relating to the fatty acid composition of PDEVs generated in vitro, although it was hypothesised that their fatty acid composition would reflect that of platelets. The fatty acid composition of the ‘parent’ platelets was not analysed in the current study due to lack of sufficient sample, but there is published data on the fatty acid composition of platelets (Rise et al., 2007), which was compared with that of circulating EVs and PDEVs in the current study. This demonstrated that the fatty acid composition of PDEVs generated in

vitro was markedly different from that of platelets and therefore contrary to the hypothesis. PDEVs generated in vitro contained a higher proportion of SFAs (both palmitic and stearic acid) and total n-3 PUFA than platelets (due to higher proportion of ALA), and the proportions of EPA, DPA and DHA were lower. The proportions of LA and AA and a proportion of oleic acid in PDEVs produced in vitro were largely lower than that in platelets. Overall, it appeared that in vitro-generated PDEVs regardless of whether they were derived from unstimulated or stimulated platelets were enriched in SFAs and total n-3 PUFA but contained lower proportions of total n-6 PUFA and MUFA, suggesting that PDEVs generated in vitro possess a unique fatty acid profile that does not resemble that of platelets and or that of circulating PDEVs. This is in line with the observation by Hu *et al* (2016) of differences in lipid classes and species of in vitro-generated PDEVs compared with 'parent' platelets (Hu et al., 2016). Also, Biró *et al* (2005) reported that the phospholipid content of PDEVs differed significantly from three platelet membrane fractions, including platelet plasma-, intracellular- and granule membranes and suggested that in vitro-generated EVs from stimulated platelets might be enriched in lipid rafts, given that PDEVs had a tendency to have a higher cholesterol content (Biró et al., 2005). The formation of EVs may involve selective enrichment of lipid rafts present in the platelet plasma membrane; these are notably more saturated because of enrichment in sphingomyelin and free cholesterol (Pollet et al., 2018, Stillwell, 2006) and is consistent with the enrichment of PDEVs with palmitic and stearic acid in the current study. Further studies examining the partitioning of fatty acids into PDEVs during their generation from platelets will be important in the future.

The fact that the fatty acid composition of EVs generated in vitro from unstimulated and stimulated platelets was also markedly different from that of circulating EVs from the same subjects was also contrary to the original hypothesis. PDEVs generated in vitro contained a lower proportion of oleic acid, linoleic acid, ETA and DHA and a higher proportion of stearic acid, ALA, DGLA, arachidonic acid, EPA and DPA than circulating EVs, suggesting that the in vitro model for EV generation may not fully replicate the in vivo process, although it is important

to note that circulating EVs represent a heterogeneous mixture of EVs, whereas the in vitro-generated PDEVs are derived exclusively from platelets. Differences in the phospholipidomes of circulating EVs compared with EVs produced in vitro from activated platelets have been reported previously (Losito et al., 2015). It is estimated that 10-40% of circulating EVs are generated by blood cells such as erythrocytes, leucocytes or endothelial cells rather than platelets (Brisson et al., 2017) and these alternative sources of circulating EVs might partly account for differences in the fatty acid composition of circulating EVs compared with PDEVs produced in vitro, although the differences are so marked that they require further investigation.

Supplementation with fish oil alters the fatty acid composition of platelet membrane phospholipids (Adili et al., 2018), which in turn may affect the ability of platelets to shed EVs (O'Donnell et al., 2014). The current study demonstrated that there was a substantial increase in the n-3 PUFA content of EVs derived from both stimulated and unstimulated platelets following supplementation, indicating that diet-induced changes in the fatty acid composition of platelets were carried through to the PDEVs generated in vitro. Fish oil supplementation resulted in an increase in the proportions of four individual fatty acids: GLA, ALA, eicosenoic acid and EPA in EVs derived from stimulated platelets whilst in EVs derived from unstimulated platelets, only ETA and EPA were elevated. These results suggested that the mode of platelet stimulation influenced the impact of fish oil on the fatty acid composition of PDEVs. Since n-3 PUFA supplementation generally increases the proportions of both EPA and DHA in platelets (Di Stasi et al., 2004, Mori et al., 1997, Larson et al., 2011), it was surprising to see that only the proportion of EPA was increased in PDEVs generated in vitro from platelets, particularly since the proportions of both EPA and DHA were increased in circulating EVs, as demonstrated by another PhD student on the HI-FIVE study and included for comparison in the results section of this chapter. This may indicate that DHA is selectively excluded from being incorporated into PDEVs generated in vitro, but this requires further investigation. The unexpected increase in GLA in EVs derived from stimulated platelets after n-3 PUFA

supplementation is difficult to explain, since this fatty acid is not detectable in platelets. The increase in proportion of ALA in PDEVs generated in vitro following fish oil supplementation is also unexpected as this n-3 PUFA is generally only observed in plasma phospholipids following substantial ALA intake (Wallace et al., 2003). In both circulating and in vitro-generated PDEVs, n-3 PUFA replaced arachidonic acid, which is a commonly observed phenomenon in plasma phospholipids, cells and platelets following fish oil supplementation (Woodman et al., 2003, Mori et al., 1997, Larson et al., 2011, Yaqoob et al., 2000, Walker et al., 2015, Arnold et al., 2017, Harris et al., 2007). The molecular mechanisms controlling the trafficking of fatty acids during the generation of PDEVs by platelets are poorly understood; nevertheless, examination of the fatty acid profile of in vitro-generated PDEVs following fish oil supplementation, as presented in this chapter, is highly novel.

PS exposure relates to negatively charged phospholipid externalisation on the EV surface, which is a feature of EV generation (Owens III and Mackman, 2011). Negatively charged phospholipids support the formation of the tenase (factors IXa, VIIIa, and X) and prothrombinase (factors Xa, Va, and prothrombin) complexes (Spronk et al., 2014), thereby supporting the coagulation cascade, resulting in the conversion of fibrinogen to fibrin (Voukalis et al., 2019, Sinauridze et al., 2007). Lower PS exposure therefore suggests a reduction in procoagulant potential (Owens III and Mackman, 2011, Zarà et al., 2019). The current study demonstrated that supplementation significantly decreased PS exposure by PDEVs generated from unstimulated platelets. Although the mechanism underlying the effect of n-3 PUFA on PS exposure is not clear, remodelling of platelet membrane phospholipids in response to fish oil supplementation may play a role. A recent study demonstrated that cholesterol depletion of platelets prevented the formation of EVs, suggesting that lipid rafts may be required for the generation of PS-exposing EVs by platelets (Wei, Malcor, & Harper, 2018). Separately, the composition and size of these lipid rafts have been shown to be disrupted by n-3 PUFA (Stillwell, 2006, Turk and Chapkin, 2013), indicating a potential

mechanism by which the generation of PS-exposing EVs might be reduced following supplementation with n-3 PUFA.

It has been reported that PDEVs derived from stimulated platelets express lower levels of negatively charged PS on their surface compared to EVs released by unstimulated platelets (Ferreira et al., 2020); this was confirmed in the current study. However, it was notable that PS exposure by EVs generated from stimulated platelets was not altered by fish oil in the current study, although there was a trend for a similar effect to the EVs derived from unstimulated platelets. If EVs derived from stimulated platelets already have lower PS expression on their surface than those derived from unstimulated platelets, this might explain the lack of a significant effect of n-3 PUFA supplementation on PS exposure by PDEVs produced by stimulated platelets. Although there are indications that different agonists mediate EV generation via distinct pathways (Schoenwaelder et al., 2009), there is still very little information from a lipid biochemistry perspective about the mechanisms underlying generation of PDEVs in vitro, and this includes a lack of understanding about potential differences between the mechanisms underlying the generation of EVs from stimulated vs unstimulated platelets, which deserves further attention.

5.6 Conclusion

The present study provides novel insight into the effect of fish oil on the generation and characteristics of PDEVs produced in vitro from platelets. In subjects with moderate risk for CVD, supplementation with n-3 PUFAs did not affect size or number of PDEVs generated in vitro from unstimulated or stimulated platelets, despite the fact that it did decrease numbers of circulating EVs and PS-positive circulating PDEVs, as reported in another PhD thesis from the research group. Supplementation with n-3 PUFA altered the fatty acid composition of both circulating and in vitro-generated PDEVs, although they were not comparable, either with each other, or with the known fatty acid composition of platelets; this requires better understanding of the remodelling of membrane lipids during the generation of EVs. Supplementation decreased the proportion of PS-exposing PDEVs produced by unstimulated platelets, indicating a potential reduction in procoagulatory activity, which will be examined in the next chapter.

Chapter 6. Effects of n-3 PUFAs on the functional activities of EVs generated in vitro from washed platelets

6.1 Introduction

EVs are proposed to play a role in coagulation and haemostasis, which rely on their procoagulant and fibrinolytic activities (Chen et al., 2018, Zarà et al., 2019). Platelet-derived EVs (PDEVs), which comprise the most abundant EV population in the circulation, at least in healthy individuals (Brisson et al., 2017), are considered to be particularly prothrombotic, supporting platelet activation, thrombin generation and thrombus formation (Nomura and Shimizu, 2015, Tripisciano et al., 2017b, Aatonen et al., 2014) playing an important role in vascular dysfunction and thrombosis (Zarà et al., 2019, Boulanger et al., 2017). Elevated numbers of EVs in the circulation are reported to be associated with a high risk of thromboembolic events, including atherosclerosis and stable coronary artery disease (CAD) (Chen et al., 2018). **Chapter 4** of this thesis described an association between higher numbers of circulating EVs and increased thrombogenic risk markers, including accelerated fibrin clot formation and thrombin generation, in agreement with other studies (Tripodi et al., 2011, Ayers et al., 2014, Nielsen et al., 2018, Sinauridze et al., 2007, Silachev et al., 2019).

PDEVs have 50- to 100-fold greater procoagulant activity than activated platelets (Sinauridze et al., 2007) due to exposure of phosphatidylserine (PS) and tissue factor on the outer surface of their membranes. During the formation of plasma membrane-derived EVs from platelets upon activation, PDEV membranes are enriched with negatively charged phospholipids, such as PS, due to flipping of PS from the inner to the outer leaflet of the platelet membrane, with these EVs shed from the plasma membrane (microvesicles) being considered as the predominant source of PS (Brisson et al., 2017, Lhermusier et al., 2011). This negatively charged phospholipid surface facilitates the assembly and activation of tenase (factors IXa, VIIIa, and X) and prothrombinase complexes (factors Xa, Va, and prothrombin), thereby supporting further activation of the coagulation cascade to generate thrombin and

subsequently convert fibrinogen to fibrin (Voukalis et al., 2019, Sinauridze et al., 2007, Aatonen et al., 2014) (for more details see **Chapter 1**). Exposure of tissue factor (Zarà et al., 2019), acts as a key activator to initiate the coagulation cascade by binding factor FVII (Zwicker et al., 2011). In addition, the exposure of PS by EVs may support the transformation of tissue factor to its active form (Tripisciano et al., 2017a, Voukalis et al., 2019). Although the mere presence of PS on the surface of EVs is suggested to be an indicator of procoagulant activity (Connor et al., 2010, Tripisciano et al., 2017b, Lipets et al., 2014), it can also be demonstrated by their influence on fibrin clot formation, thrombin generation and fibrinolysis in reconstructed systems (Tripisciano et al., 2017b, Zubairova et al., 2015b, Sinauridze et al., 2007, Berckmans et al., 2019, Del Turco et al., 2008).

Long chain n-3 PUFAs have been reported to have anti-inflammatory, anti-thrombogenic and anti-atherogenic properties (Calder, 2015, Calder, 2011, Grundt and Nilsen, 2008, Siscovick et al., 2017, Golanski et al., 2021), which have, in turn, been associated with reduced risk for cardiovascular diseases (CVDs) (Hu et al., 2019) although not all studies are consistent in this respect (Abdelhamid et al., 2018, Nicholls et al., 2020). The cardioprotective effects have been attributed to beneficial impacts on conventional and thrombogenic risk markers of CVDs and are associated with the modulation of the lipid composition of the plasma, platelets and cells (**Chapter 3, Section 2.1**) (Calder, 2014, Adili et al., 2018). It has been suggested that the intake of n-3 PUFA may alter platelet function due to the fact that n-3 PUFAs can incorporate into platelet membrane phospholipids (Adili et al., 2018) eicosanoid-generating pathways, thereby altering platelet function (Yeung et al., 2017). Moreover, the incorporation of n-3 PUFAs into EVs derived in vitro from unstimulated and stimulated platelets was described in **Chapter 5** of this thesis, and the question therefore arises as to whether n-3 PUFA could also affect the coagulatory behaviour of in vitro-generated PDEVs. Supplementation with fish oil has previously been reported to reduce the procoagulant activities of EVs in subjects with post-myocardial infarction (Del Turco et al., 2008). A study by Phang *et al* (2012) also demonstrated that acute supplementation of healthy subjects with a single dose of EPA

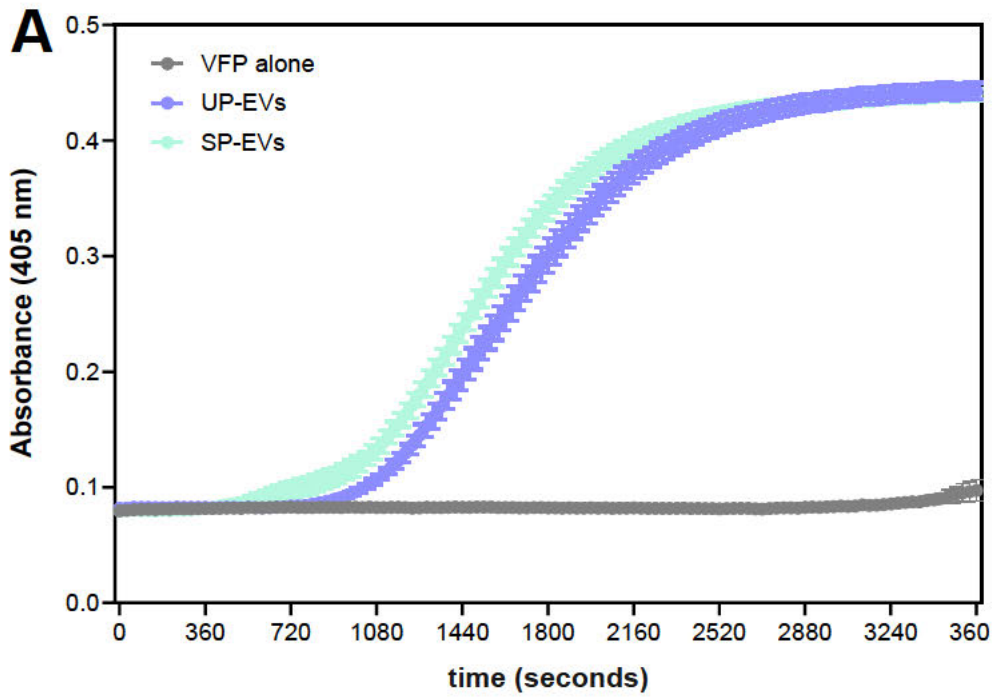
significantly decreased the procoagulant activities of PDEVs, but DHA-rich supplementation had no effect (Phang et al., 2012a). With only one chronic and one acute study exploring the effects of n-3 PUFAs on the coagulatory behaviour of EVs, a more detailed exploration of the effect of fish oil on different stages of the coagulation cascade is warranted.

The current randomized, double-blind, placebo-controlled, crossover trial aimed to investigate whether daily supplementation of subjects at moderate risk of CVDs with 1.8 g/d of fish oil-derived n-3 PUFAs altered the coagulatory behaviour of PDEVs generated in vitro by stimulated and unstimulated platelets by examining their influence on fibrin clotting, thrombin generation, fibrinolysis and ex vivo thrombus formation. The hypothesis was that supplementation with n-3 PUFAs will inhibit the procoagulant functions of in vitro-generated PDEVs.

6.2 Results

6.2.1 Differences in clot formation, thrombin generation and fibrinolysis of EVs generated in vitro from unstimulated and stimulated platelets

Pooled vesicle free plasma (VFP) from healthy subjects (n=3) was used for benchmarking purposes in the assessment of clot formation, thrombin generation and fibrinolysis induced by in-vitro-generated PDEVs in the intervention study. VFP supported virtually no fibrin clot formation, but the addition of PDEVs generated in vitro to VFP significantly enhanced all aspects of clot formation which could be determined (**Figure 6.1A**). For thrombin generation, VFP alone was significantly less able to support thrombin generation than VFP reconstituted with in vitro-generated PDEV (**Figure 6.1B**). Pooled VFP alone from healthy subjects (n=3) significantly shortened time for fibrinolysis in comparison with VFP reconstituted with in vitro-generated PDEV (**Figure 6.1C**). Procoagulant activities of PDEVs from both unstimulated and stimulated platelets were also evaluated at baseline (V1). There were no statistically significant differences in clot formation, thrombin generation and fibrinolysis between the two types of PDEVs (**Figure 6.1**).



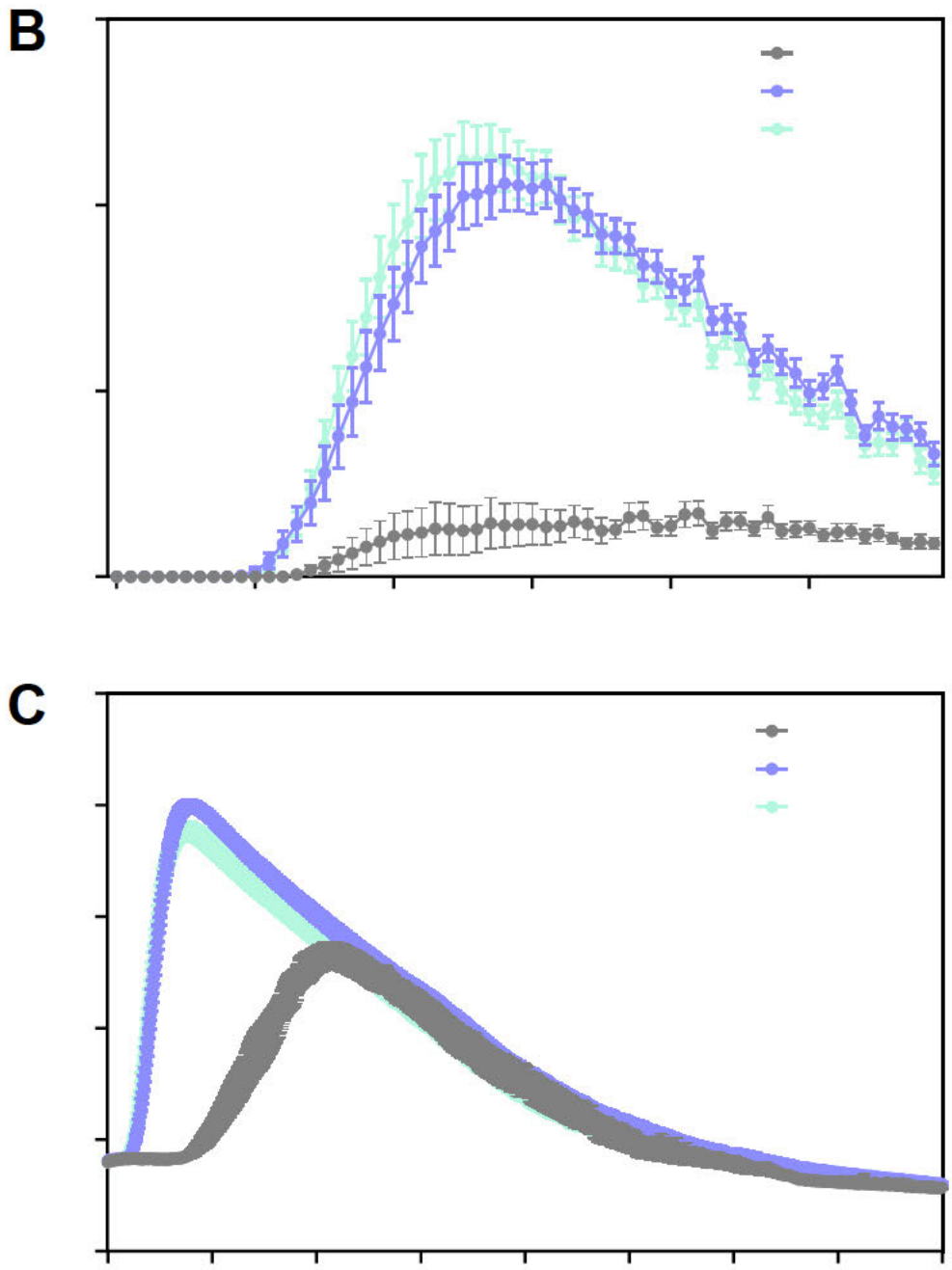
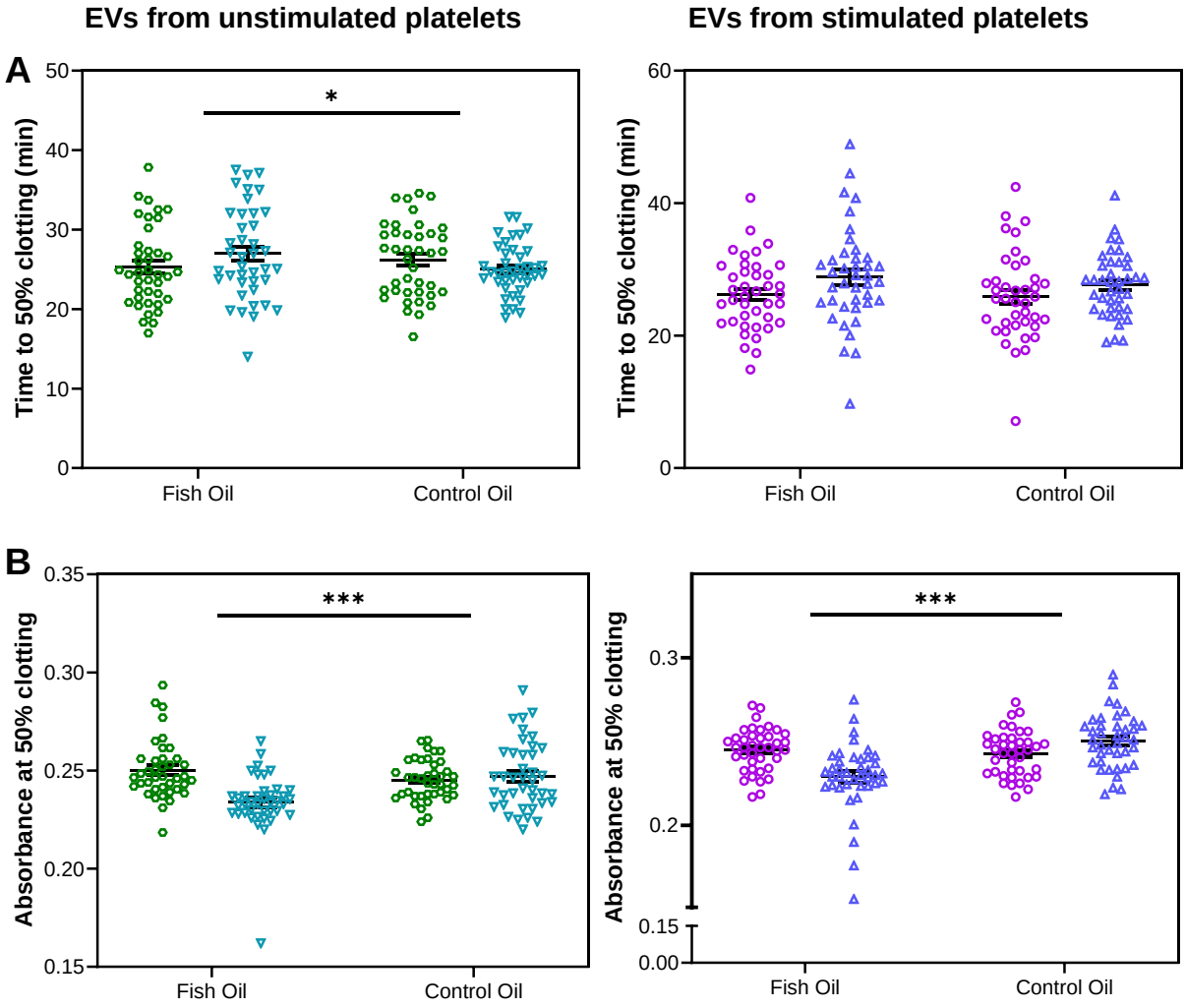


Figure 6.1. Differences in clot formation, thrombin generation and fibrinolysis of EVs generated in vitro from unstimulated and stimulated platelets. Data are mean ± SEM, (n=40). Comparisons were drawn using two-tailed paired *t*-test difference shown at $p < 0.05$. There was no difference in any EV-mediated clot formation between EVs generated in vitro from unstimulated and stimulated platelets. Addition of PDEVs into VFP significantly augmented; SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles.

6.2.2 Effect of n-3 PUFA supplementation on clot formation induced by PDEVs generated in vitro by unstimulated or stimulated platelets

PDEVs from subjects supplemented with fish oil and derived from unstimulated platelets delayed clotting time compared with those derived from the control group (**Figure 6.2A, left panel**). Fibrin clot formation induced by PDEVs from both unstimulated and stimulated platelets was also reduced by fish oil supplementation (**Figure 6.2B to 6.2D**). There was no significant effect of time and no treatment*time interaction with respect to any parameter, apart from absorbance at 50% clotting and absorbance at peak for in vitro-generated PDEVs derived from unstimulated platelets, where there were significant effects of treatment*time interaction.



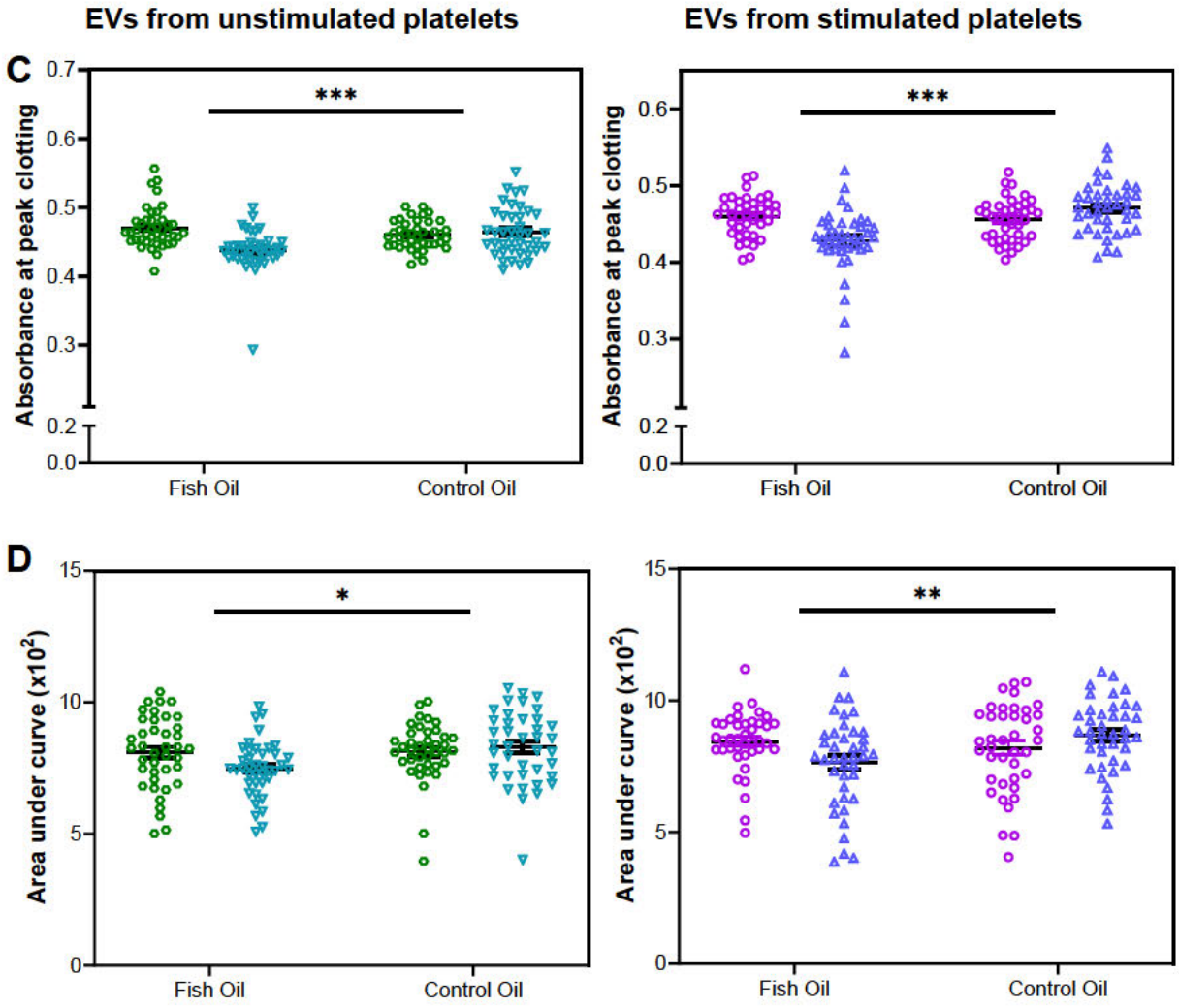
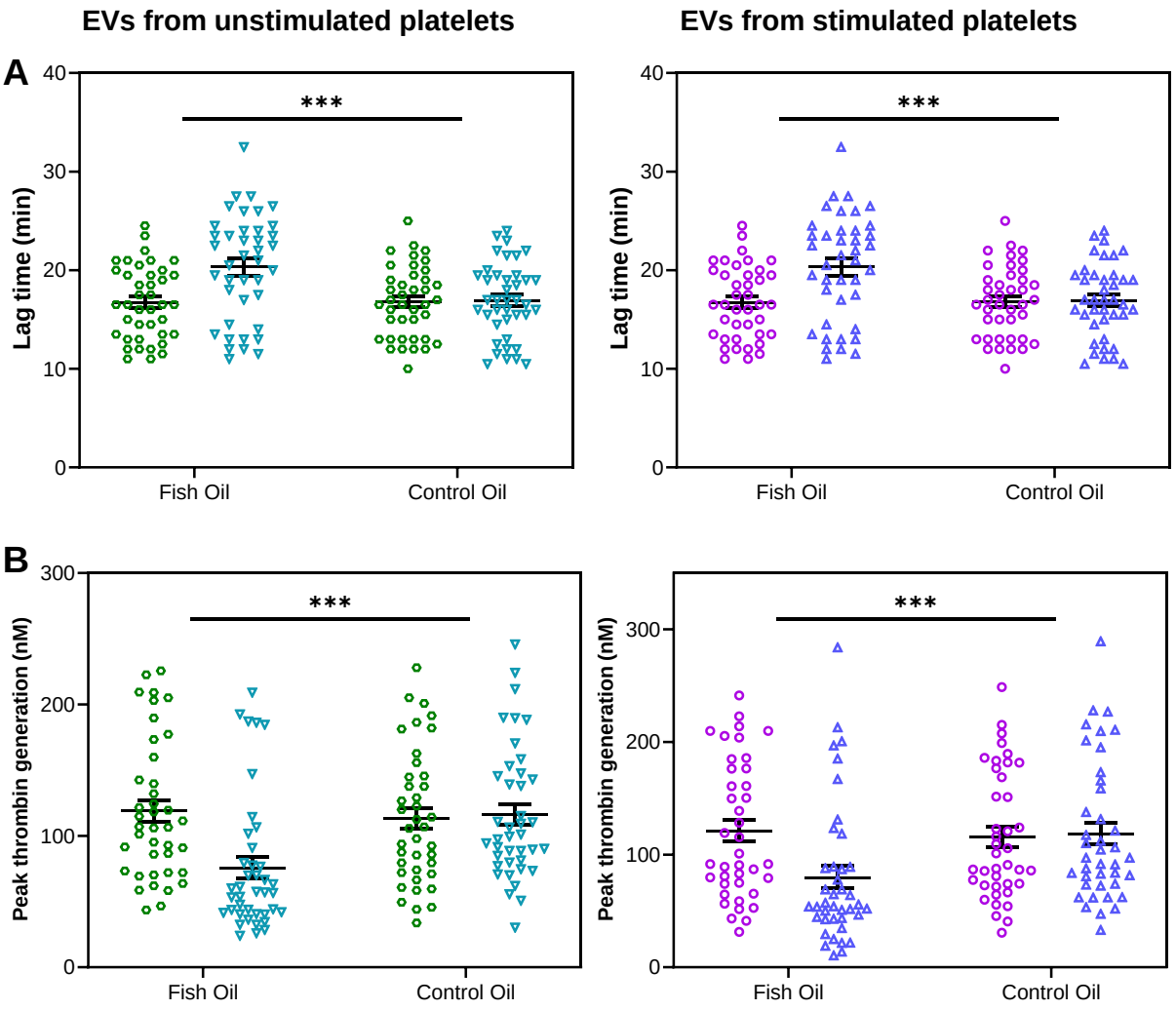


Figure 6.2. Effects of n-3 PUFA intervention on clot formation induced by EVs generated in vitro from unstimulated or stimulated platelets. Each point represents the response of baseline (green circle for UP-EVs, circle for SP-EVs) either for control oil or baseline for fish oil against intervention (turquois triangle for UP-EVs, blue triangle for SP-EVs) either for control oil or for fish oil. Data are mean ± SEM, (n=40). Comparisons after each intervention were drawn using the General Linear Model (GLM), including pairwise comparison with Bonferroni test for treatment, period and treatment*time interaction, with differences shown at $p < 0.05$. Pooled VFP from healthy subjects (n=3) was served as negative control. There was significant effect of fish oil on **(A left panel)** time to 50% clotting for only UP-EVs, while there was significant effect of fish oil on **(B)** absorbance at 50% clotting, **(C)** absorbance at peak and **(D)** area under curve for both (**left panel**) UP-EVs and (**right panel**) SP-EVs (treatment effects: $p < 0.05$; general linear model). There was no significant effect of time and no treatment*time interaction with respect to any parameter (overall effects: $p > 0.05$; general linear model), apart from a significant effect of treatment*time interaction with respect absorbance at 50% clotting and absorbance at peak for UP-EV (treatment*time effects: $p < 0.05$; general linear model). **p<0.05, **p<0.01 and ***p<0.001*; SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles.

6.2.3 Effect of n-3 PUFA supplementation on thrombin generation induced by PDEVs generated in vitro by unstimulated or stimulated platelets

Thrombin generation supported by PDEVs derived from subjects supplemented with fish oil was significantly reduced compared with that supported by PDEVs from subjects supplemented with control oil, as reflected by increased lag time and time to peak thrombin, decreased peak thrombin, velocity index and area under curve (**Figure 6.3**). There was no significant effect of time and no treatment*time interaction for any parameters.



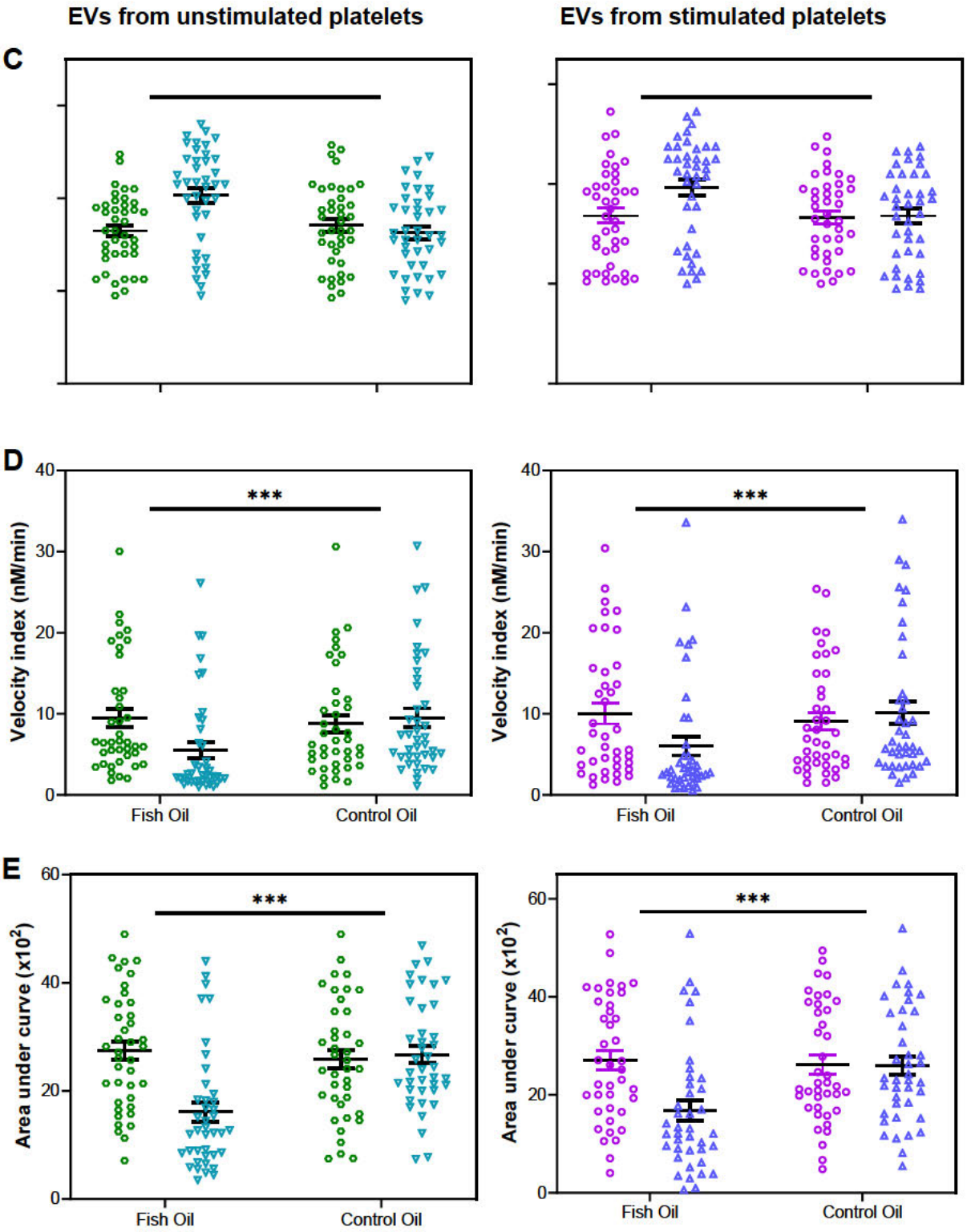
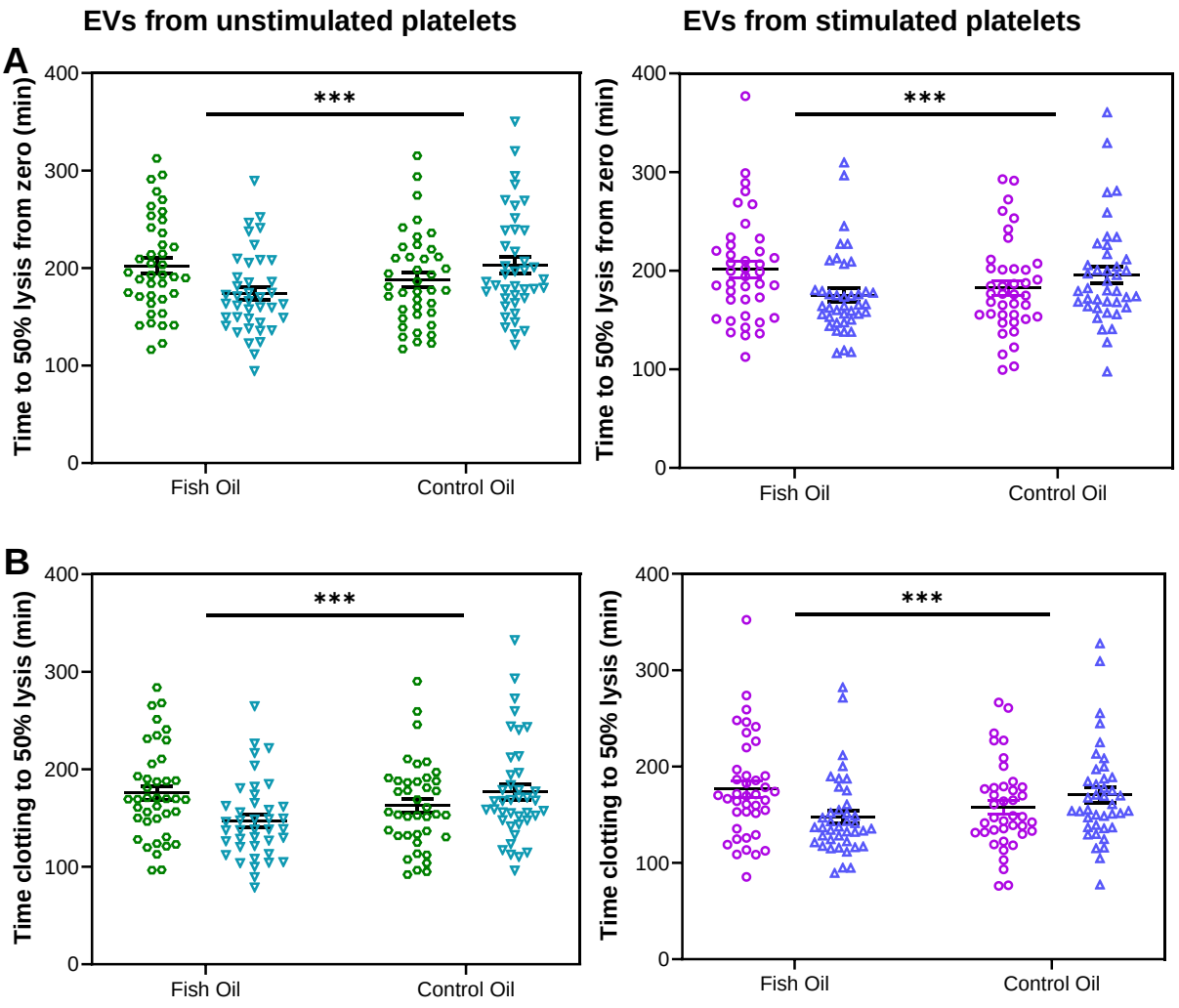


Figure 6.3. Effects of n-3 PUFA intervention in subjects at moderate risk for CVD on thrombin generation induced by EVs generated in vitro from unstimulated or stimulated platelets. Each point represents the response of baseline (green circle for UP-EVs, circle for SP-EVs) either for control oil or baseline for fish oil against intervention (turquois triangle for UP-EVs, blue triangle for SP-EVs) either for control oil or for fish oil. Data are mean ± SEM, (n=40). Comparisons after each intervention were drawn using the General Linear Model (GLM), including pairwise comparison with Bonferroni tests for treatment, period and treatment*time interaction, with differences shown at $p < 0.05$. Pooled VFP from healthy subjects (n=3) was served as negative control. There was significant effect of fish oil on **(A)**

lag time for thrombin generation, **(B)** peak thrombin concentration, **(C)** time to reach peak thrombin, **(D)** velocity index and **(E)** area under curve for both (**left panel**) UP-EVs and (**right panel**) SP-EVs (treatment effects: $p < 0.05$; general linear model). ****p<0.001*; SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles.

6.2.4 Effect of n-3 PUFA supplementation on fibrinolysis induced by PDEVs generated in vitro by unstimulated or stimulated platelets

Supplementation with fish oil resulted in a shortened time to fibrin lysis mediated by in vitro-generated PDEVs (**Figure 6.4A to 6.4C**), as well as reduced area under curve (**Figure 6.4D**). For all parameters, there was a main effect of treatment, but no significant effect of time and no treatment*time interaction.



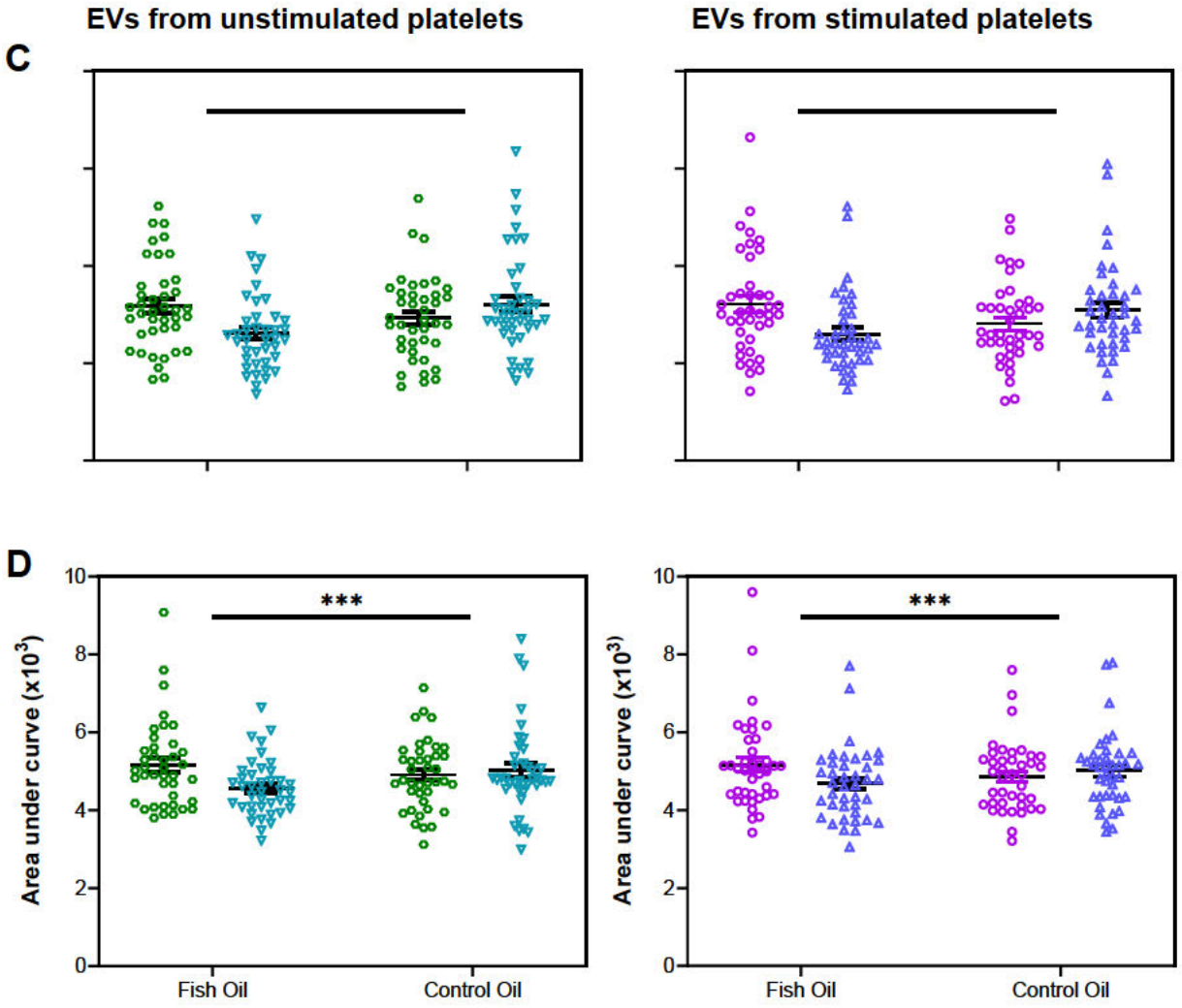


Figure 6.4. Effects of n-3 PUFA intervention in subjects at moderate risk for CVD on fibrinolysis induced by EVs generated in vitro from unstimulated or stimulated platelets. Each point represents the response of baseline (green circle for UP-EVs, EVs from unstimulated platelets; purple circle for SP-EVs, EVs from stimulated platelets) either for control oil or baseline for fish oil against intervention (turquois triangle for UP-EVs, blue triangle for SP-EVs) either for control oil or for fish oil. Data are mean ± SEM, (n=40). Comparisons after each intervention were drawn using the General Linear Model (GLM), including pairwise comparison with Bonferroni test for treatment, period and treatment*time interaction, with differences shown at $p < 0.05$. Pooled VFP from healthy subjects (n=3) was served as negative control. There was significant effect of fish oil on **(A)** time to 50% lysis from zero, **(B)** time clotting to 50% lysis, **(C)** time peak to 50% lysis and **(D)** area under curve for both UP-EVs (**left panel**) and SP-EVs (**right panel**) (treatment effects: $p < 0.05$; general linear model); SP-EVs, stimulated platelet-derived extracellular vesicles; UP-EVs, unstimulated platelet-derived extracellular vesicles.

6.2.5 Effect of n-3 PUFA supplementation on thrombus formation induced by PDEVs generated in vitro by stimulated platelets

There was no effect of the intervention on thrombus formation induced by PDEVs derived in vitro from stimulated platelets, including endpoints of thrombus formation, maximum thrombus formation and AUC, although there was a trend for a decrease in these parameters (**Figure 6.5**). There was no significant effect of time and no treatment*time interaction with respect to any parameter of thrombus formation.

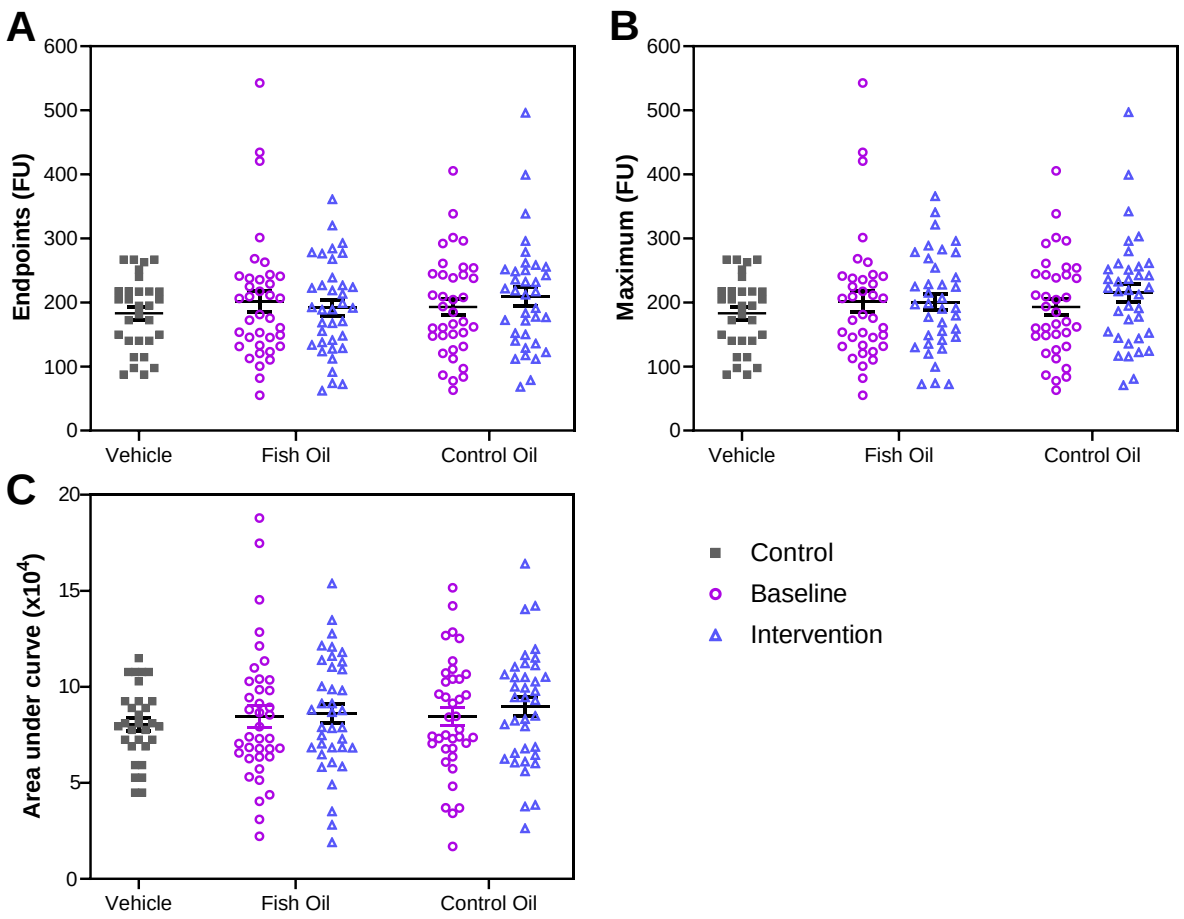


Figure 6.5. Effects of n-3 PUFA intervention on thrombus formation of s-PEVs in subjects at moderate risk for CVD. Each point represents the response of baseline (purple circle) either for control oil or baseline for fish oil against intervention (blue circle) either for control oil or for fish oil. Data are mean ± SEM, (n=40). Comparisons after each intervention were drawn using General Linear Model (GLM), including pairwise comparison test with Bonferroni for treatment, period and treatment*time interaction, with differences shown at $p < 0.05$. There was no significant effect of interventions on **(A)** endpoints of thrombus formation, **(B)** maximal thrombus formation and **(C)** area under curve (treatment effect: $p > 0.05$; general linear model).

6.3 Discussion

This randomised controlled crossover trial is the first to provide evidence that fish oil can alter the coagulatory properties of in vitro-generated PDEVs. Results demonstrated that supplementation with 1.8 g/d of n-3 PUFAs for 12 weeks reduced the ability of PDEVs produced in vitro from unstimulated and stimulated platelets to promote fibrin clot formation and thrombin generation and this was accompanied by an improvement in clot resolution (fibrinolysis). However, the intervention had no effect on thrombus formation induced by in vitro-generated EVs from stimulated platelets.

In the present study, the removal of EVs from pooled plasma resulted in a decline in fibrin clot formation kinetics in re-calcified VFP, as well as tissue factor-stimulated thrombin generation and shortened the time for fibrinolysis in the presence of tissue plasminogen activator (tPA). This confirms earlier observations in this thesis, which demonstrated greater coagulatory and thrombogenic activity in pooled PFP, which contains EVs, than in pooled VFP, which does not (**Chapter 2, Section 18.1, 18.2 and 18.3**). The current study, therefore, provided clear evidence that the presence of EVs in plasma has profound effects on coagulation, as determined by fibrin clot formation, thrombin generation and fibrinolysis. This is in accordance with previous findings, which reported a similar effect of the removal of EVs from plasma on the procoagulant activities of EVs (Aleman et al., 2011, Bidot et al., 2008, Zubairova et al., 2015b, Kaufmann et al., 2006, Tripisciano et al., 2017b). Moreover, the addition of PDEVs generated in vitro from unstimulated and stimulated platelets to pooled VFP promoted clot formation and tissue factor-stimulated thrombin generation, and prolonged time for clot lysis, once again consistent with the observation that clot formation in pooled PFP is greater than that in pooled VFP (**Chapter 2, Section 18.1, 18.2 and 18.3**). This indicates clearly that adding in vitro-generated PDEVs to pooled VFP restores the clot formation that is evident when EVs are present, most likely because of replacement of a phospholipid surface to support clot formation (Tripisciano et al., 2017b, Bidot et al., 2008, De Paoli et al., 2018). Of note, the anionic phospholipid, PS, is key to the procoagulant activity of PDEVs, since PS provides a

catalytic surface for the assembly of tenase (factors VIIIa, IXa, X) and prothrombinase (factors Va, Xa, thrombin) complexes in the presence of calcium due to its negatively charged ions, thereby supporting further activation of coagulation cascade to convert prothrombin to thrombin and fibrinogen to fibrin (Voukalis et al., 2019, Sinauridze et al., 2007, Zifkos et al., 2021, Aatonen et al., 2014, Spronk et al., 2014). Earlier studies reported that EVs derived from platelets promote coagulation under conditions allowing for factor XII-driven contact activation (Tripisciano et al., 2017b, Van Der Meijden et al., 2012). Considering their phospholipid content, EVs are also highly potent source of esterified fatty acids and eicosanoids, released by phospholipid membrane of EVs in the presence of phospholipases (Boilard, 2018). Arachidonic acid (AA), one of the key components of membrane phospholipids, can be metabolised into eicosanoids, of which can contribute to thrombosis (Yeung et al., 2017). In earlier chapter of this thesis (**Chapter 5**), the existence of AA on the phospholipid membrane of in-vitro generated PDEVs was reported in this study, indicating that the availability of AA on PDEVs may lead to the release of eicosanoids (Barry et al., 1999), which is likely to have a role in procoagulant activities of EVs. An animal study demonstrated that AA derived from PDEVs can be metabolised into eicosanoids such as thromboxane A_2 (TXA_2) under cyclooxygenase-1 (COX-1) pathway and 12-hydroxyeicosatetraenoic acid (12-HETE) under 12-lipoxygenase (12-LOX) pathway (Duchez et al., 2015), which may support to thrombin generation (Slatter et al., 2018). An alternative underlying mechanism for the procoagulant properties of EVs is the expression of tissue factor, which is an initiator of coagulation cascade by binding factor VII/VIIa, which in turn activates both factor X and factor IX (Lacroix and Dignat-George, 2012). However, the presence of biologically active tissue factor on PDEVs remains a matter of debate (Ferreira et al., 2020, Nomura and Shimizu, 2015, Aleman et al., 2011, Biro et al., 2003). For example, two studies suggested that tissue factor was not present on in-vitro produced PDEVs, of which one demonstrated that PDEVs did not support factor Xa generation (Aleman et al., 2011) and the other reported that in vitro-generated PDEVs did not support thrombin generation in the absence of exogenous tissue factor (Ferreira et al., 2020).

EV-associated fibrin clot formation could be attributed to either direct assembly of enzymatic coagulation complexes and binding to fibrin fibers (Zubairova et al., 2015b, Nabiullina et al., 2014, Lipets et al., 2014) or the changes in EV-induced thrombin generation (Zubairova et al., 2015b, Nabiullina et al., 2014) as the ability to trigger clot formation was observed in the absence of exogenous clotting activator (Zubairova et al., 2015b). The thrombogenicity of EVs primarily relies on negatively charged phospholipid externalisation on the EV surface, as demonstrated by a recent study demonstrating that greater PS exposure resulted in PDEVs promoting more thrombin generation. Furthermore, inhibition of PS exposure on EVs reduced thrombin generation in a dose-dependent manner, providing further support regarding the contribution of PS exposure to thrombin generation (Tripisciano et al., 2017b). EV-induced thrombin generation is also likely to be an underlying mechanism for changes in fibrinolysis, because thrombin formation can modulate clot stability by changing fibrin properties, including its network structure and resistance to fibrinolysis (Campbell et al., 2008, Campbell et al., 2009). These results, overall, indicated that coagulation was enhanced by the presence of EVs. The current study investigated not only the impact of EVs on thrombogenicity, but also the effect of fish oil on EV functions through different stages of coagulation cascade.

This study demonstrated that PDEVs generated in vitro from unstimulated and stimulated platelets following supplementation with fish oil for 12 weeks reduced fibrin clot formation, as indicated by effects on time for 50% clotting and overall fibrin clot formation (absorbance at 50% clotting, absorbance at peak clotting and area under curve) (Zubairova et al., 2015b, Aleman et al., 2011, Del Turco et al., 2008). Although the exact mechanism by which n-3 PUFAs reduce EV-mediated clot formation remains elusive, it is possible that it is connected with a decrease thrombin generation and reduced PS exposure, as described in **Chapter 5**. To the best of our knowledge, little is known regarding the effect of fish oil supplementation on fibrin clot formation induced by EVs. In line with the present study, supplementation of patients post myocardial infarction with 5.2 g/d of n-3 PUFAs for 12 weeks reduced fibrin generation capacity by prolonging lag time to clotting, and this was associated with a reduction

in CD61+ PDEV numbers (Del Turco et al., 2008). Moreover, in this relatively small study (n=21), both intrinsic (factor XII-dependent) and extrinsic (tissue factor-dependent) pathways accounted for the anticoagulatory effect of fish oil, since EVs following n-3 PUFA supplementation prolonged the time to clotting in either factor VII-deficient plasma or factor XII-deficient plasma.

As in clot formation assays, supplementation with fish oil had a favourable effect on tissue factor-stimulated thrombin generation of PDEVs. The current findings suggested that the consumption of fish oil significantly prolonged initiation phase of thrombin generation (lag time) and time to reach peak thrombin generation of in vitro-generated PDEVs so that overall thrombogenicity (thrombin generation and thrombin propagation) of PDEVs was reduced. These data reflect that PDEVs generated in vitro, regardless of whether they were derived from unstimulated or stimulated platelets, had less thrombogenic capability following n-3 PUFA supplementation for 12 weeks, perhaps due to incorporation of n-3 PUFAs into platelet membrane phospholipids (Adili et al., 2018) and subsequently that of PDEVs shed from those platelets (**Chapter 5, Section 2.4**) (O'Donnell et al., 2014). Thrombin generation has been also shown to be associated with phospholipid concentration in the plasma (Phang et al., 2012b) and surface exposure of phospholipids, PS in particular, on EVs are key to their coagulatory activities as they promote the assembly and the activation of prothrombinase complex of coagulation to form thrombin (Zarà et al., 2019). Tripisciano *et al* (2017) highlighted that thrombin generation induced by EVs generated from platelets is dependent on PS exposure by PDEVs as lower exposure of PS resulted in less thrombin generation (Tripisciano et al., 2017b). De Paoli *et al* (2018) also showed completely prohibited thrombin generation when the activity of PS on the PDEV surface was inhibited (De Paoli et al., 2018). The ability of n-3 PUFAs to decrease PS exposure by PDEVs may, therefore, represent the mechanism underlying the effects of fish oil on EV-induced thrombin generation and is supported by the data presented in **Chapter 5** (**Section 2.5**), which showed that fish oil significantly reduced the expression of PS by EVs generated from unstimulated platelets, with a similar trend for

EVs generated from stimulated platelets. In this regard, reduced PS exposure by PDEVs following n-3 PUFA supplementation may at least partially account for the antithrombogenic effects of fish oil on thrombin generation induced by PDEVs. Earlier chapter (**Chapter 5**) also reported that fish oil supplementation in this study achieved the replacement of AA with EPA in PDEVs produced in vitro from platelets. The reduction in AA may have a role in decreased thrombin generation since AA enable to contribute to eicosanoid biosynthesis, which may, in turn, have a role in thrombin generation (Slatter et al., 2018). Similar to the finding of the current study, Phang *et al* (2012) reported a decrease in thrombin generation 24 hours after a single 2 g dose of EPA in healthy subjects; however, they reported no alteration in numbers of circulating PS+ PDEVs. When the data were grouped by gender, antithrombogenic effect of EPA was seen in only males (Phang et al., 2012b). The reduction in procoagulant activity of EVs, but not in numbers of PS+ PDEVs after supplementation with a single dose of EPA, could be partly due to study population. This intervention was conducted in a healthy population, so it may be unlikely that there would be a reduction in already low numbers of PS+ PDEVs. Also, PS+PDEV numbers were assessed using flow cytometry, which cannot detect EVs <200 nm, which account for 96% of the EV population according to data from another PhD student on the HI-FIVE study. It is notable that smaller EVs have been reported to be more procoagulant than larger EVs (Tripisciano et al., 2017b, Jy et al., 2010) because they present dramatically tighter binding of coagulation factors than larger EVs with the same phospholipid composition (Jy et al., 2010). In addition to fibrin clot formation and thrombin generation, EVs may also modulate coagulation through fibrinolytic mechanisms, which is the end of the process (breakdown of fibrin clot) (Zarà et al., 2019). EVs support the formation of fibrin clots with smaller pores (denser fibrin clot) packed with thinner fibres, which are likely to be more resistant to lysis (Zubairova et al., 2015b). Resistance to fibrinolysis has been suggested to be associated with high risk for CVDs (Bridge et al., 2014a). In the current study, a significant shortened the time to fibrinolysis induced by in vitro-produced PDEVs was caused by fish oil rather that control oil, indicating n-3 PUFA treatment improve fibrinolysis resistance. To our knowledge, these are highly novel results, reported for the first time and the mechanism

is not elucidated, yet it assumed that the observed action of n-3 PUFAs on EV-induced fibrinolysis may be caused by the changes in thrombin generation. Thrombin has been suggested to contribute to the regulation of fibrinolysis by affecting fibrin clots in terms of density, pore size, fibre thickness and stabilisation of fibrin polymers, which in turn mediate resistance to fibrinolysis (Bridge et al., 2014b, Weisel and Litvinov, 2013, Gajos et al., 2011). The reduced PDEV-induced thrombin generation in the current study, therefore, may be likely to result in the formation of a looser fibrin clot structure, accompanied with larger pores and thicker fibres, which results in less resistant fibrin clot to lysis. Although the precise mechanism is not clear yet, it is well appreciated that the present study provides robust data showing the impact of n-3 PUFAs on the fibrinolytic activities of PDEVs.

In addition to EV-induced procoagulant and fibrinolytic activities, the present study investigated the effect of fish oil on ex vivo thrombus formation mediated by EVs produced in vitro from stimulated platelets. For this, thrombi were formed in the absence (vehicle) and presence of PDEVs from TRAP-6 stimulated platelets in whole blood and there was no difference in PDEV-induced thrombus formation compared with vehicle-treated blood. Intervention with fish oil had no effect. It is rather surprising to see that in vitro-generated PDEVs had no effect on thrombus formation in this assay as PDEVs have been suggested to be involved in several aspects of this process (Gasecka et al., 2017). Suades *et al* (2012) showed that PDEVs induce platelet deposition, platelet aggregation and shortening clotting time, with a consequent increase in thrombus formation, even in healthy subjects (Suades et al., 2012b). The role of PDEVs in thrombus formation has been explained by their ability to bind tissue factor and transfer it to the platelet outer membrane, thus supporting thrombus formation in vivo in a tissue factor-dependent manner (Biro et al., 2003). The absence of standardised methodology due to the nature of this experiment is a limitation. In this assay, fresh whole blood, collected from a healthy volunteer was reconstituted with in vitro-generated PDEVs from stimulated platelets to observe the impact of PDEVs on thrombus formation. Although the whole blood was always from the same volunteer and standardised as far as

possible, day-to-day variability is a limitation, as is the maximum concentration of PDEVs available for use in the assay. However, the effect of supplementation with fish oil on this aspect of procoagulant activity of PDEVs has never previously been investigated and although there was no effect, the same limitations apply, particularly the fact that the number of PDEVs available for use in the assay was limited. Thus, despite the fact that the fatty acid composition of PDEVs was substantially altered by n-3 PUFA, (**Chapter 5, Section 2.3**) and despite the fact that many aspects of coagulation were altered by n-3 PUFA, ex vivo thrombus formation was not altered under the conditions employed in the assay. Further work is warranted with respect to overcoming the technical limitations. Future work should also consider a study design where fresh whole blood from study participants is used in the assay, which was not the case in the current study due to considerable logistical challenges.

6.4 Conclusion

Supplementation with n-3 PUFA for 12 weeks significantly altered the coagulatory behaviour of PDEVs generated in vitro from unstimulated and stimulated platelets. This suggests a role for EVs in coagulation and fibrinolytic pathways, which is modifiable by dietary fatty acids. Although there was no significant effect of n-3 PUFA-modified PDEVs on ex vivo thrombus formation, technical limitations in the assay design mean that further exploration is warranted. Overall, this chapter presents a substantial advance in understanding of the impact of in-vitro generated PDEVs on coagulation and thrombosis, and potential inhibition of these effects of PDEVs by dietary intervention with n-3 PUFA.

Chapter 7. Final discussion and future work

7.1 Overview of HI-FIVE study outcomes

In this thesis, it was hypothesised that supplementation with 1.8 g/d of n-3 PUFAs for 12 weeks to subjects with moderate risk for cardiovascular diseases (CVDs) would improve selected conventional and thrombogenic cardiovascular risk markers, as well as emerging risk markers: extracellular vesicles (EVs), including their generation, composition and functional activities. The hypothesis was confirmed to a significant extent, as illustrated in **Figure 7.1**. The effects of fish oil supplementation on conventional cardiovascular risk markers were re-affirmed through observations of a significant decrease in blood pressure and plasma TAG concentration and a significant increase in the concentration of LDL-cholesterol. New evidence relating to thrombogenic risk markers suggested that n-3 PUFAs reduce fibrin clot formation and tissue factor-stimulated thrombin generation, but do not alter platelet aggregation or fibrinolysis, as presented in **Chapter 3**. The beneficial effect of n-3 PUFAs on thrombin generation was associated with EVs since their removal from plasma resulted in a decrease in thrombin generation and addition of isolated EVs to vesicle-free plasma (VFP) stimulated thrombin generation, suggesting a potential contribution of EVs to this aspect of coagulation.

There was a strong relationship between numbers of circulating EVs and a range of parameters of thrombogenic risk factors in subjects with moderate risk for CVDs. Higher numbers of circulating total EVs (TEVs) and EVs smaller than 200 nm (SEVs) were associated with thrombogenic risk markers, including fibrin clot formation and thrombin generation and there was a positive association between numbers of PS+ EDEVs and fibrin clot formation. Furthermore, numbers of circulating TEVs were demonstrated to be independent predictors of aspects of clot formation and thrombin generation. Subjects with higher than median circulating EV numbers exhibited greater fibrin clot formation and thrombin generation than subjects with lower than median circulating EV numbers, confirming the potential role of

increased numbers of circulating EVs in thrombosis, which might, in turn, be associated with elevated risk for CVDs (**Chapter 4**).

As indicated in **Chapter 2**, the HI-FIVE Study, of which this project was a component, was comprised of two strands; (1) the effect of n-3 PUFA supplementation on the composition and functional activities of total circulating EVs isolated from plasma and (2) the effect of n-3 PUFA supplementation on the in vitro generation of EVs by platelets and procoagulant activity of those PDEVs in functional assays. As reported by another PhD student working on strand 1 of this project, supplementation with 1.8 g/d of n-3 PUFAs for 12 weeks in subjects with moderate risk for CVD significantly decreased numbers of both circulating EVs measured by NTA and EV subpopulations measure by FCM, including circulating PS+EVs, PDEVs and EDEVs. This thesis is based on strand 2 of the HI-FIVE study and demonstrated that the intervention significantly modulated the fatty acid composition of in vitro-generated PDEVs from unstimulated and stimulated platelets and decreased the surface expression of PS in the case of PDEVs derived from unstimulated platelets; this is highly novel data, reported for the first time in **Chapter 5**. Moreover, fish oil supplementation restrained the ability of PDEVs generated in vitro from unstimulated and stimulated platelets to promote fibrin clot formation and thrombin generation and this was accompanied by an improvement in fibrinolysis, as presented in **Chapter 6**. No effect was observed on thrombus formation induced by in vitro-generated EVs from stimulated platelets. Nevertheless, this thesis provides a great deal of novel insight into the effects of n-3 PUFAs on PDEVs and their procoagulant activities.

The following discussion focusses on key areas where an advance in knowledge has been identified by the work described in this thesis and key outstanding questions for future work.

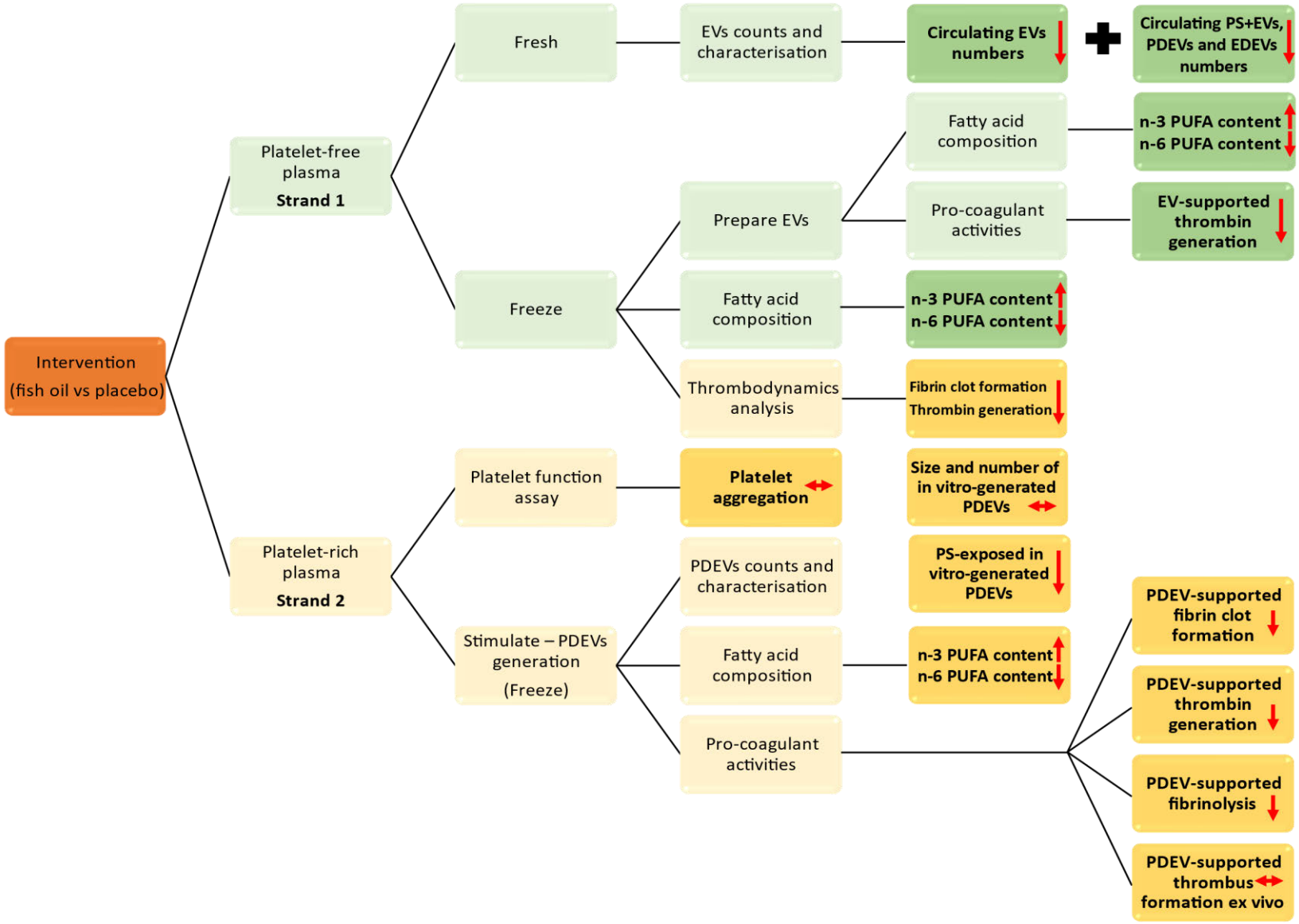


Figure 7.1. A schematic overview of outcomes from the HI-FIVE Study

7.2 Do in vitro-generated PDEVs resemble their circulating counterparts?

Circulating EVs are derived from a number of sources, including platelets (Coumans et al., 2017) and they have been suggested to play critical roles in both physiological and pathological conditions due to the fact that these vesicles carry bioactive cargo, such as protein, lipids and nucleic acids (Yáñez-Mó et al., 2015, Boulanger et al., 2017). EVs shed from platelets constitute the main EV population in the circulation, representing up to 60–90% of circulating EVs (Brisson et al., 2017, Zaldivia et al., 2017) and PDEVs are suggested to be endowed with highly procoagulant functions due to their phospholipid surface and bioactive molecules (Zarà et al., 2019, Owens III and Mackman, 2011). Although this thesis focussed exclusively on purified PDEVs generated in vitro from washed platelets, **Chapter 5** provided insight into stark differences in the fatty acid composition of in vitro-generated PDEVs compared with circulating EVs, suggesting that PDEVs derived in vitro may not resemble those in vivo, either in composition or behaviour. While it is important to note that circulating EVs represent a heterogeneous mixture of EVs, whereas the in vitro-generated PDEVs in this study were derived exclusively from platelets, the marked difference in fatty acid composition highlights the need for better understanding of the mechanisms underlying EV generation, and in particular, the lipid remodelling associated with it.

7.3 EVs and thrombogenic risk

The role of EVs as emerging risk markers and in the development of CVDs is supported by reports of higher numbers of EVs in the circulation associated with CVDs and thrombotic disease (Mooberry and Key, 2016, Nomura and Shimizu, 2015). **Chapter 3** of this thesis highlighted the potential roles of EVs in thrombogenic pathways and **Chapter 4** specifically reported a strong relationship between circulating EV numbers detected by NTA and thrombogenic risk markers, including fibrin clot formation and thrombin generation, supporting the idea that EVs possess procoagulant capacity (Yáñez-Mó et al., 2015). Previous studies

demonstrating a relationship between EVs and thrombogenic risk markers, have tended to detect circulating EVs by FCM, which is only able to capture large EVs due to the lower resolution of most flow cytometers (Ayers et al., 2014, Nielsen et al., 2014, Aatonen et al., 2014). The greater resolution of NTA demonstrated that EVs below the FCM threshold accounted for 96% of total circulating EV numbers, indicating that the majority of circulating EVs were undetectable by FCM. Therefore, it is perhaps inevitable that there were strong associations between thrombogenic risk markers and numbers of EVs determined by NTA, which were no consistently observed for those determined by FCM. In understanding these associations, it should be noted that the current work does not prove a causal relationship between EV numbers and thrombogenic risk markers; this requires further investigation.

An exception to the above was that numbers of PS+ EDEVs detected by FCM were significantly associated with clot formation and fibrinolysis, indicating their potential contribution to coagulation and fibrinolytic activities in the circulation. EVs released from endothelial cells are reported to express tissue factor (Zwicker et al., 2011, Holnthoner et al., 2017) and plasminogen activators on their surface under pathological conditions, which promote fibrinolytic activity (Lacroix and Dignat-George, 2012). Increased numbers of EDEVs have been reported to be associated with impaired endothelial function (Feng et al., 2010, Horn et al., 2014, Wang et al., 2009, Wang et al., 2007) rather than coagulation, while elevated numbers of PDEVs appear to be highly associated with arterial thrombosis (Namba et al., 2007, Gasecka et al., 2017). Further investigation is warranted to explore the link between EDEVs and thrombogenicity.

The key messages relating to EVs and thrombogenic risk include:

- Circulating EV numbers, as determined by NTA, were strongly associated with thrombogenic risk markers, but further work to understand whether there is a causal link is required.
- Numbers of PS+ EDEVs, determined by FCM, were also associated with thrombogenic risk markers, but the basis for the link is not clear.

7.4 Influence of the EV size and platelet source on coagulatory activity

The size distribution analysis of PDEVs in **Chapter 5** demonstrated that in vitro-generated PDEVs predominantly ranged from 100-250 nm in diameter and that EVs derived from unstimulated platelets were predominantly smaller than those derived from stimulated platelets. Activated platelets generate principally two subpopulations of EVs, exosomes (30-150 nm) generated via multivesicular bodies (van der Meijden and Heemskerk, 2018) and microvesicles (50-1000 nm) shed from the plasma membrane as a result of a loss of membrane phospholipid asymmetry, remodelling of cytoskeleton and exposure of phosphatidylserine on the outer membrane leaflet (Owens III and Mackman, 2011). The fact that unstimulated platelets tended to generate smaller PDEVs may have implications with respect to their composition and biological effects (Aatonen et al., 2014), although there was no evidence that the coagulatory activity of PDEVs from unstimulated platelets was different from that from stimulated platelets in the current study (**Chapter 6**) and size does not appear to be a key determinant of the coagulatory behaviour of PDEVs (Ferreira et al., 2020). **Chapter 5** demonstrated that PS exposure by EVs from unstimulated platelets was greater than that by EVs from stimulated platelets. De Paoli *et al* (2018) demonstrated that PS exposure by small PDEVs (mean size 103 nm) was double that of large ones (the mean size 350 nm) (De Paoli et al., 2018), with both observation often being interpreted as being associated with greater procoagulant activity. However, as indicated, direct evaluation of the procoagulant activities of PDEVs produced by unstimulated and stimulated platelets (**Chapter 6)**, demonstrated no difference.

The key messages relating to the influence of EV size and platelet source on coagulatory activity are:

- EVs generated from unstimulated platelets in vitro were smaller, but expressed higher levels of PS than those generated from stimulated platelets.

- Neither size nor PS exposure influenced the coagulatory activity of EVs, but further exploration of the influence of size and composition on coagulatory activity is warranted.

7.5 Influence of n-3 PUFAs on the anticoagulatory effects of EVs

Figure 7.1 demonstrates the significant impact of n-3 PUFAs on almost all coagulatory outcomes examined in this study. **Chapter 3** demonstrated that n-3 PUFA significantly decreased fibrin clot formation and tissue factor-stimulated thrombin generation, but did not affect platelet aggregation or fibrinolysis. The thrombogenic capacity of EVs was demonstrated by removal of EVs from plasma, which resulted in almost three-fold lower thrombin generation and also by an increase in thrombin generation when EVs were added to vesicle-free plasma. This supports previously published data suggesting that EVs have procoagulant activities (Zarà et al., 2019).

Chapter 5 demonstrated, for the first time, that the fatty acid composition of in vitro-generated PDEVs is altered by fish oil supplementation, with an increase in the proportion of long chain n-3 PUFAs and a decrease in the proportion of arachidonic acid. **Chapter 6** suggested that these alterations were associated with a decrease in PDEV-stimulated fibrin clot formation and thrombin generation, which supported the suggestion that beneficial effect of these fatty acids in haemostasis might be mediated at least partly through the inhibition of the coagulatory behaviour of PDEVs. However, while supplementation with n-3 PUFAs improved the fibrinolytic activity of PDEVs, it did not alter fibrinolysis in plasma, suggesting that not all aspects of the coagulatory activity of EVs in the assays translates to equivalent effects in plasma.

Two previous studies have investigated the coagulatory effects of EVs modified by fish oil supplementation. One reported a reduction in procoagulant activity of EVs in parallel with decreased platelet aggregation in healthy males following a single dose of n-3 PUFA supplementation at 2 g/d (Phang et al., 2012a). The other demonstrated decreased

coagulatory functions of EVs after supplementation with 5.2 g/d n-3 PUFAs for 12 weeks, but in the absence of alterations in coagulation markers, including plasma levels of thrombin-antithrombin (TAT) complexes and the prothrombin fragment 1+2 (F^{1+2}) (Del Turco et al., 2008).

Chapter 6 demonstrated no effect of EVs on ex vivo thrombus formation, either prior to or after fish oil supplementation (Figure 7.1), which did not reflect the observations relating to most of the in vitro coagulation assays. It is notable that in the thrombus formation assay, in vitro-generated PDEVs from stimulated platelets were reconstituted in whole blood, which itself contains high numbers of circulating EVs, and therefore experimental conditions were limited by the small number of in vitro-generated PDEVs which were available. **Chapter 6** demonstrated that 5 μg/ml protein concentration of PDEVs from TRAP-6 stimulated platelets added to whole blood had no effect on thrombus formation compared to the vehicle, but it was not possible to increase the PDEV concentration above this due to limited availability of sample.

Key questions arising from this work include the need to better understand the role of EVs in coagulation in vivo and the mechanisms underlying the modulation of this role by dietary fatty acids; therefore, in summary:

- Supplementation with n-3 PUFAs significantly altered procoagulant activity and reduced the thrombogenicity of EVs.
- PDEVs, under the conditions employed in these experiments, did not affect thrombus formation measured ex vivo, and there was no influence of n-3 PUFA intervention, but this is likely to be due to limitations in the experimental conditions and deserves further attention.
- The mechanistic roles of EVs in coagulatory pathways and their modulation by n-3 PUFAs requires further investigation.

7.6 Recommendations for future work

7.6.1 Is there a difference in the fatty acid composition between 'parent' platelets and EVs derived these platelets?

This thesis hypothesised that the fatty acid composition of EVs produced in vitro from platelets would reflect the fatty acid composition of the 'parent' platelets. However, there was insufficient sample of platelets to test this hypothesis, so the results presented in **Chapter 5** compared the fatty acid composition of PDEVs with literature values for the fatty acid composition of platelets (Rise et al., 2007), demonstrating unexpected and stark differences between the two. PDEVs generated in vitro had a higher proportion of SFAs than platelets and lower proportions of total n-6 PUFA, MUFA, EPA, DPA and DHA, suggesting that PDEVs generated in vitro possess a unique fatty acid profile that does not resemble that of platelets. It would be essential, therefore, to characterise the fatty acid remodelling that occurs when platelets are stimulated to release EVs at every stage of the process, up to and including the end product. It is intriguing to consider that there may be selective partitioning of fatty acids into PDEVs during their generation from platelets.

7.6.2 How do n-3 PUFAs modulate the coagulatory functions of PDEVs?

Intervention with n-3 PUFAs resulted in modification of the fatty acid composition of and procoagulant PS expression by PDEVs, as well as their coagulatory and fibrinolytic activities. However, it is not clear whether modification of the fatty acid composition of PDEVs is causally linked with alterations in biological function. PS exposure by in vitro-generated PDEVs may be reduced following remodelling of platelet membrane phospholipids in response to fish oil supplementation due to the impact of n-3 PUFAs on the composition and size of lipid rafts (Stillwell, 2006, Turk and Chapkin, 2013), suggesting a possible link between composition and function. However, there is still uncertainty about whether reduction in PS exposure by EVs after n-3 PUFA supplementation could at least partially account for the beneficial effect of fish oil on the coagulatory potential of PDEVs (Zarà et al., 2019). Decreased arachidonic acid (AA)

as a result of the incorporation of n-3 PUFAs into membrane phospholipids may also have a role in reduced procoagulant properties of PDEVs because of its role as a substrate for eicosanoid biosynthesis (Slatter et al., 2018, Adili et al., 2018). The expression of tissue factor on the PDEV surface has been reported to contribute to procoagulant activity (Biro et al., 2003, Nomura and Shimizu, 2015) and the externalisation of tissue factor in the outer cell membrane has been suggested to be prevented by intake of n-3 PUFAs (Elvevoll et al., 2006). The existence of tissue factor on PDEVs should be investigated and if tissue factor is indeed present on the surface, the possibility that n-3 PUFAs inhibits the activity of tissue factor exposed PDEVs should be examined. The ultimate aim of future work should be to explore underlying mechanisms for the beneficial impact of n-3 PUFAs on the coagulatory behaviour of EVs.

7.6.3 Do small and large PDEVs have different effects on the coagulatory behaviours of vesicles?

In the current work, the size distribution analysis of PDEVs showed that EVs derived from unstimulated platelets had a higher proportion of small EVs and PS-exposure compared to EVs shed from stimulated platelets. This raised several questions regarding the relationship between PDEV and procoagulant activity. Future works should focus on the targeted isolation of platelet derived-exosomes (small PDEVs) and -microvesicles (larger PDEVs) in vitro and explore whether their composition and activity differ. The question is not just one of size, of course, because the mechanisms by which exosomes and microvesicles are generated are completely different. Distinguishing between these two types of EVs is difficult due to overlapping size and density (Brisson et al., 2017, Lee et al., 2012, Aatonen et al., 2014). The targeted isolation of exosomes released by unstimulated and stimulated platelets in vitro can be achieved using the ExoQuick-TC solutions (Goetzl et al., 2016), and this technique could be used to explore differences in bioactive cargo, surface components and biological function by comparing exosomes with a mixed EV preparation (Pienimaeki-Roemer et al., 2015).

Furthermore, there is scope to evaluate the effects of n-3 PUFAs specifically in a purified exosome population in future work.

7.7 Concluding remarks

This thesis demonstrated a strong relationship between circulating EVs and thrombogenic risk markers for CVDs, as well as significant modification of the composition and thrombogenic capacity of EVs by n-3 PUFA. This was the first study to investigate the effect of n-3 PUFAs on in vitro-generated PDEVs, demonstrating that EVs released from platelets have procoagulant activity, which is reduced by n-3 PUFAs. As such, the study described in this thesis confirmed to a significant extent the original hypothesis that supplementation with n-3 PUFAs for 12 weeks significantly alters the composition and functions of PDEVs generated in vitro from subjects with moderate CVD risk. This thesis provides novel insight into the beneficial impacts of n-3 PUFAs on cardiovascular health, which may have valuable implications for the prevention of CVDs and the underlying mechanisms deserve further investigation.

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Appendix: Ethical applications and documents

School of Chemistry, Food and Pharmacy
Research Ethics Committee

Application Form for UREC Applications

SECTION 1: APPLICATION DETAILS

1.1

Project Title: Effects of fish oil-derived n-3 polyunsaturated fatty acids on the generation and functional activities of extracellular vesicles
(Simplified title to be used in communications with potential volunteers: "**H**uman **I**nvestigation of the effects of **FI**sh oil on extracellular **VE**sicles" or "**HI-FIVE STUDY**")

Date of Submission: 24/02/17 Proposed start date: October 2017 Proposed End Date: December 2018

1.2

Principal Investigator: Professor Parveen Yaqoob

Office room number: 2-55 Internal telephone: 8720

Email address: p.yaqoob@reading.ac.uk Alternative contact telephone: 0118 378 8720

(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: Dr Dionne Tannetta (Staff) Institution/Department: Food and Nutritional Sciences
Email: d.s.tannetta@reading.ac.uk

Name: Dr Plinio Ferreira (Staff) Institution/Department: Food and Nutritional Sciences
Email: Not yet assigned

Name: Professor Jon Gibbins (Staff) Institution/Department: Biomedical Sciences
Email: j.m.gibbins@reading.ac.uk

Name: Dr Chris Jones (Staff) Institution/Department: Biomedical Sciences
Email: c.i.jones@reading.ac.uk

Name: Ruihan Zhou (Student) Institution/Department: Food and Nutritional Sciences
Email: ruihan.zhou@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: 20/2/17
.	…. (Student)	Date: 16/02/17
	(Dr Dionne Tannetta)	Date: 21/02/17
	Dr Plinio Ferreira)	Date:21/02/17
…	… (Professor Jon Gibbins)	Date: 21/02/17
……	…… (Dr Chris Jones)	Date: 21/02/17

1.4

University Research Ethics Committee Applications

Projects expected to require review by the University Research 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:……

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

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

N-3 polyunsaturated fatty acids (n-3 PUFA), which are abundant in oily fish and fish oils, have been suggested to play a role in reducing the risk of cardiovascular diseases (CVDs) by modifying a wide range of risk factors, such as blood fats, blood clotting, blood vessel function and inflammation. Extracellular vesicles (EVs) are small particles released from various cells when they are activated or damaged. High numbers of EVs in the blood have been associated with a higher risk of CVDs, and it is thought that this is because they carry 'bioactive' components which can affect many processes involved in CVDs. However, very few clinical trials have investigated the relationships between the consumption of n-3 PUFA and circulating EVs, although we have previously shown that fish oil supplements decrease numbers of some EVs. This project aims to investigate the effects of dietary n-3 PUFA on the generation and functional activities of EVs, which would provide new insight into the benefits of n-3 PUFA on cardiovascular health.

(*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*).

Study Design

The proposed study will be a randomised, double-blind, placebo-controlled crossover intervention. Subjects (25-65y) at moderate CVD risk will be supplemented with either fish oil (1.8 g/d n-3 PUFA) or placebo (olive oil) for 12 weeks. Random assignment of subjects for intervention order ("1" and "2") will be performed with an online software (https://www.randomizer.org/). Also, fish oil and placebo, whose labels will be taken off and will be stored separately in two identical boxes marked with "A" or "B". Subjects assigned into "1" will receive "A" for the first 12 weeks while subjects with "2" will receive "B" firstly. After a 12-week washout and then cross-over to the other intervention for another 12 weeks. In order to be double-blind, boxes labelling will be conducted by Dr Michelle Weech (Hugh Sinclair Manager), who will also put the information on the correspondence between label and intervention in a sealed envelope and will keep it in the site file. In this way, any involved investigator will not have access to know the intervention order before the end of study. Blood samples will be collected before and after each intervention (blood will be frozen for analysis and the Department of Food and Nutritional Sciences has a licence for storing such material for the purpose of research obtained from the Human Tissue Authority. The licence holder is Professor Glenn Gibson). A food frequency questionnaire (Appendix F) will be administered to assess the subject's habitual intake of n-3 PUFA. Subjects will also be expected to maintain a low consumption of n-3 fatty acids, refrain from the use of all supplements, and maintain their body weight during the study. Baseline n-3 PUFA status will be established at the start of each arm of the study.

Objectives

Since platelet-derived extracellular vesicles (PEVs) account for approximately 70-90% circulating EVs, the study will investigate **Strand 1**: the influence of n-3 PUFA supplementation on the number and profile of EVs and their activity in blood clotting assays. **Strand 2**: the influence of n-3 PUFA on the production of EVs by platelets taken from subjects and generated *in vitro* as well as the activity of the platelet-derived EVs in blood-clotting assays.

Strand 1:

The numbers and cell origin of EVs in platelet-free plasma will be analysed by flow cytometry and fluorescence nanoparticle tracking analysis (fl-NTA) with fluorescently labelled antibodies. After enumeration and characterisation, EVs will be isolated from platelet-free plasma and be assessed for fatty acid composition and activity in blood clotting assays (prothrombinase activity, thrombin generation, *ex vivo* thrombus formation).

Strand 2:

Platelets will be stimulated *in vitro* and analysed for fatty acid composition (gas chromatography), membrane exposure and intracellular concentration of calcium (flow cytometry). PEVs will be prepared from the supernatants of stimulated platelets and assessed for fatty acid composition and activity in blood clotting assays, as described above.

Screening Procedures

Interested volunteers will be assessed for their initial eligibility for the study by completing a medical and lifestyle questionnaire (Appendix E) via email or phone. The participant information sheet (Appendix D), which outlines the details of the study, will be sent with questionnaire as well. Potential participants indicating an interest in the study will be invited to attend a screening visit at the Hugh Sinclair Unit of Human Nutrition at the University of Reading. During the screening visit, all the procedures will be explained in detail and participants will be offered the opportunity to ask questions. If they are willing to proceed they will give their consent by completing the Consent Form (Appendix C), which will be also signed by the researcher. During this screening session, anthropometric measurements (weight, height, waist-hip circumference and blood pressure) and biochemical test (blood count, lipid profile, glucose levels and markers of liver & kidney function, all of which will be sent to Royal Berkshire Hospital Pathology Department for analysis) will be taken to identify individuals at moderate risk of CVDs. QRISK2, a prediction algorithm using traditional risk factors (age, systolic blood pressure, smoking status and ratio of total serum cholesterol to high-density lipoprotein cholesterol) together with body mass index, ethnicity, measures of deprivation, family history, will be applied for risk evaluation. The online QRISK2 calculator (details please see https://qrisk.org/2016/) provides a percentage of risk of having a heart attack or stroke within the next 10 years, and subjects with 10%-20% will be regarded as being at moderate risk. Following the screening session, suitable volunteers will be contacted by one of the study investigators and informed that they are eligible to attend study. If the screening results of participants indicate any cause for concern, they will be advised to discuss this with their GP.

Exclusion Criteria:

- BMI: <18.5 kg/m^2
- Anaemia (haemoglobin concentration <12.5 g/L in men and <11.5 g/L in women)
- Hyperlipidaemia (total cholesterol concentration >8 mmol/L)
- Diabetes (diagnosed or fasting glucose concentration >7 mmol/L) or other endocrine disorders
- Angina, stroke, or any vascular disease in the past 12 months
- Renal, gastrointestinal, respiratory, liver or bowel disease
- Inflammatory disease

- Take drug treatment for hypertension, hyperlipidaemia, inflammation, depression or thyropathy.
- Take aspirin, ibuprofen or other nonsteroidal anti-inflammatory drugs (NSAIDs) > 4 times per month, or once in the week preceding the study
- Take any other anti-platelet or anti-coagulant drugs, like triflusal, clopidogrel and warfarin.
- Have allergies
- Smoking (including e-cigarettes and nicotine products)
- Alcohol misuse or intakes ＞21 units/wk for men and ＞15 units/wk for women or have a history of alcohol misuse
- Regularly consume oily fish and/or dietary supplements
- Planning to start or on a weight reducing regimen
- Intense aerobic exercise (＞20 min, three times a week)
- Females who are pregnant, lactating, or if of reproductive age and not using a reliable form of contraception (including abstinence)
- Have participated in another clinical trial within the last three months

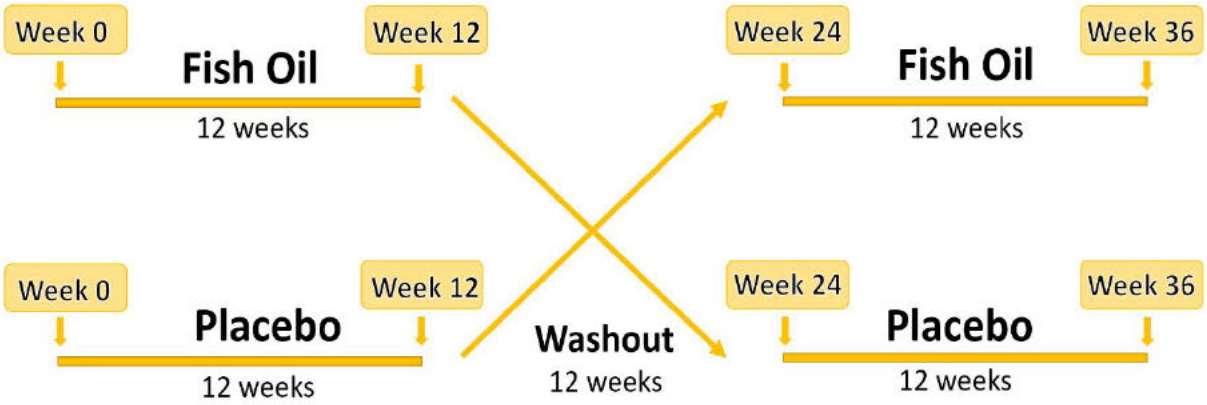
Study Day Procedures

During the study, there will be four 'intervention' visits, which will take place at the beginning and end of each 12-week intervention period (weeks 0, 12, 24 and 36). Before each study visit, subjects will be asked to abstain from alcohol and strenuous exercise during the 24 hours prior to the study day. On each visit day, subjects will be asked to come to the nutrition unit in an unfed state (fasted, not eating or drinking anything but water from 8 pm the night before). After detecting the weight and blood pressure, a blood sample of approximately 100 ml (volume equivalent to six tablespoons) will be collected from subjects as well (blood will be processed to obtain platelet-free plasma (PFP) for the counting and characterisation of EVs, then PFP will be stored at -80℃ for further analysis). Each visit will last approximately 30 minutes.

In week 0, subjects will be supplemented with capsules containing either fish oil (1.8 g/d n-3 PUFA) or placebo (olive oil) and be asked to consume them with breakfast, lunch and dinner for a period of 12 weeks. There will be a 12-week washout period between week 12 and week 24. In week 36, another intervention will be provided to subjects for another 12 weeks (Fig 1).

Subjects will be given a food frequency questionnaire (Appendix F) as well after screening if they are eligible for the study, which will be used to assess their normal diet. They need to complete this questionnaire at home before their first visit and again during each arm of the intervention study (weeks 0, 12, 24 and 36). The questionnaire can take up to one hour to complete.

Figure 1. Study Outline



Subjects will be provided with capsules in excess of requirements (3 capsules/d x 12 weeks), and remaining capsules at the end of each treatment period will be counted. Compliance will be monitored by capsule counts and changes in the plasma fatty acid composition. A good compliance by capsule counts is supposed to be reflected in a significant increase in plasma n-3 PUFA after fish-oil supplementation than with the placebo.

Both fish oil capsule and placebo capsule of identical appearance that contains olive oil will be provided by Wiley's Finest Company (wild alaskan fish oil). The product have an excellent track record for safety and had independent confirmation of high quality based on our previous studies. They were also used in a recent study featured on Trust Me I'm A Doctor with no adverse effects. Also, the compositions and stability of two capsules will be confirmed before the start of study.

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

2.3

Where will the project take place?

The study will be conducted at the Hugh Sinclair Unit of Human Nutrition, Department of Food and Nutritional Sciences, University of Reading where numerous human studies have been safely and successfully completed.

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 (Dr Michelle Weech, m.weech@reading.ac.uk)

Signed…… … (Hugh Sinclair Unit Manager) Date: 16/02/17

2.4

Funding

Is the research supported by funding from a research council or other *external* sources (e.g. charities, business)?
Yes/~~No~~ ~~(delete)~~

If Yes, please give details:
This project is funded by BBSRC-DRINC (Diet & Health Research Industry Club)

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.

There are no reported severe adverse effects of the study treatment as the intervention and placebo products to be used in this study are widely consumed and tested. However, some mild side effects like nausea, dizziness and stomach discomfort have been seldom reported. So during each intervention visit, subjects will be given the opportunity to discuss any issues with the capsules. Any adverse effect will be recorded and discussed with nurse and subjects themselves to estimate whether intervention should be terminated. Any drop-out due to adverse effect will be followed up and their GPs will be informed as well. All procedures, including venepuncture, will be performed by trained researchers. Moreover, in the case of an emergency (e.g. fainting, blood spillage), researchers will take immediate action on the basis of the relevant Hugh Sinclair Unit of Human Nutrition SOPs, which describe the necessary actions needed in emergency.

(*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)?

[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]

No. We will provide participants with all the necessary information about the study precisely and comprehensively.

2.7

Payment

Will you be paying your participants for their involvement in the study? Yes/No (delete)

If yes, please specify and justify the amount paid

Each participant will receive an honorarium of £200 in order to cover time and travel expenses. There is no payment for the initial screening visit. Early dropouts will be paid on a pro-rata basis (£50 per visit).

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?

A coding system will be applied for all participants and their samples. All personal data will be stored in a computer specifically allocated for the study with a specific password, known only by the principal investigator and key

researchers. A secure shared drive storing all data will be applied as well for monitor. The names of the volunteers will not be saved on the same file with the rest of the data. Also, all the completed consent forms will be saved in a safe locker. In this way, the access to confidential information is restricted to the project researchers only. The University is currently implementing enhanced storage infrastructure for research projects, which will be available by the proposed start date for this project, and the intention is to use this for both data storage and archiving; costs were included for this in the proposal. Whilst control of the data will be retained by the applicants for the publication and dissemination of results, after publication the entire experimental dataset will be made freely available online, in an appropriately anonymised form and in widely used open/generic formats, in accordance with both BBSRC-DRINC policy and the University's Research Data Management Policy. This dataset will be preserved and made publicly available for a minimum of 10 years after the completion of the project using the University's Research Data Archive (http://www.reading.ac.uk/internal/reas-RDArchive.aspx). Data will be identifiable only by study ID codes and thus will be completely anonymised.

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 potential participants will be provided with a participant information sheet (Appendix F), which outlines the details of the study. Participants indicating an interest in the study will be invited to attend a screening visit at the Hugh Sinclair Unit of Human Nutrition at University of Reading. During the screening visit, all of the procedures will be explained in detail and participants will be offered the opportunity to ask questions. If they are willing to proceed, they will give their consent by completing the Consent Form (Appendix C), which will also be signed by the researcher. A copy of these forms will be kept by the participant and in a secure place at The University of Reading (for a period of 5 years).

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.

The sample size calculation was performed for the main endpoints: EV numbers, function and platelet function. A total of 34 subjects will be required and we aim to recruit 40 volunteers to allow for a 15% dropout.

Based on previous study (Szu-Yun Wu et al., 2014), 34 subjects is a sufficient sample size to detect a 10% reduction in the number of EVs following fish oil supplementation with a two-sided significance level of 5% and a power of 95%. This calculation is based on the assumption that the standard deviation is 2.4 (Szu-Yun Wu et al. observed standard deviations between 1.38 and 2.4). Published data on thrombus formation suggests that 22 subjects are required to detect a 10% change in thrombus formation (Vaiyapuri et al., 2012) and 30 subjects would detect a significant effect of n-3 PUFA on platelet aggregation and PS exposure (Melinda et al., 2012).

***References**:*

*Szu-Yun Wu, Mayneris-Perxachs, J., Lovegrove, J., Todd, S. and Yaqoob, P. (2014). Fish oil supplementation alters numbers of circulating endothelial progenitor cells and micro particles independent of eNOS genotype. Am J Clin Nutr, **100**, 1232–1243.*

*Vaiyapuri, S., Jones, C.I., Sasikumar, P., Moraes, L.A., Munger, S.J. et al. (2012). Gap Junctions and Connexin Hemichannels Underpin Haemostasis and Thrombosis. Circulation, **125(20)**, 2479–2491.*

*Melinda, P., Lisa, L., Michae,l S., Manohar, L.G. (2012). Acute supplementation with eicosapentaenoic acid reduces platelet microparticle activity in healthy subjects. Journal of Nutritional Biochemistry, **23**, 1128–1133.*

3.2

Will the research involve children or vulnerable adults (e.g. adults with mental health problems or neurological conditions)? ~~Yes~~/No ~~(delete)~~

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? ~~Yes~~/No ~~(delete)~~
Will your research involve children under the age of 5 years? ~~Yes~~/No ~~(delete)~~

3.4

Will your research involve NHS patients, Clients of Social Services or will GP or NHS databases be used for recruitment purposes? ~~Yes~~/No ~~(delete)~~

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.

•Use of our department's volunteer database containing approximately 2000 volunteers (Appendix G).
•Email advertisement to staff and students of University of Reading, to members of local community group such as Women's Institute and to staff members in large local organisations and companies such as Reading Borough Council, The Prudential, Oracle and ING Direct (Appendix H).
•Posters (Appendix I) and leaflets (Appendix J) in public places, such as around the university campus and/or in community centres and shops. Also at public events (e.g. Royal County Berkshire Show).
•Advertisement in local newspapers, magazines, social media (such as Facebook and Streetlife) and websites (Appendix K).

Important Notes

1. The Principal Investigator must complete the Checklist in Appendix A to ensure that all the relevant steps and 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 Form in Appendix B.

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

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 (please specify): Poster	☑	N/A	☐

Expected duration of the project (months) 14

Name (print)…PARVEEN YAQOOB… **Signature** ....

Appendix B

Project Submission Form

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

Principal Investigator: Professor Parveen Yaqoob

School: School of Chemistry, Food and Pharmacy

Title of Project: Effects of fish oil-derived n-3 polyunsaturated fatty acids on the generation and functional activities of extracellular vesicles (Simplified title to be used in communications with potential volunteers: "**HI-FIVE STUDY**").

Proposed starting date: October 2017

Brief description of Project:

A randomised, double-blind, placebo-controlled crossover intervention detecting the effects of dietary n-3 PUFA on the generation and functions of extracellular vesicles in a population at moderate cardiovascular diseases risk.

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: 20/2/17

……. (Head of Department) Date……

…. (Student) Date: 16/02/17

(Dr Dionne Tannetta) Date: 21/02/17

Dr Plinio Ferreira) Date: 21/02/17

(Professor Jon Gibbins) Date: 21/02/17

…… (Dr Chris Jones) Date: 21/02/17

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……

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; ☐

   OR

   (b) 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.

Professor Parveen Yaqoob
Phone: 0118 378 8720
Email: p.yaqoob@reading.ac.uk

School of Chemistry, Food & Nutritional Sciences and Pharmacy

Whiteknights
PO Box 266, Reading RG6 6AP, UK
phone +44 (0)118 378 8453
fax +44 (0)118 378 6331

Appendix C Consent Form

Consent Form for HI-FIVE STUDY

Please initial boxes

1. I confirm that I have read and understand the Participant Information Sheet dated _____for the study, **Effects of fish oil-derived n-3 polyunsaturated fatty acids on the generation and functional activities of extracellular vesicles (HI-FIVE 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 my participation is voluntary and that I am free to withdraw at any time without giving any reason. ☐
3. I authorise the Investigator to inform my General Practitioner of my participation in the study. ☐
4. I have received a copy of this Consent Form and of the accompanying Participant Information Sheet. ☐
5. I consent to an initial blood sample being taken for screening purposes, followed by a series of blood samples throughout the study at the times indicated 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 blood test results, such as level of cholesterol, triacylglycerol, and glucose) being stored on the Nutrition Unit Volunteer Database. ☐ Yes ☐ No
- I wish to receive a summary of the overall results once the study is complete and analysed statistically. ☐ Yes ☐ No

- I consent to all of the data I provide being preserved over the long term, and being available in anonymised form, either openly or subject to appropriate safeguards, so that data can be consulted and re-used by oth ☐ Yes ☐ No

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: _____

General Practitioner (GP) details

Name: _____

Address: _____

_____

Telephone: _____

Witnessed by

Name of researcher taking consent: _____

Signature: _____ Date: _____

Appendix D Participant Information Sheet

Participant information sheet

HI-FIVE STUDY

UREC 17/18 (10th April 2017)

Thank you for your interest in the study.

You have been invited to take part in a study investigating the effect of fish oil on platelets and on tiny particles called extracellular vesicles in the blood (HI-FIVE STUDY). Before you decide to participate please take time to read the following information carefully. Please ask us if there is anything that is not clear or if you would like more information.

Contact Name: Ruihan Zhou (PhD student, Study Researcher)
Room 2-01, Food and Nutritional Sciences Department,
School of Chemistry, Food and Pharmacy
The University of Reading, PO Box 226, Whiteknights
Reading RG6 6AP United Kingdom
Email: hi-five@reading.ac.uk

Background of study

It is now well established that the role of diet is significant in the prevention and treatment of cardiovascular diseases (CVDs). There are many risk factors which can affect the risks of developing CVD. Some are not modifiable, such as genetics and age, while others are modifiable, such as smoking and diet. Fish oil, containing omega-3 (or n-3) polyunsaturated fatty acids (n-3 PUFA), is suggested to have a favourable effect on several risk factors associated with CVDs, but there is still a great deal that is not understood. In this study, we wish to investigate the effects of fish oil on newly emerging risk factors for CVD, extracellular vesicles (EVs). EVs are tiny particles released from many types of cells and from platelets, particularly when they are stimulated, activated or damaged. High numbers of EVs in the blood have been associated with a higher risk of CVD, but it is not clear exactly what EVs do in the body.

A few studies, including our own, suggest that fish oil reduces numbers of EVs, but research is limited.

What is the purpose of the study?

This study aims to investigate whether fish oil supplements alter (i) the number and profile of EVs in the blood, (ii) the way the EVs in the blood behave, (iii) production of platelet-derived EVs (PEVs) by platelets taken from blood and (iv) the behaviour of the PEVs in the blood.

Am I suitable to take part?

We are aiming to recruit male and female participants between the ages of 40 and 70 years, non-smokers, who are generally healthy, but may be considered to have moderate risk for heart disease (risk will be evaluated by an online calculator called “QRISK2”).

Suitable volunteers should have a normal liver and kidney function and haematology and a weekly alcohol intake of <21 units (men) / <15 units (women). A 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).
You will not be able to take part if you:

- BMI: ＜18.5 kg/m^2
- Have hyperlipidemia
- Have diabetes mellitus or other endocrine disorders.
- Have heart problems.
- Have renal, gastrointestinal, respiratory, liver or bowel disease
- Have inflammatory disease.
- Take drug treatment for hypertension, hyperlipidaemia, inflammatory conditions, depression or thyropathy.
- Take aspirin, ibuprofen or other nonsteroidal anti-inflammatory drugs (NSAIDs) > 4 times per month, or once in the week preceding the study.
- Take any other anti-platelet or anti-coagulant drugs, like triflusal, clopidogrel and warfarin.
- Have allergies.
- Smoking (including e-cigarettes and nicotine products)
- Have a history of alcohol misuse.
- Regularly consume oily fish and/or dietary supplements.
- Planning to start or on a weight reducing regimen.

- Participate in intense aerobic exercise (＞20 min, three times a week).
- Pregnant, lactating, or if of reproductive age and not using a reliable form of contraception (including abstinence).
- Have participated in another clinical trial within the last three months.

The medical and lifestyle questionnaire that you have already completed and some blood tests will be used to screen for the eligibility of study.

Do I have to take part?

It is up to you to decide whether to take part or not. If you decide to take part, you will be given this information sheet to keep and asked to sign a consent form. Your participation remains purely voluntary. You may withdraw at any stage and without giving a reason.

What would happen to me if I take part?

If you are willing to participate in the study after reading this information sheet, your initial eligibility will be determined via the medical and lifestyle questionnaire sent together with this sheet. If you meet the criteria, you will be invited to attend a screening visit at the Hugh Sinclair Unit of Human Nutrition at the University of Reading.

Screening visit

You will need to come in the morning in an unfed state (fasted, not eating or drinking anything but water from 8 pm the night before). All of the procedures of this study will be explained in detail to you and you will be offered the opportunity to ask questions. After your consent for participation being taken, we will measure your height, weight, waist-hip circumference and blood pressure. A small blood sample (~15 ml, volume equivalent to one tablespoon) will be collected as well. The screening visit should take approximately 30 minutes.

If you are found suitable for the study and are willing to proceed, we will confirm your participation in the study and inform your GP of your wish to take part. If your screening results indicate any cause for concern, we will advise you to discuss this with your GP.

Study visit

If you agree to participate and are suitable for the study, you will need to consume capsules containing one oil per day (with breakfast, lunch and dinner) for a period of 12 weeks. This will be followed by a 12-week 'washout' period when you will have no treatment. The final phase will be a 12-week period when you will be asked to consume capsules containing similar dose of another oil per day again. During one of the periods, the capsules will contain fish oil at a dose of 1.8g n-3 PUFA per day, and during another, they will contain a 'placebo' or 'dummy' oil, which consists of olive oil. Some individuals will receive the fish oil capsules first, while

others will receive the placebo first, but all subjects will receive both types of capsule during the study. However, neither you nor researchers will know the order in which you are receiving the capsules.

During the study, there will be four 'intervention' visits, which will take place at the beginning and end of each 12-week intervention period (weeks 0, 12, 24 and 36). Before each study visit, you will be asked to abstain from alcohol and strenuous exercise during the 24 hours prior to the study day. On each visit day, you will be asked to come to the nutrition unit in an unfed state (fasted, not eating or drinking anything but water from 8 pm the night before). After detecting the weight and blood pressure, a blood sample of approximately 100 ml (volume equivalent to six tablespoons) will be collected from you. During each intervention visit, you will be given the opportunity to discuss any issues with the study or the capsules. Each visit will last approximately 30 minutes.

Following screening, if you are eligible for the study, you will be given a food frequency questionnaire, which will be used to assess your normal diet. We will need you to complete this questionnaire at home before your first visit and again during each arm of the intervention study (weeks 0, 12, 24 and 36). The questionnaire can take up to one hour to complete.

What will be measured in the blood samples collected?

The blood sample collected at the screening visit will be used to measure levels of blood fats, glucose, markers of kidney and liver function and to perform a full blood count. This is necessary to further determine your suitability for participation in the study.

The blood samples collected during the study visits will be used to measure (i) the number and profile of EVs in the blood, (ii) the way the EVs in the blood affect blood clotting, (iii) production of platelet-derived EVs (PEVs) by platelets taken from blood, (iv) the way the PEVs in the blood affect blood clotting and (v) levels of n-3 PUFA in your blood.

Blood will be frozen for analysis and the Department of Food and Nutritional Sciences has a licence for storing such material for the purpose of research, which has been obtained from the Human Tissue Authority.

Do I have to modify my diet or lifestyle in any way?

During the study period, you will be asked to maintain your normal diet, exercise normally and carry out your usual activities. Volunteers are also advised not to drink alcohol and do strenuous exercise 24 hours before the study day. We do ask that you inform us if at any time

during the study period you are prescribed any medication or if you are advised to stop any medication that you are taking at the start of the study period.

What are the possible disadvantages of taking part?

There are no reported severe adverse effects of the study treatment as fish oil and olive oil are widely consumed and tested. However, some mild side effects like nausea, dizziness and stomach discomfort have been seldom reported. Therefore, during each intervention visit, you are encouraged to discuss any issues with the study or the capsules. Any adverse effect will be recorded and discussed with you and our nurses to estimate whether intervention should be terminated. Any dropout due to adverse effect will be followed up and your GPs will be informed as well.

Blood sampling is an invasive procedure, so there can be a small discomfort as any blood sampling which may affect some people more than others. You should not experience any pain during or after this procedure. You may develop a small bruise at the site of the blood sample, but this will fade like any bruise. This procedure will be performed by a fully trained researcher or nurse in accordance with the University of Reading guidance on research involving blood samples collection and first aid. The volume of blood collected, approximately 100 ml, will cause no adverse consequences.

What are the possible benefits of taking part?

The knowledge gained from the study will help us to identify whether there are any new means by which fish oil could have a beneficial effect on the risk of developing heart disease.

Would my taking part in this study be kept confidential?

Confidential information will be stored securely and can only be accessed by the study investigators. All information collected during the study will be treated in strict confidence in accordance with the relevant data protection legislation. Your data will only be identifiable by a unique volunteer number, not by your name, so information will be disclosed in any way which will allow the identification of yourself. Information obtained from the study may be published in scientific journals but only in the form of average values for the group. No results for the individual subjects will be published or presented in scientific meetings.

Will the results be available to me?

You will be supplied with your screening results. Once the study is completed and analysed statistically, we can provide you with some feedback about what we have found in the study

and what it may mean for future research.

What would happen to the results of the research?

The results of this study will contribute to the PhD thesis of a postgraduate student and will be published anonymously in scientific journals, oral presentations or other scientific contributions. The results of this study will also be provided to the Biotechnology and Biological Sciences Research Council Diet & Health Research Industry Club (BBSRC-DRINC), who are funding this study.

What will happen if I don't want to carry on with the study?

If you do decide to take part, you will be asked to sign a consent form during the screening visit and you will be given a copy of this to keep. However, you will still be free to withdraw from the study at any time and without giving a reason. This will not affect your participation in future studies. While you are participating in the study, it is important for you to attend all visits to the best of your ability. If the appointment is not

convenient on a particular date, please contact the study investigators as soon as possible so that an alternative date can be offered to you.

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. If you remain unhappy and wish to complain formally, you can do this through Professor Richard Frazier (Head of Department) (see contact details at the end of this Participant Information Sheet).

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 that provides cover against negligence, error or omission for the activities of its employers.

Will I get paid for taking part?

An honorarium will be paid as an inconvenience allowance of £200 upon completion of the study, which includes any travel costs you may incur.

Who is organising and funding the research?

This research is being organised by the University of Reading's Hugh Sinclair Unit of Human

Nutrition, and funded by BBSRC-DRINC.

Who reviewed this study?

This project has been reviewed by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.

Contact Information

Main point of contact:	*For formal complaints:*
Ruihan Zhou (PhD student, Study Researcher) Email: hi-five@reading.ac.uk Address: Department of Food and Nutritional Sciences, PO Box 266, University of Reading, Whiteknights Campus, Reading, RG6 6AP Office: 2-01, Harry Nursten Building	**Professor Richard Frazier (Head of Department)** Email: r.a.frazier@reading.ac.uk Tel: 0118 378 8709 Address: Department of Food and Nutritional Sciences, PO Box 266, University of Reading, Whiteknights Campus, Reading, RG6 6AP Office: 2-41, Harry Nursten Building

Thank you for reading this information sheet. If you decide to take part in this study you will be given a copy of the information sheet and a signed consent form to keep.

Appendix E Medical and Lifestyle Questionnaire

HI-FIVE STUDY
Medical and Lifestyle Questionnaire

Name:		Title:
Address:		Date of Birth: Age: Ethnicity: Sex:
Daytime Telephone:	Evening Telephone:	Best time to call:
Weight (kg):	Height (m):	BMI (kg/m^2):
E-mail: Do you use emails on a regular basis? YES/NO		

How did you hear about the study?

_____

Please cross out as appropriate

Medical questions

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

a) High blood cholesterol YES/NO

b) Thyroid disorder YES/NO

c) Diabetes or other endocrine disorders YES/NO

d) Heart problems, stroke or any vascular disease in the past 12 months YES/NO

e) Inflammatory diseases (e.g. rheumatoid arthritis) YES/NO

f) Renal, gastrointestinal, respiratory, liver or bowel disease YES/NO

g) Cancer YES/NO

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

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? How many and how long have you taken*)

a) Are you presently on any long term medication? YES/NO
   (*If 'YES', what are they and for what reasons?*)

b) Do you take any pain killing medication for example: aspirin, paracetemol, nurofen, ibuprofen, or any non-steroid anti-inflammatory drugs (NSAID), more than 4 times per week, or once in the week preceding the study? YES/NO

c) Do you take any other anti-platelet or anti-coagulant drugs, like triflusal, clopidogrel and warfarin? YES/NO

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

5. Have you ever suffered from a pulmonary embolism, deep vein thrombosis, blood clots or had a blood transfusion? YES/NO
   (*If 'YES', please give details*)

6. Do you have a pacemaker? YES/NO

7. Do you suffer from any type of allergies including food and pollen? YES/NO
   (*If 'YES', please give details*)

8. Has any of your first degree relatives (father, mother, brother, sister) suffered from angina or heart attack before the age of 60 years? YES/NO

9. This question is **only to female** participants.

a) Are you premenopausal, perimenopausal or postmenopausal? Please cross out as appropriate.

If you are premenopausal:

b) Are you using contraception? YES/NO
If 'YES', please give details (including the name of the contraceptive pill or device)

c) Are you pregnant, lactating or planning a pregnancy in the next year? YES/NO

If you are postmenopausal:

d) Do you remember when your final menstrual cycle was?

Less than 1 year ago	☐
1-2 years ago	☐
2-5 years ago	☐
More than 5 years ago	☐
Can't remember	☐

e) Are you on hormone replacement therapy (HRT)? YES/NO
(*If 'YES', how long have you been on HRT?*)

Dietary questions

10. Do you regularly consume oily fish? YES/NO
(Examples include salmon, mackerel, herring, fresh tuna (not canned tuna), sardine, trout, pilchard, kipper, eel, whitebait, anchovy, swordfish, bloater, carp.)
(If 'YES', please give details: what kind of fish, how often and amount)

11. Are you vegetarian or vegan? YES/NO
(*If 'YES', please specify*)

12. 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*)

If user of fatty acids or vitamin/mineral supplements,

If you were to participate in our study, would you be willing to give up taking the supplements during the months of the study?

YES/NO

(A wash-out period of 4 weeks is required for those taking vitamin/mineral supplements and a wash-out period of 8-10 weeks for those taking fatty acid supplements.)

13. 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).

14. Have you ever suffered from alcohol misuse? YES/NO

15. Are you following or planning to start a restricted diet, e.g. to lose weight? YES/NO

If 'YES', would you be willing to postpone this until after your final study visit? YES/NO

Lifestyle questions

16. a) Do you smoke (including e-cigarettes and nicotine products)? YES/NO
(*If 'YES', please give details.*)

b) If not smoking, how long has it been since you last smoked?

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

But currently do a manual job of baggage loading 4 days a week but do not work up a sweat

18. 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*)
RISSCI ends on 20th December 2017

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

20. Are you a blood donor? YES/NO
(*If 'YES', when was the last time you gave blood?*)

If you are eligible to participate in the study, are you willing to postpone further blood donations until 3 months after your final study visit? YES/NO

21. Do you plan to take a holiday in the next 6 months? YES/NO

List holiday period:

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

Appendix F Food Frequency Questionnaire

FOOD FREQUENCY QUESTIONNAIRE

HI-FIVE STUDY

How to answer the questions

There are several types of question in this booklet. Most of them can be answered by ticking a box.

For example:

FOODS & AMOUNTS	AVERAGE USE IN THE LAST YEAR								
FISH *(medium serving)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Fried fish in batter, as in fish and chips	1	2	√ 3	4	5	6	7	8	9

Please put a tick or cross in the appropriate box on each line to indicate how often, **on average**, you have eaten each food **during the past year**.

- Answer every question by putting a tick on every line
- Do not leave **ANY** lines blank.

Another example of questions requiring boxes to be ticked:

Q. Do you usually add salt to food while cooking?

Yes.....☑1
No.....❒2

Some of these questions have several boxes and you may be asked to tick ONE only.

For example:

What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter……❒1
Lard/dripping……❒2
Solid vegetable fat……❒3
Margarine……❒4
Vegetable oil……❒5
Olive oil……☑6
Walnut Oil……❒7
Soya Oil……❒8
None……❒9
Other……❒10

Some of these questions have several boxes and you may be asked to tick all the boxes you think apply to you.

For example:

Do you follow a special diet?

Please tick <u>ALL</u> that apply.

No……❒1
Yes, because of a medical condition/allergy……❒2
Yes, to lose weight……☑3
Yes, because of personal beliefs (religion, vegetarian)…… ☑4
Yes,other ❒5

What do I do if I make a mistake?

Cross out the incorrect answer, and put a tick where you think the right answer should be.
If you have any problems filling in this dietary questionnaire, we will discuss them at your next visit.

==========

FOODS & AMOUNTS	AVERAGE USE IN THE LAST YEAR								
1. MEAT ***(medium serving)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: e.g. roast, steak, mince, stew, casserole, curry, bolognese	1	2	hm 3	4	5	6	7	8	9
Beefburgers	1	2	3	4	5	6	7	8	9
Corned beef, Spam, luncheon meats	1	2	3	4	5	6	7	8	9
Lamb: e.g. roast, chops, stew, curry	1	2	3	4	5	6	7	8	9
Chicken, turkey or other poultry: e.g. casserole, sliced, curry	1	2	3	4	5	6	7	8	9
Breaded or fried poultry products: e.g. chicken nuggets, deep fried chicken pieces	1	2	3	4	5	6	7	8	9
Pork: e.g. roast, chops, stew, curry	1	2	3	4	5	6	7	8	9
Bacon and ham	1	2	3	4	5	6	7	8	9
Sausages	1	2	3	4	5	6	7	8	9
Savoury pies, e.g. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls, scotch egg	1	2	3	4	5	6	7	8	9
Game and Wild-fowl: e.g. duck, rabbit, grouse	1	2	3	4	5	6	7	8	9
Kidneys or liver; including liver pate, liver sausage	1	2	3	4	5	6	7	8	9

FOODS & AMOUNTS	AVERAGE USE IN THE LAST YEAR								
2. FISH and SEAFOOD ***(medium serving)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Fresh or frozen mackerel, kippers, herring	1	2	3	4	5	6	7	8	9
Canned mackerel, kippers, herring, anchovies	1	2	3	4	5	6	7	8	9
Fresh or frozen tuna, salmon, sardines, eel, trout, swordfish, mullet, spratts	1	2	3	4	5	6	7	8	9
Canned salmon, sardines, pilchards	1	2	3	4	5	6	7	8	9
Canned tuna in brine or oil	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

FOODS & AMOUNTS	AVERAGE USE IN THE LAST YEAR								
2. FISH and SEAFOOD (continued) *(medium serving)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Fish products from oily fish e.g. salmon en croute, whitebait, salmon/tuna pate	1	2	3	4	5	6	7	8	9
Other fish, fresh or frozen, e.g. cod, haddock, plaice, sole, halibut, turbot, coley, skate, monkfish	1	2	3	4	5	6	7	8	9
Fried fish in batter, as in fish and chips	1	2	3	4	5	6	7	8	9
Fish products from non-oily fish: e.g. fish fingers / cakes, scampi, boil in the bag fish, breaded baked fish, fish paste	1	2	3	4	5	6	7	8	9
Fish based dishes e.g. fish pie, fish curry, kedgeree	1	2	3	4	5	6	7	8	9
Fresh or frozen shellfish, e.g. crab, lobster, prawns, mussels, oyster, scallops	1	2	3	4	5	6	7	8	9
Canned / bottled or "snack" shellfish, e.g. mussels, seafood cocktail, crabsticks, shrimps, whelks	1	2	3	4	5	6	7	8	9
Roe and roe products including taramasalata, caviar	1	2	3	4	5	6	7	8	9
Fresh squid and octopus, or squid or octopus products	1	2	3	4	5	6	7	8	9
Other fish / seafood or fish products not mentioned (please state and tick for frequency) 1	1	2	3	4	5	6	7	8	9
2	1	2	3	4	5	6	7	8	9
3	1	2	3	4	5	6	7	8	9
3. BREAD & SAVOURY BISCUITS *(one slice or biscuit)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls, white pitta bread	1	2	3	4	5	6	7	8	9
Scones, teacakes, crumpets, muffins or croissants	1	2	3	4	5	6	7	8	9
Brown bread and rolls, brown pitta bread	1	2	3	4	5	6	7	8	9
Wholemeal bread and rolls	1	2	3	4	5	6	7	8	9
Cream crackers, cheese biscuits	1	2	3	4	5	6	7	8	9
Naan bread, chapati	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

FOODS & AMOUNTS	AVERAGE USE IN THE LAST YEAR								
3. BREAD & SAVOURY BISCUITS (continued) ***(one slice or biscuit)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Garlic bread	1	2	3	4	5	6	7	8	9
4. CEREALS ***(one bowl)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Porridge, Readybrek	1	2	3	4	5	6	7	8	9
Sugar coated cereals e.g. Sugar Puffs, Cocoa Pops, Frosties	1	2	3	4	5	6	7	8	9
Non-sugar coated cereals e.g. Cornflakes, Rice Crispies	1	2	3	4	5	6	7	8	9
Muesli	1	2	3	4	5	6	7	8	9
Bran containing cereals e.g. All Bran, Bran Flakes	1	2	3	4	5	6	7	8	9
Wholegrain cereals e.g. Cheerios, Weetabix, Shredded Wheat	1	2	3	4	5	6	7	8	9
5. POTATOES, RICE & PASTA ***(medium serving)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Boiled, mashed, instant or jacket potatoes	1	2	3	4	5	6	7	8	9
Chips, potato waffles	1	2	3	4	5	6	7	8	9
Roast potatoes	1	2	3	4	5	6	7	8	9
Yorkshire pudding, pancakes, dumpling	1	2	3	4	5	6	7	8	9
Potato salad	1	2	3	4	5	6	7	8	9
White rice	1	2	3	4	5	6	7	8	9
Brown rice	1	2	3	4	5	6	7	8	9
White or green pasta, e.g. spaghetti, macaroni, noodles, tortellini	1	2	3	4	5	6	7	8	9
Tinned pasta, e.g. spaghetti, ravioli, macaroni	1	2	3	4	5	6	7	8	9
Super noodles, pot noodles, pot savouries	1	2	3	4	5	6	7	8	9
Wholemeal pasta	1	2	3	4	5	6	7	8	9
Pasta dishes e.g. Lasagne, moussaka, cannelloni	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

FOODS & AMOUNTS	AVERAGE USE IN THE LAST YEAR								
5. POTATOES, RICE & PASTA (continued) *(medium serving)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Pizza	1	2	3	4	5	6	7	8	9
6. (a) DAIRY PRODUCTS & FATS	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream *(tablespoon)*	1	2	3	4	5	6	7	8	9
Double or clotted cream *(tablespoon)*	1	2	3	4	5	6	7	8	9
Low fat yoghurt, fromage frais *(125g carton)*	1	2	3	4	5	6	7	8	9
Full fat or Greek yoghurt *(125g carton)*	1	2	3	4	5	6	7	8	9
Dairy desserts *(125g carton)*, e.g. mousse	1	2	3	4	5	6	7	8	9
Cheese, e.g. Cheddar, Brie, Edam *(medium serving)*	1	2	3	4	5	6	7	8	9
Cottage cheese, low fat soft cheese *(medium serving)*	1	2	3	4	5	6	7	8	9
Eggs as boiled, fried, scrambled, omelette etc. *(one)*	1	2	3	4	5	6	7	8	9
Quiche *(medium serving)*	1	2	3	4	5	6	7	8	9
6.(b) DAIRY PRODUCTS & FATS used on bread or vegetables *(teaspoon)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Butter	1	2	3	4	5	6	7	8	9
Blended spreads, e.g. I can't believe it's not butter	1	2	3	4	5	6	7	8	9
Block margarine, e.g. Stork, Krona	1	2	3	4	5	6	7	8	9
Polyunsaturated margarine, e.g. Flora, sunflower	1	2	3	4	5	6	7	8	9
Other soft margarine, dairy spreads, e.g. Blue Band, Clover	1	2	3	4	5	6	7	8	9
Margarine containing olive oil	1	2	3	4	5	6	7	8	9
Low fat spread (less than 60g/100g fat), e.g. Gold	1	2	3	4	5	6	7	8	9
Very low fat spread (less than 50g/100g fat) e.g. Flora light	1	2	3	4	5	6	7	8	9
Cholesterol lowering spreads e.g. Flora Pro-Active, Benecol	1	2	3	4	5	6	7	8	9
	Never or less than	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

	once/ month								
FOODS & AMOUNTS	**AVERAGE USE IN THE LAST YEAR**								
7. SWEETS & SNACKS	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chocolate coated sweet biscuits, e.g. Penguin, kit-kat, chocolate digestive *(one)*	1	2	3	4	5	6	7	8	9
Sweet biscuits, plain, e.g. Nice, ginger *(one)*	1	2	3	4	5	6	7	8	9
Cakes e.g. fruit, sponge, sponge pudding *(medium serving)*	1	2	3	4	5	6	7	8	9
Sweet buns & pastries e.g. flapjacks, doughnuts, Danish pastries, cream cakes *(medium serving)*	1	2	3	4	5	6	7	8	9
Fruit pies, tarts, crumbles *(medium serving)*	1	2	3	4	5	6	7	8	9
Milk puddings, e.g. rice, custard, trifle *(medium serving)*	1	2	3	4	5	6	7	8	9
Ice cream, choc ices *(one)*	1	2	3	4	5	6	7	8	9
Chocolates *(medium bar of chocolate)*	1	2	3	4	5	6	7	8	9
Chocolates snack bars e.g. Mars, Crunchie *(one)*	1	2	3	4	5	6	7	8	9
Sweets, toffees, mints *(one packet)*	1	2	3	4	5	6	7	8	9
Sugar added to tea, coffee, cereal *(teaspoon)*	1	2	3	4	5	6	7	8	9
Crisps or other packet snacks e.g. Wotsits *(one packet)*	1	2	3	4	5	6	7	8	9
Peanuts *(one packet)*	1	2	3	4	5	6	7	8	9
Walnuts (medium serving)	1	2	3	4	5	6	7	8	9
Other nuts not mentioned (please state and tick for frequency) 1. 2. 3.	1	2	3	4	5	6	7	8	9
8. SOUPS, SAUCES AND SPREADS	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Vegetable soups *(bowl)*	1	2	3	4	5	6	7	8	9
Meat soups *(bowl)*	1	2	3	4	5	6	7	8	9
Sauces, e.g. white sauce, cheese sauce, gravy *(medium serving)*	1	2	3	4	5	6	7	8	9
Tomato based sauces e.g. pasta sauces *(medium serving)*	1	2	3	4	5	6	7	8	9
	Never or less than	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

	once/ month								

FOODS & AMOUNTS	AVERAGE USE IN THE LAST YEAR								
8. SOUPS, SAUCES AND SPREADS (continued)	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tomato ketchup, brown sauce *(tablespoon)*	1	2	3	4	5	6	7	8	9
Relishes e. g. pickles, chutney, mustard *(tablespoon)*	1	2	3	4	5	6	7	8	9
Low calorie, low fat salad cream or mayonnaise *(tablespoon)*	1	2	3	4	5	6	7	8	9
Salad cream, mayonnaise *(tablespoon)*	1	2	3	4	5	6	7	8	9
French dressing *(tablespoon)*	1	2	3	4	5	6	7	8	9
Other salad dressing *(tablespoon)*	1	2	3	4	5	6	7	8	9
Marmite, Bovril *(teaspoon)*	1	2	3	4	5	6	7	8	9
Jam, marmalade, honey, syrup *(teaspoon)*	1	2	3	4	5	6	7	8	9
Peanut butter *(teaspoon)*	1	2	3	4	5	6	7	8	9
Chocolate spread, chocolate nut spread *(teaspoon)*	1	2	3	4	5	6	7	8	9
Dips e.g. houmous, cheese and chive *(tablespoon)*	1	2	3	4	5	6	7	8	9
9. DRINKS (continued)	Never or less than once/ month	1-3 per month	Once A Week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea *(cup)*	1	2	3	4	5	6	7	8	9
Coffee, instant or ground *(cup)*	1	2	3	4	5	6	7	8	9
Coffee whitener, e.g. Coffee-mate *(teaspoon)*	1	2	3	4	5	6	7	8	9
Cocoa, hot chocolate *(cup)*	1	2	3	4	5	6	7	8	9
Horlicks, Ovaltine *(cup)*	1	2	3	4	5	6	7	8	9
Wine *(glass)*	1	2	3	4	5	6	7	8	9
Beer, lager or cider *(half pint)*	1	2	3	4	5	6	7	8	9
Port, sherry, vermouth, liqueurs *(glass)*	1	2	3	4	5	6	7	8	9
Spirits, e.g. gin, brandy, whisky, vodka *(single)*	1	2	3	4	5	6	7	8	9
Low calorie or diet fizzy soft drinks *(glass)*	1	2	3	4	5	6	7	8	9
Fizzy soft drinks, e.g. Coca cola, lemonade *(glass)*	1	2	3	4	5	6	7	8	9

	Never or less than once/ month	1-3 per month	Once A Week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

FOODS & AMOUNTS	AVERAGE USE IN THE LAST YEAR								
9. DRINKS	Never or less than once/ month	1-3 per month	Once A Week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Pure fruit juice (100%) e.g. orange, apple juice *(glass)*	1	2	3	4	5	6	7	8	9
Fruit squash or cordial *(glass)*	1	2	3	4	5	6	7	8	9
10. FRUIT *(1 fruit or medium serving)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
*For very seasonal fruits such as strawberries, please estimate your average use when the fruit is in season									
Apples	1	2	3	4	5	6	7	8	9
Pears	1	2	3	4	5	6	7	8	9
Oranges, satsumas, mandarins, tangerines, clementines	1	2	3	4	5	6	7	8	9
Grapefruit	1	2	3	4	5	6	7	8	9
Bananas	1	2	3	4	5	6	7	8	9
Grapes	1	2	3	4	5	6	7	8	9
Melon	1	2	3	4	5	6	7	8	9
*Peaches, plums, apricots, nectarines	1	2	3	4	5	6	7	8	9
*Strawberries, raspberries, kiwi fruit	1	2	3	4	5	6	7	8	9
Tinned fruit	1	2	3	4	5	6	7	8	9
Dried fruit, e.g. raisins, prunes, figs	1	2	3	4	5	6	7	8	9
Other fruit or fruit dishes not mentioned (state and tick for frequency) 1	1	2	3	4	5	6	7	8	9
2	1	2	3	4	5	6	7	8	9
3	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

FOODS & AMOUNTS	AVERAGE USE IN THE LAST YEAR								
11. VEGETABLES Fresh, frozen or tinned *(medium serving)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots	1	2	3	4	5	6	7	8	9
Spinach	1	2	3	4	5	6	7	8	9
Broccoli	1	2	3	4	5	6	7	8	9
Brussels sprouts	1	2	3	4	5	6	7	8	9
Cabbage	1	2	3	4	5	6	7	8	9
Peas	1	2	3	4	5	6	7	8	9
Green beans, broad beans, runner beans	1	2	3	4	5	6	7	8	9
Marrow, courgettes	1	2	3	4	5	6	7	8	9
Cauliflower	1	2	3	4	5	6	7	8	9
Parsnips, turnips, swedes	1	2	3	4	5	6	7	8	9
Leeks	1	2	3	4	5	6	7	8	9
Onions	1	2	3	4	5	6	7	8	9
Garlic	1	2	3	4	5	6	7	8	9
Mushrooms	1	2	3	4	5	6	7	8	9
Sweet peppers	1	2	3	4	5	6	7	8	9
Beansprouts	1	2	3	4	5	6	7	8	9
Green salad, lettuce, cucumber, celery	1	2	3	4	5	6	7	8	9
Mixed vegetables (frozen or tinned)	1	2	3	4	5	6	7	8	9
Watercress	1	2	3	4	5	6	7	8	9
Tomatoes	1	2	3	4	5	6	7	8	9
Sweetcorn	1	2	3	4	5	6	7	8	9
Beetroot, radishes	1	2	3	4	5	6	7	8	9
Coleslaw	1	2	3	4	5	6	7	8	9
Avocado	1	2	3	4	5	6	7	8	9
Baked Beans	1	2	3	4	5	6	7	8	9
Dried lentils, beans, peas	1	2	3	4	5	6	7	8	9

	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

FOODS & AMOUNTS	AVERAGE USE IN THE LAST YEAR								
11. VEGETABLES Fresh, frozen or tinned (continued) *(medium serving)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tofu, soya meat, TVP, Vegeburger	1	2	3	4	5	6	7	8	9
Other vegetables or vegetable dishes / products not mentioned (please state and tick for frequency) 1. 2. 3.	1	2	3	4	5	6	7	8	9

YOUR DIET IN THE LAST YEAR, *continued*

12. (a) What type of milk did you most often use?

Select one only

Full cream.....❒1
Channel Islands.....❒2
Dried milk.....❒3
Semi-skimmed.....❒4
Skimmed.....❒5
Soya.....❒6
Other.....❒7
None.....❒8

13. (b) Approximately, how much milk did you drink each day, including milk with tea, coffee, cereals etc?

None.....❒1
Quarter of a pint (roughly 125mls).....❒2
Half a pint (roughly 250mls) .....❒3
Three quarters of a pint (roughly 375mls) .....❒4
One pint (roughly 500mls) .....❒5
More than one pint (more than 500mls) .....❒6

4

14. What kind of fat did you use for cooking?

Please tick <u>all</u> that apply

Butter……❒1
Lard/dripping……❒2
Solid vegetable fat……❒3
Margarine……❒4
Vegetable oil……❒5
Olive oil……❒6
Walnut Oil……❒7
Soya Oil……❒8
None……❒9
Other……❒10

If "other" selected in question 14, please state……

15. Do you usually add salt to food while cooking?

1. Yes……❒1
2. No……❒2

3. 4

16. Do you usually add salt to any food at the table?

4. Yes……❒1
5. No……❒2

17. Do you usually eat the fat on cooked meats?

6. Yes……❒1
7. No……❒2

18. Do you usually eat the skin on cooked meats?

Yes……❒1
No……❒2

4

19. Do you usually add sugar to drinks i.e. tea/coffee?

Yes……❒1
No……❒2

20. On average, how many portions of fruit and vegetables do you eat per DAY?

Please estimate:….……

21. On average, how many portions of fish and seafood do you eat per WEEK?

Please estimate:…….……

22. Do you follow a special diet?

Please tick all that apply.

No……❒1
Yes, because of a medical condition/allergy……❒2
Yes, to lose weight……❒3
Yes, because of personal beliefs (religion, vegetarian)……❒4
Yes,other……❒5

If "other" please state ……

23. Have you taken any of the following during the past year?

	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day
Vitamins (e.g. multivitamins, vitamin B, vitamin C, folic acid)	1	2	3	4	5	6	7
Minerals (e.g. iron, calcium, zinc, magnesium)	1	2	3	4	5	6	7
Fish oils (e.g. cod liver oil, omega-3)	1	2	3	4	5	6	7
Other food supplements (e.g. oil of evening primrose, starflower oil, royal jelly, ginseng)	1	2	3	4	5	6	7
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day

- Do you use other food supplements? Please state below:

1.
2.

3.
4.

Thank you for filling in such a detailed questionnaire, your efforts are appreciated.

For office use only, please leave this section blank.

Researcher notes:

Appendix G Email-contact to potential volunteers from the Hugh Sinclair database

Re: HI-FIVE STUDY

Dear Volunteers,

When you took part in a previous human nutrition research study at the Hugh Sinclair Unit of Human Nutrition (University of Reading), you expressed an interest in hearing about new studies taking place, for which you might be eligible. We are currently recruiting for a new nutrition study:

The effect of fish oil on cardiovascular health

If you are **aged between 40 and 70 years & a non-smoker**, we would like your help.

You will be recruited to take fish oil supplements and provide blood samples for analysis. The study will involve five 30 min visits: one screening visit and four study visits over 36 weeks.

You will be reimbursed for your time and travel expenses.

If you would like to find out more, please contact **Ruihan Zhou** on **hi-five@reading.ac.uk**.

King regards,

Ruihan Zhou

Appendix H Email-contact to other potential volunteers

Dear ***xxx***,

We are seeking volunteers willing to take part in a human nutrition study:

The effect of fish oil on cardiovascular health

If you are **aged between 40 and 70 years & a non-smoker**, we would like your help.

You will be recruited to take fish oil supplements and provide blood samples for analysis. The study will involve five 30 min visits: one screening visit and four study visits over 36 weeks.

You will be reimbursed for your time and travel expenses.

If you would like to find out more, please contact **Ruihan Zhou** on **hi-five@reading.ac.uk**.

King regards,

Ruihan Zhou

Appendix I Poster

HI-FIVE STUDY:

Can you help us to test the effects of fish oil on cardiovascular health?

We are looking for volunteers who are:

- **Male or female aged 40-70 years**
- **Non-smoker**
- **Willing to take fish oil supplements**
- **Willing to attend 5x 30 min visits at the *Hugh Sinclair Unit of Human Nutrition (University of Reading)***

✧ ***You will be reimbursed for your time and travel***

To find out more:

- Contact **Ruihan Zhou** (PhD student, Study Researcher):
- Email: **hi-five@reading.ac.uk**
- Mobile phone: **07410861109**

HI-FIVE STUDY (Ruihan Zhou) **hi-five@reading.ac.uk**	HI-FIVE STUDY (Ruihan Zhou) **hi-five@reading.ac.uk**	HI-FIVE STUDY (Ruihan Zhou) **hi-five@reading.ac.uk**	HI-FIVE STUDY (Ruihan Zhou) **hi-five@reading.ac.uk**	HI-FIVE STUDY (Ruihan Zhou) **hi-five@reading.ac.uk**	HI-FIVE STUDY (Ruihan Zhou) **hi-five@reading.ac.uk**	HI-FIVE STUDY (Ruihan Zhou) **hi-five@reading.ac.uk**	HI-FIVE STUDY (Ruihan Zhou) **hi-five@reading.ac.uk**	HI-FIVE STUDY (Ruihan Zhou) **hi-five@reading.ac.uk**	HI-FIVE STUDY (Ruihan Zhou) **hi-five@reading.ac.uk**

Department of Food and Nutritional Sciences

University of Reading

HUGH SINCLAIR Unit of Human Nutrition

Volunteers Needed Aged 40-70yrs

Can you help us to test the effects of fish oil on cardiovascular health?

We are looking for volunteers who are:

- Male or female aged 40-70 years, Non-smoker
- Willing to take fish oil supplements
- Willing to 5x 30 min visits at the Hugh Sinclair Unit of Human Nutrition (University of Reading)

You will be reimbursed for your time and travel expenses

HI-FIVE STUDY

To find out more:

- Contact **Ruihan Zhou** (PhD student, Study Researcher):
- Email: **hi-five@reading.ac.uk**
- Mobile phone: **07410861109/07419538429**

Appendix K Advertisement in local newspapers, magazines and websites

The University of Reading is recruiting men and women, aged 40-70, non-smoker for a human nutrition study investigating the effect of fish oil on heart health (HI-FIVE STUDY). Volunteers should be willing to take fish oil supplements and attend five 30 min visits: one screening visit and four study visits over 36 weeks. You will be reimbursed for your time and travel expenses.

For more information please contact: Ruihan Zhou (HI-FIVE STUDY)

Email: **hi-five@reading.ac.uk**