University of Reading

Effects of Fish Oil-Derived N-3 Polyunsaturated Fatty Acids on the Generation and Functional Activities of 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 materal from other sources has been properly and fully acknowledged.

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Abstract

Background: Extracellular vesicles (EVs) are submicron membrane-bound vesicles released from almost all cells, which affect many pathophysiological processes involved in cardiovascular diseases (CVDs) and may therefore be considered as a novel marker for CVDs. N-3 polyunsaturated fatty acids (n-3 PUFA) have been suggested to play a role in protecting cardiovascular health. However, there is little information about the effect of n-3 PUFA on circulating EV numbers, composition and functional activity in the context of CVDs.

Objective: This project investigated the relationships between circulating EVs, cardiovascular risk markers and CVD risk, as well as the chronic effects of fish oil-derived n-3 PUFA on the numbers, fatty acid composition and procogulatory activity of circulating EVs.

Design: Subjects (n=40) aged 40-70yrs with moderate risk of CVDs were recruited into a double-blind, randomised crossover trial of fish oil (1.8g/d n-3 PUFA) or control oil (high-oleic safflower oil) for 12 weeks with a 12-week washout. EVs were analysed by Nanoparticle Tracking Analysis (NTA) and fluorescence flow cytometry (FCM). Total lipid fatty acid composition and procoagulatory activity of circulating EVs were analysed by gas chromatography and a thrombin generation assay, respectively.

Results: Circulating EV numbers were positively associated with body mass index (BMI), blood pressure (BP), plasma triacylglycerol (TAG) concentration and overall CVD risk. Furthermore, plasma TAG concentration and diastolic blood pressure (DBP) independently predicted total EV numbers based on a multivariate regression model. Fish oil supplementation significantly decreased numbers of circulating EVs, modified their total lipid fatty acid composition and decreased their thrombogenic potential.

Conclusion: Circulating EVs are associated with CVD risk and are particularly strongly related with plasma TAG concentration and DBP. They could therefore potentially serve as a novel biomarker for CVD prediction. Dietary n-3 PUFA modifies the numbers, fatty acid composition and function of circulating EVs in a manner which suggests beneficial effects of n-3 PUFA on CVDs.

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Abbreviations

AA	arachidonic acid
ADP	adenosine diphosphate
ALA	α-linolenic acid
ALP	alkaline phosphatase
ALT	alanine transaminase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
APC	Allophycocyanin
Apo	apolipoprotein
BP	blood pressure
BSA	bovine serum albumin
CAD	coronary artery disease
CHD	coronary heart disease
CI	confidence interval
CM	chylomicrons
COXs	cyclooxygenases
CVDs	cardiovascular diseases
DBP	diastolic blood pressure
DGC	density gradient centrifugation
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EDEVs	endothelial-derived extracellular vesicles
EM	electron microscopy
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid
EPIC	European Prospective Investigation of Cancer
ErDEVs	erythrocyte-derived extracellular vesicles

ESCRT	Endosomal Sorting Complex Required for Transport
ETA	eicosatetraenoic acid
ETP	endogenous thrombin potential
EVs	extracellular vesicles
FAMEs	fatty acid methyl esters
FCM	flow cytometry
FETA	FFQ EPIC Tool for Analysis
FFQ	food frequency questionnaires
FITC	fluorescein isothiocyanate
FRS	Framingham Risk Score
FSC	forward scatter
GC	gas chromatography
GGT	Gemma glutamyl transferase
GP	glycoprotein
HDHA	hydroxydocosahexaenoic acids
HDL(-C)	high-density lipoprotein (cholesterol)
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
HRP	horseradish peroxidase
HSP	heat shock proteins
ICAM-1	intercellular-cell adhesion molecule-1
IDL	intermediate-density lipoproteins
IL	interleukin
ILVs	intraluminal vesicles
IQR	inter-quartile distance
ISEV	International Society for Extracellular Vesicles
JBS2	Joint British Societies' guidelines
LDEVs	leukocytes-derived extracellular vesicles
LDL(-C)	low-density lipoprotein (cholesterol)

LEVs	large total extracellular vesicles
LNCaP-EVs	left supraclavicular lymph node-derived extracellular vesicles
LOXs	lipoxygenases
MCP-1	monocyte chemoattractant protein-1
MDEVs	monocyte-derived extracellular vesicles
MHC	major histocompatibility complex
MI	myocardial infarction
MISEV	Minimal Information for Studies of Extracellular Vesicles Guidelines
MS	metabolic syndrome
MUFA	monounsaturated fatty acids
MVBs	multivesicular bodies
N/A	not applicable
NO	nitric oxide
NTA	Nanoparticle Tracking Analysis
PAH	pulmonary arterial hypertension
PAI-1	plasminogen activator inhibitor type 1
PB	Pacific Blue
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PC3-EVs	prostate cancer-derived extracellular vesicles
PC7	phycoerythrin cyanine 7
PDEVs	platelet-derived extracellular vesicles
PE	phosphatidylethanolamine
PE	phycoerythrin
PECAM-1	platelet endothelial cell adhesion molecule-1
PerCP	Peridinin-Chlorophyll-Protein
PFP	platelet-free plasma
PPP	platelet-poor plasma
PS	phosphatidylserine

RCTs	randomised clinical trials
SBP	systolic blood pressure
SCORE	systematic coronary risk evaluation
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	standard error
SEC	size exclusion chromatography
SEM	standard error of the mean
SEVs	small total extracellular vesicles
SFA	saturated fatty acids
SM	sphingomyelin
SSC	side scatter
T2D	type 2 diabetes
TAG	triacylglycerol
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TC	total cholesterol
TEVs	total extracellular vesicles
TF	tissue factors
TNF	tumour necrosis factor
TSG101	tumour-susceptibility gene 101
TX	thromboxane
UC	ultracentrifugation
VCAM-1	vascular cell adhesion molecule-1
VFP	vesicle-free plasma
VLDL	very-low-density lipoprotein
vWF	von Willebrand factor
WHO	World Health Organisation

Accomplishments

Oral presentation

International Society for Extracellular Vesicles Annual Conference 2020

Philadelphia, USA (virtual)

Title: Cardiovascular risk markers are strongly related to numbers of circulating extracellular vesicles

UK Society for Extracellular Vesicles Forum 2019

London, United Kingdom

Title: Cardiovascular risk markers are strongly related to numbers of circulating extracellular vesicles

Food and Nutritional Sciences Research Seminar 2018

University of Reading, United Kingdom

Title: Methodological considerations for the isolation, characterisation and storage of extracellular vesicles

Diet and Health Research Industry Club Dissemination Workshop 2017

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Title: Isolation methods of extracellular vesicles

Food and Nutritional Sciences Research Symposium 2017

University of Reading, United Kingdom (won the first prize)

Title: Effects of fish oil-derived n-3 polyunsaturated fatty acid on the generation and functional activities of extracellular vesicles

Posters

International Society for Extracellular Vesicles Annual Conference 2019

Kyoto, Japan

Title: Influence of cardiovascular risk markers on numbers and characterisation of circulating extracellular vesicles

UK Society for Extracellular Vesicles Forum 2018

Sheffield, United Kingdom

Title: Association of circulating extracellular vesicles with cardiovascular risk markers

Food and Nutritional Sciences Research Symposium 2018

University of Reading, United Kingdom (won the first prize)

Title: Methodological considerations for the isolation, characterisation and storage of extracellular vesicles

Doctoral Research Conference 2017

University of Reading, United Kingdom

Title: How does fish oil protect our cardiovascular health: some new insights into extracellular vesicles

Diet and Health Research Industry Club Dissemination Event (Annual)

United Kingdom

Papers (manuscripts in preparation)

Association of conventional cardiovascular risk markers with numbers and functions of circulating extracellular vesicles

Effects of fish oil-derived n-3 PUFA on numbers, composition and functions of circulating extracellular vesicles

Chapter 1 Introduction and literature review

1.1 Cardiovascular diseases (CVDs)

1.1.1 Introduction to CVDs

Cardiovascular diseases (CVDs) are the leading cause of worldwide mortality, which accounted for 31% of global deaths in 2015 and are expected to cause more than 23.6 million deaths annually by 2030. CVDs are defined by the World Health Organisation (WHO) as disorders of the heart and blood vessels, which encompass coronary heart disease (CHD), rheumatic heart disease, cerebrovascular disease, peripheral arterial disease, deep-vein thrombosis and pulmonary embolism (WHO, 2017).

1.1.2 Cardiovascular risk factors

As a multifactorial disease, there are various risk factors, which are linked with the subsequent occurrence of CVDs. Some of these are non-modifiable, including age, gender and family history, while others could be controlled by making certain lifestyle and diet changes (Frayn *et al*., 2019). There are some so-called conventional risk factors, such as total cholesterol (TC) concentration, blood pressure (BP) and obesity, whose effects on CVDs have been well-established in recent decades. There is growing interest in newly emerging risk factors such as endothelial dysfunction, oxidative stress and extracellular vesicles formation. The common unmodifiable and modifiable risk factors are summarised in **Table 1.1** (Frayn *et al*., 2019). The WHO has estimated that over 75% of premature CVDs are preventable, and efforts to control or treat risk factors can help to decrease the morbidity and mortality of CVDs (WHO, 2017; Cannon, 2008).

Table 1.1 Unmodifiable and modifiable risk factors for cardiovascular disease

Risk factors	**Direction of association**	**Comments**
Unmodifiable risk factors		
Age	Positive	Increased prevalence as population ages

Gender	Males at higher risk	Risk in men and women is equalised after the menopause, possibly due to protection by oestrogens in younger women or to increases in male pattern (central) obesity amongst post-menopausal women
Ethnic group	People from the Indian subcontinent at higher risk	May reflect abdominal obesity and insulin resistance
Modifiable risk factors (established)		
Serum TC concentration	Higher blood TC concentration increases risk	Uptake of cholesterol by macrophages is the origin of the core of the atherosclerotic plaque
Serum high HDL-C concentration	Lower HDL-C increases risk, particularly amongst women	HDL may transport excess cholesterol to the liver for excretion
BP	Higher BP increases risk	Hypertension increases the risk of haemorrhagic/ischaemic stroke, induces endothelial dysfunction, exacerbates the atherosclerotic process and contributes to the instability of the atherosclerotic plaque
Modifiable risk factors (emerging)		
Vascular(endothelial) dysfunction	Impaired vascular function increase risk	Impaired endothelial function may allow entry of monocytes and LDL particles to subendothelial space, and/or may reflect injury to endothelium.
Inflammation-related factors	High proinflammation factors increase risk	May reflect atherosclerosis as an inflammatory process
Coagulation-related factors	High procoagulation factors increase risk	Thrombus formation leads to myocardial infarction or stroke

Table adapted and modified from Frayn *et al*., 2019. *BP, blood pressure; CHD,* coronary heart disease; *CVDs, cardiovascular diseases*; *HDL-C, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; TC, total cholesterol.*

1.1.3 Pathogenesis of CVDs

Atherosclerosis, which has been proved as the primary origin of CVDs, begins in early life and progresses gradually throughout adulthood (McGill *et al*., 2000). This chronic inflammatory disease is first initiated by the adhesion of circulating leukocytes and platelets to a dysfunctional endothelium. The normal role of the endothelium is to maintain vascular homeostasis by protecting vessel wall integrity, regulating vascular tone, preventing cellular adhesion, controlling smooth muscle cell proliferation and

vessel wall inflammation (Deanfield *et al*., 2007; De Caterina, 2000). The most critical mediator involved in the endothelial homeostasis is nitric oxide (NO). This endogenous vasodilator molecule, produced by endothelial cells, can prevent unexpected cellular adhesion and inflammation (Tousoulis *et al*., 2012). However, endothelial dysfunction, resulting from the impairment of NO production and activity, attracts circulating monocytes, platelets and lipids to the damaged endothelium (De Caterina, 2000). This recruitment is facilitated by the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular-cell adhesion molecule-1 (ICAM-1). Other adhesion molecules, such as E-selectin and P-selectin, promoting the attachment of monocytes to the endothelial surface, are also upregulated (Falk, 2006). The recruited monocytes then infiltrate into the intima and mature into macrophages under the action of monocyte chemoattractant protein-1(MCP-1). Next, macrophages recognise and phagocytose oxidised low-density lipoprotein (LDL), and ultimately, elaborate foam cells (Libby *et al*., 2002). The early fatty streak, containing foam cells, aggregated platelets and a few T cells evolves into the advanced raised lesion, which consists of the replicated smooth muscle cells and apoptotic macrophages covered by a fibrous cap (McGill *et al*., 2000). As it matures, the atherosclerotic plaque develops a lipid-rich core. The size of the lipid-rich core, the degree of infiltration of inflammatory macrophages and the thickness of the fibrous cap influence the stability of the atherosclerotic plaque; in general, a plaque with a large lipid core, a large number of inflammatory macrophages and a thin fibrous cap is more unstable and likely to rupture. Plaque rupture can result in thrombosis, which can completely occlude the arterial lumen, leading to clinical events, such as unstable angina, myocardial infarction (MI), or a cerebrovascular event, such as stroke (Falk, 2006). The process of atherosclerosis development is shown in **Figure 1.1**.

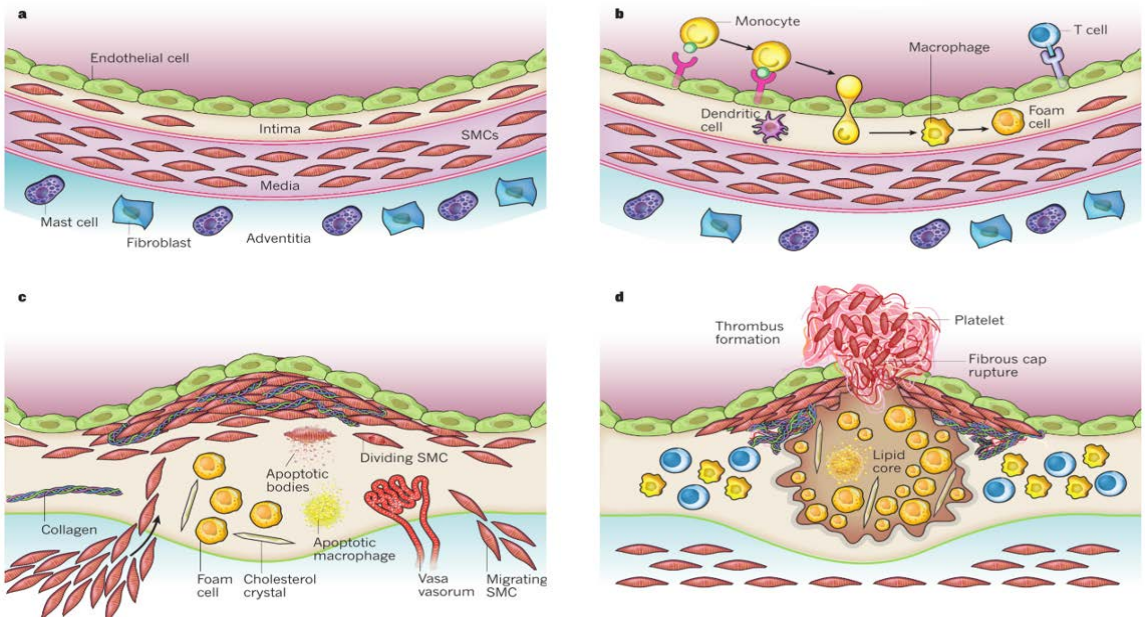


Figure 1.1. Process of the development of atherosclerosis. a. the normal artery wall; **b**. the initial steps of atherosclerosis include the adhesion of monocytes, platelets and lipids to the damaged endothelium, the entry of recruited monocytes into the intima, and the yield of foam cells; **c**. the formation of atherosclerotic plaque involves the accumulation of foam cell and smooth muscle cells, and the synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans; **d**. thrombus formation due to the rupture of plaque (Libby *et al.,* 2011).

1.1.4 Role of platelets, coagulation and thrombosis in development of CVDs

Under normal physiological conditions, activated platelets triggering the coagulation cascade play a pivotal role in arresting blood loss. However, in pathological conditions, such as atherosclerosis, these processes have been recognised as important contributors to the development of atherothrombosis (Kaplan & Jackson, 2011).

After the disruption of the endothelium, von Willebrand factor (vWF) is highly expressed on the surface of activated endothelial cells and then recruits platelets from flowing blood (Savage *et al.*, 1996). After adhering to the endothelium, platelets are activated and undergo a series of changes, including the activation of integrin $\alpha_{IIb}\beta_3$ and the externalisation of phosphatidylserine (PS) (Kaplan & Jackson, 2011). Activated $\alpha_{IIb}\beta_3$ can engage more vWF and fibrinogen, leading to the aggregation of platelet and the formation of a loose platelet plug (Jackson, 2007). Aggregated platelets then

regulate Factor XII activation, which is involved in the intrinsic pathway of the coagulation cascade (Müller *et al.*, 2009). Tissue factors (TF), secreted by macrophages and smooth muscle cells in plaques, simultaneously initiate the extrinsic pathway of coagulation by stabilising the active conformation of coagulation Factor VIIa (Persson & Olsen, 2010). Concomitantly, thrombin is produced both at the damaged vessel wall and on the surface of activated platelets. Thrombin can not only cleave fibrinogen to fibrin but also, in turn, activate platelets. Finally, platelet activation and aggregation culminate, which attract more platelets, red blood cells, leucocytes to the site of ruptured plaque to form more and larger thrombus (Badimón *et al.*, 2009). Considering the above pathophysiological mechanisms, many procoagulation related factors such as platelet function, thrombin generation and thrombus formation have been investigated as significant contributors to the development of atherosclerosis and potential markers to predict the risk of CVDs. Several studies have reported that platelet function tests were able to predict cardiovascular events (Frossard *et al*., 2004; Matetzky *et al*., 2004; Heeschen *et al*., 2003). Increased thrombin generation has also been found to be associated with a higher risk of CVDs (Carcaillon *et al*., 2011; Loeffen *et al*., 2015).

1.2 Extracellular vesicles (EVs)

1.2.1 Introduction to EVs

EVs are characteristically a heterogeneous group of submicron membrane-bound, particles that are released into extracellular space by cells and are widely presented in biological fluids. EVs were first described as 'platelet dust' by Wolf in 1967, in the study where membrane fragments with procoagulant activities were detected to be generated by activated platelets (Wolf, 1967). The fact that vesicles can arise from almost all cell types under both physiological and pathological conditions has been revealed over the past 50 years (Berckmans *et al*., 2001; György *et al*., 2011; Raposo & Stoorvogel, 2013). Moreover, EVs were initially regarded as cell debris without biological function; however, the stimulatory activity of EVs with respect to adaptive

immune responses was demonstrated by Raposo and co-workers in 1996 (Raposo *et al*., 1996) and since then, growing evidence indicates that EVs can act as a key mediator in cellular communications in many different scenarios by transferring biological cargo such as lipids, proteins, RNAs and specific receptors from parental cells to target recipient cells (Zaborowski *et al*., 2015; Paudel *et al*., 2016; Kalra *et al*., 2016). Therefore, EVs are now being considered as a potential novel biomarker in many diseases due to their physiological and pathological properties (Baron *et al*., 2012; Burger *et al*., 2013).

1.2.2 Classification of EVs

The nomenclature of EVs has, until recently, lacked standardisation, but this is now undergoing review, and the use of unspecific terms such as "microparticle" is declining. EVs are now classified into three main categories based on their size and current knowledge of biogenesis: exosomes, microvesicles and apoptotic bodies (Kalra *et al*., 2016). The common denominator of these three EV subtypes is that they are lipid bilayered particles, carrying bioactive cargo reflective of their cell of origin, which includes proteins, RNA, and/or cellular debris (Mathivanan *et al*., 2010). However, exosomes, ranging from 30nm to 150nm, are initially generated intracellularly within multivesicular bodies (MVBs) and are subsequently secreted into the extracellular space after the fusion of MVBs with the plasma membrane (Théry *et al*., 2002). In contrast to exosomes, microvesicles, whose diameters range from 100nm~1µm, are formed by the direct outward budding and fission of plasma membrane (Hugel *et al*., 2005). Apoptotic bodies (1µm~5µm) are shed as blebs of cells undergoing apoptosis (Elmore, 2007) (**Figure 1.2**). A significant challenge in the field is to establish methods to distinguish between three types of EVs, especially between exosomes and microvesicles, which overlap in size. In the interest of standardisation, researchers are encouraged to use the term "extracellular vesicles" and to specify the size range of particles when referring to exosomes, microvesicles and apoptotic bodies. In this thesis, the term "extracellular vesicles" will refer to both exosomes and microvesicles

unless otherwise stated; this is mainly because the current isolation and characterisation methods focus on the analysis of exosomes and microvesicles smaller than 1μm.

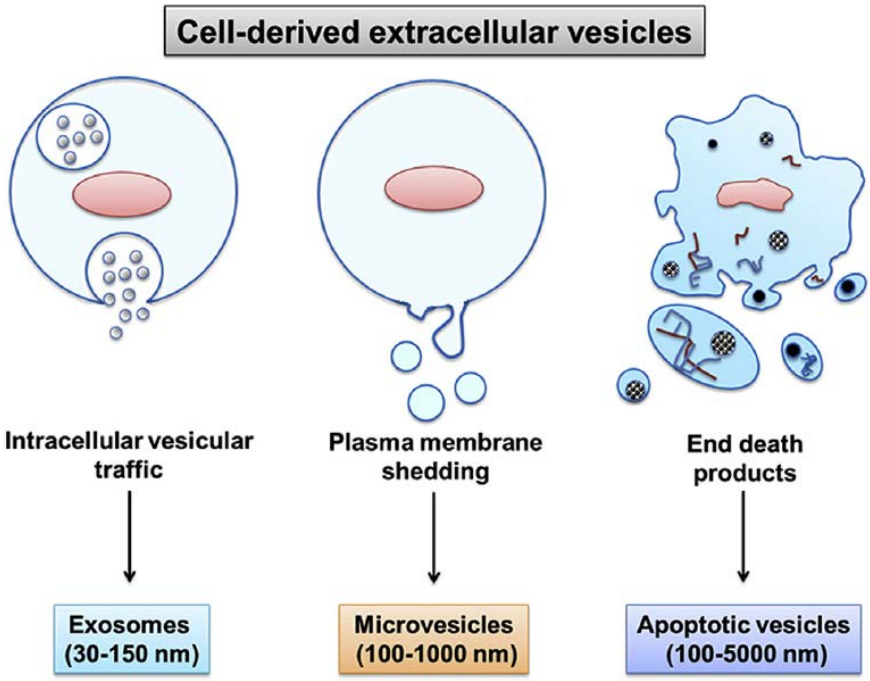


Figure 1.2. Classification of EVs based on their size and biogenesis. Exosomes (30nm~150nm) are produced intracellularly from multivesicular bodies and released by exocytosis. Microvesicles (100nm~1μm) are generated by the direct budding from plasma membrane. Apoptotic vesicles (1μm~5μm) are released during apoptotic cell death (Devhare & Ray, 2018).

1.2.3 Biogenesis of EVs

a. Exosomes

Exosomes were first discovered in 1983 when vesicles with 50~75nm in diameter transferring transferrin receptor for reticulocytes were reported (Pan & Johnstone, 1983). Johnstone *et al.* (1987) then applied the name "exosome" to refer to these secreted membrane vesicles. Exosomes are generated by the endolysosomal pathway, which involves the inward budding of MVBs membrane and the formation of intraluminal vesicles (ILVs) within MVBs (Kowal *et al*., 2014). Although the processes under the biogenesis of MVBs and ILVs are still incompletely understood, the Endosomal Sorting Complex Required for Transport (ESCRT) machinery composed of four separate protein ESCRTs 0-III, is generally accepted to be responsible for MVBs

formation, ILVs budding and protein cargo sorting (Babst, 2006; Henne *et al*., 2011). The ESCRT-0 complex recognises ubiquitinated proteins and receptors in the endosomal membrane and then buds with sequestered cargo with the cooperation of ESCRT-I and -II complexes. The assembly of ESCRT-III promotes the reverse budding of nascent ILVs within MVBs. Finally, closed mature ILVs containing packaged cargo are generated after the oligomerisation of ESCRT-III (Henne *et al*., 2011; Andreu & Yáñez-Mó, 2014; Zhang *et al*., 2019). However, some studies suggest that there may be other ESCRT-independent pathways for the formation of exosomes, which involve tetraspanins or heat shock proteins (HSP) since the silencing of some ESCRT genes had no influence on the formation of MVBs (Kowal *et al*., 2014; Andreu & Yáñez-Mó, 2014; Stuffers *et al*., 2009). This pathway is proposed to be based on the conversion of ceramide from sphingomyelin (SM) via sphingomyelinases in lipid rafts (Trajkovic *et al*., 2008; Stuffers *et al*., 2009). Once ILVs are generated, MVBs dock and fuse with the plasma membrane and then secrete exosomes into the extracellular environment (Babst, 2006; Henne *et al*., 2011; Zhang *et al*., 2019).

b. Microvesicles

Unlike exosomes, microvesicles are shed directly from the plasma membrane by blebbing during cell activation and/or apoptosis (Hugel *et al*., 2005). The process of microvesicle formation involves the reorganisation of membrane phospholipids, the remodelling of cytoskeleton and reverse budding. The plasma membrane has an asymmetrical phospholipid composition between the two leaflets of the membrane bilayer. In general, the outer leaflet is dominated by SM and phosphatidylcholine (PC), while PS and phosphatidylethanolamine (PE) are largely segregated in the inner one. This distribution is achieved by three major proteins: flippase, floppase and scramblase. Flippase, often referred to as aminophospholipid translocase, exclusively transports aminophospholipids (PS and PE) from the outer to the inner leaflet against their electrochemical gradient. Floppases allow the opposite translocations of both amino- and choline-phospholipids (SM and PC) and especially, are responsible for the transport of PS to the outer membrane. Scramblase facilitates random bidirectional

movements of PS (Burger *et al*., 2013; Morel *et al*., 2011). Under resting conditions, only flippase is active in maintaining an asymmetrical distribution, while floppase and scramblase are inactive. During cell activation, calcium is released from cytosolic stores, leading to an increase in intracellular calcium concentration. This process contributes to the activation of floppase and scramblase, as well as the inhibition of flippase and finally the externalisation of PS (Baron *et al.,* 2012). The outward expression of PS has been regarded as a fundamental process in the formation of microvesicles (Burger *et al*., 2013). In addition to PS exposure, the increase in intracellular calcium also activates proteases, such as calpain, which play an important role in the redistribution of cytoskeleton. After cytoskeletal reorganisation, plasma membrane will proceed to outward blebbing and finally form microvesicles (Morel *et al*., 2011). The mechanism for microvesicle formation is shown in **Figure 1.3**.

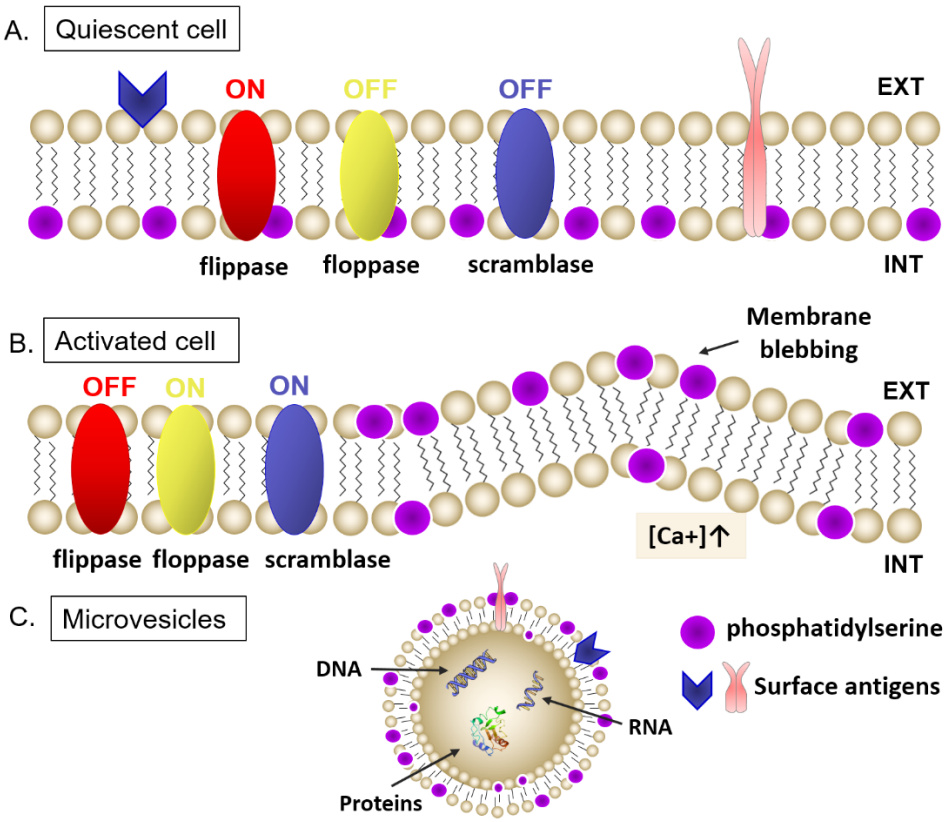


Figure 1.3. Mechanism of microvesicles formation. A. in quiescent cells, only flippase is active, allowing PS localised in the inner leaflet; **B**. Floppase and scramblase is activated due to the increased calcium concentrations, leading to the PS externalisation, cytoskeleton and membrane blebbing to generate microvesicles; **C**. microvesicles express PS and exhibit proteins and nucleic acids from the parent cells (modified based on Baron *et al.,* 2012).

c. Apoptotic bodies

Although apoptotic bodies are also formed by the outward blebbing of the apoptotic cell membrane, the underlying mechanisms are distinct. Apoptotic cells undergo chromatin contraction, nuclear rupture and mitochondrial distortion before cytoskeletal reorganisation (Elmore, 2007). Cleavage of the cytoskeleton is driven by caspases-3, -6 and -7 (Pop & Salvesen, 2009). Also, the exposure of PS is triggered by a Ca^{2+}-independent scramblase. After the expansion and retraction of the membrane, cellular debris is packed into the lumen of blebs and subsequently released as apoptotic bodies (Kalra *et al*., 2016). Although microvesicles can also be released by apoptotic cells during the early stages of apoptosis, apoptotic bodies are generated at the end of apoptosis (Distler *et al*., 2005b). The presence of PS on apoptotic bodies exhibits an 'eat me' signal, leading to the rapid elimination by phagocytes, while microvesicles will express signal like CD47 to prevent phagocytosis (Kalra *et al*., 2016).

1.2.4 Composition of EVs

The biochemical composition of EVs includes proteins, lipids and nucleic acids derived from the original cells.

a. Protein

The protein composition of EVs depends on the mode of biogenesis and cell type. For example, B lymphocyte-derived exosomes carry major histocompatibility complex class I and II (MHC I and II) (Pan & Johnstone, 1983; Kleijmeer *et al*., 2001) and tetraspanins CD63, CD81, and CD82 (Escola *et al*., 1998). HSP70 and 90, tumour-susceptibility gene 101 (TSG101) protein have been observed in the membranes of exosomes from dendritic cells (Théry *et al*., 1999). Moreover, proteins involved in MVB formation (e.g., tetraspanin CD9 and Alix) and release (e.g., GTPases and Annexins) are commonly identified in exosomes as well (Théry *et al*., 2002; Babst, 2006; Henne *et al*., 2011). In contrast to exosomes, microvesicles tend to be enriched in plasma-derived proteins including integrins, glycoprotein (GP) Ib and P-selectin, which is unsurprising,

given that microvesicles are derived directly from the plasma membrane of the parent cell (Palmisano *et al*., 2012). Apoptotic bodies carry a distinct set of proteins, including Annexin A6, HSP β6, histone-related (e.g., Histone H1 and H2A) and immune-related proteins (Lleo *et al*., 2014). Furthermore, the protein expression profile of EVs are also affected by the activation state or the stimulus causing EV release. Activated T lymphocytes-released microvesicles carried signalling proteins and proteins of the actin-myosin cytoskeleton, whereas HSP70 and TSG101 were highly expressed within microvesicles after apoptosis induction (Tucher *et al*., 2018). Therefore, EV subtypes can, to some degree, be classified according to the proteins they contain. However, many proteins, especially MHC II, tetraspanins CD9 and CD63, TSG101 and HSP70, are common to all EVs and regardless of the underlying release stimulus, and can consequently be regarded as general EV markers when analysing vesicles within a given size range (Tauro *et al*., 2012).

b. Lipids

EVs feature a lipid bilayer which comprises multiple membrane lipids, including PS, PC, PE and SM, but the proportions of these lipids in EVs are altered relative to the parent cells (Zaborowski *et al*., 2015). In general, exosomes exhibit an enrichment in PS and SM, with less PC and PE than plasma membranes (Laulagnier *et al*., 2004; Llorente *et al*., 2013). However, microvesicles exhibit greater similarity of lipids to plasma membranes than exosomes (Bicalho *et al*., 2013). A higher content of SM and disaturated lipids in exosomes and microvesicles contributes to a tighter packing of lipid bilayers and greater rigidity and stability, and therefore resistance to degradation and to carry bioactive cargo (Laulagnier *et al*., 2004; Zaborowski *et al*., 2015). Moreover, the enrichment of PS on EV membranes facilitates membrane division, merging, and internalisation by recipient cells (Chernomordik & Kozlov, 2003). SM and PS also influence the functions of EVs. SM is believed to exhibit a proangiogenic activity, which promotes endothelial cell migration and neovascularisation (Kim *et al*., 2002), while PS provides a surface for the binding of the clotting complex and therefore has a procoagulatory property (Owens & Mackman, 2011).

1.2.5 Physiological and pathological properties of EVs

The numbers, cellular origin, composition and functional properties of EVs depend on the individual status being studied (Yuana *et al*., 2013). In peripheral blood of healthy subjects, the approximate EV concentrations range from 10^7 to 10^{10} per ml blood varying according to different isolation and detection methods (Esposito *et al*., 2006; Gardiner *et al*., 2013; Yuana *et al*., 2015; Jamaly *et al*., 2018; Berckmans *et al*., 2019). The majority of EVs are derived from platelets and erythrocytes, which together account for over 80%~90%; the proportions of EVs released from endothelial cells and leukocytes range from 5% to 15% respectively, while monocytes and lymphocytes generally contribute to 1%~5% of circulating EVs (Berckmans *et al*., 2001; Kuo *et al*., 2017; Hromada *et al*., 2017; Berckmans *et al*., 2019). However, increased numbers, altered cellular origin and composition of EVs are observed in several disease conditions such as sepsis, CVDs and cancer (Nieuwland *et al*., 2000; Simak *et al*., 2006; Sinning *et al*., 2011; Tseng *et al*., 2013). Moreover, EVs are believed to mediate intercellular communication by transferring membrane and/or cytosolic components between cells, or by interacting with receptors on the surface of cells, and thus are critically involved in multiple physiological and pathological activities (Ridger *et al*., 2017). EVs have been found to not only regulate some normal physiological processes, such as haemostasis and tissue regeneration (Owens & Mackman, 2011; Gatti *et al*., 2011; Willms *et al*., 2018), but also contribute to the pathogenesis of various diseases, including atherosclerosis and CVDs (VanWijk *et al*., 2003; Owens & Mackman, 2011; Baron *et al*., 2012; Zarà *et al*., 2019).

a. Roles of EVs in coagulation and thrombosis

During the process of formation, EVs present PS on their outer membranes, which facilitates the assembly of coagulation complexes. Externalised PS provides a surface for the binding of many components involved in the clotting cascade, such as Factor VII, Factor IX, Factor X and prothrombin, leading to procoagulatory properties of EVs (Owens & Mackman, 2011). In fact, platelet-derived EVs (PDEVs) are suggested to

present 50~100-fold higher procoagulant ability than activated platelets (Sinauridze *et al*., 2007). TF, as another key player in coagulation and thrombosis, is also suggested to be expressed on the surface of EVs from monocytes, endothelial cells and platelets (Owens & Mackman, 2011). TF binding of Factor VIIa activates Factor X and then initiates the extrinsic pathway of coagulation cascade (Persson & Olsen, 2010). The procoagulatory activity of TF positive (TF+) EVs isolated from atherosclerotic plaques has been reported by Mallat and colleagues (1999). P-selectin expressed on PDEVs upregulates the expression of TF on monocytes and subsequent accumulation of TF+ monocytes into a developing thrombus (Falati *et al*., 2003). Clinical studies also support procoagulatory properties of EVs *in vivo*. Patients presenting with various forms of hypercoagulation, such as in acute coronary syndrome (Mallat *et al*., 2000), lupus anticoagulant (Combes *et al*., 1999) and sepsis (Nieuwland *et al*., 2000), have increased numbers of EVs. Elevated TF activity associated with EVs has been specifically suggested in acute coronary syndrome patients as well (Huisse *et al*., 2009).

b. Roles of EVs in CVDs

The levels of circulating EVs are not only increased in patients with cardiovascular events such as stable CAD (Jansen *et al*., 2014; Sinning *et al*., 2011), acute ischemic stroke (Simak *et al*., 2006; Pawelczyk *et al*., 2017) but also are significantly associated with cardiovascular risk factors such as increasing age (Berezin *et al*., 2016), smoking (Gordon *et al*., 2011), obesity (Koga *et al*., 2006), hypertension (Preston *et al*., 2003) or hyperlipidaemia (Ferreira *et al*., 2004; Pawelczyk *et al*., 2017). Furthermore, proteomic analysis of EVs in plasma from obese patients suggested an increased content of fibrinogen and a reduced content of adiponectin (Barrachina *et al*., 2019). The level of exosomal microRNA-223 was also positively associated with acute ischemic stroke occurrence and stroke severity (Chen *et al*., 2017). Therefore, both the numbers and compositions of EVs may be regarded as novel biomarkers for CVDs, which may further extend to predictive and diagnostic potential (Jansen *et al*., 2017). Potential mechanisms underlying the contribution of EVs to the development of atherosclerosis and CVDs are summarised in **Figure 1.4**. In addition to the

procoagulatory and prothrombotic properties mentioned above, circulating EVs have been reported to reduce the generation and bioavailability of NO, leading to the impairment of endothelial-dependent vasodilation (Densmore *et al*., 2006; Liu *et al*., 2013). PDEVs and endothelial-derived EVs (EDEVs) induce the expression of intercellular ICAM-1, VCAM-1 and E-selectin on the endothelium, which promote inflammatory processes involved in CVDs (Gidlöf *et al*., 2013; Hijmans *et al*., 2018). EVs also confer proinflammatory activity by inducing the secretion of inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumour necrosis factor α (TNF-α) (Distler *et al*., 2005a; Bretz *et al*., 2013). These properties exhibited by EVs contribute to the potential role of EVs in the aetiology of CVDs.

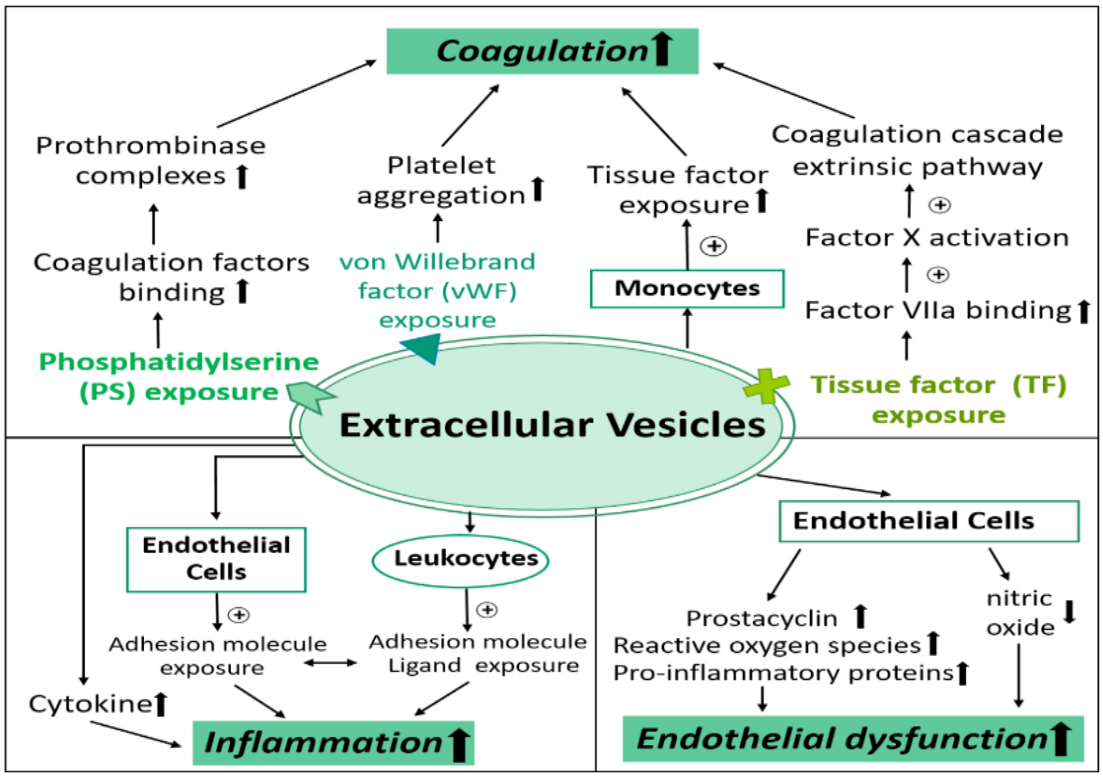


Figure 1.4. Potential mechanisms of EVs involved in the development of CVDs. Top: the effect of EVs on coagulation promotion; **left bottom**: the effect of EVs on inflammation response; **right bottom**: the effect of EVs on endothelial dysfunction (modified based on VanWijk *et al.,* 2003).

1.2.6 Methods to study EVs

a. Methods to isolate EVs

There are several techniques currently available for the isolation and purification of EVs based on either physical or molecular properties of EVs including ultracentrifugation (UC) (sometimes combined with density gradient centrifugation (DGC)), size exclusion chromatography (SEC), ultrafiltration, field-flow fractionation, organic solvent precipitation and immunoaffinity isolation (Taylor & Shah, 2015; Willms *et al*., 2018). However, there is still a lack of consensus and standardisation of EV isolation protocols as every method has limitations, including the aggregation and destruction of EVs, co-isolation of some non-vesicular contaminants or bias towards subsets of EVs (Monguió-Tortajada *et al*., 2019). The common isolation methods for EVs are illustrated in **Figure 1.5**.

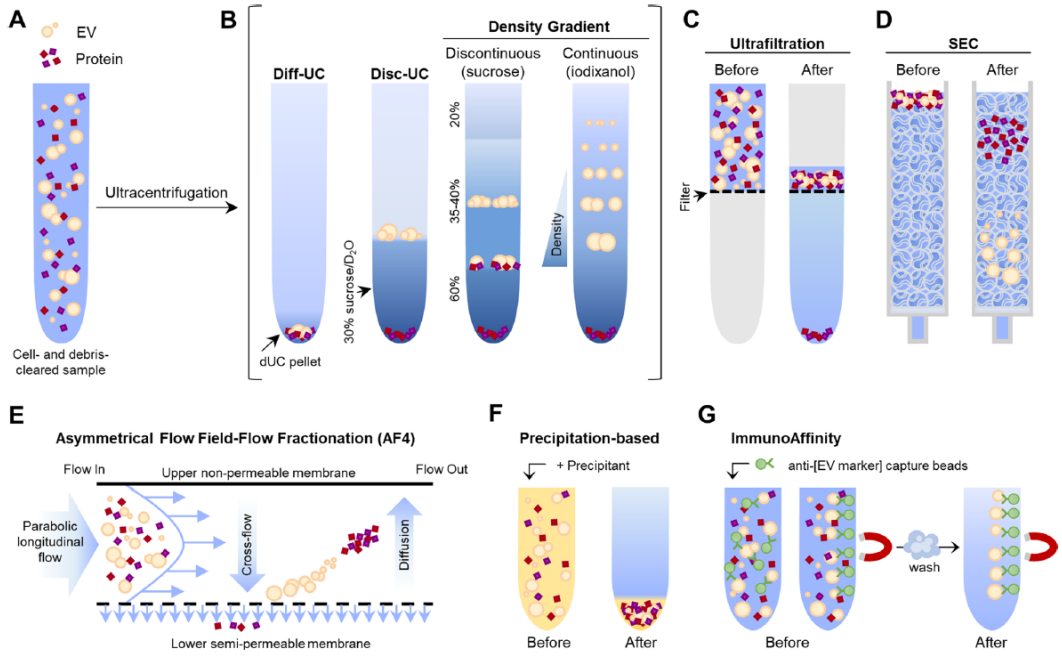


Figure 1.5. Common methods for the isolation of EVs. A. Starting sample as a cell- and debris-cleared biofluid containing EVs and proteins; **B**. UC combined with DGC; **C**. ultrafiltration; **D**. SEC; **E**. asymmetrical flow field-flow fractionation; **F**. precipitation; **G**. immunoaffinity. *DGC, density gradient centrifugation; EVs, extracellular vesicles; SEC, size exclusion chromatography; UC, ultracentrifugation* (Monguió-Tortajada *et al*., 2019).

UC is the most widely used method to isolate EVs from body fluids such as plasma, urine and saliva, as well as cell culture media. Although there are variations in the protocols employed by different research centres, it generally involves multiple centrifugation steps based on the methods published by Raposo *et al*. (1996). In some studies, the sequential centrifugations are replaced by a single filtration step with a 0.22μm filter (Théry *et al*., 2006). However, the methods developed by Raposo *et al.* (1996) focused on the isolation of exosomes from conditioned culture media, so the protocol has since been adapted to extract other subpopulations of microvesicles and apoptotic bodies (Théry *et al*., 2006; Szatanek *et al*., 2015). A particular challenge for isolation of EVs from body fluids is the high level of contamination with soluble proteins, protein aggregates, lipoproteins and microbes (Tauro *et al*., 2012). The pellet can be washed in a large volume of phosphate-buffered saline (PBS), followed by the same ultracentrifugation again to remove these contaminants (Théry *et al*., 2006). However, an alternative approach to address this issue is to combine with a DGC, which separates vesicles based on their buoyant density (Tauro *et al*., 2012). There are two main types of density medium: sucrose and iodixanol (Optiprep), both of which should allow separation of EVs from the majority of lipoproteins on the basis of density (Keller *et al*., 2011; Tauro *et al*., 2012). One of the most obvious drawbacks of UC is that it is relatively laborious, time-consuming and requiring a specialised ultracentrifuge and training (Monguió-Tortajada *et al*., 2019). Also, repeated centrifugation at such high speeds has been reported to cause co-isolation of vesicles with contaminants such as soluble proteins present in body fluids, or even aggregation and rupture of vesicles, leading to a relatively low yield of EVs and affecting the downstream analysis (Lamparski *et al*., 2002; Ismail *et al*., 2013; Yuana *et al*., 2015). The quantity and quality of EVs are also easily affected by multiple parameters such as the *g* force, the rotor type, the k-factor of rotor and solution viscosity, leading to enormous variability in the recovery rate of EVs (2%~80%) among studies (Taylor & Shah, 2015).

SEC enables the separation between EVs and contaminants based on their different sizes. SEC is performed to obtain sequential EV fractions from a single column

containing porous beads. The smaller particles in the solution will enter the pores in the beads and thus move more slowly, while the larger ones move between the beads, and therefore travel through the column more quickly. EVs, therefore elute before most contaminants, such as soluble proteins and high-density lipoprotein (HDL). The removal of 99% of soluble proteins and > 95% of HDL has been demonstrated in the EV-rich fractions separated by SEC (Baranyai *et al*., 2015). However, some very large proteins, such as vWF and large lipoproteins such as very-low-density lipoproteins (VLDL: 30~60 nm) and chylomicrons (CM: 100~1200 nm), may still co-elute with EVs (chiefly CM) because of their overlapping size. Contamination with CM can be avoided by collecting blood from overnight fasted participants (Sódar *et al*., 2016). Another potential method of eliminating lipoproteins is to combine SEC with other purification methods, such as bind-elute chromatography (Corso *et al*., 2017) or flotation in a density cushion (Karimi *et al*., 2018). An advantage of SEC compared to UC is the much reduced negative effect on the integrity and function of EVs (Baranyai *et al*., 2015). The recovery of EVs from SEC is rapid (~30 min) and appears to be more reproducible, reportedly ranging from 40%~90% (Coumans *et al*., 2017). Moreover, SEC is flexible, since the size cut-off can be adjusted by choosing variable gels with different pore sizes (Monguió-Tortajada *et al*., 2019). Column length and diameter can also be modified in order to increase resolution and sample volumes if required (Taylor & Shah, 2015).

Other approaches for isolation of EVs include field-flow fractionation, precipitation and immunoaffinity capture. Field-flow fractionation is performed based on a channel with parabolic longitudinal flow combined with an external gradient or 'field' (Monguió-Tortajada *et al*., 2019). The separation of vesicles can depend on their distinct hydrodynamic diameters in the field generated by cross/tangential flow through one or two semipermeable membranes or their distinct electrophoretic mobility in the field generated by electrical field-flow fractionation (Kang *et al*., 2008; Zhang *et al*., 2018; Petersen *et al*., 2018; Monguió-Tortajada *et al*., 2019). Precipitation as a user-friendly and relatively cheap method has also been applied to EV isolation. This commonly relies on polyethylene glycol-based volume exclusion precipitation and

often employs kits such as Exo-Spin and Invitrogen (Lane *et al*., 2015; Monguió-Tortajada *et al*., 2019). Although the good recovery of EVs has been reported after precipitation, the purity may be compromised due to the co-precipitation of EVs and proteins. Furthermore, the precipitant may not be suitable for EV characterisation and functional analysis (Osteikoetxea *et al*., 2016). Immunoaffinity capture is based on combinations of antibody-coated magnetic or latex beads with specific ligands on the surface of EVs. This method reportedly achieves a relatively high purity of EV subpopulations (Coumans *et al*., 2017). However, there is bias resulting from the choice of affinity beads as only EVs exposing the corresponding receptors can be separated (Osteikoetxea *et al*., 2016). All of the existing isolation methods have their own merits and limitations, and therefore the choice of methods should be guided by the required EV recovery, purity and by the intended downstream applications based on the Minimal Information for Studies of Extracellular Vesicles Guidelines (MISEV) proposed by the International Society for Extracellular Vesicles (ISEV) in 2014 (Lötvall *et al*., 2014).

b. Methods to detect and characterise EVs

One of the most commonly employed techniques for EV enumeration is flow cytometry (FCM). On scatter mode, the light is scattered either along the path of the laser, which is referred to as forward scatter (FSC) or at a ninety-degree angle relative to the laser, which is called side scatter (SSC). In general, FSC relates to particle size, whereas the SSC relates to the inner complexity (granularity) of the particles. On fluorescence mode, fluorescently labelling specific EV components enables the identification and characterisation of EVs (van der Pol *et al*., 2010). The advantages of FCM are a high counting speed (at a rate of thousands per second) and wide availability in most laboratories. However, the relatively high detection threshold (around 200~500nm) of conventional FCM leads to the limited application for smaller EVs; exosomes cannot be detected by FCM at all (Orozco & Lewis, 2010). For some time, polystyrene beads have been employed to calibrate FCM and to set gates for EVs based on particle diameter. However, it has recently been discovered that the refractive index

of EVs is lower than that of beads, resulting in a > 10-fold lower degree of light scattering by EVs than similar-sized polystyrene beads (van der Pol *et al*., 2014, 2016). This is corroborated by the fact that 400nm beads were only able to resolve EVs of around 1000nm a few years earlier (Chandler *et al*., 2011). Another major complication associated with FCM is 'swarm detection'. Compared to cells, single EVs are small but present at high concentrations, which easily cause the simultaneous detection of multiple EVs in the laser beam. The signal of single EVs may be too weak to generate a detectable signal, while multiple EVs can be detected more easily. This phenomenon can be reduced or eliminated by evaluating serial sample dilutions (van der Pol *et al*., 2016; Coumans *et al*., 2017). Also, conventional FCM uses scatter as a thresholding parameter, in which the light intensities of background noise are often higher than those of EVs. One of the protocols to increase the sensitivity of FCM is to trigger detection on a fluorescence parameter, which can reduce background noise and prevent false scatter signals caused by non-EVs (Coumans *et al*., 2017; Arraud *et al*., 2016). There are still very significant challenges associated with the characterisation of EVs by FCM since there is no generic ligand which can label all types of EVs (van der Pol *et al*., 2016). One of the commonly used EV markers is PS, which is externalised in the outer leaflet of the cell membrane during cell activation, resulting in membrane blebbing and EV shedding. (Bevers *et al*., 1983; Zwaal & Schroit, 1997; Boersma *et al*., 2005; Morel *et al*., 2011). Accordingly, Annexin V has been adopted as a general EV marker based on its availability to binding to PS in a calcium-dependent manner (Boersma *et al*., 2005). However, studies have recently demonstrated that not all circulating EVs are Annexin V positive, which may be due to its insufficient binding of Annexin V to PS or the presence of EVs which do not appear to express PS (Connor *et al.*, 2010; Ayers *et al*., 2011; Arraud *et al.*, 2014). Therefore, other markers, such as lactadherin, with a higher affinity for PS than Annexin V have been used as a substitute for Annexin V (Shi *et al*., 2004; Dasgupta *et al*., 2006). Also, PE can be expressed at levels several-fold higher than PS in the membrane depending on cell type and agonist, so some groups have been exploring it as another possible general marker for EV detection (Fadeel & Xue, 2009; Clark *et al*., 2013; Larson *et al*., 2012). Regarding

phenotyping of circulating EVs, most studies used single- or double-antibody labelling and the most widespread antibodies used for common EV subpopulations are summarised in **Table 1.2** (Burnier *et al*., 2009; Yuana *et al*., 2013; França *et al*., 2015).

Table 1.2 Common antibodies used to label EV subpopulations on fluorescence FCM

	Common antibodies
Platelet-derived EVs (PDEVs)	CD31, CD41, CD42, CD61, CD62P, CD63
Endothelial-derived EVs (EDEVs)	CD31, CD51, CD54, CD62E,CD105, CD106, CD144, CD146
Erythrocytes-derived EVs (ErDEVs)	CD235a
Leukocytes-derived EVs (LDEVs)	CD45
Monocytes-derived EVs (MDEVs)	CD14, CD54
Lymphocytes-derived EVs (LyDEVs)	CD3, CD4,CD8,CD20

Table adapted from Burnier *et al*., 2009; Yuana *et al*., 2013; França *et al*., 2015

Nanoparticle Tracking Analysis (NTA) is a relatively novel technique to measure EV concentration and size distribution. This technique, consisting of a laser light scattering microscope and a camera, allows particles in the fluid to be illuminated by the laser beam and to scatter light visualised by microscope. The movement of particles under Brownian motion is tracked by the video and their speed of motion at the known temperature, pressure and viscosity is determined, which can be finally related to particle size by software based on the Stokes-Einstein Equation (van der Pol *et al*., 2010; Filipe *et al*., 2010). NTA outperforms FCM in small EV measurement due to the lower detection threshold ranging from about 30~100nm, although NTA is limited in its ability to phenotype EVs and discriminate EVs from other contaminants. Fluorescence NTA has been developed to overcome this, but only one fluorescent antibody can be applied per sample and it is extremely difficult to acquire an efficient labelling signal for detection due to the photobleaching of the probe, either before EVs appear in the field-of-view of the microscope or before EVs can be recorded for a sufficient length of time for accurate size calculation due to the relatively longer analysis time compared to FCM (van der Pol *et al*., 2016). A synchronisation cable or a foil-covered syringe can,

to some degree, protect the dye from the illumination source and decrease bleaching (Gardiner *et al*., 2013). Another promising alternative is to employ quantum dot-conjugated antibodies for fluorescent NTA, which exhibit superior stability and brightness to traditional fluorophores (Dragovic *et al*., 2011). However, the high background caused by these unbound quantum dot-conjugated antibodies may affect the final measurement. Also, the availability and applications are currently very limited, as this methodology is just emerging (Dragovic *et al*., 2015). Another factor which needs to be considered is the proper dilution of samples so that the NTA camera can capture all the vesicles present in a sample without overlaying the effect of larger vesicles masking smaller ones (Szatanek *et al*., 2017). The recommended concentration range of vesicles is $1\sim10*10^8$ vesicles/ml (Gardiner *et al*., 2013; Vestad *et al*., 2017; Dragovic *et al*., 2011).

Other common techniques used for the characterisation of EVs include electron microscopy (EM), dynamic light scattering and tunable resistive pulse sensing. EM, including scanning EM, transmission EM and cryogenic EM, applies an electron beam to obtain high-resolution images of nanoscale objects and is used to visualise the morphology and size of EVs (Hartjes *et al*., 2019). EM combined with immune-gold labelling can also identify the subtypes of EVs and reveal the presence of aggregates and other contaminants (Arraud *et al*., 2014; Linares *et al*., 2015). However, one of the main challenges of using EM on biological objects is sample preparation, in which fixation and drying of samples are usually required for the imaging in vacuum, and such steps may complicate the translation of observed structures to the native morphology of vesicles (Hartjes *et al*., 2019). Therefore, cryogenic electron microscopy, which is based on the formation of ultra-thin vitrified film by flash-freezing thin liquid film in EV suspension rather than chemical fixation, has been developed as a reliable method to characterise EVs as it allows EVs to be imaged in their native state (Issman *et al*., 2013; Cizmar & Yuana, 2017). Tunable resistive pulse sensing is performed to measure individual particles by determining their electrical current changes when each particle passes through a nanopore (Hartjes *et al*., 2019). Tunable resistive pulse

sensing has been used to determine the size and concentration of EVs after the calibration with known standards nanoparticles of well-defined size (Maas *et al*., 2017) and to detect surface charge (zeta potential) of EVs by measuring the time each vesicle spends within the nanopore (Vogel *et al*., 2017). Dynamic light scattering is a technique that measures the size distribution of vesicles by detecting the collective mobility of scattering vesicles based on the laser beam. Dynamic light scattering allows the detection of vesicles ranging from 1nm to 6μm simply and quickly (several minutes), which can provide a more complete picture of all EV populations, including apoptotic bodies compared to NTA or FCM (Szatanek *et al*., 2017; Hartjes *et al*., 2019). However, this method is more accurate when detecting monodisperse samples, and therefore the analysis of EVs in some minimally-processed biofluids with particles varying in size may become problematic (Hoo *et al*., 2008; Palmieri *et al*., 2014).

Although various techniques have been rapidly developed for the enumeration and characterisation of EVs, challenges associated with every technique remain and further efforts in standardising these protocols are still needed. Also, it is clear that a combination of detection techniques is important for the reliable analysis of EVs based on MISEV (Lötvall *et al*., 2014).

c. Methods to measure the composition and procoagulatory activity of EVs

Standard colourimetric protein assays, such as the Bradford protein assay, can simply measure the total protein content of EVs, although its accuracy may be compromised by protein contaminants present in samples (Hartjes *et al*., 2019). The presence of specific proteins associated with EVs is commonly detected by immunoblotting, where purified EVs are first lysed to release their proteins and then either directly spotted on a membrane (in a dot blot assay), or separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (in a Western blot assay) before final detection using labelled antibodies targeting the proteins of interest (Hartjes *et al*., 2019). The potential markers for EVs include tetraspanins CD9 and CD63, Alix, TSG101 and HSP70 (Théry *et al*., 2002; Witwer *et al*., 2013). However, immunoblotting is of

limited use for quantifying EVs and is unable to provide information on individual EVs. Also, it typically needs a large sample volume to purify and concentrate EV samples (Witwer *et al*., 2013; Hartjes *et al*., 2019). There are several techniques available for evaluating the lipid composition of EVs isolated from tissue culture or biological fluids, including mass spectrometry, thin-layer chromatography and gas chromatography (GC) (Wubbolts *et al*., 2003; Kreimer *et al*., 2015; Skotland *et al*., 2017; Chen *et al*., 2019). Mass spectrometry, converting samples into gaseous ions and then separating and characterising lipid classes based on their specific mass-to-charge ratio and relative abundance, has become one of the best methods for lipidomics due to its high throughput, high sensitivity and high accuracy (Li *et al*., 2014; Cajka & Fiehn, 2016). Lipid separation by thin-layer chromatography is achieved on the different ascending rates of lipid classes on stationary phase (normally silica gel) due to their polarity differences. Thin-layer chromatography is quite inexpensive, simple and fast, but the potential oxidation of lipid (mainly unsaturated fatty acids) caused by the exposure to atmospheric oxygen raise concerns against its wider application (Fuchs *et al*., 2011). GC, which identify and quantify fatty acid composition between a liquid stationary phase and a gas mobile phase, is another highly established technique for lipid analysis of EVs, although preparative derivatisation of lipid classes is required because only volatile compounds can be analysed. (Fisk *et al*., 2014; Connolly *et al*., 2015).

Procoagulatory features of EVs can be assessed using several assays. One of the commonly used approaches is to detect the time and rate of clotting in plasma containing EVs compared to EV-free plasma (Aleman *et al*., 2011; Marchetti *et al*., 2014; Shustova *et al*., 2017). Another approach is to apply a thrombin generation test, which determines whether the lag phase for thrombin generation, the time to reach peak thrombin generation, the peak height and the total amount of thrombin formed are altered by the presence of EVs (Aleman *et al*., 2011; Van Der Meijden *et al*., 2012; Tripisciano *et al*., 2017). Effects of EVs on other coagulation related parameters, such as prothrombin time and activated partial thromboplastin time, the rate and amount of thrombus formation, fibrin network structure and resistance to fibrinolysis, can also

be analysed (Biró *et al*., 2003; Aleman *et al*., 2011; Durrieu *et al*., 2018; Nielsen *et al*., 2018). The presence of PS and TF on EVs measured immunologically using related antibodies can also reflect their coagulation ability. The activity of TF can be measured by either directly evaluating its ability to activate factor X or checking the effect on the coagulation parameters with the blocking anti-TF antibodies (Khaspekova *et al*., 2016; Shustova *et al*., 2017; Aleman *et al*., 2011; Tripisciano *et al*., 2017).

1.3 Fish oil and n-3 polyunsaturated fatty acids (n-3 PUFA)

1.3.1 Introduction to n-3 PUFA

Dietary fats, represented by more than 98% of triacylglycerol (TAG), consist of three fatty acids esterified to a glycerol backbone. Fatty acids can be classified into three groups based on whether there are any double bonds between adjacent carbon atoms; n-3 PUFA has their first double bond at the third carbon atom from the methyl end. There are three key n-3 PUFA in human physiology: α-linolenic acid (ALA, C18:3 n-3), as the simplest n-3 PUFA, can be obtained from plants such as flaxseed, rapeseed and soybean. Eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) exhibiting longer carbon chains are found in abundant amounts in oily fish and fish oil (Barry, 2009). ALA is an essential fatty acid, which cannot be produced by humans and thus requires consumption in the diet, and although DHA and EPA can be synthesised from ALA as a result of the actions of desaturation and elongation enzymes, this process is limited and inhibited by the metabolism of n-6 PUFA linoleic acid (C18:2 n-6) to arachidonic acid (AA, C20:4 n-6), since they share the same enzymes (Harris *et al*., 2008). Fatty acids play important roles in energy provision, storage, membrane integrity and fluidity and cellular signalling in physiological and pathological processes (Barry, 2009).

1.3.2 Recommendations for n-3 PUFA intake

The Scientific Advisory Committee on Nutrition Advice on Fish Consumption has suggested a desirable intake for fish is two portions fish per week, at least one of which is oily (one portion is approximately 140g), equating to 0.45g n-3 PUFA per day (Scientific Advisory Committee on Nutrition [SACN], 2004). According to the United Kingdom National Diet and Nutrition Survey from 2012 to 2014, the mean intake of oily fish in all age groups was well below the recommendation. The mean consumption was only 54~87g per week in adults and 13~29g per week in children (National Diet and Nutrition Survey [NDNS]: results from Years 5 and 6, 2016), and only 25.2% of adults in the UK are oily fish consumers (Derbyshire, 2019). Similarly, the estimated EPA and DHA consumption in the United States is only 0.05g/d and 0.08g/d, respectively (Harika *et al*., 2013).

1.3.3 Effects of n-3 PUFA on CVD risk

a. Overview of n-3 PUFA and CVDs

Since the pioneering studies suggesting that Greenland Eskimos exhibit reduced coronary mortality due to a high consumption of oily fish (Hans & Jørn, 1980), the relationship between n-3 PUFA and CVDs has been extensively explored during the past three decades. The benefit of n-3 PUFA in the secondary prevention of CVDs has been suggested by a number of epidemiological and randomised clinical trials (RCTs). For instance, the Nurses Health Study of 84,688 nurses without CVDs at baseline revealed a decrease of up to 45% of CHD mortality due to a higher intake of fish oil or n-3 PUFA after a 16-year follow up (Oh *et al*., 2005). Another large prospective cohort study, including 41,578 Japanese subjects initially free of CVDs demonstrated an inverse association between n-3 PUFA consumption and the risk of MI and nonfatal coronary events (Iso *et al*., 2006). For RCTs, the Diet and Reinfarction Trial, randomising 2033 MI patients to intake 200~400g/week fish, indicated a 29% decline of total mortality in the intervention group (Burr *et al*., 1989). A similar reduction in mortality was reported in the GISSI-HF trial, in which patients with chronic heart failure were

provided with n-3 PUFA 1g daily and were followed up for 3.9 years (Tavazzi *et al*., 2008). However, secondary prevention studies conducted most recently (in the last decade) have not been supportive of earlier data (Rizos *et al*., 2012; Balk *et al*., 2016; Walz *et al*., 2016). A meta-analysis by Rizos *et al*. (2012) analysed 20 RCTs published between 1989 and 2012 with a total of 68,680 cardiovascular patients and reported that there was no significant impact of n-3 PUFA supplementation on the incidence of stroke, MI or sudden death. More recently, a systematic review of 37 observational studies and 61 RCTs concluded that the evidence for the association between n-3 PUFA and CVD outcomes and/or risk factors was insufficient. Only two out of 17 RCTs based on CVD patients reported significantly reduced risk of all-cause death following fish oil supplementation, and no significant effect of fish oil on sudden cardiac death was reported by nine RCTs (Balk *et al*., 2016). The lack of effect in recent trials can be at least partly explained by the widespread use of statins and the significant improvement in the medical management of CVDs (Bilato, 2013). Although there are some controversies about the effect of n-3 PUFA on CVD patients, the information about healthy subjects (i.e. primary prevention) or those at moderate risk of CVDs (i.e. with cardiovascular risk factors) is still limited. N-3 PUFA has been well-characterised about the beneficial effects on blood TAG, but there are still many questions regarding other risk factors, particularly those associated with endothelial dysfunction, inflammation and coagulation. Moreover, mechanistic data explaining the effects of n-3 PUFA on these parameters are lacking and require further exploration.

b. Effects of n-3 PUFA on platelet aggregation, coagulation and thrombosis

Krishnan and associates (2007) reported that platelet aggregation after adenosine diphosphate (ADP) and collagen stimulation, as well as fibrinogen levels in diabetic patients significantly declined after they received 1.2g/d of n-3 PUFA for six weeks compared to a placebo group. A significant reduction in fibrinogen and factor V levels, as well as thrombin generation in plasma after taking 3g/d of n-3 PUFA for four weeks, has been reported in healthy subjects who were borderline overweight (Vanschoonbeek *et al*., 2004). Also, a meta-analysis of RCTs summarised by Gao *et al*.

(2013) suggested a significant inverse association between n-3 PUFA supplementation and platelet aggregation among subjects with poor health status. Some *in vivo* studies also indicated that n-3 PUFA inhibit platelet aggregation and coagulation in a rat model (Yamada *et al*., 1997; Leray *et al*., 2001). However, there is some inconsistency in the recent literature. One study suggested that platelet aggregation in response to AA was not significantly altered after the intake of fish oil in patients undergoing antiplatelet therapy (Gajos *et al*., 2010). Furthermore, there was no difference in platelet aggregation, thrombin generation and fibrin clot properties after taking n-3 PUFA at a dose of 2g per day for three months in patients with atherosclerosis and type 2 diabetes (T2D) (Poreba *et al*., 2017). Similar results were reported by Bagge *et al*. (2018), in which daily consumption of 2520mg of n-3 PUFA for ten days did not affect platelet aggregation or coagulation in healthy subjects. Again, the discrepancy of those studies is believed to be partly due to the antithrombotic actions of statins, which might overwhelm the effects of n-3 PUFAs on coagulation and thrombosis (Macchia *et al*., 2012; Undas *et al*., 2014; Poreba *et al*., 2017).

One of the mechanisms underlying the proposed antiplatelet and antithrombotic effects of n-3 PUFA is attributed to the bioactive lipid mediators of n-3 PUFA produced by fatty acid oxygenases such as cyclooxygenases (COXs) and lipoxygenases (LOXs) (Calder, 2012; Isobe & Arita, 2014). Under the COX-2 pathway, AA acts as a substrate to produce thromboxane A2 (TXA2), which can potently promote platelet aggregation. One of the characteristic effects of n-3 PUFA supplementation is to incorporate into platelet membrane phospholipids, to subsequently replace AA and to compete with the metabolism of AA via COX-1, leading to the inhibition of TXA2 synthesis (von Schacky *et al*., 1985; Kim *et al*., 1995). Since inflammation can activate the coagulation system (Levi *et al*., 2003; Esmon, 2005), novel lipid mediators of n-3 PUFA with potent antiinflammatory properties could represent another potential mechanism for its anticoagulatory properties. They include the E-series resolvins derived from EPA, and the D-series resolvins, protectins and maresin derived from DHA, (Kohli & Levy, 2009; Calder *et al*., 2012; Isobe & Arita, 2014). Resolvin D1 and E1, as well as protectin D1,

have been reported to reduce transendothelial migration of neutrophils and to prevent the penetration of neutrophils during the inflammation (Sun *et al*., 2007; Campbell *et al*., 2007; Schwab *et al*., 2007). Some proinflammatory cytokines, such as TNF-α and IL-1β, are also inhibited by resolvin D1 and/or protectin D1 (Serhan *et al*., 2002; Ariel *et al*., 2005). Finally, the increased production of NO from endothelial cells promoted by n-3 PUFA could also reduce platelet aggregation (Abeywardena & Head, 2001).

c. Effects of n-3 PUFA on EVs

To our knowledge, there are ten human studies examining the effects of n-3 PUFA on numbers and/or functions of EVs. A summary of these studies is presented in **Table 1.3**. Nomura and co-works conducted four trials, of which two provided hyperlipidaemic patients (diabetic and non-diabetic) with EPA at a dose of 1.8g/d for four weeks (Nomura *et al*., 2003) or six months(Nomura *et al*., 2009a). The other two explored the effect of either EPA (1.8g/d) or pitavastatin (2mg/d) or a combination on numbers of PDEVs in hyperlipidaemic patients with T2D for six months (Nomura *et al*., 2009b) or the effect of pitavastatin (2mg/d) with either EPA (1.8g/d) or sarpogrelate (300mg/d) in patients with T2D for 12 months (Nomura *et al*., 2018). They demonstrated significant decreases in numbers of PDEVs and MDEVs (Nomura *et al*., 2003; 2009b), and EDEVs (Nomura *et al*., 2009a) after EPA supplementation. The effect of EPA combined with pitavastatin on reducing numbers of PDEVs measured by ELISA was also reported (Nomura *et al*., 2018). However, a major limitation is that the two parallel trials investigating EPA were uncontrolled and did not consider confounding factors such as age and BMI (Nomura *et al*., 2003; 2009a). Another study, providing 5.2g/d n-3 PUFA to post-MI patients for 12 weeks, reported that the levels of PDEVs and MDEVs, but not EDEVs and TF+EVs were significantly diminished after n-3 PUFA intervention compared to the placebo oil. Reduced procoagulatory activity of EVs was also reported (Del Turco *et al*., 2008).

There are five studies focusing on healthy volunteers, including one only with young men (Englyst *et al*., 2007) and another only with elderly men (Marin *et al*., 2011). Englyst and colleagues (2007) reported a significant increase in PDEVs after providing healthy young men with 6g/d n-3 PUFA for 12 weeks. In Marin's study (2011), healthy elderly men were randomised to receive three diets, including Mediterranean diet enriched in monounsaturated fatty acids (MUFA) with virgin olive oil, saturated fatty acids (SFA) diet or a low-fat, high-carbohydrate diet enriched in n-3 PUFAs, each for four weeks and there was a significant decline in total EVs, activated EDEVs and apoptotic EDEVs in n-3 PUFA enriched group compared to SFA-rich one. However, these two trials are of limited within specific subjects, may leading to some degrees of bias. Another two studies regarding healthy individuals, both conducted by Phang *et al.* (2012; 2016) have compared the different effects of EPA and DHA on EVs. The acute one, in which healthy volunteers have received EPA-rich, DHA-rich or placebo treatment for 24 hours with a one-week washout period between treatments, has suggested that the PDEVs activities but not the numbers have been improved after EPA-rich oil exclusively, and this beneficial effect only existed in males after separation by gender (Phang *et al*., 2012). A follow-up study by the same group involved more subjects on the same interventions for four weeks, but in that study, there were no effects of n-3 PUFA on the numbers of circulating PS+EVs or EV subsets, either in the whole population or within gender groups (Phang *et al*., 2016). Finally, a double-blind, randomised crossover study examining the effects of either n-3 PUFA (1.8g/d) or placebo on EVs in subjects at moderate risk of CVDs for eight weeks including an eight-week washout was conducted by Wu *et al.* (2014). It reported a significant reduction in numbers of EDEVs, but not PDEVs, an effect which was independent of endothelial nitric oxide synthase (eNOS) genotype (Wu *et al*., 2014). It is notable that all the mentioned studies applied FCM or ELISA for analysis of EVs. The detection limit of FCM may result in an underestimation of the levels of EVs, since smaller EVs predominate and yet escape detection by FCM (van der Pol *et al*., 2016). Also, their FCM gating strategies have commonly used polystyrene beads over a different range of sizes, which have a higher refractive index than EVs and are able to scatter more light

compared to similar-sized biological particles, leading to the possibility that EV gate in all previous studies captured not only EVs but also platelets and apoptotic bodies (Chandler *et al*., 2011; van der Pol *et al*., 2012, 2016).

Table 1.3 Effects of n-3 PUFA on EVs generation and function

Study	Subjects	Intervention/ Design	Durations	Measured markers and techniques	EVs related outcomes
Nomura *et al.* (2003)	Hyperlipidaemia with T2D subjects (n=18); hyperlipidaemia without T2D subjects (n=23); Healthy controls (n=20)	EPA 1.8g/d (parallel study)	4 weeks	**PDEVs**: labelled with anti-GPIX-FITC **MDEVs**: labelled with anti-CD14-PE *Flow cytometry (PDEVs)* *Cell counter analyser (MDEVs)*	**PDEVs/MDEVs**: Significantly increased in hyperlipidaemic with T2D+ subjects compared with control subjects (p< 0.01 for both); Significantly decreased in hyperlipidaemic with T2D+ subjects after the EPA intervention (p=0.01 for PDMP; p=0.05 for MDEVs)
Englyst *et al.* (2007)	Healthy young men (mean age: 25.4±8.3yrs)(n=35)	n-3 PUFA 6g/d (open study without control)	12 weeks	**PDEVs**: labelled with anti-CD61 *ELISA*	**PDEVs**: Significantly increased (↑12%, p=0.012) (n=25)
Del Turco *et al.* (2008)	Post-MI patients (n=42); healthy controls (n=10)	n-3 PUFA 5.2g/d or olive oil (randomised, controlled study)	12 weeks	**PS+EVs**: labelled with Annexin V-FITC **TF+EVs**: labelled with anti-TF 4508CJ **PDEVs**: labelled with anti-CD61; **EDEVs**: labelled with anti-CD62E **MDEVs**: labelled with anti-CD14 *Flow cytometry*	**PS+EVs**: significantly increased in post-MI patients compared with healthy subjects (p=0.021); **PDEVs/MDEVs**: significantly decreased in post-MI patients after the treatment with n-3 PUFA (↓49%, p=0.016 and ↓34%, p=0.010, respectively), but not after olive oil intervention; **EDEVs/ TF+EVs**: no differences after either treatment with n-3 PUFA (p=0.159 and p=0.249, respectively) or olive oil; **EVs activity**: a significant increase in fibrin generation time (↑12%) and time to clotting (↑11%) after treatment with n-3 PUFA compared with pre-treatment, but no significant change after olive oil intervention

Table 1.3 Effects of n-3 PUFA on EVs generation and function (continued)

Study	Subjects	Intervention/ Design	Durations	Measured markers and techniques	EVs related outcomes
Nomura *et al.* (2009a)	Hyperlipidaemia with T2D subjects (n=76); hyperlipidaemia without T2D subjects (n=50);	EPA 1.8g/d (parallel study)	6 months	**EDEVs**: labelled with Annexin V-FITC and anti-CD51-PE *Cell counter analyser*	**EDEVs**: Significantly increased in hyperlipidaemic with T2D subjects compared to subjects without T2D ($p< 0.01$); Significantly decreased in hyperlipidaemic with T2D subjects ($p< 0.05$) whereas no change in subjects without T2D after the EPA intervention
Nomura *et al*. (2009b)	Hyperlipidaemia with T2D subjects (n=191); healthy controls (n=30)	Group A: pitavastatin 2mg/d (n = 64); Group B: EPA 1.8g/d (n=55); Group C: both (n=72) (parallel study)	6 months	**PDEVs** *ELISA*	**PDEVs**: Significantly increased in hyperlipidaemia with T2D subjects compared with healthy controls ($p< 0.0001$); Significantly decreased in group B ($p< 0.01$) and C ($p<0.001$), but not in group A after intervention; Decrease in group C was significantly greater than that in group B ($p< 0.05$)

Table 1.3 Effects of n-3 PUFA on EVs generation and function (continued)

Study	Subjects	Intervention/ Design	Durations	Measured markers and techniques	EVs related outcomes
Marin *et al.* (2011)	Healthy elderly subjects (age> 65yrs) (n=20)	Diet 1: Mediterranean diet enriched in MUFA with virgin olive oil (24% en MUFA, <10% en SFA, 4% en PUFA, of which 0.4% was ALA); Diet 2: SFA diet (12% en MUFA, 22% en SFA, 4% en PUFA, of which 0.4% was ALA) Diet 3: low-fat, high-carbohydrate diet enriched with n−3 PUFAs (<10% en SFA, 12% en MUFA, 8% en PUFA, of which 2% was ALA) (randomised, crossover study)	Each diet for 4 weeks	**PS+EVs**: labelled with Annexin V-FITC **Apoptotic EDEVs**: labelled with Annexin V-FITC and anti-CD31E-phycoeritrine **Activated EDEVs**: labelled with anti-CD144-phycoeritrine and anti-CD62E-FITC *Flow cytometry*	**PS+EVs/ Apoptotic EDEVs/ Activated EDEVs**: Significantly decreased after the intervention with both diet 1 and diet 3 compared to diet 2; Decrease after diet 1 was greater than that after diet 3
Phang *et al.* (2012)	Healthy subjects (n=30)	EPA-rich oil 1g (EPA:DHA=5:1) or DHA-rich oil 1g (EPA:DHA=1:5) or sunola oil 2g (blinded, placebo-controlled study)	24 hours + 1-week washout	**PDEVs**: labelled with Annexin V-FITC and anti- CD41a-PE. *Flow cytometry*	**PDEVs**: no significant differences in numbers among three oils; EVs activity: a significant decrease in thrombin generation only after the treatment with EPA-rich oil (↓19.4%, p=0.003) and still existed only in males after separation by gender (↓22%, p=0.008)

Table 1.3 Effects of n-3 PUFA on EVs generation and function (continued)

Study	Subjects	Intervention/ Design	Durations	Measured markers and techniques	EVs related outcomes
Wu *et al.* (2014)	Subjects with moderate risk of CVDs (n=84)	n-3 PUFA 1.5g/d (EPA 0.9g+ DHA 0.6g) or corn oil (double-blind, placebo-controlled crossover study)	8 weeks+ 8 weeks wash-out+ 8 weeks	**PDEVs/EDEVs**: labelled with anti-CD31-PE and anti-CD42b-FITC (**PDEVs**: CD31+/CD42b+; **EDEVs**: CD31+/CD42b-) *Flow cytometry*	**EDEV**: significantly decreased after the treatment with n-3 PUFA compared to corn oil (p=0.001) **PDEVs**: no significant changes after the treatment with n-3 PUFA compared to corn oil (p=0.217) Genotype: no influence of genotype for PDEVs and EDEVs
Phang *et al.* (2016)	Healthy subjects (n=94)	EPA-rich oil (1000mg EPA+ 200mg DHA) or DHA-rich oil 1g (200mg EPA+1000mg DHA) or sunola oil 2g (double-blinded, placebo-controlled study)	4 weeks	**PS+EVs:** labelled with Annexin V-APC **EVs subsets:** labelled with anti-CD36-DyLight-488 and anti-CD41-PE (CD36+EVs; CD41+EVs;CD36+/CD41+ EVs; Annexin V+/CD41+; Annexin V+/CD36+) *Flow cytometry*	**PS+EVs/ EVs subsets**: no significant change after the treatment with either EPA or DHA compared to sunola oil either in the whole population or within gender groups (CD36+EVs: p= 0.158 for all subjects; p=0.187 for male; p=0.552 for female; other EVs subsets: data not shown)
Nomura *et al*. (2018)	T2D patients (n=84); Nondiabetic controls (n=50)	Pitavastatin 2mg/d with either EPA 1800mg/d or with sarpogrelate 300mg/d (randomised, non-blinded, parallel-group study)	12 months	**PDEVs** *ELISA*	**PDEVs**: Significantly decreased after the treatment with pitavastatin + EPA in T2D patients (p<0.01), but no significant change after the treatment with pitavastatin + sarpogrelate

ALA, alpha-linolenic acid; APC, allophycocyanin; CVDs, cardiovascular diseases; DHA, docosahexaenoic acid; EDEVs, endothelial-derived extracellular vesicles; en, energy; EPA, eicosapentaenoic acid; FITC, fluorescein isothiocyanate; GPIX, glycoprotein IX; MDEVs, monocyte-derived extracellular vesicles; MI, myocardial infarction; MUFA, monounsaturated fatty acids; N-3 PUFA, n-3 polyunsaturated fatty acids; PDEVs, platelet-derided extracellular vesicles; PE, phycoerythrin; PS+ EVs, phosphatidylserine positive extracellular vesicles; SFA, saturated fatty acids; T2D, Type 2 diabetes; TF+EVs, tissue factor positive extracellular vesicles.

1.4 Aims and objectives of the thesis

1. To develop and refine protocols for the isolation, characterisation and storage of circulating EVs from plasma, and to indicate final methods involved in the intervention study. (**Chapter 2** and **Chapter 3**)
2. To evaluate the association of conventional cardiovascular risk markers with numbers of circulating EVs in subjects with moderate risk of CVDs. (**Chapter 4**)
3. To investigate the influence of fish oil-derived n-3 PUFA on numbers and fatty acid composition of circulating EVs in subjects with moderate risk of CVDs. (**Chapter 5**)
4. To evaluate the influence of conventional cardiovascular risk markers and fish oil-derived n-3 PUFA on the procoagulatory activity of circulating EVs in subjects with moderate risk of CVDs. (**Chapter 6**)

1.5 Hypothesis

It is hypothesised that conventional cardiovascular risk markers will be positively associated with numbers and procoagulatory activity of circulating EVs, and that supplementation with n-3 PUFA will decrease numbers of circulating EVs, alter the fatty acid composition of circulating EVs and inhibit their procoagulatory function.

Chapter 2 Methodological considerations for the isolation, characterisation and storage of EVs

2.1 Introduction

EVs are characteristically a heterogeneous group of submicron membrane-bound, particles that are secreted by almost all cells in various biological fluids. Although EVs were firstly discovered 50 years ago and their roles in both normal biological and pathological processes have been explored for decades, there is a lack of consensus on 'gold-standard' methods to isolate and characterise EVs (Sáenz-Cuesta *et al*., 2015; Ridger *et al*., 2017). In order to cope with ambiguity in appropriate methods of EVs and to improve the reliability and reproducibility of studies in this field, ISEV has developed a set of guidelines and criteria for studying EVs, which was first published in 2014 and updated in 2018 (Lötvall *et al*., 2014; Théry *et al*., 2018). Based on the guidelines, the absolute isolation of total pure EVs from plasma to date is technically impractical and challenging. One of the challenges is that contaminants such as soluble proteins, protein aggregates and lipoproteins in plasma may interfere with the analysis due to size overlap (Witwer *et al*., 2013; Serrano-Pertierra *et al*., 2019). Moreover, it is also difficult to distinguish between categories of EVs because of their overlapping size and relative lack of definitive markers (Witwer *et al*., 2013; Heath *et al*., 2018). In general, plasma-derived EVs are isolated based on their size and/or density and many techniques have been applied, including UC, SEC, precipitation with kits and immunoaffinity capture (Lane *et al*., 2015). Regarding EVs characterisation, the guidelines recommend using at least two different technologies. One of the most commonly employed techniques is FCM, while NTA is a relatively novel technique to measure EV concentration and size distribution. Other methods, such as western blotting and EM, have also been applied for the specific identification of EVs (details see **Chapter 1 Section 1.2.6**). However, all of the existing isolation and characterisation methods have their own advantages and limitations, and therefore it is crucial when selecting a method to consider the specific questions addressed and the subsequent application required (Lötvall *et al*., 2014; Serrano-Pertierra *et al*., 2019). Furthermore, attempts have been made to standardise the collection, handling and storage of EVs

(Yuana *et al*., 2015; Lötvall *et al*., 2014; Théry *et al*., 2018). Most studies to date store EV samples at − 80°C, yet information about the effects of storage on EV numbers or function is still limited (Jeyaram & Jay, 2017). This thesis is based on a human intervention study investigating the effects of n-3 PUFA on the generation and function of EVs, and therefore the aim of the work described in this chapter was to select suitable methods and to refine protocols for the isolation, characterisation and storage of EVs based on the ISEV guidelines and the previous protocols used in our laboratory, which were then applied in the human intervention study. These EV-related protocols were explored from the following perspectives:

I. Comparison of UC and SEC for isolation of EVs (n=4) (including a pilot trial of two different types of SEC columns with n=1 only as this had been trialled by other members of the group);
II. Trials of FCM and NTA protocols for characterisation and phenotyping of EVs (n=4);
III. Trials of western blotting to detect the presence of specific proteins associated with EVs (n=2 and performed in duplicate);
IV. Trials evaluating the effect of sample freezing on EV enumeration and analysis (n=5).

2.2 Subjects and methods

2.2.1 Subjects

Blood samples from healthy subjects were collected at the Hugh Sinclair Unit of Human Nutrition, University of Reading after obtaining written informed consent for the study, which was approved by the University of Reading Research Ethics Committee and in accordance with guidelines detailed in the Declaration of Helsinki. For trial I and II, samples were collected from four subjects (two females and two males in total, aged 26~32yrs), and two subjects (two females, 24yrs and 50yrs) were involved in trial III. Trial IV involved five subjects (two females and three males in total, aged 24~30yrs).

2.2.2 Preparation of platelet-free plasma (PFP)

The first step required for isolation of EVs is the preparation of PFP, since platelets would interfere with EV analysis because their size is close to that of the largest EVs and platelet will produce EVs during the freeze-thaw cycle (Lacroix *et al*., 2012; Mitchell *et al*., 2016). Blood samples from individuals after overnight fasting was collected into vacutainer tubes containing 3.2% sodium citrate (Greiner Bio-One, Gloucestershire, United Kingdom). The tourniquet was removed once starting blood collection, and the first 2 to 3ml of blood was discarded. Blood samples were kept at room temperature and processed within one hour. Blood was transferred into a falcon tube (Fisher Scientific, Loughborough, United Kingdom) and volume was accurately recorded, followed by centrifugation at 1500xg for 15 minutes at room temperature (Heraeus Labofuge 400R Centrifuge, Thermo Scientific, United Kingdom). After recording the volume of plasma, two-thirds of the platelet-poor plasma (PPP) was transferred into a clean falcon tube. Every 1ml of this PPP was transferred into nonsticky microcentrifuge tubes (Alpha Laboratories Ltd, Hampshire, United Kingdom), and then centrifuged at 13,000xg for 2 minutes at room temperature (Eppendorf Centrifuge 5415R, DJBlabcare, Newport Pagnell, United Kingdom). Finally, 900µl PFP per microcentrifuge tube was pooled into a clean falcon tube, and 600µl aliquots were stored in microtubes (SARSTEDT AG & Co. KG, Nümbrecht, Germany) labelled “PFP”.

2.2.3 SEC for isolation of EVs

a. Preparation of columns

Two different types of column, iZON qEV columns and hand-poured sepharose columns, were used in a pilot trial to check their ability to isolate EVs. The iZON qEV column (Izon Science Ltd, Oxford, United Kingdom) containing beads with an estimated 75nm pore size, was rinsed with 30ml of PBS (Sigma-Aldrich, Gillingham, United Kingdom). The time for 5ml of PBS to flow through was recorded, and a time of approximately 5 minutes indicated that the column was clean and ready to use. For sepharose column preparation, sepharose CL-2B (GE Healthcare, Amersham, United Kingdom) was first vacuumed for 10 minutes to remove air bubbles. Then the tip of a 10ml plastic syringe (BD Biosciences, Wokingham, United Kingdom) was stuffed with a

luer tip cap, and the bottom filter was placed into the syringe, followed by the slow stacking of 12.5ml sepharose CL-2B. After placing the top filter, the column was cleaned with 10ml of PBS and was ready to use.

b. Collection of fractions

After pipetting out the PBS above the top filter, 500µl of PFP was loaded onto the column. The bottom luer tip cap was removed, and PBS was added until the last of the PFP just entered the column top-filter to allow the collection of eluate in 36 (or 30) sequential factions of 0.5ml. Once eluted, 30ml of PBS and 10ml of 20% ethanol (Sigma-Aldrich, Gillingham, United Kingdom) were applied for cleaning, and columns were finally stored in 20% ethanol at 4~8°C.

2.2.4 UC with DGC for isolation of EVs

a. UC

PFP (2ml) was loaded into ultracentrifuge tubes and topped with PBS. Then the tubes were ultracentrifuged at 29,600xg for 2 hours at 4°C (Optima L-90K Ultracentrifuge, Beckman Coulter Life Sciences, United Kingdom). Subsequently, the supernatant was discarded, and the pellet was re-suspended with PBS and ultracentrifuged again under the same conditions as before. Finally, the supernatant was discarded, and PBS was added to 200µl; samples were then covered and stored in the fridge overnight.

b. DGC

OptiPrep™ (60% (w/v) aqueous iodixanol; Axis-Shield PoC, Oslo, Norway) was diluted with 0.25 M sucrose/10 mM Tris (pH 7.5) (Sigma-Aldrich, Gillingham, United Kingdom) to obtain a discontinuous gradient, 40% (w/v), 20% (w/v), 10% (w/v) and 5% (w/v) solutions of iodixanol. To make the gradient, 3 ml of 40% iodixanol solution was first added to a polyallomer tube (Beckman Coulter Life Sciences, United Kingdom), followed by the careful layering of 3ml each of 20% and 10% solutions, and 2.5ml of 5% solution. The EV suspension acquired by UC was gently overlaid onto the top or bottom of the gradient and then centrifuged in the ultracentrifuge at 100,000xg for 18 hours at 4°C (Optima L-90K Ultracentrifuge, Beckman Coulter Life Sciences, United

Kingdom). Twelve fractions were collected based on their density (1.13~1.19g/ml), diluted with PBS and centrifuged again at 100,000xg for 2~3 hours at 4°C.

2.2.5 Characterisation of EVs using NTA after isolation

NTA, consisting of a laser light scattering microscope and a camera, allows the capture and recording of particles (EVs in the current study) moving under Brownian motion. The speed of motion of EVs at a known temperature, pressure and viscosity is determined in two dimensions and is related to particle size by software based on the Stokes-Einstein Equation:

$$D = \frac{4k_B T}{3\pi\eta d}$$

where K_B is the Boltzmann constant and particle diameter d can be calculated as function of the diffusion coefficient D at a temperature T and a viscosity η of the liquid (van der Pol *et al*., 2010; Filipe *et al*., 2010; Particle Metrix). The NanoSight 300 (Malvern, Amesbury, United Kingdom), equipped with a high sensitive sCMOS camera and a 488nm blue laser, was used to assess the size distribution and concentrations of all types of particles. Fractions collected by SEC or the pellet obtained after UC were diluted with PBS to maintain the numbers of particles in the field of view below 200/screen. For each analysis, five videos, each of 60 seconds duration, were captured with the camera level at 12. A higher camera level could detect more particles but also has a higher risk to introduce unexpected background noise. The setting up of camera level has been trialled, and camera level at 12 or 13 is optimal for the detection of EVs (data not shown). Data were analysed using the instrument software NTA 3.20. In each captured video, individual particles are presented as bright dots, and their motions are tracked as red lines (**Figure 2.1A**). The size distribution profile (blue line) was generated based on the Stokes-Einstein Equation by the software, and the mean concentrations and size of vesicles shown in **Figure 2.1B** represent the analysis of five separate videos. Finally, a lower threshold of 70nm was set for NTA to ensure the minimal interference by lipoproteins.

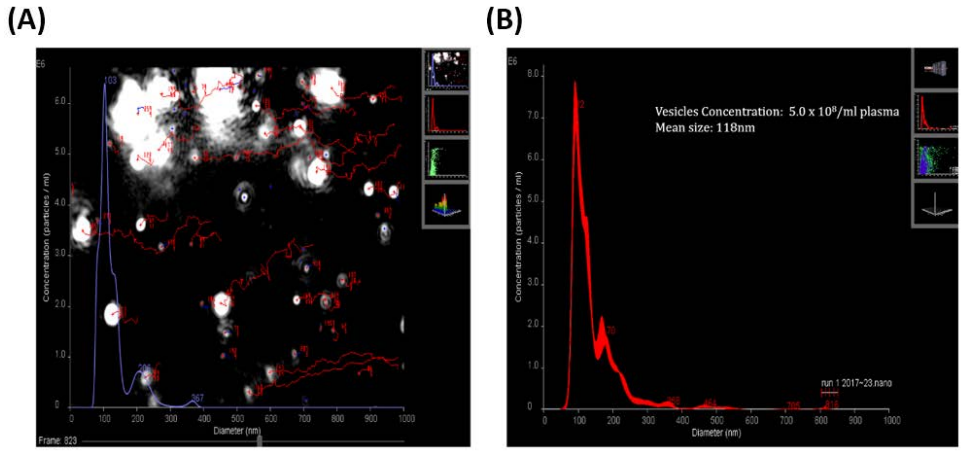


Figure 2.1. Analysis of EVs by NTA. (A) individual particles presented as bright dots, motions tracked as red lines and size distribution profile (blue line) based on the Stokes-Einstein Equation; **(B)** mean concentrations and size of vesicles calculated after the analysis of five videos.

i. PFP: The number of EVs in the PFP was unable to be detected by NTA directly due to the presence of large numbers of interfering particles.

ii. SEC: The number of EVs per ml of analysed fractions was calculated as (formula 1):

Events indicated in NTA (particles/ml) × dilution factor= EVs/ml of neat fraction

The total EV concentration isolated from SEC was summed from fractions 7~9, and this value represented the total numbers of EVs per ml of PFP. The total number of EVs per ml of blood was calculated as (formula 2):

$$\text{Total EVs/ml of PFP} \times \frac{\text{Total volume of blood}}{\text{Total volume of plasma}} = \text{Total EVs/ml of blood}$$

iii. UC: The number of EVs per ml of neat UC was calculated as (formula 3):

Events indicated in NTA (particles/ml) × dilution factor= EVs/ml of neat UC

The number of EVs per 200µl of neat UC was calculated as (formula 4):

$$\frac{\text{EVs/ml of neat UC}}{5} = \text{EVs/200µl of neat UC}$$

EVs/200µl of neat UC is equivalent to EV/2ml of PFP, so the total number of EVs per ml of PFP was calculated as (formula 5):

$$\frac{\text{EVs/2ml of PFP}}{2} = \text{Total EVs/ml of PFP}$$

The total number of EVs per ml of blood was calculated by formula 2.

2.2.6 Characterisation of EVs using FCM after isolation

a. Flow cytometer set-up

The flow cytometer detects particles (EVs in the current study) flowing in a fluid through a beam of light. On scatter mode, the light is scattered either along the path of the laser, which is referred to as FSC, or at a ninety-degree angle relative to the laser, which is called SSC. In general, FSC relates to particle size, whereas the SSC relates to the inner complexity (granularity) of the particles. Furthermore, particles are detected based on the fluorescent signal exhibited by a fluorescent reagent, which labels specific EV components and can then be detected using fluorescence mode (van der Pol *et al*., 2010). The BD FACSCanto II flow cytometer (BD Biosciences, Wokingham, United Kingdom) equipped with a violet (405nm), a blue (488nm), a red (633nm) laser was used for EV numeration and characterisation.

An initial EV size gate was set using ApogeeMix beads (Apogee flow systems, Hemel Hempstead, United Kingdom), which consist of non-fluorescent silica beads with diameters of 180nm, 240nm, 300nm, 590nm, 880nm, and 1300nm, and additionally fluorescent latex beads with defined sizes of 110nm and 500nm. The silica beads were analysed on FSC and SSC by BD FACSCanto II, and the exact sizes of the beads and the gating are illustrated in **Figure 2.2**. The 240nm silica beads corresponded to the lowest reliable detection limit of the flow cytometer; any particles below this size were excluded (on SSC) to minimise background noise. In order to exclude platelets and cellular material, the upper detection gate would ideally be set at 1µm. However, 1µm silica beads were unavailable, so the cut-off gate was set just above 880nm silica beads to classify all particles within this gate as being ≤ 1µm in size. The fluorescent polystyrene beads were not used to set gates on fluorescence mode in the current protocol, but were used as a general guide to contextualise fluorescently-labelled EVs. The gating for EVs on scatter mode was therefore set to include all particles ranging in

size between 240nm and 1µm, while the visualisation of EVs on fluorescence mode does not require a threshold for debris exclusion, because the analysis is based on fluorescence rather than size, and therefore all fluorescently labelled particles down to the flow cytometer instrument threshold can be detected.

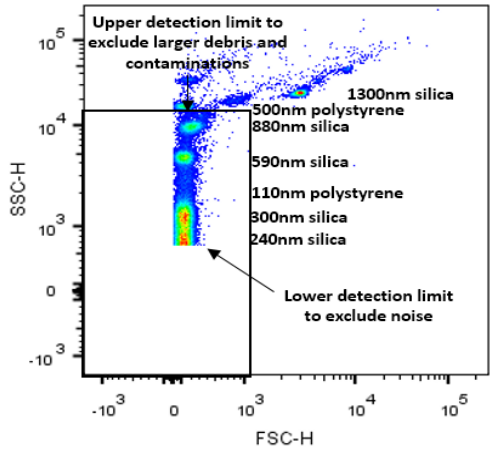


Figure 2.2. FACSCanto II flow cytometer set up. FSC vs SSC plot to show the EV analysis gate established by ApogeeMix beads. The gate included all particles of size 240nm-1µm, in which the lower detection limit was set by 240nm silica beads to exclude noise, while the upper detection limit was set just above 880nm silica beads to exclude larger debris and contaminations (scatter mode).

Before each analysis day, one drop of Cytometer Setup and Tracking beads (BD Biosciences, Wokingham, United Kingdom) was diluted with 350µl FACS flow solution and was used for the quality control of the instrument's optics, electronics, and fluidics. Routine calibration and verification of the performance of FACSCanto II was conducted by running 500µl FACS flow solution containing a mixture of rainbow calibration particles dyed with eight different fluorescent intensities (BD Biosciences, Wokingham, United Kingdom).

b. EV analysis by FCM

FCM can detect both scattered light and fluorescence, so EV detection can be achieved on the basis of both scatter (size/granularity) and fluorescence (specific markers). Samples (PFP, fractions collected by SEC or the pellet obtained after UC) diluted with PBS were first analysed on scatter mode, acquiring 500,000 events at an event rate of approximately 400~500 event/second at a low flow rate, in which each single particle

was measured under the combination of FSC light and SSC light. For fluorescence mode, there is a lack of standardisation in EV analysis by fluorescence FCM since there is no generic ligand which can label all types of EVs. One of the commonly used EV markers is PS, which is asymmetrically distributed in the inner leaflet of the plasma membrane bilayer in resting cells. The externalisation of PS in the outer leaflet occurs during cell activation, resulting in membrane blebbing and EV shedding. Therefore, PS-exposing EVs represent the majority of circulating EVs (Bevers *et al*., 1983; Zwaal & Schroit, 1997; Boersma *et al*., 2005; Morel *et al*., 2011). In the current protocol, the diluted sample was labelled with 8µl of lactadherin conjugated to fluorescein isothiocyanate (FITC) (Haematologic Technologies, Vermont, United States), which binds to PS, and incubated for 15 minutes in the dark before the detection on fluorescence mode. EVs were defined as vesicles that stained positive for lactadherin (Shi *et al*., 2004; Dasgupta *et al*., 2006). Unstained samples and lactadherin-FITC alone served as controls. Trucount tubes (BD Biosciences, Wokingham, United Kingdom) containing 500µl PBS were used to calculate the absolute numbers of EVs. In general, the sample should be added directly to Trucount tube. However, there are many contaminating particles in Trucount tubes, and their size range overlaps with that of EVs; therefore the Trucount beads were run separately to minimise their interference with particle detection when samples were run on light scatter mode.

On scatter mode, FSC represents particle size and is plotted on the x-axis, whereas SSC represents the inner complexity of the particles and plotted on the y-axis. As **Figure 2.3A** shows, each dot represents one particle and light scatter allows for easy differentiation of small particles from larger particles and differentiation of particles from similarly sized but significantly more complex ones. For fluorescence mode, particles are detected based on the fluorescent signal exhibited by lactadherin-FITC (**Figure 2.3B**). The detection threshold was set by ApogeeMix beads as mentioned above, so the total count of EVs are those particles inside the gated region. In order to reduce background noise, a threshold of 240nm was set on scatter mode so that debris could be excluded. However, smaller particles (below 240nm) are missed when this threshold is set, leading to a lower EV result than for NTA, where the threshold is 70nm. On the other hand, the visualisation of EVs using lactadherin-FITC labelling on

fluorescence mode does not require a threshold for debris exclusion and therefore EVs smaller than 240nm, but within flow cytometer instrument threshold, can be captured.

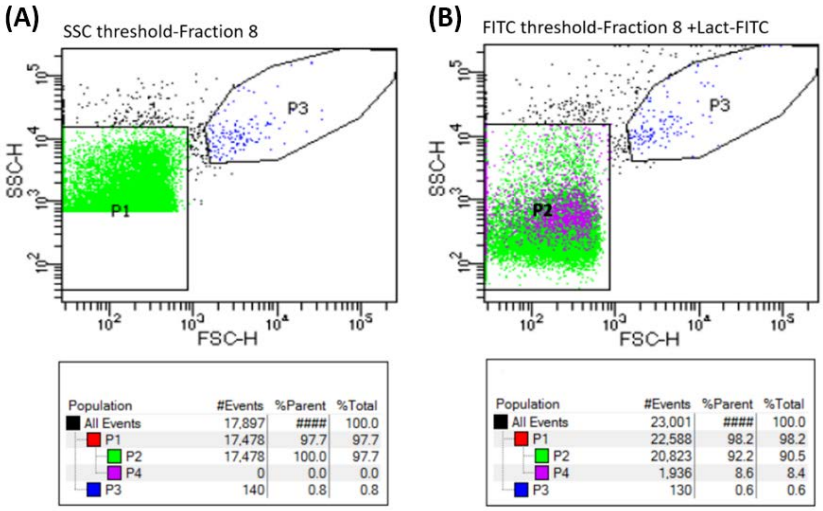


Figure 2.3. Analysis of EVs by FCM. The detection gate for EVs was set via ApogeeMix beads; (**A**) the particle events detected on scatter mode are presented as green dots (P2), and blue dots (P3) are platelets; (**B**) the lactadherin-positive particles on fluorescent mode are presented as green dots (P2), and blue dots (P3) are platelets and purple dots (P4) were lactadherin events. *FITC, fluorescein isothiocyanate; SSC, side scatter.*

c. Analysis of EV numbers on scatter mode

i. PFP: The number of EVs per FCM-analysed volume of neat PFP was calculated as (formula 6):

(Sample events indicated in FCM− PBS events) × dilution factor= EVs/FCM-analysed volume of neat PFP

The real volume that FCM analysed was calculated as (formula 7):

$$\frac{\text{Trucount events indicated in FCM}\times 500\mu l}{\text{Trucount beads (packet)}} = \text{FCM-analysed volume (µl)}$$

The total number of EVs per ml of PFP was calculated as (formula 8):

$$\frac{\text{EVs/FCM−analysed volume of neat PFP}\times 1000\mu l}{\text{FCM−analysed volume}} = \text{Total EVs/ml of PFP}$$

The total number of EVs per ml of blood was calculated by formula 2.

ii. SEC: The number of EVs per FCM-analysed volume of neat fraction was calculated as (formula 9):

(Sample events indicated in FCM− PBS events) × dilution factor= EVs/FCM-analysed volume of neat fraction

The total EV concentration isolated from SEC was summed from fractions 7~9, and this value represented the total numbers of EVs per FCM-analysed volume. After calculating the volume that FCM analysed by formula 7, the total number of EVs per ml of PFP was calculated as (formula 10):

$$\frac{\text{EVs/FCM-analysed volume of neat fraction} \times 1000\mu l}{\text{FCM-analysed volume}} = \text{Total EVs/ml of PFP}$$

The total number of EVs per ml of blood was calculated by formula 2.

iii. UC: The number of EVs per FCM-analysed volume of neat UC was calculated as (formula 11):

(Sample events indicated in FCM− PBS events) × dilution factor= EVs/FCM-analysed volume of neat UC

After calculating the volume that FCM analysed by formula 7, the number of EVs per ml of neat UC was calculated as (formula 12):

$$\frac{\text{EVs/FCM-analysed volume of neat UC} \times 1000\mu l}{\text{FCM-analysed volume}} = \text{Total EVs/ml of neat UC}$$

The total number of EVs per ml of PFP was calculated by formula 4 and 5; the total number of EVs per ml of blood was then calculated by formula 2.

d. Analysis of EV numbers on fluorescence mode

PFP/SEC/UC: The number of EVs per FCM-analysed volume of neat PFP /fraction/UC was calculated as (formula 13):

(Events of samples labelled lactaherin-FITC − events of samples alone−events of lactaherin-FITC alone+events of PBS alone) ×dilution factor= EVs/FCM-analysed volume of neat PFP/fraction/UC

The total number of EVs per ml of PFP was calculated by formula 7~8 (PFP); formula 9~10 (SEC) and formula 12&4~5 (UC); the total number of EVs per ml of blood was then calculated by formula 2.

2.2.7 Analysis of protein, plasma cholesterol and TAG after isolation of EVs

In order to quantify the potential contaminants presented in isolated EVs samples, the concentrations of contaminants such as protein, plasma cholesterol and TAG (the latter two are abundant in lipoproteins) were assessed in either fractions collected by SEC or the pellet obtained after UC. The protein concentration was determined using a Bradford protein assay, according to the manufacturer's instructions (Pierce, Thermo Scientific, United Kingdom). The absorbance was detected at 540nm on a microplate reader (Tecan GENios Microplate Reader, Global Methane Initiative, United States). The cholesterol and TAG concentrations were determined by iLab (iLab 600 Clinical Chemistry System, Diamond Diagnostics, United States) using 160µl of each sample with cholesterol and TAG reagent (Werfen Limited, Warrington, United Kingdom).

2.2.8 Comparison between different isolation methods of EVs

In general, there are two aspects to evaluate the efficiency of isolation methods for EVs. One is the yield/recovery of EVs after isolation. The yield is expressed as total EV numbers after isolation and the recovery is defined as total EV numbers after isolation compared to total EV numbers in original PFP, both of which suggest how many EVs remained after isolation. Another factor is the purity/enrichment of isolated EVs compared to contaminants. The purity is expressed as the ratio of total EV numbers to the levels of contaminants, where greater purity indicates a lower degree of contaminants in the samples. Normally, the purity will increase after isolation as contaminants will be excluded and separated from EV-contained samples, which can be regarded to enrich EVs somehow. Thus, the enrichment of EV numbers by minimising contaminants, which is calculated as the purity of EVs after isolation divided by the purity of EVs in the PFP, is able to quantify how contaminants are minimised effectively by isolation (Webber & Clayton, 2013; Böing *et al*., 2014; Xu *et al*., 2016). EV numbers in the above fractions 7~9 collected by SEC or the pellet obtained after UC were detected by both NTA and FCM (on both scatter and fluorescence mode). However, the numbers of EVs in PFP samples was only measured by FCM because of the difficulty with analysing PFP by NTA directly as mentioned above, thus in this trial, the yield/recovery and purity/enrichment of EVs exhibited by

different isolation methods were only calculated and compared based on the numbers of EVs analysed by FCM.

The recovery of EVs from SEC/UC was calculated as:

$$\frac{\text{Total number of EVs from SEC/UC}}{\text{Total number of EVs from PFP}} = \text{Recovery of EVs from SEC/UC}$$

The purity of isolated vesicles from SEC/UC, which indicates how pure isolated EVs are, was calculated as:

$$\frac{\text{Total numers of EVs from SEC/UC}}{\text{Proteins /Cholesterol/TAG concentrations from SEC/UC}} = \text{Purity of EVs}$$

The enrichment factor of vesicles to protein/cholesterol/TAG from SEC/UC, which was used for the evaluation of isolation ability and purity of EVs after isolation was calculated as:

$$\frac{\text{Purity of EVs from SEC/UC}}{\text{Purity of EVs from PFP}} = \text{Enrichment of EVs from SEC/UC}$$

TAG: triacylglycerol.

2.2.9 Western blotting of EVs isolated by SEC

After isolation by SEC, fractions 5~12 were subjected to western blotting to determine whether proteins known to be associated with EVs eluted in the same fractions as EVs characterised by FCM and NTA, providing additional confidence about the presence of EVs in these fractions. For each fraction, 25µl samples were lysed in 2X Laemmli sample buffer (Bio-Rad Laboratories Inc, United States), and heated for 5 minutes at 95°C. The proteins were separated by gel electrophoresis by using 4~15% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories Inc, United States) in running buffer (3.03g Trizma base, 14.42g glycine, 1g sodium dodecyl sulphate, with distilled water to 1 litre) at constant 180V for 1 hour. After transferring proteins onto polyvinylidene difluoride membrane applying the iBlot™ Dry Blotting System (Invitrogen, Thermo Scientific, United Kingdom), the membrane was blocked with 1% (w/v) skim milk powder in Tris-buffered saline (TBS: 2.42g Tris base, 8g NaCl, with distilled water to 1 litre, pH 7.6) with 0.1% (v/v) Tween-20 (TBST: 1 litre TBS, 1ml Tween 20) (Sigma-Aldrich, Gillingham,

United Kingdom) for 1 hour at room temperature with agitation. After three washes with TBST, the membrane was incubated with the primary antibody, mouse anti-CD63 (1:1000, BD Biosciences, Wokingham, United Kingdom) in 2% TBST overnight at 4°C with agitation. The incubation of secondary anti-mouse immunoglobulins horseradish peroxidase (HRP) (1:1500, Dako, California, United States) in 2% TBST for 1 hour at room temperature was then conducted. The membrane was washed three times in TBST for 10 min after each incubation step and finally visualised on ImageQuant LAS 4000 (GE Healthcare, Amersham, United Kingdom) after incubation with Amersham™ ECL™ Prime detection reagent (GE Healthcare, Amersham, United Kingdom).

2.2.10 Effect of freezing on EVs

EVs were isolated from 500µl PFP by SEC using qEV columns and the pooled fractions 7~9 were analysed immediately by NTA and FCM as described above. Further samples of PFP were deposited and stored as 600µl aliquots at − 80°C. Frozen PFP aliquots were thawed at room temperature after freezing for one week, one month, three months, four months and six months and the above isolation and detection processes were repeated.

2.2.11 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). The difference between UC and SEC in isolating EVs (trial I) and the difference between NTA and FCM in characterising EV numbers (trial II) were examined by paired sample t-tests. One-way analysis of variance (ANOVA) was performed to determine the effect of sample freezing on EV analysis (trial IV). All statistical analyses were performed with SPSS Statistics version 25 and a p-value < 0.05 was considered statistically significant.

2.3 Results

2.3.1 Comparison between SEC and UC for isolation of EVs

Two options for SEC to isolate EVs were available for this project: qEV columns, which are industrially manufactured by Izon Science, or hand-poured sepharose columns, which are manually packed before use. A previous in-house experiment had already compared the ability of qEV columns and hand-poured sepharose columns to isolate EVs, demonstrating qEV columns to be superior. The current pilot trial was therefore simply performed to ensure technical competence and ability to reproduce the observation.

In the pilot trial, a blood sample was collected from one female aged 50 yrs and was analysed twice. The 36 sequential fractions were obtained by running a sample of PFP through a qEV or sepharose column, and these fractions were analysed by NTA to determine the presence of EVs and other small particles. The distribution of EVs, proteins and cholesterol after being isolated by two columns were shown in **Figures 2.4**. In this sample, EVs (mean size: 95nm) eluted using qEV columns were present in fractions 7 to 10, while both protein and cholesterol significantly increased from fraction 10 onwards. When hand-poured sepharose columns were used to isolate EVs, they were mainly detected from fractions 6 to 9, while protein and cholesterol eluted from fraction 9 onwards. Also, the total yield of EVs isolated using qEV column (fractions 7~10) was higher than that using hand-poured sepharose column (fractions 6~9) (mean numbers: $2.0*10^{10}$/ml PFP vs $8.2*10^{9}$/ml PFP; $7.2*10^{10}$/ml blood vs $3.0*10^{10}$/ml blood). Also, the purity of EVs compared to protein obtained from qEV column was $3.2*10^{11}$/μg, which was approximately 5.2-fold higher than that from hand-poured sepharose column (purity of EVs to protein: $6.2*10^{10}$/μg); similarly, the purity of EVs compared to cholesterol after qEV column isolation was $1.7*10^{8}$/mmol, which was also approximately 5-fold higher than that after hand-poured sepharose column isolation (purity of EVs to cholesterol: $3.4*10^{7}$/mmol).

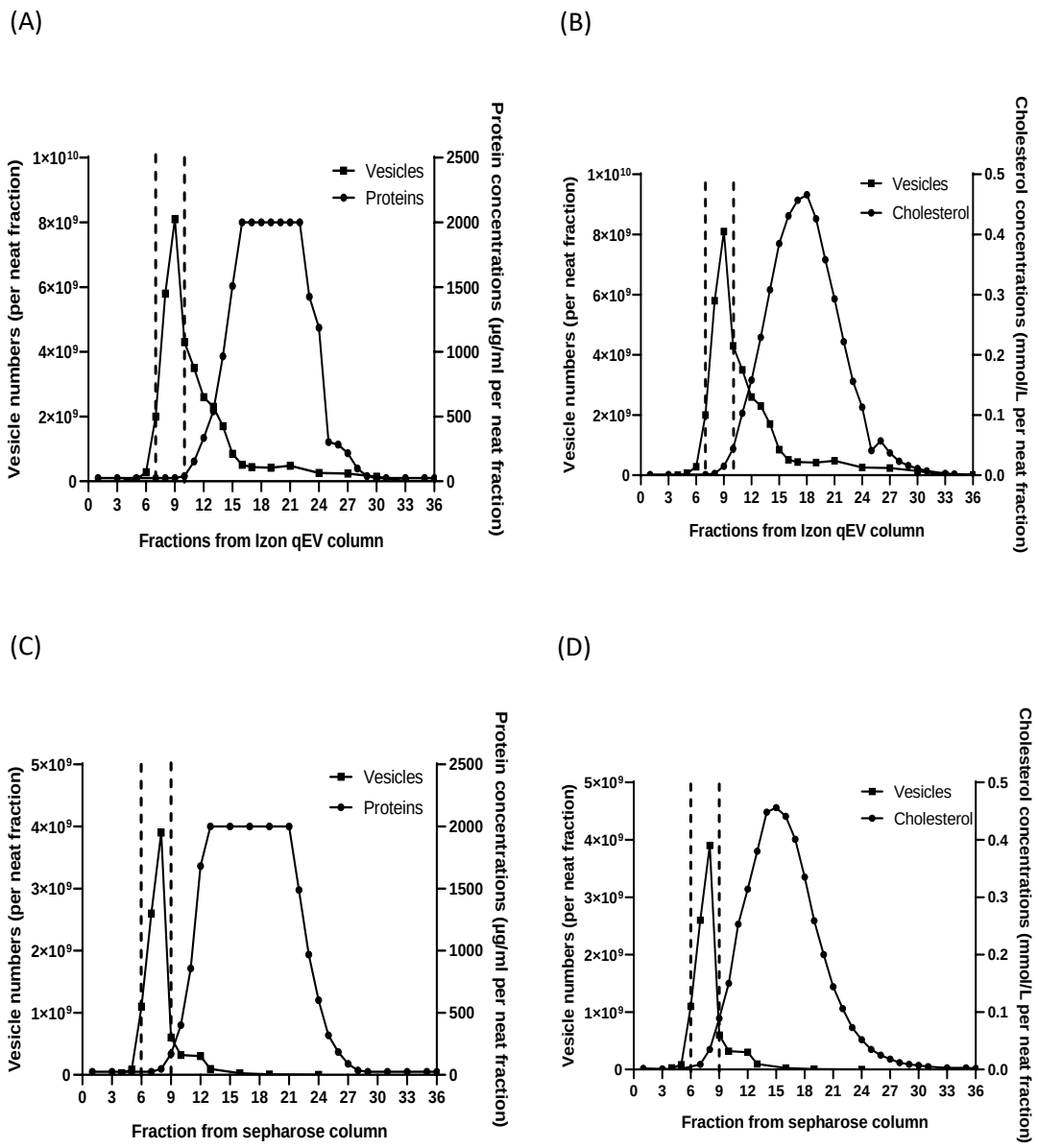


Figure 2.4. The distribution of EVs, proteins and cholesterol after being isolated by qEV or hand-poured sepharose column. The separation between EVs and protein (**A**)/cholesterol (**B**) in fractions isolated by qEV column and the separation between EVs and protein (**C**)/cholesterol (**D**) in fractions isolated by hand-pour sepharose column. Between dotted lines: vesicles-rich fractions.

In trial I, four samples of PFP from four separate volunteers were used to compare SEC (with qEV columns) and UC. The above result suggested that fractions 7 to 9 obtained from qEV columns contains a high concentration of EVs almost free of proteins and cholesterol, which was confirmed by the further repeated practice trial on qEV (data not shown), and is in agreement with the finding reported by Gaspar *et al*. (2020); thus fractions 7 to 9 were finally selected for EVs detection. Fractions 7~9 collected by SEC

or the pellet obtained after UC or PFP were analysed respectively by FCM on both scatter and fluorescence mode. There were attempts to separate EVs using UC combined with DGC, but this proved to be technically difficult and to yield much lower EV numbers (the results are therefore not presented in this chapter). The results obtained by FCM suggested that EV numbers generated from SEC were significantly greater than those generated from UC, regardless of whether FCM scatter (p=0.002) or fluorescence mode (p<0.001) was used for analysis (**Figure 2.5**). Moreover, there was ~63% recovery of EVs detected on scatter mode compared with that in PFP, whereas only ~7% were recovered after UC (p<0.001). Similarly, when fluorescence FCM was used to detect the lactadherin-positive (lactadherin+) EVs, the recovery after SEC was ~57% compared to only ~8% after UC (p<0.001) (**Figure 2.6**).

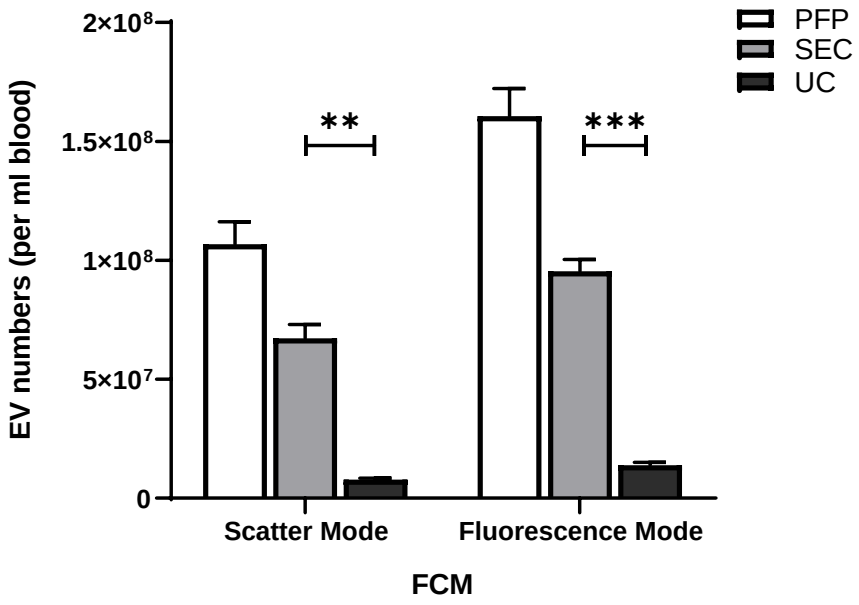


Figure 2.5. Numbers of EVs after isolation using SEC or UC (analysed by FCM). Data are mean ± SEM (n=4). The yield of EV numbers after SEC isolation was significantly higher than that from UC isolation on both FCM scatter (p=0.002) and fluorescence mode (p<0.001). **p<0.01 and ***p<0.001. *FCM, flow cytometry; PFP, platelet-free plasma; SEC, size exclusion chromatography; UC, ultracentrifugation.*

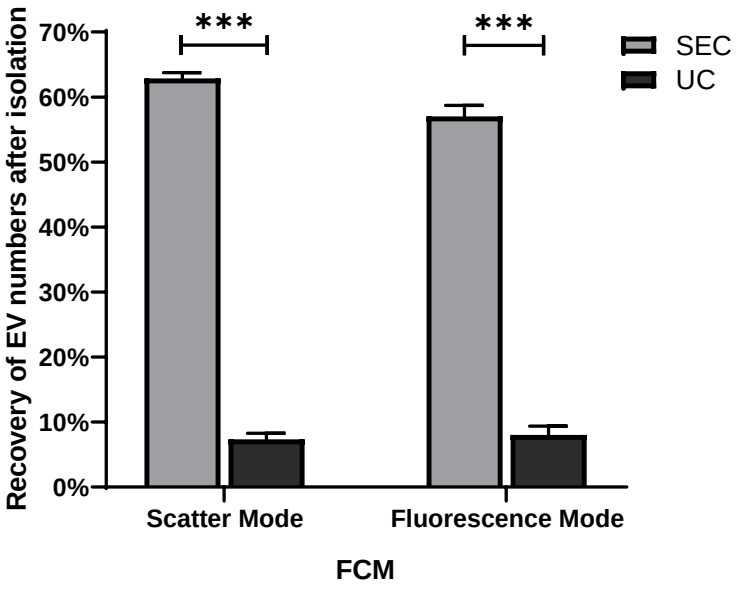


Figure 2.6. Recovery of EVs after isolation using SEC or UC (analysed by FCM). Data are mean ± SEM (n=4). The recovery of EV numbers after SEC isolation was significantly higher than that from UC isolation on both FCM scatter (p<0.001) and fluorescence mode (p<0.001). ***p<0.001. *FCM, flow cytometry; PFP, platelet-free plasma; SEC, size exclusion chromatography; UC, ultracentrifugation.*

Regarding purity, SEC resulted in a significantly higher purity of EVs compared to protein and compared to cholesterol than those from UC on both FCM scatter (protein: p=0.015; cholesterol: p=0.026) and fluorescence mode (protein: p=0.007; cholesterol: p=0.034). Furthermore, a significantly higher purity of EVs compared to TAG was found after the isolation by UC compared to after the isolation by SEC, regardless of whether FCM scatter (p=0.013) or fluorescence mode (p=0.004) was used for analysis (**Figure 2.7**).

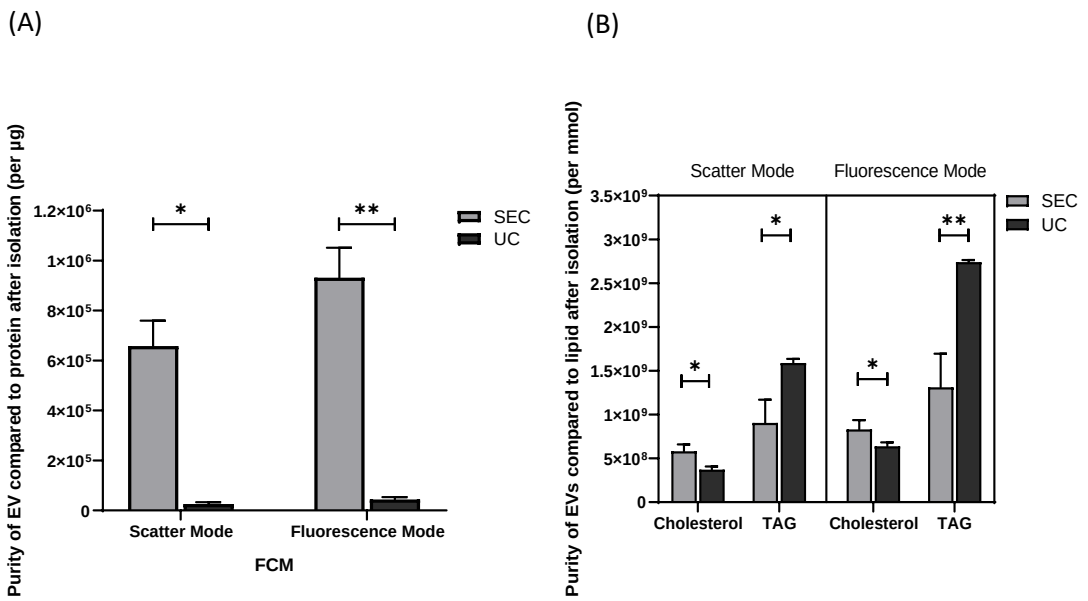


Figure 2.7. Purity of EVs to contaminants after isolation by SEC or UC (analysed by FCM). Data are mean ± SEM (n=4).The purity of EV compared to protein after SEC isolation was significantly higher than that from UC isolation on both FCM scatter (p=0.015) and fluorescence mode (p=0.007) (**A**). The purity of EV compared to cholesterol after SEC isolation was significantly higher than that from UC isolation on both FCM scatter (p=0.026) and fluorescence mode (p=0.034), while UC isolation presented a significantly higher purity of EV compared to TAG compared to SEC on both FCM scatter (p=0.013) and fluorescence mode (p=0.004) (**B**). *p<0.05 and **p<0.01. *FCM, flow cytometry; SEC, size exclusion chromatography; TAG, triacylglycerol; UC, ultracentrifugation.*

Moreover, a 318-fold (scatter mode) and 289-fold (fluorescence mode) enrichment of EV numbers by minimising contamination of protein compared to PFP were observed after SEC isolation compared to PFP, which were significantly higher than those exhibited after UC isolation (scatter mode: 14-fold, p=0.011; fluorescence mode: 15-fold, p=0.012) (**Figure 2.8A**). Regarding lipids, SEC resulted in a significantly higher enrichment of EV numbers by minimising contamination of cholesterol (26-fold on scatter mode and 24-fold on fluorescence mode) compared to UC (15-fold on scatter mode and 16-fold on fluorescence mode) (scatter mode: p=0.012; fluorescence mode: p=0.025), while the significantly higher enrichment of EV numbers by minimising contamination of TAG was found after the isolation by UC compared to after the isolation by SEC (scatter mode: 20-fold vs 15-fold, p=0.031; fluorescence mode: 21-fold vs 14-fold, p=0.021) (**Figure 2.8B**).

(A) (B)

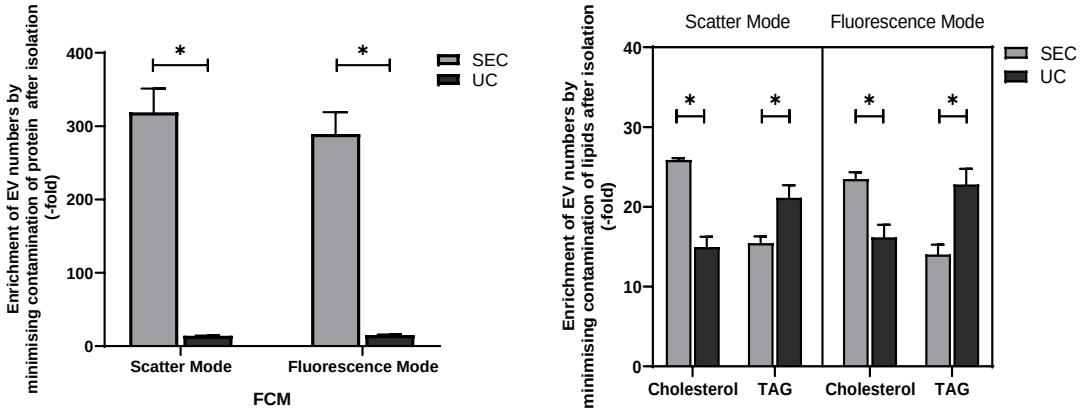


Figure 2.8. Enrichment of EV numbers by minimising contaminants after isolation using SEC or UC (analysed by FCM). Data are mean ± SEM (n=4). The enrichment of EV numbers by minimising contamination of protein was significantly higher than that from UC isolation on both FCM scatter (*p*=0.011) and fluorescence mode (*p*=0.012) (**A**). The enrichment of EV numbers by minimising contamination of cholesterol after SEC isolation was significantly higher than that from UC isolation on both FCM scatter (*p*=0.012) and fluorescence mode (*p*=0.025), while UC isolation presented a significantly higher enrichment of EV numbers by minimising contamination of TAG compared to SEC on both FCM scatter (*p*=0.031) and fluorescence mode (*p*=0.021) (**B**). **p*<0.05. *FCM, flow cytometry; SEC, size exclusion chromatography; TAG, triacylglycerol; UC, ultracentrifugation.*

On the other hand, NTA was also applied for detecting total EV numbers in the above fractions 7~9 collected by SEC or the pellet obtained after UC, but not in samples of PFP due to the presence of large numbers of interfering particles as mentioned above. Thus, the number of EVs obtained from NTA were not used for the isolation method comparison, but the data provided information about particle sizes and indicated that the vesicles detected by NTA following UC were significantly larger than those prepared using SEC (mean sizes: 171±5.7nm vs 89±8.0nm, *p*=0.005; mode sizes: 134±15.7nm vs 72±5.3nm, *p*=0.05). Taken together, SEC outperformed UC for the isolation of EVs; thus SEC using qEV columns was selected as the preferred option for all EV isolation throughout this project.

2.3.2 Comparison between NTA and FCM for detection of EVs

In the above trial, EV numbers in either fractions 7~9 isolated by SEC or the pellet obtained after UC detected by NTA were significantly higher than that those detected by scatter FCM (SEC: $5.3*10^{10}$/ml blood vs $6.7*10^{7}$/ml blood, p=0.002; UC: $3.0*10^{9}$/ml blood vs $7.8*10^{6}$/ml blood, p=0.003), which is expected as NTA is able to detect very small particles down to a size of 70nm, and scatter FCM requires a size threshold of 240nm to exclude debris. Also, the mean numbers of EVs after SEC and UC isolation were $9.5*10^{7}$/ml blood and $1.4*10^{7}$/ml blood, respectively when analysed by fluorescence FCM, which were also significantly higher than EV numbers detected on scatter mode (SEC: p<0.001; UC: p=0.04). Scatter FCM detected all particles larger than 240nm, whereas fluorescence FCM detected all fluorescently labelled particles (lactadherin-labelled particles in this chapter) down to the instrument limit.

2.3.3 Western blotting to identify proteins associated with EVs

In order to further check the performance of SEC in the isolation and purification of EVs, the presence of CD63 and albumin in fractions 5 to 12 obtained after SEC were identified by western blotting. CD63, as a membrane protein, has been described as the most frequently identified protein in EVs and thus is widely used in western blotting for the determination of EVs (Latysheva *et al*., 2006; Andreu & Yáñez-Mó, 2014; Baranyai *et al*., 2015; Xu *et al*., 2015). Albumin is the most abundant protein existed in plasma, and thus is widely used for a general marker for detecting protein contamination (Nicholson *et al*., 2000). The results suggested that CD63 was present in fractions 7 to 9, while albumin signal was detected from fractions 10 to 12. As mentioned above, the earlier results have suggested that fractions 7~9 contained a high concentration of EVs almost free of proteins, which was consistent with the presence of CD63 and albumin observed in the corresponding fractions (**Figure 2.9**).

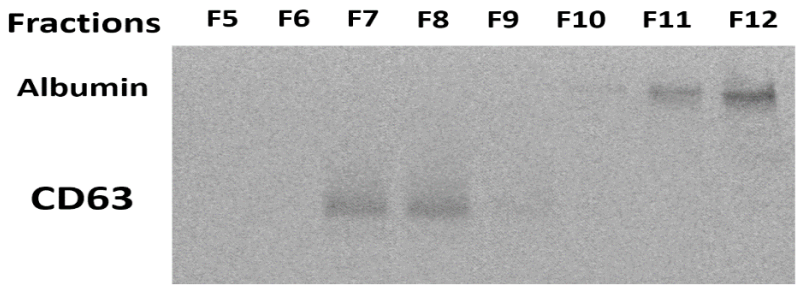


Figure 2.9. An example of identification of CD63 and albumin in fractions obtained from SEC. CD63 was observed in fractions 7~9, while albumin appeared from fractions 10 to 12.

2.3.4 Effects of freezing on EV isolation and analysis

Five healthy volunteers were recruited to provide samples which were used to evaluate the impact of freezing at − 80°C, for up to 6 months, on EV enumeration and characteristics.

a. Effect of freezing on EV numbers and average size detected by NTA

There was no influence of freezing at − 80°C for one week or one month on numbers of total EVs assessed by NTA (**Figure 2.10**). However, numbers of EVs significantly increased after storage at − 80°C for three months, four months and six months (**Figure 2.10**). There were no significant changes in EV numbers from three to six months of freezing (**Figure 2.10**).

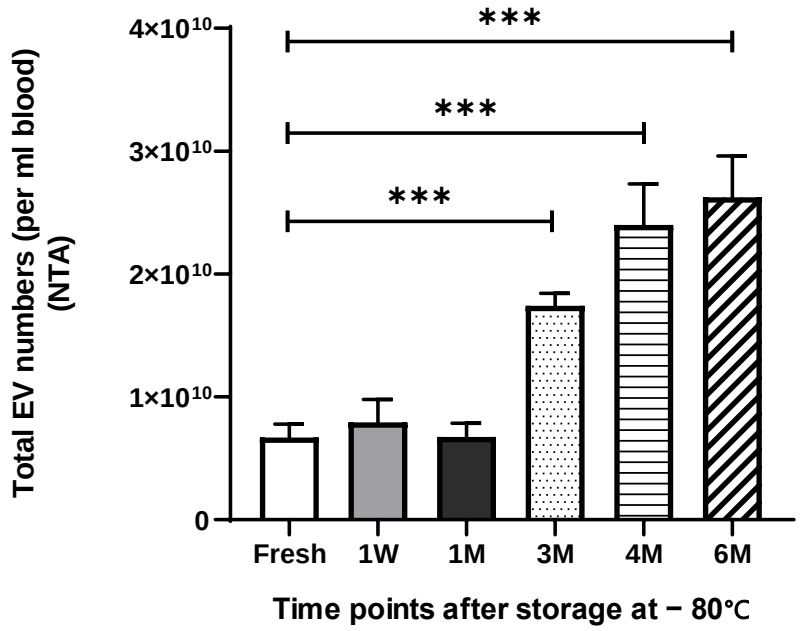


Figure 2.10. Numbers of fresh and frozen-thawed EVs detected by NTA. Data are n=5 (n=4 at 1 week and 6 months due to some missing samples). There were no changes in EV numbers after storage at −80°C for 1 week and 1 month compared to fresh samples, but they were significantly increased after freezing for 3, 4 and 6 months. ***p<0.001. *NTA, nanoparticle tracking analysis; W, week; M, month(s).*

The mean and mode size of EVs did not significantly change after freezing at − 80°C for one week or one month compared to fresh samples, but freezing for three months or more decreased EV size. Changes in EV size were stable after three months of freezing (**Figure 2.11**)

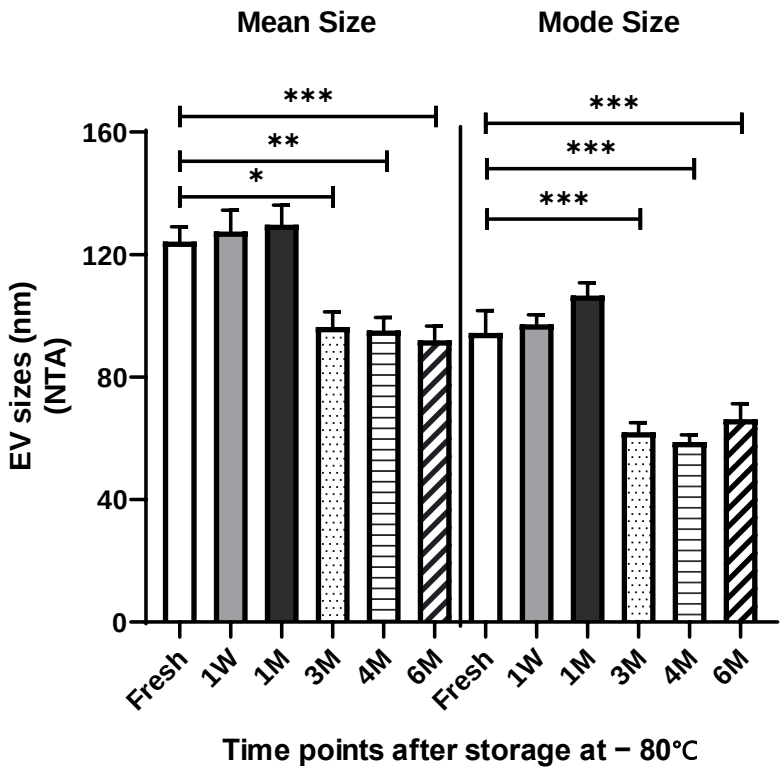


Figure 2.11. Size of fresh and frozen-thawed EVs detected by NTA. Data are n=5 (n=4 at 1 week and 6 months due to missing samples). There were no changes in either mean or mode size of EV numbers after storage at −80°C for 1 week or 1 month compared to fresh samples; however, they were significantly decreased after freezing for 3, 4 and 6 months. *p<0.05, **p<0.01, ***p<0.001. *NTA, nanoparticle tracking analysis; W, week; M, month(s).*

b. Effect of freezing on EV numbers detected by FCM

There was no influence of freezing at − 80°C for one week or one month on numbers of EVs assessed by scatter FCM (**Figure 2.12**) and fluorescence FCM (**Figure 2.13**). However, numbers of EVs significantly increased after storage at − 80°C for three months, four months and six months, and there were no significant changes in EV numbers from three to six months of freezing (**Figure 2.12** and **Figure 2.13**).

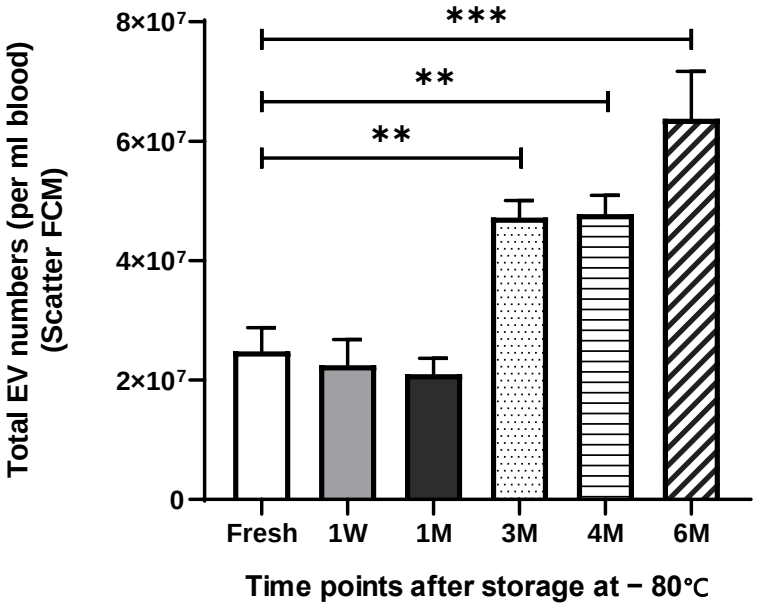


Figure 2.12. Numbers of fresh and frozen-thawed EVs detected by FCM on scatter mode. Data are n=5 (n=4 at 1 week and 6 months due to missing samples). There were no changes in EV numbers after storage at −80°C for 1 week or 1 month compared to fresh samples, but they were significantly increased after freezing for 3, 4 and 6 months. ***p*<0.01, ****p*<0.001. *FCM, flow cytometry; W, week; M, month(s).*

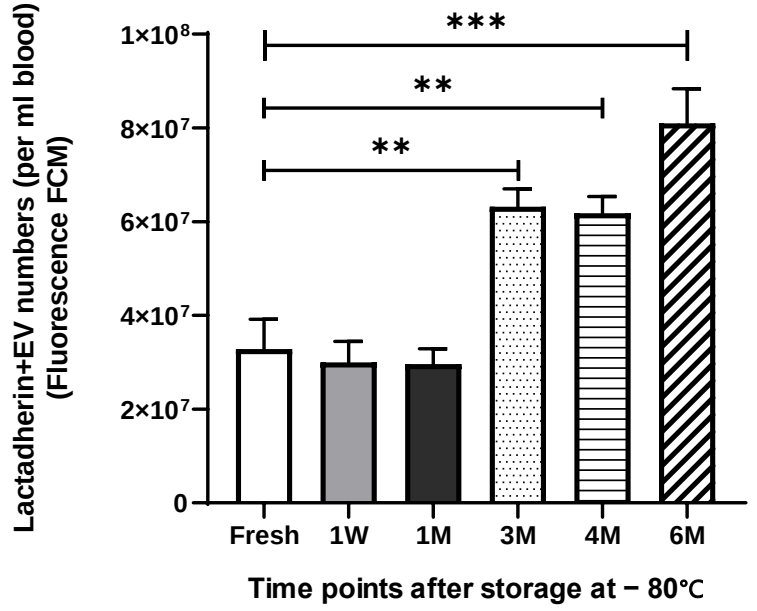


Figure 2.13. Numbers of fresh and frozen-thawed EVs detected by FCM on fluorescence mode. Data are n=5 (n=4 at 1 week and 6 months due to missing samples). There were no changes in EV numbers after storage at −80°C for 1 week or 1 month compared to fresh samples, but they were significantly increased after freezing for 3, 4 and 6 months. ***p*<0.01, ****p*<0.001. *FCM, flow cytometry; Lactadherin+EV: lactadherin-positive extracellular vesicles; W, week; M, month(s).*

2.4 Discussion

The trials presented in this chapter have contributed to the development of the protocols for the isolation, characterisation and storage of EVs, which were applied to the human study described in this thesis. Regarding the isolation of EVs, the pilot trial repeated the previous in-house experiment and confirmed that commercially available qEV columns performed better than hand-poured sepharose columns when applying SEC. Moreover, SEC has been found to outperform UC with respects to the yield/recovery of EVs and the separation of EVs from contaminants. Western blotting also confirmed the presence of EVs in the correct fractions following SEC by identifying the presence of a general EV marker, CD63 in fractions 7~9. Therefore, SEC by using qEV columns was finally selected for all EV isolation involved in the human study. Both NTA and FCM have been trialled for the enumeration and characterisation of EVs. NTA is able to detect smaller vesicles, but is unable to distinguish vesicles from some potential contaminants, even after SEC isolation. FCM analysis mainly focuses on larger vesicles, and fluorescence FCM allows the specific detection of labelled EVs and the phenotyping of EV subpopulations, which is described in **Chapter 3**. This chapter demonstrated that NTA and FCM were finally decided to be used in combination for all the numeration and characterisation of EVs, accepting the protocol that NTA and FCM are still not optimal and have some limitations (details see **Chapter 3**). Finally, there appeared to be no significant effect of freezing samples at − 80°C for less than one months on EV numbers; however, while storage at − 80°C for more than three months could induce the increased EV numbers. Thus freezing EVs for more than one month at − 80°C should either be avoided or factored into any subsequent analysis. Furthermore, it has been found to be practical and feasible to enumerate and characterise circulating EVs immediately after blood collection in the human study, thus finally only fresh samples obtained from the human study were subjected to EV numbers analysis. Although the majority of this work was not new to the field, it was (i) important in establishing competence with the technique and (ii) provided critical information for planning the intervention study, particularly around aspects where the literature was unclear.

2.4.1 Comparison between SEC and UC for isolation of EVs

Commonly used methods for the isolation and separation of EVs include UC, DGC (combined with UC or used individually), SEC, precipitation and immunoaffinity capture, each varying in the yield of EVs, the depletion of contaminants, labour-intensity, and the cost of the procedure (Brennan *et al*., 2020). As mentioned above, there are two aspects to evaluate the efficiency of the isolation method for EVs generally: one is the yield/recovery of EVs after isolation; another factor is the purity of EVs compared to contaminants or the enrichment of EV numbers by minimising contaminants. The current trial only applied the numbers of EVs detected by FCM to evaluate isolated EVs because NTA cannot be used to enumerate EVs in PFP (due to high level of interfering small particles in this medium), although some researchers have reported the recovery and enrichment results from NTA by using a different instrument version with a threshold setting (Böing *et al*., 2014; Nordin *et al*., 2015).

The difference in elution times between qEV columns and hand-poured sepharose columns is because qEV columns are prepared under pressure and are therefore more tightly packed compared to hand-poured ones. Furthermore, there is published evidence that qEV columns perform better than hand-poured sepharose columns with respect to yields of EVs and the ability to separate EVs from protein and cholesterol. The n=1 experiment to test this in the current chapter simply represented a demonstration of the author's ability to reproduce the observation as there was already sufficient confidence in the superiority of qEV columns. Various studies have reported that qEV columns separate EVs from contaminants with high efficiency (Lobb *et al*., 2015; Vogel *et al*., 2016; Gaspar *et al*., 2020; Ter-Ovanesyan *et al*., 2020; Brennan *et al*., 2020), and in agreement with the current findings, Ter-Ovanesyan *et al*. (2020) reported that qEV columns resulted in higher purity of EVs compared to albumin contamination than that from sepharose CL-2B columns. Another advantage of qEV columns is that they are rapid and ready-made, while sepharose columns are cheaper, but need to be packed before use every time and will result in variations from column to column.

Regarding the comparison between SEC and UC, the current findings suggested that SEC by qEV columns resulted in EVs of higher yield and recovery. Also, the higher purity of EVs compared to protein and cholesterol, and the enrichment of EV numbers by minimising protein and cholesterol were observed after SEC isolation by qEV columns compared to UC. In agreement with the current findings, one of the study examining rat plasma demonstrated that SEC-isolated EVs had higher particle yield and purity of particle compared to protein ratio than UC-isolated EVs, although SEC resulted in greater amounts of apolipoprotein (Apo) B positive lipoproteins and larger quantities of non-EVs protein (Takov *et al*., 2018). Brennan *et al*. (2020) also reported that a higher number of particles, especially particles < 60nm in serum was yield and a higher enrichment of particles by minimising protein was obtained after the isolation of SEC than that of UC, although there was once again co-isolation of more lipoproteins labelled by ApoB and ApoE after SEC compared to UC. In contrast, another study suggested that there was no difference in EVs yield or protein content after SEC compared with UC isolation (Mol *et al*., 2017). The reasons for this are not clear, but this discrepancy may because that the details of SEC and UC protocols vary in the different studies, such as the combination of ultrafiltration with SEC, or the different speed /duration of UC applied. UC is the most widely used method for EV isolation to date; however, a number of important disadvantages of this technique have been indicated. A key disadvantage of UC for EV isolation is the substantial destruction and aggregation of EVs after repeated centrifugations, leading to a relatively low recovery (Lamparski *et al*., 2002; Ismail *et al*., 2013). Taylor & Shah (2015) demonstrated that the recovery of UC-purified EVs can vary from 2%~80% based on published studies, but most of them reported very low yields. Yuana *et al*. (2015) indicated a < 30% EVs recovery after UC separation. Only 5% recovery of EVs isolated by UC was also reported in both Momen-Heravi's (2012) and Welton's (2015) studies, which is similar to the recovery reported in the current trial (~7% on scatter FCM and ~8% on fluorescence FCM). Furthermore, the suggestion that repeated centrifugation may induce the aggregation and rupture of EVs has been visualised by EM and fluorescence correlation spectroscopy (Yuana *et al*., 2015; Linares *et al*., 2015; Nordin *et al*., 2015). The current trial also demonstrated that EVs isolated by UC had a larger average diameter than those isolated by SEC. The larger size of UC-isolated EVs may be the result of the

aggregation of EVs during UC, which was also reported by other studies (Linares *et al*., 2015; Mol *et al*., 2017). The second major challenge is that the pellet contains potentially high levels of contamination with soluble proteins, protein aggregates, lipoproteins and microbes (Tauro *et al.,* 2012). In agreement with the current study, Coumans *et al.* (2017) reported that UC only resulted in 1~15-fold enrichment of EV numbers by minimising contamination relative to protein contaminants compared to the original material. Besides protein, the current trial indicated that the enrichment of EVs relative to numbers by minimising contamination of cholesterol was also relatively low.

There were attempts to separate EVs using UC combined with DGC, which is commonly used to separate EVs with a high yield of EVs and to overcome the co-isolation of contaminants exhibited in UC-isolated samples (Ramirez *et al*., 2018). Theoretically, DGC should allow the majority of lipoproteins to be separated from exosomes (1.10~1.11g/ml) and microvesicles (1.18~1.19g/ml) on the basis of their density, with only HDL potentially overlapping (1.06~1.21g/ml) (Xu *et al.*, 2015). However, the trial resulted in an even lower yield of EVs than UC, which is consistent with the findings reported by Brennan *et al*. (2020). Also, it proved to be technically challenging as the amount of material that can be loaded onto the density gradients is extremely small and requires western blotting for the detection of EVs because of very low yields (Lobb *et al.,* 2015). Therefore, UC combined with DGC was not considered any further.

EV isolation by SEC effectively excludes most contaminants based on their different sizes. Larger particles cannot enter the pores and can only travel along with fluid to elute earlier, while smaller particles travel through the pores and thus move more slowly through the column (Baranyai *et al*., 2015). This explains why EVs elute before the proteins, cholesterol and TAG. Coumans *et al*. (2017) indicated that the recovery of EVs from SEC can range from 40% to 90%. The better recovery is likely to be due to the fact that SEC relies on gravity flow and therefore results in less damage to EVs (Benedikter *et al*., 2017). In the current trial, FCM analysis indicated a recovery of approximately 50%~60%. A 44% recovery of lactadherin-binding EVs has been reported by Böing and colleagues (2014). Another study suggested a 70% recovery of

EVs after SEC isolation combining ultrafiltration (Nordin *et al*., 2015). Since SEC results in an only minimal overlap of EVs with protein, cholesterol and TAG, there is excellent enrichment of EV numbers with minimal contamination. The current results suggested that compared to the starting material, there was a 318-fold increase in the purity of EVs relative to protein and a 26-fold increase in the purity of EVs relative to cholesterol after SEC isolation. Similar results were reported by Böing *et al*. (2014), who noted a 70-fold increase in the purity of EVs compared to protein and an 8-fold increase in the purity of EVs compared to HDL after purification by SEC. The divergence in the figures for the recovery and enrichment of EVs following SEC may arise from the slight differences in the materials and protocols among these studies. For example, Böing *et al*. (2014) employed sepharose columns, while Nordin *et al*. (2015) used sephacryl columns to isolate EVs, which may lead to variation in the efficiency of isolation as both are different from the qEV columns employed in the current trial. Besides the better efficiency of SEC isolation, another major advantage of SEC is the rapid operation time and relatively low cost. In general, it takes only ~30min for SEC to isolate one sample, but UC requires several hours. Also, SEC does not require any specialised equipment, whereas an ultracentrifuge is needed for UC isolation.

Taken together, SEC clearly outperforms UC in terms of a higher yield of EVs and better separation of EVs from contaminants. Also, SEC is more practical for the large number of samples generated by the human intervention due to the easy operation and relatively low cost. For these reasons, SEC by using qEV columns was the EV isolation method of choice throughout this project.

2.4.2 Comparison between NTA and FCM for detection of EVs

In the current trial, samples were subjected to SEC before analysis by NTA or FCM, and therefore the analysis was performed on identical samples. EV concentrations determined by NTA were much higher than those determined by FCM, which is in line with the expectation, since FCM lacks the ability to resolve smaller particles, whereas NTA can resolve particles as small as 70nm. Some studies in the literature employed NTA to detect EV numbers, and these numbers of EVs vary considerably as a result of

differences in methodology. One study suggested that the number of SEC-isolated EVs in PFP was 4.2 *10^{10} /ml blood (Brennan *et al*., 2020), while another study reported EV number after SEC isolation as 2*10^{10}/ml blood (Gámez-Valero *et al*., 2016). Several studies reported EV numbers in healthy subjects to range from 0.5~5.0*10^{10} EVs per ml blood after UC isolation (Gardiner *et al*., 2013; Yuana *et al*., 2015; Jamaly *et al*., 2018). FCM is more widely used to enumerate circulating EVs. In the study of van der Pol *et al*. (2014), the numbers of EVs in normal individuals analysed by scatter FCM were 1.8*10^{7}/ml, whereas those detected by NTA were 300-fold higher. Yuana and co-workers (2015) reported 10^{4}-fold higher numbers of EVs after isolation by centrifugation and detection by NTA compared with analysis by scatter FCM. The numbers of fluorescently labelled EVs after (ultra)centrifugation in healthy subjects have been reported to range from 10^{7} to 10^{8} per ml blood when analysed by fluorescence FCM (Preston *et al*., 2003; Esposito *et al*., 2006; Yuana *et al*., 2015; Berckmans *et al*., 2019). In the current study, the numbers of EVs after SEC isolation detected by NTA, scatter and fluorescence FCM were 5.3*10^{10}/ml blood, 6.7*10^{7}/ml blood and 9.5*10^{7}/ml blood, respectively, and the numbers of EVs after UC isolation detected by NTA, scatter and fluorescence FCM were 3.0*10^{9}/ml blood, 7.8*10^{6}/ml blood and 1.4*10^{7}/ml blood, respectively.

NTA can detect much smaller EVs than FCM and can provide information about particle size distribution. However, the potential overestimation of EV concentrations by NTA has been discussed due to the size overlap between EVs and some contaminants, such as soluble proteins and lipoproteins. Previous studies using NTA have reported EV numbers in PPP (without isolation of EVs by SEC or UC) as approximately 1.5*10^{12}/ml, but this number of EVs was decreased by more than 98% when only particles labelled with a cell tracker dye were determined, indicating the presence of large numbers of contaminants when NTA analysis is applied directly to PPP (Dragovic *et al*., 2011). The range for the detection of particles by NTA is 30nm to 2000nm, so the smallest lipoproteins, namely HDL (7~12nm) and LDL (18~25nm) may escape detection as they are at the lower limits of resolution. However, intermediate-density lipoprotein cholesterol (IDL: 23~27nm) and larger lipoproteins, such as VLDL (30~60nm) and CM (100~1200nm) are a cause for concern as they may interfere with the analysis of EVs

by NTA (Colhoun *et al*., 2002). In order to minimise this possibility, SEC was performed to purify EVs from PFP before NTA analysis. The removal of 99% of the soluble proteins and > 95% of HDL by SEC has been demonstrated in the EV-enriched fractions by SEC (Baranyai *et al*., 2015). The results from the current study indicated that SEC enables the effective separation of EVs from contaminants. Although some large lipoproteins (chiefly CM) will still co-elute with EVs during SEC, contamination with CM can be avoided by collecting blood from at least 12 hours overnight fasted subjects (Sódar *et al*., 2016). An in-house experiment conducted by my colleague confirmed that the level of CM labelling using an ApoB48 kit was negligibly low in fractions 7~9 after isolating EVs from PFP using SEC in fasted subjects (unpublished data). Finally, a lower threshold of 70nm was set for sample analysis on NTA software, which would have excluded most of the IDL and VLDL, although also clearly excluded the very smallest exosomes (< 70nm) (Böing *et al*., 2014).

Gating of EVs using FCM has previously employed polystyrene beads mix containing 0.5µm, 0.9µm and 3µm beads as size calibrators (Wu *et al.,* 2014). Unlike cells, EVs scatter much less light as they are smaller than the laser wavelength, often generating signals even smaller than the background (Nolan & Duggan, 2018). Also, it was only recently discovered that the polystyrene beads have a higher refractive index (1.59) than EVs (1.39), leading to a > 10-fold lower degree of light scattering by EVs than similar-sized polystyrene beads (van der Pol *et al*., 2016). One study reported that 400nm polystyrene beads scattered light similarly to that scattered by EVs with a diameter of 1000nm (Chandler *et al*., 2011). Another study also noted that 500nm beads were only able to resolve EVs of around 800nm (van der Pol *et al*., 2012). Therefore, it is highly likely that the EV gate in previous studies captured not only EVs, but also platelets and apoptotic bodies. The silica beads applied in the current study have a comparable refractive index (1.43) to EVs (1.39) and have been reported to scatter light in a manner which more closely resembles cellular particles of the same size, therefore providing a significantly more accurate estimation of particle size than polystyrene beads. On the other hand, it has been well-established that the externalisation of PS in the outer leaflet of the plasma membrane during the early step of the cell activation process triggers membrane blebbing and EV shedding (Bevers *et*

al., 1983; Zwaal & Schroit, 1997). Accordingly, most studies investigating circulating EVs consider that PS-exposing EVs represent the majority of EVs (Morel *et al*., 2011; Boersma *et al*., 2005). Another reason why PS-exposing EVs have attracted great interest is due to the procoagulatory properties of PS on the EVs, since PS provides a surface for the binding of many components involved in the coagulation cascade, potentially contributing to the pathological process of atherosclerosis and cardiovascular events (Owens & Mackman, 2011; Tripisciano *et al*., 2017). Considering the theory of EV formation, Annexin V has been adopted as a general EV marker based on its availability to binding to PS on the cell membrane in a calcium-dependent manner (Boersma *et al*., 2005). However, many studies have recently demonstrated that not all circulating EVs are Annexin V positive and this may be because (i) these particles do not express PS; (ii) PS is expressed, but not sufficiently to be detectable by Annexin V; (iii) other PS-binding proteins have a higher affinity for PS and compete for Annexin V binding (Connor *et al*., 2010; Ayers *et al*., 2011; Arraud *et al*., 2014). Lactadherin, which also binds PS and has a higher affinity than Annexin V (Shi *et al*., 2004; Dasgupta *et al*., 2006), and therefore lactadherin was used to label PS-positive (PS+) EVs in the current trial. Its main advantage over FCM triggered on scatter is that the background noise and false scatter signals caused by non-EVs particles are minimised since they do not bind, are not fluorescent, and therefore will not be detected (Coumans *et al.,* 2017). Similarly, Arraud *et al.* (2016) reported 40-fold more EVs when triggering on fluorescence compared to scattering mode. Fluorescence FCM can also phenotype EVs derived from different types of cells by multicolour labelling. Although lactadherin performed well for staining total circulating EVs, an in-house experiment conducted by my colleague suggested that the co-detection of EV subpopulations was unsatisfactory, especially the co-detection of CD41+ for PDEVs, which was involved in the multicolour-labelling panel of EVs for the human study (unpublished data). Multicolour labelling in FCM is challenging because the fluorescence intensity of probes is limited for EVs and because there is spillover amongst fluorescence reagents. Lactadherin was conjugated to FITC and Annexin V was conjugated to APC in the trials conducted as part of this study. APC is brighter than FITC and has less spectral overlap with the PE used for CD41 detection compared to FITC. Considerable spillover of FITC into the PE detector will compromise resolution

sensitivity in the PE detector and result in suboptimal resolution of CD41; therefore, Annexin V instead of lactadherin was finally selected to label PS+EVs in the FCM protocols of phenotyping EVs applied in the human study (**Chapter 3**).

2.4.3 Western blotting to identify proteins associated with EVs

Western blotting, which can detect the presence of specific proteins associated with EVs, has been employed to confirm the existence of EVs. The potential markers for EVs include tetraspanins CD9 and CD63, Alix, TSG101 and HSP70 (Théry *et al.,* 2002; Witwer *et al.,* 2013). CD63, which directly binds to syntenin-1 and is involved in the biogenesis of EVs (Latysheva *et al.,* 2006), has been widely used in western blotting for the determination of EVs (Böing *et al.*, 2014; Baranyai *et al.,* 2015; Xu *et al*., 2015). Several studies have described CD63 as the most frequently identified protein in EVs (Andreu & Yáñez-Mó, 2014). Proteomic analyses have indicated that CD63 is enriched > 100-fold in B cell-derived EVs (Escola *et al.,* 1998). Crescitelli and associates (2013) also detected CD63 by FCM in EVs secreted by three different cell lines. In the current trial, CD63 was detectable between fractions 7 and 9 after SEC, which corresponds to another report in the literature (Böing *et al.,* 2014). The existence of albumin in later fractions also suggested the good separation of EVs from contaminating protein; again, this was consistent with reports in the literature (Baranyai *et al.,* 2015).

2.4.4 Effects of freezing on EV isolation and analysis

The current results indicated that the numbers of EVs remained constant after freezing at − 80°C for up to one month, but storage at − 80°C for three months increased EV numbers and decreased EV size. Beyond the three month point, EV numbers and size were stable.

The results suggested that EVs should ideally be analysed immediately in fresh samples in order to minimise alterations in EVs numbers and characteristics. However, most human studies require freezing of EVs for logistical reasons, and it is therefore important that the particles are not damaged or altered by the freeze-thaw process. Many studies have explored the optimal conditions for the storage of EVs, and they

indicated that the ideal temperature for storing EVs is − 80°C (Jeyaram & Jay, 2017); however, the effect of storage on EVs is still not consistent in the literature. Kreke *et al*. (2015) and Sarker *et al.* (2014) have reported that the numbers of exosomes measured by NTA were not altered following one week or one month of freezing at − 80°C. Lőrincz and co-workers (2014) similarly reported that neutrophilic granulocyte-derived EVs detected by FCM were stable after freezing at − 80°C for one day, one week and one month, which is consistent with the current findings. However, one study demonstrated that numbers of CD31+CD42b- and CD62E+ EDEVs significantly increased after freezing for both one week and one month, while numbers of CD144+EDEVs decreased (van Ierssel *et al*., 2010). Moreover, another study reported that Annexin V-positive (Annexin V+) EV numbers were significantly elevated after 1 month of freezing at − 80°C (Shah *et al*., 2008). Dey-Hazra and co-workers (2010) also reported that Annexin V+EV numbers were significantly increased within the first two weeks and then significantly decreased from one to two months after freezing at −80°C compared to fresh samples. It has been suggested that proteases can be activated by freezing and digest EVs into smaller particles (Dey-Hazra *et al*., 2010). The increased EV numbers with reduced average EV sizes after three months of freezing in the current trial would support this. Thus, it may explain why the numbers of AnnexinV+EVs reduced after storage of one to two months in the Dey-Hazra's study (2010) as they only focused on larger EVs detected by fluorescent FCM with 0.8μm and 3.0μm latex beads and they were therefore unable to detect freezing-generated smaller EVs. In agreement with the current findings, Yuana *et al.* (2015) found that the numbers of lactadherin+EVs increased 7-fold after the storage of six months at − 80°C compared to freshly analysed samples, although they also reported that numbers of CD61+PDEVs and CD235+erythrocyte-derived EVs (ErDEVs) detected by FCM, and EVs detected by NTA were unaffected by freezing for six months. Furthermore, some studies have reported that no significant change in EV numbers was observed after storage at − 80°C for one year (Lacroix *et al*., 2012; Jayachandran *et al*., 2012). Although there is no clear consensus regarding the optimal storage conditions prior to EV analysis, it should be kept in mind that fresh samples are ideal, if feasible. For further human work conducted as part of this project, where practical, counting and phenotyping of EVs was performed immediately after the collection of blood samples

on the visit day, while the remainder were stored at − 80°C for fatty acid composition and functional analysis (which could not be performed on fresh samples for logistical reasons).

There are some limitations which need to be considered. First, some of the pilot experiments presented were very limited in sample number, largely because they had been reproduced many times by other members of the research team and their purpose was mainly to establish technical competence. Second, only CD63 and albumin were targeted in western blotting to check the performance of SEC isolation for EVs, while the guidelines recommended that three or more different types of proteins should be assessed in EV isolates (Lötvall *et al*., 2014; Théry *et al*., 2018). Finally, trial IV only determined the effect of sample freezing on EV numbers, but not on fatty acid composition or functional analysis of EVs, both of which were assessed after freezing in the human study for logistical reasons.

2.5 Conclusion

Interest in EVs is greatly increasing due to their potential role in multiple pathological conditions and aetiology of several diseases, such as atherosclerosis and CVDs, and thus a potential role in diagnostic and therapeutic aspects of these diseases. A set of guidelines and criteria has been established for the standardisation of pre-analytical treatments, such as sample collection, isolation, storage, and analytical protocols for the enumeration and characterisation of EVs. This chapter explored options for the isolation, characterisation and storage of EVs based on guidelines and previous in-house experiments to enable decisions for a future human intervention study. The human study (HI-FIVE STUDY) aimed to investigate the effects of fish oil-derived n-3 PUFA on the generation and functional activities of EVs, and is described in the following chapters.

Chapter 3 Methods

3.1 Introduction

The HI-FIVE (**H**uman **I**nvestigation of the effects of **FI**sh oil on extracellular **VE**sicles) study investigated the effect of fish oil-derived n-3 PUFA on the generation and functional activities of EVs. Additionally, it evaluated the relationships of traditional cardiovascular risk markers with numbers and functional activities of circulating EVs in subjects with moderate CVD risk. The study (clinicaltrials.gov: NCT03203512) was conducted at the Hugh Sinclair Unit of Human Nutrition, University of Reading, in accordance with guidelines detailed in the Declaration of Helsinki, and also approved by the University of Reading Research Ethics Committee (UREC 17/18). The study design, recruitment, screening and methods for sample analysis are described in this chapter.

3.2 Study design

The study was a randomised, double-blind, controlled crossover intervention. A total of 40 subjects aged between 40 and 70 years with moderate CVD risk (see criteria below) were recruited and randomly allocated to consume either fish oil or control oil (high-oleic safflower oil) in the first 12-week intervention period. After a 12-week washout, they then crossed over to the other intervention for a further 12 weeks. Random assignment of subjects for intervention order ("1" and "2") was performed using online software (https://www.randomizer.org/). Fish oil and control oil capsules were of identical appearance and blinded by an individual not involved in the study. All investigators involved in the study remained blind until all sample and statistical analysis had been completed. The length of the intervention and washout periods was based on evidence that incorporation of n-3 PUFA in all cell types reaches a plateau after 12 weeks' intervention and that a 12-week washout period is sufficient to avoid carryover effects (Walker *et al*., 2015).

During the study, there were four intervention visits, which took place at the beginning and the end of each 12-week intervention period (weeks 0, 12, 24 and 36). Before each study visit, subjects were asked to abstain from alcohol and strenuous exercise during the 24 hours prior to the study day. On each visit day, subjects were asked to come to the Hugh Sinclair Unit of Human Nutrition, University of Reading in an unfed state (fasted, not eating or drinking anything but water from 8 pm the night before). Subjects had their height and weight measured via stadiometer, their BMI was calculated using a Tanita (Tanita MC-780MA P, Tanita Europe BV, Netherlands) and BP was measured using a blood pressure monitor (Omron M2 Upper Arm Blood Pressure Monitor, OMRON Healthcare Europe BV, United Kingdom). A blood sample of approximately 100ml was then collected into vacutainer tubes containing 3.2% sodium citrate (Greiner Bio-One, Gloucestershire, United Kingdom) for EV-related analysis (details see below). After the first visit, subjects were asked to start to consume six capsules per day of either fish oil (Wiley's Finest Easy Swallow Minis, Canada), providing a total daily intake of 1.8g n-3 PUFA, or high-oleic safflower oil (Wiley's Finest, Canada), providing 740mg oleic acid + 120mg linoleic acid per day. Subjects were advised to take capsules with breakfast, lunch and dinner (two capsules at each meal). Weeks 12-24 represented a washout period and then subjects crossed over to the other intervention product until week 36 (**Figure 3.1**).

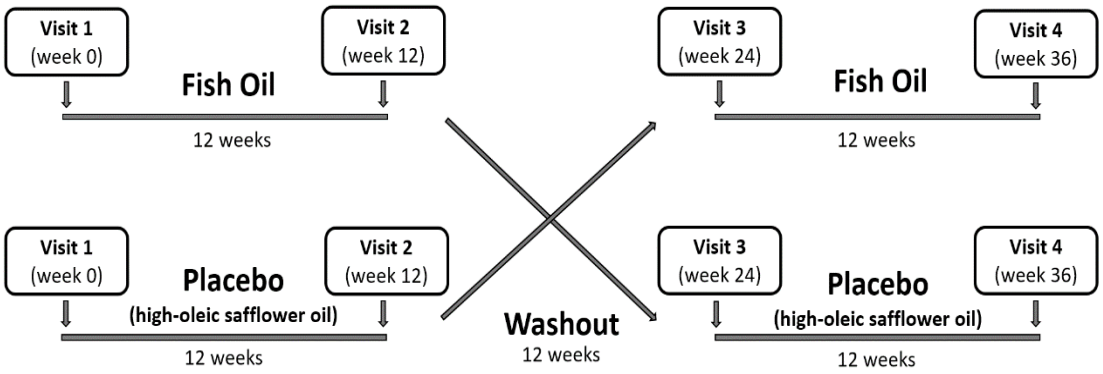


Figure 3.1. Study timeline

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

3.3 Selection of an appropriate control oil

A total of 212 papers published during the last decade were reviewed to identify the nature of the control used in fish oil supplementation trials and key reasons for the choice. Olive oil is by far the most commonly used control, followed by corn oil and high-oleic safflower oil. One of the reasons why olive oil and high-oleic safflower oil are commonly selected is because their overall fatty acid composition is reasonably representative of the background fat composition of the United Kingdom diet. Although olive oil and safflower oil contains a high proportion of MUFA, some studies have suggested that the consumption of high proportions of MUFA (19%~60% of total food energy per day), particularly when they replace saturated fat in the diet, can have effects on numbers of EVs (Weech *et al*., 2018; Vafeiadou *et al*., 2012). However, this does not appear to be the case with small increases in dietary MUFA (12%~14% of total food energy per day) (Vafeiadou *et al*., 2012). This suggests that a small dose of MUFA as a control in a trial would not add to the background intake of MUFA in the diet to a significant degree. The average intake of MUFA in the United Kingdom is ~26g, accounting for 12.5% total food energy per day for United Kingdom adults (Pot *et al*., 2012), so the addition of 2.7~3 g/d from the control supplements would have minimal impact on overall intake. Unlike olive oil, safflower oil contains few polyphenol compounds, which have been associated with bioactivity and potential effects on EVs (Rein *et al*., 2000; Gröne *et al*., 2020) and other CVD risk markers (Vita, 2005; Kishimoto *et al*., 2013). Some studies have used corn oil, containing a high proportion of linoleic acid, as a control (Wu *et al*., 2014). However, dietary intake of linoleic acid is lower than that of oleic acid (~15g/d, accounting for 6.5% of total food energy per day), so the use of corn oil as a control would result in a greater proportionate increase in the intake of n-6 PUFA. Furthermore, linoleic acid-rich oils are claimed to have proinflammatory effects (Farvid *et al*., 2014); although these claims have not been well substantiated, there is perhaps enough reason to be cautious about using them as a control. Some studies have employed mixtures of oils, such as palm oil combined with soybean oil, in order to optimise the ratio of saturated, monounsaturated and polyunsaturated fat to closely mimic that of the United Kingdom diet. However, blended oils tend to be more unstable and the use of palm oil is not only viewed

negatively from a sustainability point of view, but also results in fat which is semi-solid and a capsule which is opaque, making it difficult to match in appearance. For these reasons, and because high-oleic safflower oil contains a ratio of oleic to linoleic acid which better matches that in the United Kingdom diet, it was selected as the control oil. The fatty acid compositions of the fish oil and high-oleic safflower oil used in the study are shown in **Table 3.1**.

Table 3.1. The fatty acid compositions of capsules used in the study

	Fish oil (wt%)	High-oleic safflower oil (control oil) (wt%)
Palmitic acid (C16:0)	0.02	5.3
Stearic acid (C18:0)	0.07	2.0
Oleic acid (C18:1 n-9)	0.7	77.0
Linoleic acid (C18:2 n-6)	0.2	13.4
ALA (C18:3 n-3)	0.2	0.1
DGLA (C20:3 n-6)	0.1	0
AA (C20:4 n-6)	0.9	0
ETA (C20:4 n-3)	4.7	0.01
EPA (C20:5 n-3)	48.2	0.03
DPA (C22:5 n-3)	2.8	0
DHA (C22:6 n-3)	35.5	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 acid (wt%) in either fish oil or control oil capsules. *AA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid.*

3.4 Sample size

The sample size calculation was performed for the main endpoints: EV numbers, thrombus formation and platelet function. Based on a previous study (Wu *et al*., 2014), 34 subjects were sufficient 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 counts/µl (Wu *et al*. (2014) 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 be required to detect a significant effect of n-3 PUFA on platelet aggregation and PS

exposure (Phang *et al*., 2012). A total of 34 subjects was therefore considered to be the minimum required, but 40 subjects were recruited to allow for a 15% dropout.

3.5 Recruitment

Potential volunteers were recruited by using the Hugh Sinclair Unit of Human Nutrition volunteer database, emailing advertisements to staff and students of the University of Reading, to members of local community groups, such as the Women's Institute and to staff members in large local organisations and companies, such as Reading Borough Council and The Oracle. Study information was also advertised in local newspapers (The Reading Chronicle and The Wokingham Paper), magazines (50 Plus) and websites (Facebook and Nextdoor). Posters and leaflets were distributed in public places, such as around the University campus and/or in community centres and shops. Interested volunteers were assessed for their initial eligibility for the study by completing a medical and lifestyle questionnaire via email or phone. Exclusion criteria are listed below. A participant information sheet, which outlined the details of the study, was provided to all potential participants and those indicating an interest and fulfilling the criteria were invited to attend a screening visit at the Hugh Sinclair Unit of Human Nutrition.

Exclusion Criteria:

- Underweight (BMI: < 18.5kg/m^2)
- Anaemia (haemoglobin concentration < 12.5g/L in men and < 11.5g/L in women)
- hyperlipidaemia (TC concentration > 8mmol/L)
- Diabetes (diagnosed or fasting glucose concentration > 7mmol/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 > four 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 > 21units/week for men and > 15units/week for women or have a history of alcohol misuse
- Regularly consume oily fish and/or dietary supplements (it will be considerable if subjects are willing to stop or reduce oily fish consumption no more than one portion per month for three months before the study and keep it during the whole study period)
- Planning to start or on a weight-reducing regimen
- Intense aerobic exercise (> 20 minutes, 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

3.6 Screening

During the screening visit, all procedures were explained in detail and participants were offered the opportunity to ask questions. They gave their consent by completing the consent form, which was also signed by the researcher. After subjects had their height, weight (Tanita MC-780MA P, Tanita Europe BV, Netherlands) and BP (Omron M2 Upper Arm Blood Pressure Monitor, OMRON Healthcare Europe BV, United Kingdom) measured, a blood sample of approximately 5ml was taken into a K_3EDTA tube (Greiner Bio-One, Gloucestershire, United Kingdom) for full blood counts at the Royal Berkshire Hospital Pathology Department, and a sample of approximately 9ml was taken into a serum-separating tube (Greiner Bio-One, Gloucestershire, United Kingdom) for analysis of blood lipids, glucose levels and markers of liver & kidney function. Serum-separating tubes were kept upright at room temperature for 30

minutes (and no more than 60 minutes) and then centrifuged at 1700xg for 15 minutes at room temperature (Heraeus Labofuge 400R Centrifuge, Thermo Scientific, United Kingdom). The plasma (160µl) was collected and analysed by iLab (iLab 600 Clinical Chemistry System, Diamond Diagnostics, United States) for lipid profile (TC, TAG and HDL-C), glucose, alanine transaminase (ALT), alkaline phosphatase (ALP) and Gemma glutamyl transferase (GGT), kidney and liver function (total bilirubin, creatinine and uric acid) using standard reagent kits (Werfen Limited, Warrington, United Kingdom).

3.7 Selection of eligible subjects with moderate risk of CVDs

There are four common scoring systems used for selecting subjects classified to be at mild or moderate CVD risk, as follows: the Framingham Risk Score (FRS) (D'Agostino *et al*., 2008), the Joint British Societies' guidelines (JBS2) (British Cardiac Society [BCS] *et al*., 2005), systematic coronary risk evaluation (SCORE) (Perk *et al*., 2012), QRISK 2 (Hippisley-Cox *et al*., 2008). Some studies employ scoring systems which are loosely based on a combination of the above, and this has been the case for previous studies conducted in our group, including a recent study examining the effects of fish oil supplementation on endothelial progenitor cells and microparticles (Wu *et al*., 2014). However, it was considered important to re-visit this to check whether there were other options available. A detailed review of ~25 papers using different screening methods was conducted and the screening data from Wu's study (2014) was used to compare the screening systems. The evaluation of these scoring systems is shown in **Table 3.2**.

Table 3.2. The characterisation of different scoring systems for CVDs risk

Scoring system	Assessment risk factors	Moderate risk*	Advantages	Disadvantages
JBS2 (2005)	Age; Gender; TC/HDL-C ratio; SBP; Smoking; Diabetes	10%~20%	• Straightforward • Available for > 65yrs	• Lacks several traditional risk factors • Overestimates risk for <69yrs and underestimates risk for > 70yrs

FRS (2008)	Age; Gender; HDL-C; TC; SBP; Smoking; Diabetes; Family history	10%~19%	• Commonly used • Available for > 65yrs • Guidance for CVDs treatment	• Relatively complex • Underestimates risk in women • Based on American population
QRISK 2 (2008)	Age; Gender; Ethnicity; TC/HDL-C ratio; BMI; SBP; Family history; Smoking; Clinical information (Diabetes; chronic kidney disease; atrial fibrillation; rheumatoid arthritis; BP treatment)	10%~20%	• Straightforward • Includes BMI, family history, ethnicity and clinical disease • Based on United Kingdom population	• Only available as online calculator, therefore subject to updates, making earlier versions unavailable
SCORE (2012)	Age; Gender; TC; SBP; Smoking;	1%~5%	• Straightforward • Based on United Kingdom population	• Only suitable for 40~65yrs • Lack many traditional risk factors
Wu *et al.* (2014)	Gender; TC; HDL-C; BMI; SBP; DBP; Waist circumference; Family history	≥ 2 points	• Relatively straightforward Includes BMI, family history and waist circumference	• Based on American population • No upper limit for high risk • Underestimates risk

*percentage risk of having a heart attack or stroke within ten years as moderate CVD risk. *BMI: body mass index; DBP: diastolic blood pressure; FRS: Framingham Risk Score; HDL-C: high-density lipoprotein cholesterol; JBS2: Joint British Societies' guidelines; SBP: systolic blood pressure; SCORE, systematic coronary risk evaluation; TC, total cholesterol.*

Four key factors were considered important in the selection of the scoring tool: (i) inclusion of the most important risk factors for CVD, (ii) close alignment of inclusion/exclusion decisions when compared to screening data from previous cohorts, (iii) straightforward and easy to assess, (iv) suitable for the United Kingdom/European population. JBS2 and SCORE may incorrectly categorise subjects because they lack some important risk factors, such as family history and BMI, although they are very easy to apply. The charts provided in JBS2 are for three age ranges: < 50yrs, 50~59yrs, and ≥ 60yrs, whereas the risks given for these three age ranges are based on the actual ages of 49, 59, and 69yrs, respectively. Therefore, the JBS2 tends to result in an overestimation of risk within the two younger age bands (except in people aged exactly 49 or 59yrs), and an overestimation for people aged < 69yrs and an

underestimation for those aged > 70yrs are expected in the older age band (British Cardiac Society [BCS] *et al*., 2005). The FRS, which also does not include BMI as a risk factor, is still the most commonly used system in recent decades. However, a systematic review, which analysed 27 studies with data on predicted and observed risk for CVDs, indicated that FRS underestimated in a high risk population and overestimated in a low risk population (Brindle *et al*., 2006). The scoring system applied in Wu's study (2014) was based on the FRS, but modified to include BMI and waist circumference. QRISK2 incorporates important factors such as ethnicity, clinical diseases and treatment information, and is regarded to perform well for discriminating different levels of risk. In order to compare QRISK2 and the scoring system employed by Wu *et al*. (2014), data from 20 individuals screened as being at moderate risk and 20 excluded individuals (low risk) in the Wu's study (2014) were re-evaluated using QRISK2. The result was that of the 20 included subjects, ten individuals remained with a moderate risk categorisation, but four were deemed to be low risk and six were scored as high risk according to QRISK 2. For the 20 excluded subjects, 17 were indeed at low risk and three were evaluated as moderate risk. One of the limitations of the approach used in the study by Wu *et al*. (2014) is its inability to discriminate between moderate and high risk, which is a clear advantage of the QRISK2 system. Furthermore, QRISK2 is thought to be better calibrated to the United Kingdom population than the FRS and modified versions of the FRS, which were originally based on an American population (Hippisley-Cox *et al*., 2008). Therefore, the QRISK2 system was selected as the screening tool for the HI-FIVE study.

Using QRISK2, age, gender, ethnicity, United Kingdom postcode, smoking status, disease information, family history, the ratio of plasma TC to HDL-C, SBP, height and weight were included for risk evaluation and provided a percentage of risk of having a heart attack or stroke within the next ten years (**Figure 3.2**, https://qrisk.org/2017/). Eligible subjects were required to have a risk score between 10% and 20%, which was considered to represent moderate risk of CVDs.

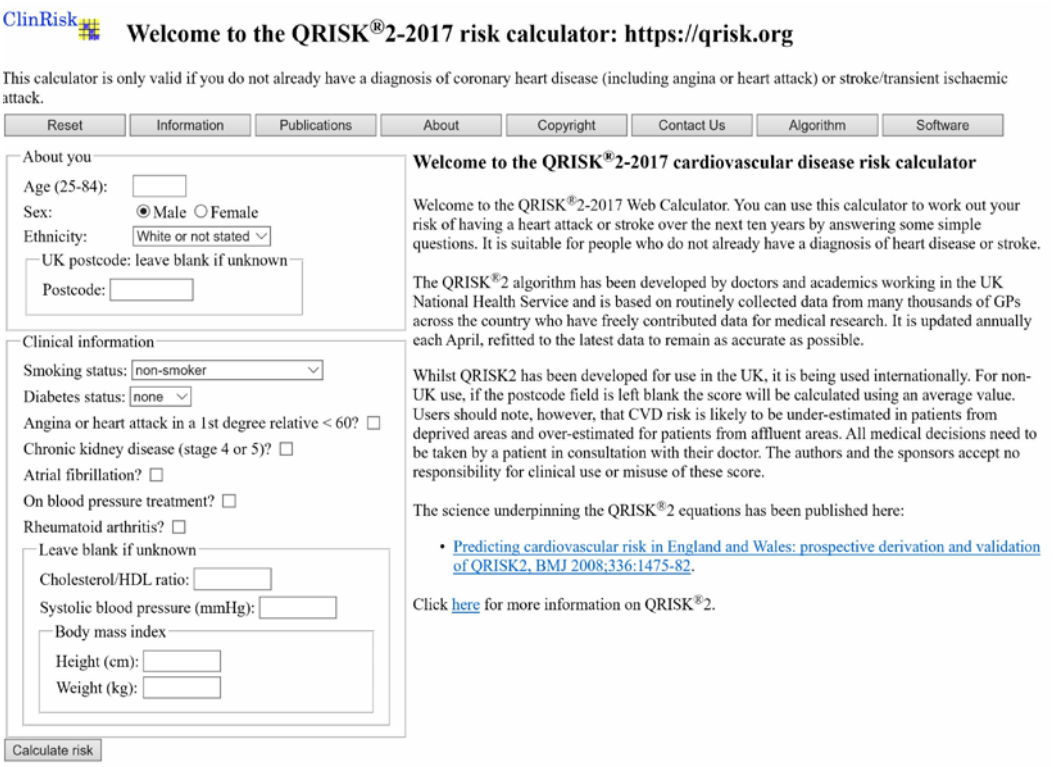


Figure 3.2. Features of the QRISK2 scoring system for selection of eligible subjects.
Subjects were enrolled if their risk score was between 10% and 20%.

Unsuitable subjects were excluded based on the above exclusion criteria. In addition, the kidney and liver function of subjects measured by iLAB had to be within the normal range (**Table 3.3**). Following the screening session, suitable volunteers were contacted by one of the study investigators and informed that they were eligible to attend the study. If the screening results of participants indicated any cause for concern, they were advised to discuss this with their GP.

Table 3.3. Reference ranges for ILAB screening tests

Parameter	Reference range Men	Reference range Female
Liver enzymes		
ALT	0-55 IU/L	0-55 IU/L
ALP	38-126 U/L	38-126 U/L
GGT	12-58 IU/L	12-58 IU/L
Kidney and Liver Function		
Total bilirubin	3-22 µmol/L	3-22 µmol/L
Creatinine	62-106 µmol/L	44-80 µmol/L
Uric acid	208-506 µmol/L	149-446 µmol/L

Reference ranges obtained from Paul Robinson at the Royal Berkshire Hospital. *ALP, alkaline phosphatase; ALT, alanine transaminase; GGT, Gemma glutamyl transferase.*

The intervention started in November 2017 and finished in March 2019. A participant flow diagram is illustrated in **Figure 3.3**. Of the 416 subjects contacted, a total of 58 subjects were screened, 42 individuals were enrolled on the trial and 40 completed the study.

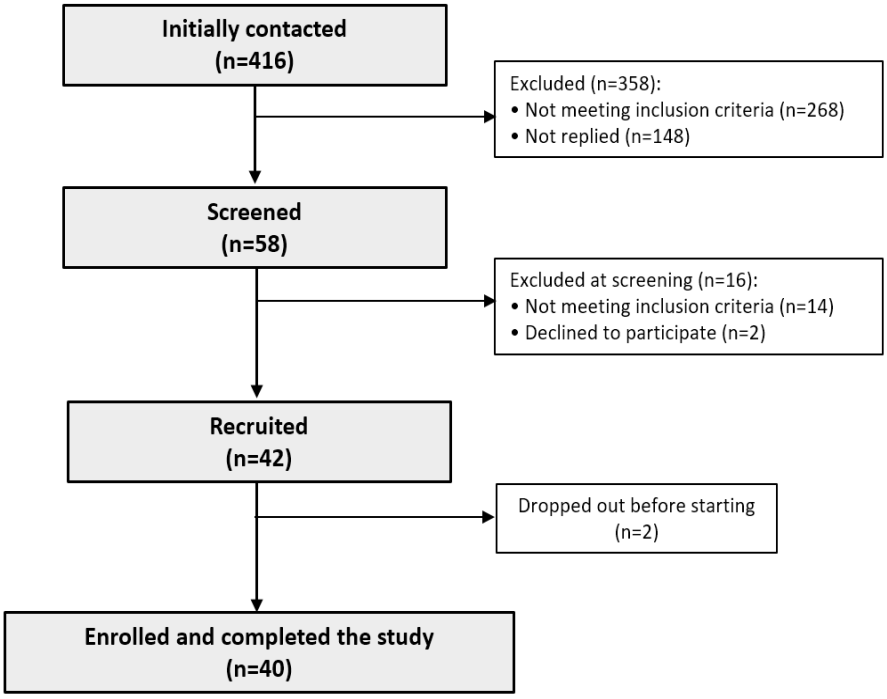


Figure 3.3. Participant flow diagram.

3.8 Study objectives

The objectives of the study were divided into two strands and this PhD project focused on strand 1, while strand 2 was largely conducted by other members in the research group (**Figure 3.4**).

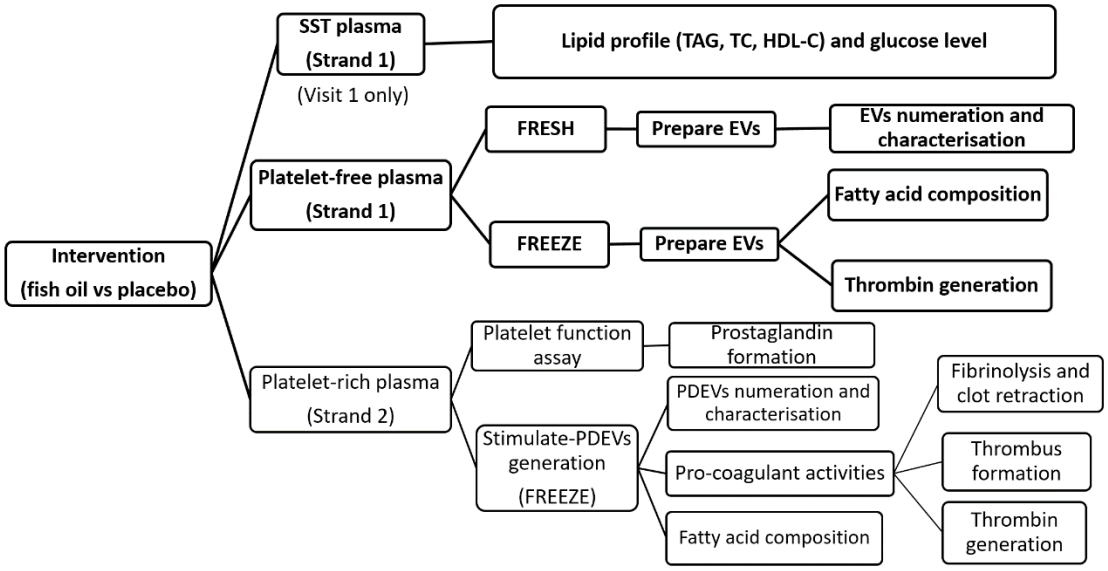


Figure 3.4. Overview of the experimental work. Strand 1 focused on the numeration and functional analysis of circulating EVs; strand 2 focused on the numeration and functional analysis of PDEVs. *EVs, extracellular vesicles; HDL-C: high-density lipoprotein cholesterol; PDEVs, platelet-derived extracellular vesicles; TAG, triacylglycerol; TC, total cholesterol.*

Strand 1:

a. the relationships between cardiovascular risk markers and numbers and procoagulatory activities of total circulating EVs (preintervention data).

b. the influence of n-3 PUFA supplementation on the numbers of total circulating EVs and EV subpopulations, and their fatty acid composition and procoagulatory activities.

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

3.9 FFQ analysis

The FFQ used in this study was modified from European Prospective Investigation of Cancer (EPIC)-Norfolk FFQ and analysed by FFQ EPIC Tool for Analysis (FETA) software, which can provide general food group and nutrient intake data.

3.10 Blood collection and processing

On the visit day, subjects were asked to come to the Hugh Sinclair Unit of Human Nutrition in an unfed state (fasted, not eating or drinking anything but water from 8 pm the night before). After subjects had their height, weight (Tanita MC-780MA P, Tanita Europe BV, Netherlands) and BP (Omron M2 Upper Arm Blood Pressure Monitor, OMRON Healthcare Europe BV, United Kingdom) measured, a blood sample of approximately 100ml was collected into vacutainer tubes containing 3.2% sodium citrate (Greiner Bio-One, Gloucestershire, United Kingdom). The tourniquet was removed once starting blood collection, and the first 2 to 3ml of blood was discarded. Blood samples were kept at room temperature and processed within 1 hour. Approximately 35ml of blood was processed to generate PFP for the analysis of circulating EVs (strand 1), while the rest was used to generate platelet-derived EVs for the analysis in strand 2.

3.11 Preparation of PFP

The first step required for EV analysis was the preparation of PFP (**Figure 3.5**). Blood samples were transferred into four 15ml falcon tubes (Fisher Scientific, Loughborough, United Kingdom) and PFP was prepared as shown in **Figure 3.5**. One of the aliquots was immediately used for the numeration and characterisation of circulating EVs by both NTA and FCM while the remainder were stored at − 80°C for fatty acid composition and functional analysis.

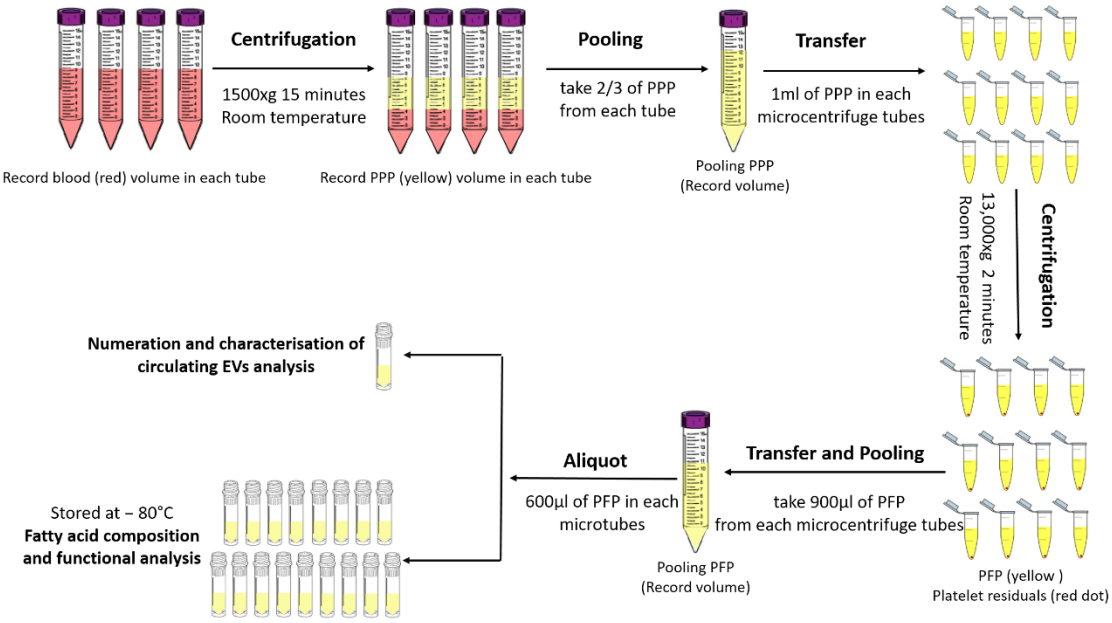


Figure 3.5. Preparation of platelet-free plasma. *PPP, platelet poor plasma; PFP, platelet free plasma.*

Although blood cells and platelets were removed by the process described above, PFP is still a very complex solution, potentially containing contaminating soluble proteins, protein aggregates and lipoproteins. Therefore, PFP was subjected to SEC to isolate EVs prior to NTA analysis. Although SEC was performed prior to FCM in **Chapter 2**, this was not the case in the intervention study, where PFP was simply diluted 50-fold and subjected to fluorescent labelling. This was partly because contamination is not an issue for flow cytometric analysis, but also because the aim was to ensure maximum capture of circulating EVs and SEC by nature only allows capture of EVs which elute within fractions 7-9. In retrospect, it would have been valuable to have conducted the FCM on samples with and without prior SEC.

3.12 EV isolation and NTA analysis

The processes of EV isolation by Izon qEV columns and characterisation EV in fractions 7~9 after SEC by NTA were described in **Chapter 2 Section 2.2**. The final population obtained was referred to as total EVs (TEVs) in the current study. Although NTA is more sensitive in detecting small EVs and more accurate in measuring EV sizes (within

5% of the expected size) (Gardiner *et al*., 2013), it is still limited in its ability to phenotype EVs and discriminate EVs from other contaminants. Attempts in our laboratory to label EVs with duramycin (which specifically binds PE) conjugated to Atto488 (a fluorescent dye, which has superior stability and brightness to traditional fluorophores) and analyse them using fluorescence NTA were unsuccessful as it was extremely difficult to achieve sufficient labelling signal for detection. This may due to photobleaching of the probe, either before EVs appear in the field-of-view of the microscope or before EVs can be recorded for a sufficient length of time for accurate size calculation, since NTA requires a relatively longer analysis time compared to FCM (van der Pol *et al*., 2016). Therefore, despite the fact that the NanoSight 300 has fluorescence capability, phenotyping of EVs by fluorescence NTA is not currently achievable. Instead, multicolour fluorescence FCM to characterise EVs was applied together with NTA in the current study, based on the MISEV Guidelines proposed by the ISEV in 2014, which recommended a combination of detection techniques for the accurate enumeration and phenotyping of EVs (Lötvall *et al*., 2014).

3.13 FCM analysis

The details of flow cytometer set-up and gating strategy were described in **Chapter 2 Section 2.2**. The current study employed a three-antibody panel in fluorescence FCM consisting of Annexin V, anti-CD41 and anti-CD105 to characterise EVs by fluorescence triggering, which was improved compared to the protocols used in **Chapter 2**. Annexin V is a general EV marker to label the majority of PS-exposing EVs (Morel *et al*., 2011; Boersma *et al*., 2005). CD41 (GPIIb receptor), which is an integrin complex only abundantly expressed on platelets and binding fibrinogen and vWf during platelet activation was used to identify PDEVs (Floyd & Ferro, 2012). Finally, CD105 (endoglin), which is a type I membrane glycoprotein highly expressed on endothelial cells and regarded as an optimal identifier of activation and proliferation of endothelial cells (Simak *et al*., 2006; Kapur et al., 2013) was used to stain EDEVs. Therefore PDEVs were double-stained as Annexin V-positive and CD41-positive (Annexin V+/CD41+) and EDEVs were identified as Annexin V-positive and CD105-positive (Annexin V+/CD105+).

Before being incubated with samples, all antibodies and isotype-matched controls (**Table 3.4**) were filtered through 0.1µm pore size centrifugal filter units (Millipore UK Limited, Hertfordshire, United Kingdom) by centrifugation at 1000xg for 10~20 minutes at room temperature (Eppendorf Centrifuge 5415R, DJBlabcare, Newport Pagnell, United Kingdom). Annexin V buffer was prepared by adding 1µl of argatroban (a direct thrombin inhibitor used to prevent plasma clotting caused by the calcium in the Annexin V buffer) into the 10 times diluted Annexin V Binding Buffer (10X) (Cambridge Bioscience Ltd, Cambridge, United Kingdom).

Table 3.4. A summary of fluorescent antibodies and isotype-matched controls used for FCM

Parameter	Fluorochrome	Clone	Final concentration	Specificity
Annexin V [a]	APC	N/A	8 µl/ml	PS
CD41 [b]	PE	PL2-49	8 µl/ml	Platelets
CD105 [c]	eFluor450	SN6	16 µl/ml	Endothelial cells
IgG1 [b]	PE	2DNP-2H11/2H12	8 µl/ml	Isotype control
IgG1 [c]	eFlour450	P3.6.2.8.1	4 µl/ml	Isotype control

[a] Fisher Scientific, Loughborough, United Kingdom; [b] Diagnostica Stago LTD, Theale, United Kingdom; [c] Life Technologies LTD (Invitrogen division), Paisley, United Kingdom. *APC, Allophycocyanin; N/A, Not applicable; PE, phycoerythrin*; *PS, phosphatidylserine.*

Fluorescence labelling allows EVs of interest to be discriminated without the need for isolation before FCM analysis. Therefore, 5µl of PFP was added into six nonsticky microcentrifuge tubes (Alpha Laboratories Ltd, Hampshire, United Kingdom), which contained 5µl FcR blocking reagent (Miltenyi Biotec Ltd, Surrey, United Kingdom) and Annexin V buffer (amounts are presented in **Table 3.5**) and incubated for 15 minutes in the dark at room temperature. Antibodies and isotype-matched controls were then added and samples incubated for another 15 minutes in the dark at room temperature. A summary of sample tubes, including antibody amounts and types, is shown in **Table 3.5**. After incubation, samples were diluted with 200µl Annexin V buffer and transferred into FACS flow tubes (BD Biosciences, Wokingham, United Kingdom), ready to be analysed by flow cytometry. PBS was run through the flow cytometer until a rate of < 100 events/second was achieved to ensure that the flow cytometer was

clean enough for this sensitive analysis. Each sample was then analysed successively on both light scatter and fluorescence mode for 1 minute at a low flow rate.

Table 3.5. A summary of sample tubes analysed by FCM

Number	Tubes	PFP (µl)	FcR (µl)	AnnV buffer(µl)	AnnV (µl)	CD41PE (µl)	IgG1-PE (µl)	CD105-eF450(µl)	IgG1-eF450(µl)
1	**AnnV buffer alone**	-	5	45	-	-	-	-	-
2	**PFP alone**	5	5	40	-	-	-	-	-
3	**CD41-PE Control**	5	5	32	2	-	2	4	-
4	**CD105-eFluor450 Control**	5	5	35	2	2	-	-	1
5	**Main sample**	5	5	32	2	2	-	4	-
6	**AnnV control**	5	5	32(PBS)	2	2	-	4	-

AnnV, Annexin V; PBS, phosphate-buffered saline; PE, phycoerythrin; PFP, platelet-free plasma.

A typical series of plots resulting from analysis of samples by both light scatter triggering (i.e. based on size and granularity) and fluorescence triggering (i.e. based on positive staining for a specific antibody) is presented in **Figure 3.6**. Fluorescence signals used were Allophycocyanin (APC) (λex. = 650nm, λem. = 660nm) and Pacific Blue (PB) (λex. = 405nm, λem. = 450nm). Particle detection on fluorescence mode (**Figure 3.6 right columns**) is higher than that on scatter mode (**Figure 3.6 left columns**) because scatter mode requires a size threshold of 240nm to exclude debris, whereas fluorescence can detect all fluorescently labelled particles down to the instrument threshold. **Figure 3.6A1~5/B1~5** present an example of the typical flow cytometric analysis of negative controls, and **Figure 3.6A6~B6** present that of the main sample on both light scatter and fluorescence mode.

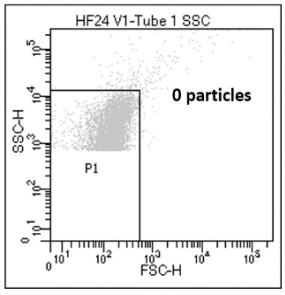
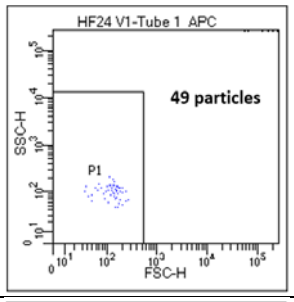
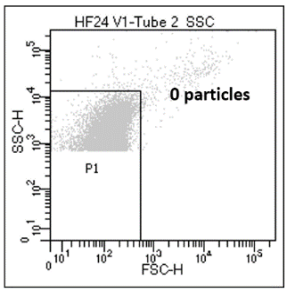
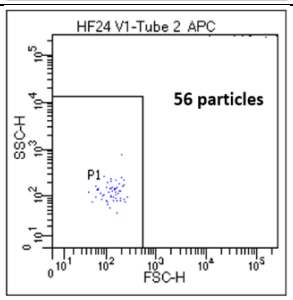
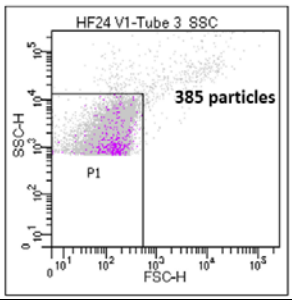
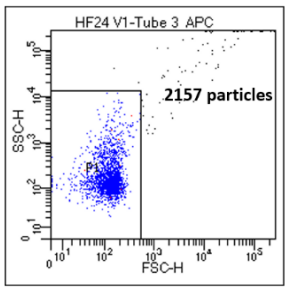
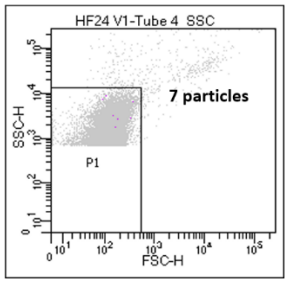
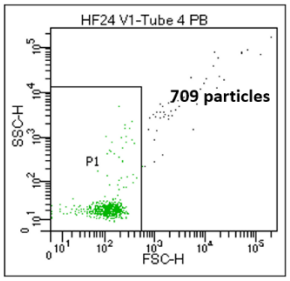
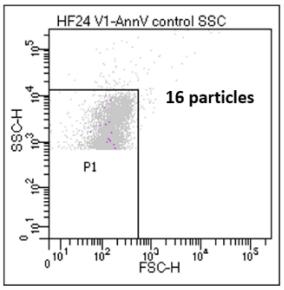
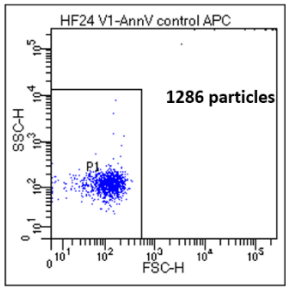
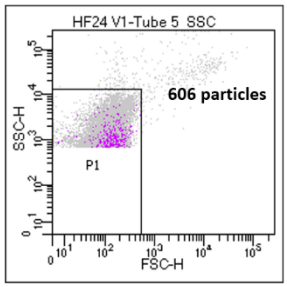
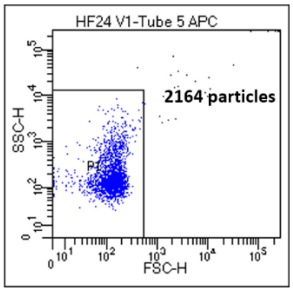
Light Scatter Triggering		Fluorescence Triggering	
(A1)		(B1)	
(A2)		(B2)	
(A3)		(B3)	
(A4)		(B4)	
(A5)		(B5)	
(A6)		(B6)	

Figure 3.6 Flow cytometric analysis of sample tubes on both light scatter and fluorescence mode. All plots in the left columns (**A1~6**) represent the labelled particles detected by light scatter triggering (in purple) and plots in the right columns (**B1~6**) represent the labelled particles detected by fluorescence triggering (APC: in blue and PB: in green); P1 is an EV gate that was set by using ApogeeMix beads to identify all particles above the SSC threshold of 240nm and up to a size of 1000nm, and the particles within this gate are presented in grey. Tube 1 contained Annexin V buffer only; there were therefore no particles detected by light scatter triggering (**A1**) and 49 particles detected on fluorescence APC triggering (**B1**), which represents background fluorescence of Annexin V buffer. Tube 2 contained unlabelled PFP only; no particles were detected by light scatter triggering (**A2**) and 56 particles were detected by fluorescence APC triggering (**B2**), again representing background fluorescence of unlabelled PFP. Tube 3 was a CD41-PE control, which contained all antibodies except for anti-CD41-PE; 385 particles were detected by light scatter triggering (**A3**) and 2157 particles by fluorescence APC triggering (**B3**), demonstrating all APC fluorescently labelled particles excluding CD41 labelled particles. Tube 4 was a CD105-eFluor450 control, which contained all antibodies except for anti-CD105-eFluor450; 7 particles were detected on light scatter triggering (**A4**) and 709 particles detected on fluorescence PB triggering (**B4**), demonstrating all PB fluorescently labelled particles. The Annexin V control tube contained Annexin V antibody only; 16 particles were detected by light scatter triggering (**A5**) and 1286 particles were detected by fluorescence APC triggering (**B5**), representing the background fluorescence of Annexin V. Tube 5 was the main sample, which contained all antibodies; 606 particles were detected by light scatter triggering (**A6**) and 2164 particles were detected by fluorescence APC triggering (**B6**), demonstrating all APC fluorescently labelled particles including CD41 labelled particles. *AnnV, Annexin V; APC, Allophycocyanin; PB, Pacific Blue; SSC, side scatter.*

The characterisation of EVs by analysis in fluorescence mode is illustrated in **Figure 3.7**. When triggering on APC fluorescence, only APC fluorescently labelled particles are visible and the particles within the P1 gate were therefore identified as Annexin V+EVs (PS+EVs) (**Figure 3.7Ai**). Annexin V+EVs which also stained positive for CD41-PE were identified as PDEVs and displayed in an APC vs PE quadrant plot to identify the double-positive population (**Figure 3.7Aii**). However, there were only a few Annexin V labelled EVs co-expressing CD105 (**Figure 3.7Aiii**), suggesting that CD105-labelled EVs should be triggered on PB fluorescence signal. When the sample was analysed on PB fluorescence, PB fluorescently labelled vesicles within P1 were only CD105-positive particles (**Figure 3.7Bi**). Annexin V+EVs which also stained positive for CD105-eFluor450 were identified as EDEVs and therefore the APC vs PB quadrant plot was used to identify the double-positive population (**Figure 3.7Biii**). The APC vs PE quadrant plot (**Figure 3.7Bii**) confirmed that few Annexin V labelled EVs co-expressing CD41 were observed on the PB fluorescence signal.

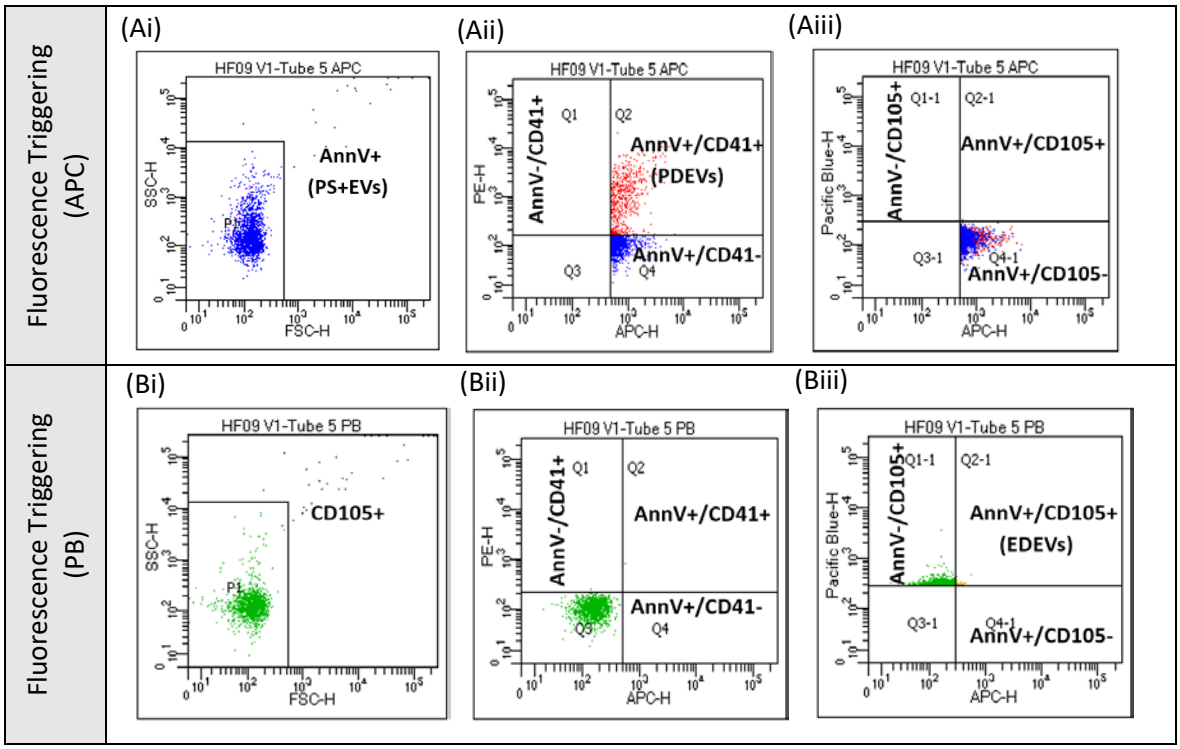


Figure 3.7 Flow cytometric analysis of a typical sample of PFP labelled with Annexin V-APC, anti-CD41-PE and anti-CD105-eFluor450. EVs were characterised as follows: **Ai**. All Annexin-APC fluorescently labelled particles were identified as PS+EVs (in blue); **Aii**. APC vs PE quadrant plot were set to identify both Annexin-APC and CD41-PE labelled particles as PDEVs (in red); **Aiii**. APC vs PB quadrant plot were set to check very few of PS+ EVs coexpressed CD105 on APC fluorescence signal (in green, hardly visible). **Bi**. All CD105-eFluor450 fluorescently labelled particles to display only CD105 labelled particles (in green); **Bii**. APC vs PE quadrant plot were set to check very few of PS+EVs coexpressed CD41 on PB fluorescence signal (in red, hardly visible); **Biii**. APC vs PB quadrant plot to identify both Annexin-APC and CD105-eFluor450 labelled particles as EDEVs (in yellow). *AnnV, Annexin V; APC, Allophycocyanin; EDEVs, endothelial-derived extracellular vesicles; PB, Pacific Blue; PDEVs, platelet-derived extracellular vesicles; PE, phycoerythrin;PS, phosphatidylserine.*

In order to minimise the background caused by nonspecific antibody binding, a panel of negative controls were included in the analysis. For Annexin V labelling, the negative control was PFP with Annexin V-APC, but Annexin V did not bind to PS due to the absence of Ca^{+}; negative controls for CD41 and CD105 were PFP staining with the matching isotype controls (IgG1-PE and IgG1-eFlour450, respectively). The final numbers of EVs were obtained by events recorded in the main sample (**Figure 3.8 right columns**), subtracting events recorded in the corresponding isotype control tube (**Figure 3.8 left columns**). The total number of EVs per ml of blood was calculated as **Chapter 2 Section 2.2** described.

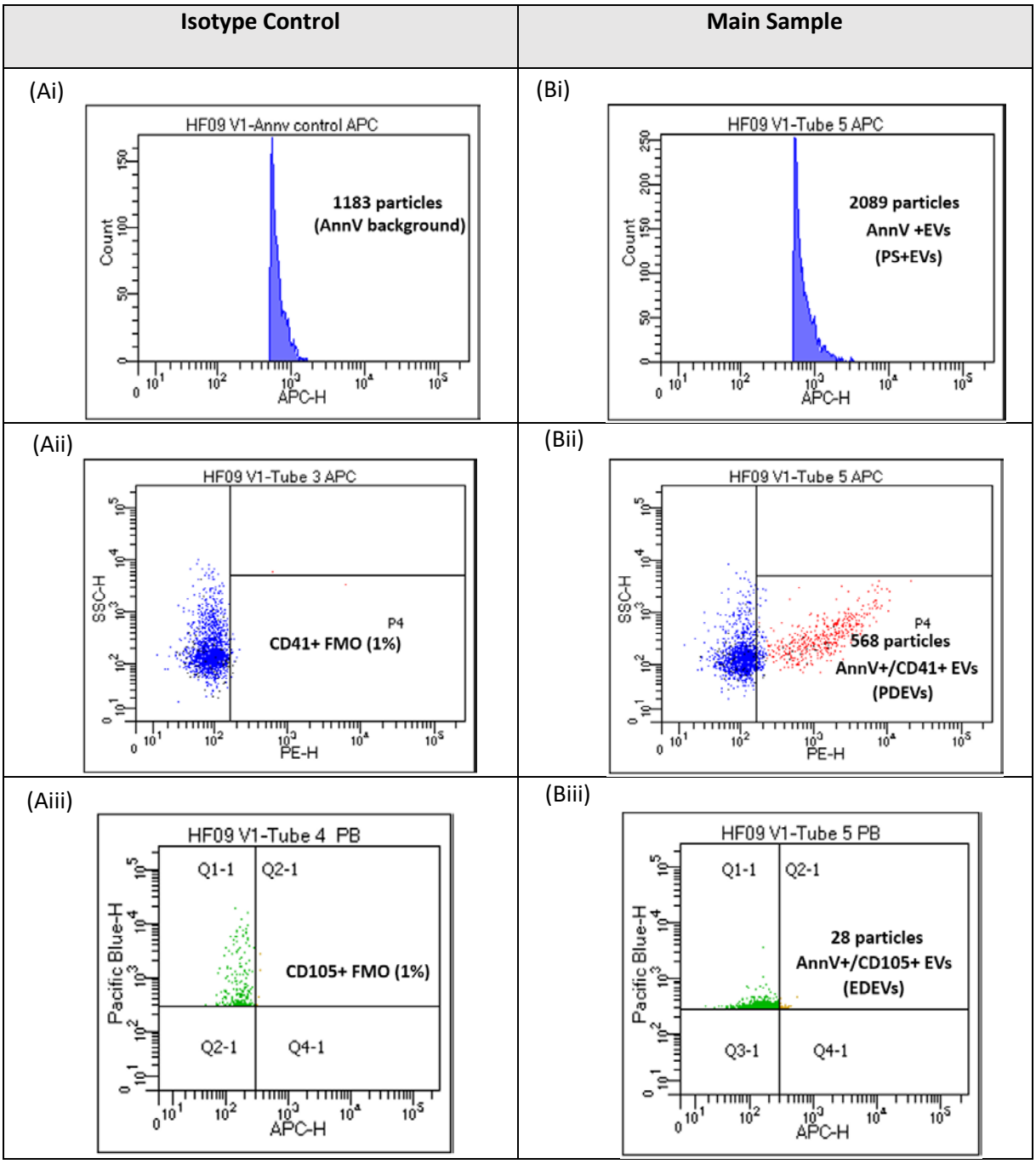


Figure 3.8 Flow cytometer analysis of isotype control tubes. In this particular sample, the final total number of PS+EVs (906 particles) were obtained by Annexin V+EV numbers displayed in main sample (**Bi:** 2089 particles) subtracting particle numbers displayed in Annexin V-APC negative control (**Ai:** 1183 particles); A cut-off as CD41+ particles to 1% was set on PE vs SSC plot for CD41 isotype control (**Aii**), and then 62.7% of Annexin V+ EVs coexpressing CD41+ were identified as Annexin V+/CD41+EVs (PDEVs) (568 particles) (**Bii**); A cut-off as CD105+ particles to 1% was set on APC vs PB quadrant plot for CD105 isotype control (**Aiii**), and then 3.1% of Annexin V+EVs coexpressing CD105+ were identified as Annexin V+/CD105+EVs (EDEVs) (28 particles) (**Biii**). *AnnV, Annexin V; APC, Allophycocyanin; EDEVs, endothelial-derived extracellular vesicles; PB, Pacific Blue; PDEVs, platelet-derived extracellular vesicles; PE, phycoerythrin; PS+EVs, phosphatidylserine extracellular vesicles.*

Lactadherin has been trialled as an EV PS-binding marker in **Chapter 2**, but Annexin V outperformed lactadherin when combing other antibodies to phenotype EVs; therefore, Annexin V was finally selected to label PS+EVs. Similar to PS, phosphatidylethanolamine (PE) is also a major phospholipid predominantly segregated in the inner leaflet of resting cells and exposed on the outer leaflet when cells are activated or undergo apoptosis. PE can be expressed at levels several-fold higher than PS in the membrane depending on cell type and agonist (Fadeel & Xue, 2009; Clark *et al*., 2013), so some groups have been exploring it as another possible general marker for EV detection. Larson and co-workers (2012) have noted that significantly higher numbers of EDEVs, MDEVs and breast cancer EVs stained positive for duramycin, which stains PE, than for Annexin-V, which stains PS. Preliminary experiments from our research team suggested that duramycin staining of purified PDEVs derived from washed platelets was greater than that of Annexin-V staining (unpublished data). However, duramycin staining of circulating EVs was very low and detection was hampered by variable background noise, particularly when combining with CD41 for phenotyping circulating PDEVs.

In the current study, PDEVs were defined as Annexin V+/CD41+ particles and EDEVs were defined as Annexin V+/CD105+ particles. Other studies have used different antibodies, such as CD42b (GPIb receptor) with or without co-expression of CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1)) and/or CD61 (GPIIIa receptor) for PDEVs (Ueba *et al*., 2010; Suštar *et al*., 2011; Diehl *et al*., 2011) and CD62E (E-selectin), CD144 (vascular endothelial cadherin), CD31 with CD42b negative for EDEVs (Landers-Ramos *et al*., 2018; Diehl *et al*., 2011; Amabile *et al*., 2014; Nozaki *et al*., 2009; Esposito *et al*., 2006; Suštar *et al*., 2011). Compared to the current antibodies, anti-CD61 has lower specificity for platelets and low fluorescence intensity and has been shown to be present on macrophages (Kieffer & Phillips, 1990) and monocytes (Law *et al*., 2000). Anti-CD62E and anti-CD144 have high specificity for endothelial cells, but these markers are expressed weakly (Nieuwland *et al*., 2000; Abid Hussein *et al*., 2003). Following a number of in-house trials prior to this project, anti-CD41 and anti-CD105 were deemed to be the best performing antibodies when

considering both minimisation of background and the specificity and intensity relative to the isotype control.

Another improvement in the current protocol compared to that in **Chapter 2** was the consideration of negative controls, which help to ensure the detected labelling is due to specific antibody binding to the target EVs rather than an artefact. For Annexin V labelling, the negative control was PFP with Annexin V antibody but Annexin V did not bind to PS due to the absence of Ca^{+}. Negative controls for anti-CD41 and anti-CD105 were PFP staining with the matching isotype controls. Furthermore, Annexin V buffer alone was run to ensure a low background and PFP alone (sample in buffer without antibodies) was run to control the flow rate, which limited the numbers of events to 400~500 event/second at a low flow rate so that the cytometer could accurately identify particles.

There are some limitations with the work described in this chapter. The FCM protocols focused on EVs derived from platelets and endothelial cells, but not EV of other origins, such as erythrocytes or leukocytes, which are also a key source of circulating EVs and contribute to many physiological and pathological conditions. Also, samples were only isolated by SEC before NTA analysis but were directly analysed by FCM, although the reasons for this have been explained. Analysis of samples by FCM with and without prior SEC would have been valuable and will certainly be considered in future work.

In conclusion, this chapter sets out some of the detailed refinement of methodology that was required for application to the HI-FIVE intervention study. The outcomes of that intervention study are described in the remainder of this thesis.

Chapter 4 Association of conventional cardiovascular risk markers with numbers of circulating EVs in subjects with moderate risk of CVDs

4.1 Introduction

CVDs, as a group of diseases of the heart and blood vessels, have rapidly grown as the leading cause of worldwide mortality and as a significant public health problem over the past decades (WHO, 2017). One of the most remarkable advances in the prevention and treatment of CVDs is the appreciation of the role of risk factors in CVDs. The underlying cause of most CVDs is atherosclerosis, which is a multifactorial process influenced by various risk factors (McGill *et al*., 2000). Two major pieces of research, the Framingham Heart Study (Dawber *et al*., 1951) and the Seven Countries Study (Keys *et al*., 1986), have significantly contributed to the identification of some risk factors, which are now widely accepted as "conventional risk factors". Some of them are non-modifiable, including age, gender, ethnicity and family history, while others could be controlled by making certain lifestyle and diet changes. The proposed mechanisms for these risk factors in the pathogenesis of CVDs are described in **Chapter 1 Section 1.1**.

Cigarette smoking, hypertension, hyperlipidaemia, obesity and diabetes have been well established as the major modifiable cardiovascular risk factors, whose importance in the development of CVDs is evidenced by the decline in the risk of future cardiovascular events when they are treated. However, the absence of risk factors should not be regarded as a guarantee of being free from CVDs (Canto *et al*., 2011). In the past, it was thought that more than 50% of patients with CHD lacked any of the conventional risk factors (Hennekens, 1998; Braunwald, 1997; Futterman & Lemberg, 1998). More recent epidemiological studies suggest that approximately 85%~95% of patients have at least one of the conventional risk factors (Khot *et al*., 2003; Hozawa *et al*., 2007; Canto *et al*., 2011). However, these risk factors are still insufficient to fully predict the development of cardiovascular events. For example,

Khot *et al*. (2003) analysed the data for four conventional risk factors (smoking, diabetes, dyslipidaemia and hypertension) from 122,458 patients with CHD enrolled in 14 international trials and found that there were still 10% to 15% of patients experiencing cardiovascular events despite lacking any of the four conventional risk factors. Another study investigated 10,460 white subjects aged 45 to 64yrs and classified the same four CVD risk factors into optimal (low), borderline, or elevated categories according to national guidelines. The results suggested that risk factors in the 'elevated' category accounted for 65% of CVD incidence and a further 11.4% of CVD incidence was explained when borderline risk factor levels were included, leaving > 20% unexplained (Hozawa *et al*., 2007). Canto *et al*. (2011) examined five risk factors (smoking, diabetes, dyslipidaemia, hypertension and family history of CHD) in 542,008 patients with initial MI and similarly identified that 14.4% of MI was unable to be attributed to any of the five risk factors.

Furthermore, since there are multiple risk factors, treatments that are highly effective in altering a single marker do not eliminate risk entirely; for instance, the drug statin used to lower cholesterol level reduces CVD risk by approximately 30% (Maron *et al*., 2000). A decrease of 5~6 mmHg in BP results in a 20~25% reduction in CHD and a 14% reduction in MI (Collins *et al*., 1990). In this regard, there is an increasing interest in discovering new risk factors, contributing to the explanation of the additional risk and occurrence of cardiovascular events that were not attributable to conventional risk markers. Various emerging risk factors reflecting the processes involved in the development of atherosclerosis and CVDs have been found to be directly correlated with increased risk of CVDs, and therefore potentially to improve the assessment of CVD risk beyond the current risk stratification (Zethelius *et al*., 2008). These include endothelial dysfunction markers (e.g. endothelial progenitor cells), inflammation-related factors (e.g. C-reactive protein and interleukins) and factors associated with coagulation and thrombosis (e.g. fibrinogen and vWf antigen). A table of emerging risk factors was summarised in **Chapter 1 Section 1.1.2**.

EVs are characteristically a heterogeneous group of submicron membrane-bound, particles that are secreted by almost all cells under both physiological and pathological conditions. Although the mechanism of EV generation is still under debate, EV release can be caused by endothelial dysfunction and platelet hyperreactivity. Having been disregarded as cellular debris for decades, EVs are now recognised as mediators of cell-to-cell communication in various pathological activities including atherosclerosis and CVDs, and thus have been investigated as an emerging candidate for cardiovascular risk prediction (for details see **Chapter 1**). Numerous studies report the elevated levels of circulating EVs in both patients with cardiovascular events, such as stable CAD (Jansen *et al*., 2014; Sinning *et al*., 2011), acute ischemic stroke (Simak *et al*., 2006; Pawelczyk *et al*., 2017) and in obese subjects without diagnosed CVDs (Koga *et al*., 2006), subjects with hypertension (Preston *et al*., 2003), or hyperlipidaemia (Ferreira *et al*., 2004; Pawelczyk *et al*., 2017). A significantly higher probability of further cardiovascular events and death was also demonstrated in subjects with higher EV numbers (Nozaki *et al*., 2009). However, there is little information about the association of circulating EV numbers with the presence of conventional cardiovascular risk markers in relatively healthy subjects (e.g. subjects with moderate risk of CVDs); the potential role of EVs in predicting CVD risk independently is also insufficiently explored. Furthermore, most of the studies investigating the relationship between EV numbers and cardiovascular risk only assessed EV levels using FCM, which mainly captures large EVs (e.g. microvesicles) and misses smaller EVs (e.g. exosomes) due to the limited detection threshold. In the current study, therefore, NTA, which has a wider threshold and higher sensitivity for detection of smaller EVs was applied in addition to FCM, providing more comprehensive enumeration of circulating EVs.

The aim of this chapter is to investigate the relationship between conventional cardiovascular risk markers and numbers of circulating EVs (including large and small EVs), and to examine whether an elevated concentration of circulating EVs could potentially serve as a novel quantitative biomarker for predicting future cardiovascular events in subjects with moderate risk of CVDs.

4.2 Subjects and methods

4.2.1 Subjects

A total of 40 subjects (aged 40~70yrs), with moderate risk of CVDs, were involved in the current study. Details about the recruitment and screening of participants are described in **Chapter 3**. Of the 416 subjects contacted, a total of 58 subjects were screened, 42 individuals were enrolled on the trial and 40 completed the study.

4.2.2 Study process and sample processing

The HI-FIVE study was a randomised, double-blind, placebo-controlled crossover intervention. The results and findings discussed in this chapter relate only to the baseline data prior to any intervention. Approximately 100ml of blood was collected from individuals after overnight fasting for all EV-related analysis, 35ml of which was processed for the analysis of circulating EVs. One of the prepared PFP (600µl) was immediately analysed for the enumeration and characterisation of circulating EVs. Another approximately 9ml of blood sample was taken for analysis of blood lipids and glucose levels for the conventional cardiovascular markers evaluation. The processes of anthropometric measurement, blood collection, sample processing and sample analysis were described in **Chapter 2** and **3**. In order to compare relationships between small and large EVs with CVD risk markers, an additional threshold of 200nm was set on NTA software after sample analysis to capture particles ranging from 70nm to 200nm; these were defined as small total EVs (SEVs), whereas particles larger than 200nm were defined as large total EVs (LEVs). Also, FCM identified PS+EVs, PDEVs and EDEVs via a three-antibody panel.

4.2.3 Statistical analysis

Data are expressed as mean ± SEM if normally distributed and as median with inter-quartile range when not. The Kolmogorov-Smirnov test was applied to assess the normal distribution of continuous variables. HDL-C concentration, numbers of TEVs, SEVs and LEVs were logarithmically transformed for analysis due to their positive-skewed distribution. The strength of the correlations between EV numbers and risk

parameters were calculated by Pearson's correlation coefficient. Those significantly associated variables BMI, systolic BP (SBP), diastolic BP (DBP) and TAG concentration were first entered into univariate regression models separately, and then into a multivariate regression model to check the interference of each associated variable on the explanation of EV numbers. In order to further determine the independent predictors of EV numbers, all variables with *p*-values < 0.05 in the univariate regression were then incorporated into a stepwise multivariate regression model, in which parameters of F≤ 0.05 were entered and F ≥ 0.10 were removed. In the categorical analysis, the median of EV numbers was used as the cut-off value and means between two categories were compared with the use of a two-tailed unpaired Student's t-test. The association between the quartile range of EV numbers and 10-yr CVD risk detected by QRISK2 was examined by analysis of covariance (ANCOVA). All statistical analyses were performed with SPSS Statistics version 25 and a *p*-value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Characteristics of the study population

Subject characteristics (n=40) are shown in **Table 4.1**. The median age was 64 years, and 24 out of 40 subjects were males. The mean and median values for SBP, DBP, TAG, HDL-C and glucose levels were within normal reference ranges (SBP was within parameters for pre-hypertension and glucose concentration was within parameters for pre-diabetes). Circulating EV numbers (detected by NTA) and HDL-C did not present as normal distributions, so common logarithmic transformation was applied for analysis. The mean size of EVs was 97.9±11.4 nm and SEVs accounted for 96% of TEVs. Also, the mean ± SEM of numbers of circulating EVs after log-transformation was 10.57±0.05 (TEVs), 10.55±0.05 (SEVs), 9.16±0.05 (LEVs) per ml blood. Approximately 63% of PS+EVs was PDEVs, while EDEVs only accounted for approximately 4% of PS+EVs.

Table 4.1. Characteristics of the Study Subjects

Sample Characteristic	All Subjects (n=40)
Age (years)	64 (5)
Male: Female ratio	24:16
BMI (kg/m^2)	25.4±0.5
SBP (mmHg)	134±2.2
DBP(mmHg)	79±1.4
TC (mmol/L)	6.0±0.2
TAG (mmol/L)	1.3±0.1
HDL-C(mmol/L)	1.6(0.3)
TC/HDL-C ratio	3.9±0.1
Glucose (mmol/L)	5.8±0.1
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±8.0
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)	$1.3*10^{6}$±$6.3*10^{5}$
10-yr CVD risk score (%) (QRISK2)	12.9±0.01

Values in normal distribution are shown as mean ± SEM, and the values in non-normal distribution as the median (inter-quartile range). *BMI, body mass index; BP, blood pressure; CVD, cardiovascular disease; DBP, diastolic blood pressure; EDEVs, endothelial-derived extracellular vesicles; FCM, flow cytometry; HDL-C, 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.*

4.3.2 Association of traditional cardiovascular risk markers with numbers and size of circulating EVs

There were statistically significant positive correlations between BMI, SBP, DBP, TAG concentration and both circulating TEV and SEV numbers. For LEVs, the only significant associations were with BMI and SBP. LEVs followed the same general pattern of associations as TEVs and SEVs with other CVD markers, but these failed to reach significance (**Table 4.2**). The mean size of EVs overall, as assessed by NTA, was inversely associated with BMI and TAG concentration (**Table 4.2**). TC concentration was positively correlated with PDEVs, but there were no other associations of risk markers with any numbers of PS+EVs, PDEVs or EDEVs (**Table 4.2**). Furthermore, there were no significant difference in EVs numbers between male and female (data not shown).

Table 4.2. Correlations between EV numbers, EV size and cardiovascular risk markers

		Correlations											
				Age	BMI (kg/m^2)	SBP (mmHg)	DBP (mmHg)	TC (mmol/L)	TAG (mmol/L)	Log HDL-C (Log mmol/L)	TC/HDL-C ratio	Glucose (mmol/L)	Risk (%)
	NTA	Log TEVs/ml blood	*r*	-.135	**.602****	**.359***	**.550****	-.037	**.703****	-.268	.186	.220	**.559****
			p	.406	**.000**	**.023**	**.000**	.821	**.000**	.095	.250	.173	**.000**
		Log SEVs/ml blood	*r*	-.136	**.596****	**.350***	**.553****	-.034	**.710****	-.265	.187	.217	**.557****
			p	.404	**.000**	**.027**	**.000**	.837	**.000**	.098	.249	.179	**.000**
		Log LEVs/ml blood	*r*	-.112	**.485****	**.365***	.306	-.181	.279	-.199	.021	.206	**.383***
			p	.492	**.002**	**.021**	.055	.265	.082	.218	.896	.202	**.015**
	FCM	PS+EVs/ml blood	*r*	-.052	.265	.114	.206	.258	.231	-.018	.206	.259	.191
			p	.750	.099	.484	.203	.108	.151	.913	.203	.107	.237
		PDEVs/ml blood	*r*	-.014	-.144	-.042	.054	**.330***	.037	.031	.245	.162	.068
			p	.932	.376	.795	.740	**.038**	.819	.849	.128	.319	.675
		Log EDEVs/ml blood	*r*	.040	-.136	.234	.245	-.120	.108	.023	-.092	.128	.260
			p	.808	.403	.146	.127	.460	.506	.887	.574	.430	.106
	NTA	Mean size (nm)	*r*	.168	**-.316***	-.162	-.302	.105	**-.383***	.181	-.097	-.247	.163
			p	.301	**.047**	.319	.058	.519	**.015**	.264	.553	.125	.314
		Mode size (nm)	*r*	.110	-.220	-.226	-.239	.135	-.100	.158	-.052	-.146	.285
			p	.498	.173	.162	.138	.407	.540	.331	.751	.367	.074

Pearson's correlation coefficient was conducted to examine the correlation between the numbers and size of EVs and cardiovascular risk parameters. *. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed). *BMI, body mass index; DBP, diastolic blood pressure; EDEV, endothelial-derived extracellular vesicles; FCM, flow cytometry; HDL-C, high-density lipoprotein cholesterol; LEVs, large total extracellular vesicles; NTA, nanoparticle tracking analysis; PDEVs, platelet-derived extracellular vesicles; PS+EVs, phosphatidylserine positive extracellular vesicles; SBP, systolic blood pressure; SEVs, small total extracellular vesicles; TAG, triacylglycerol; TC, total cholesterol; TEVs, total extracellular vesicles.*

Univariate regression analysis demonstrated that BMI, SBP, DBP and TAG were significantly correlated with both circulating TEV and SEV numbers (**Table 4.3**). However, when data were entered into a multivariate regression model, only plasma TAG concentration remained significantly associated with EV numbers.

Table 4.3. Univariate and multivariate regression analysis for the association between EV numbers and cardiovascular risk factors

	Log TEVs (per ml blood)				**Log SEVs (per ml blood)**			
	Univariate analysis		Multivariate analysis R^2=0.630 p<0.001		Univariate analysis		Multivariate analysis R^2=0.634 p<0.001	
	β	***p***	**β**	***p***	**β**	***p***	**β**	***p***
BMI (kg/m²)	0.063	p<0.001	0.023	0.094	0.063	p<0.001	0.022	0.114
SBP (mmHg)	0.008	0.023	0.003	0.360	0.008	0.027	0.003	0.432
DBP (mmHg)	0.018	p<0.001	0.005	0.376	0.019	p<0.001	0.006	0.311
TAG (mmol/L)	0.358	p<0.001	0.261	p<0.001	0.366	p<0.001	0.268	p<0.001

In univariate analysis, BMI, SBP, DBP and TAG levels were significant factors for both TEV numbers and SEV numbers, while only TAG concentration was still significantly correlated with TEV and SEV numbers when entered into multivariate model (R^2=0.630, p<0.001 and R^2=0.634, p<0.001, respectively). *BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; SEVs, small total extracellular vesicles; TAG, triacylglycerol; TEVs, total extracellular vesicles.*

In order to further determine the independent predictors of EV numbers, stepwise regression analysis was applied. The results showed that TAG concentration explained 49.4% of the variance for TEV numbers and 50.5% of the variance for SEV numbers, respectively. An additional 9.3% of the variance in both TEV numbers and SEV numbers, respectively were predicted by DBP (**Figure 4.1**).

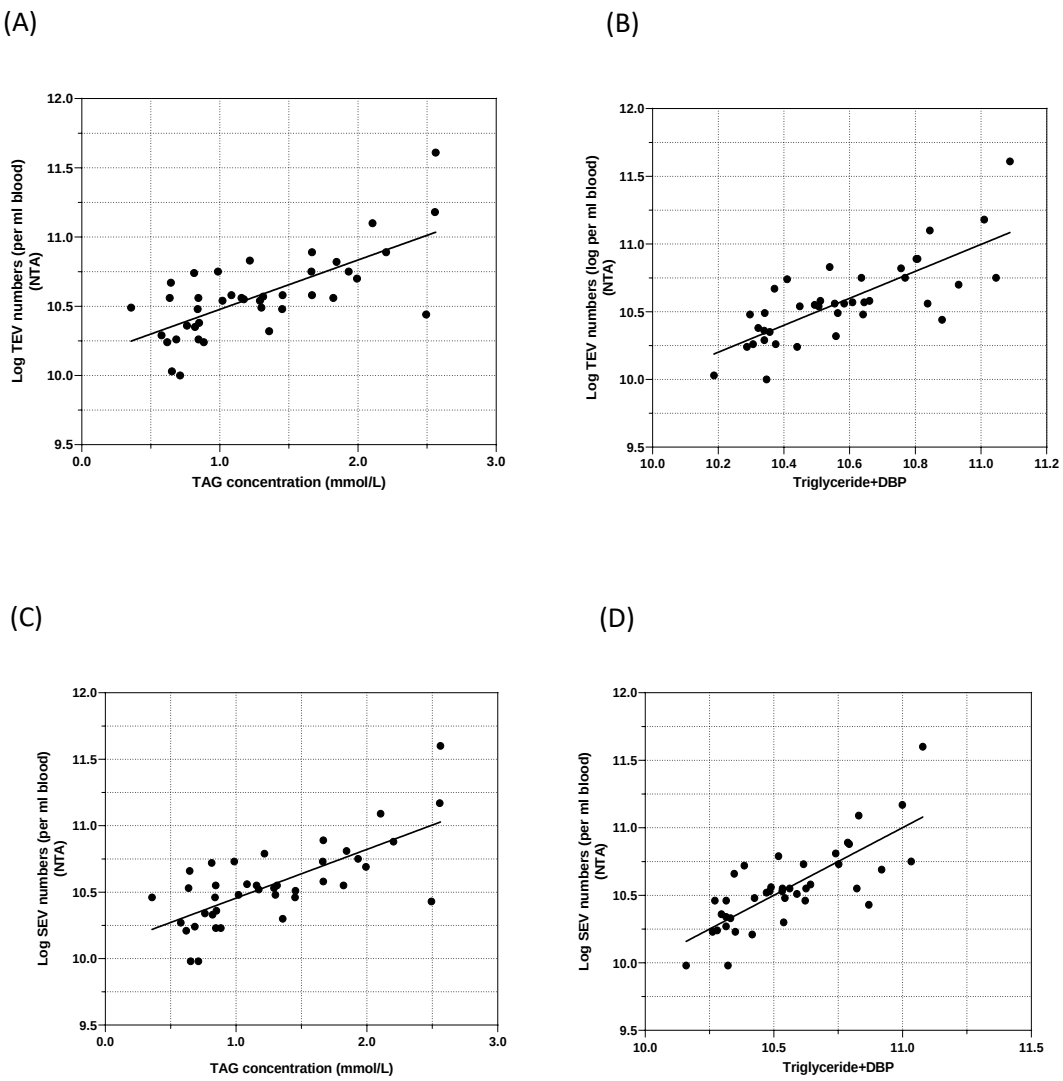


Figure 4.1. Scatter plots showing the log EV numbers detected by NTA were predicted by traditional cardiovascular risk factors. (A) TAG concentration explained 49.4% of the variance for total EV numbers (R^2= 0.494); **(B)** TAG adding DBP (predicted values from multivariate regression model) explained 58.7% of the variance for total EV numbers (R^2= 0.587); **(C)** TAG concentration explained 50.5% of the variance for SEV numbers (R^2= 0.505); **(D)** TAG adding DBP (predicted values from multivariate regression model) explained 59.8% of the variance for small EV numbers (R^2= 0.598). *DBP, diastolic blood pressure; SEVs, small total extracellular vesicles; TAG, triacylglycerol; TEVs, total extracellular vesicles.*

In this model, each 1-standard deviation (SD) (0.6mmol/L) of increase in TAG concentration was associated with 0.577 SD (0.30/log ml blood) of increase in log TEV numbers and 1-SD (9.1mmHg) higher of DBP level resulted in an additional of 0.330SD of logTEV numbers (**Table 4.4**). Regarding SEVs, each 1-SD of increase in TAG concentration was associated with 0.584 SD of increases in logSEV numbers (0.31/log

ml blood) and 1-SD higher of DBP level resulted in an additional of 0.331 SD of log SEV numbers (**Table 4.5**).

Table 4.4. Independent predictors of TEV number determined by stepwise regression

	Log TEVs (per ml blood)				
	Model	**b (95% CI)**	**SE (B)**	**β**	***p*-value**
1	(Constant)	10.120 (9.954, 10.286)	0.082		<0.001
	TAG	0.358 (0.239, 0.477)	0.059	0.703	<0.001
2	(Constant)	9.337 (8.767, 9.906)	0.281		<0.001
	TAG	0.294 (0.176, 0.412)	0.058	0.577	<0.001
	DBP	0.011 (0.003, 0.019)	0.004	0.330	0.006

Unstandardised coefficients (b) indicates that as the independent variables (TAG and DBP) change by one unit, the dependent variable (log TEV numbers) change by b units. Regression coefficient (β) indicates that as the independent variables (TAG and DBP) change by 1 SD, the dependent variable (log TEV numbers) change by β SD. For reference, 1-SD of TAG concentration is 0.6mmol/L; 1-SD of DBP level is 9.1mmHg; 1-SD of log TEV numbers is 0.30/ log ml blood. *CI, confidence interval; DBP, diastolic blood pressure; SD*, *standard deviation; SE, standard error; TAG, triacylglycerol; TEVs, total extracellular vesicles.*

Table 4.5. Independent predictors of SEV number determined by stepwise regression

	Log SEVs (per ml blood)				
	Model	**b (95% CI)**	**SE (B)**	**β**	***p*-value**
1	(Constant)	10.089 (9.922, 10.256)	0.082		<0.001
	TAG	0.366 (0.247, 0.485)	0.059	0.710	<0.001
2	(Constant)	9.295 (8.725, 9.864)	0.281		<0.001
	TAG	0.301 (0.183, 0.419)	0.058	0.584	<0.001
	DBP	0.011 (0.003, 0.019)	0.004	0.331	0.006

Unstandardised coefficients (b) indicates that as the independent variables (TAG and DBP) change by one unit, the dependent variable (log SEV numbers) change by b units. Regression coefficient (β) indicates that as the independent variables (TAG and DBP) change by 1 SD, the dependent variable (log SEV numbers) change by β SD. For reference, 1-SD of TAG concentration is 0.6 mmol/L; 1-SD of DBP level is 9.1mmHg; 1-SD of log TEV numbers is 0.31/ log ml blood. *CI, confidence interval; DBP, diastolic blood pressure; SD*, *standard deviation; SE, standard error; SEVs, small total extracellular vesicles; TAG, triacylglycerol.*

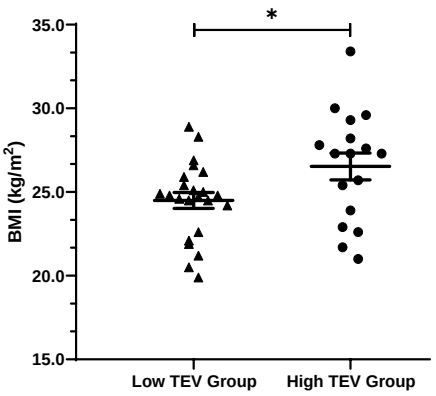
4.3.3 Comparison of traditional cardiovascular risk markers between high EV and low EV group

Subjects were categorised into 'high' and 'low' EV groups based on the median of EV numbers detected by either NTA (log-transformed) (TEVs: 10.56/log ml blood; SEVs: 10.53/log ml blood; LEVs: 9.12/log ml blood) or FCM ($3.3*10^7$/ml blood).

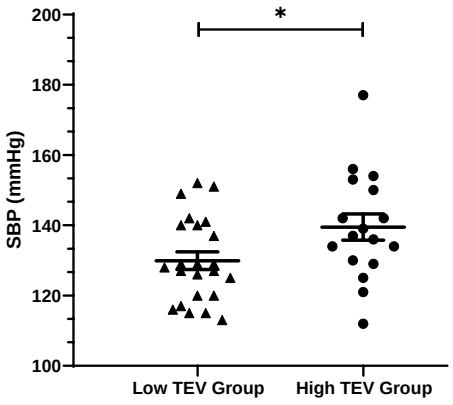
4.3.3.1 Comparison of CVD risk markers in the high TEV and low TEV groups

Individuals with high numbers of TEVs had a significantly higher BMI than those with low TEV numbers (26.5±0.8 kg/m^2 vs 24.5±0.5 kg/m^2, *p*=0.027) (**Figure 4.2A**). The high TEV group also had higher SBP (140±3.7 mmHg vs 130±2.5mmHg, *p*=0.039) (**Figure 4.2B**) and DBP (83±1.9mmHg vs 75±1.8mmHg, *p*=0.004) (**Figure 4.2C**) compared to low TEV group. A significantly higher concentration of plasma TAG (**Figure 4.2D**) and a higher TC/HDL-C ratio (**Figure 4.2E**) were observed in high TEV group (TAG: 1.6±0.1 mmol/L vs 1.0±0.1 mmol/L, *p*=0.001; TC/HDL-C ratio: 4.2±0.2 vs 3.7±0.2, *p*= 0.026), and subjects with high TEV numbers also had a significantly lower log HDL-C concentration (0.17±0.02 mmol/L vs 0.21±0.01 mmol/L, *p*=0.05) (**Figure 4.2F**).

(A)



(B)



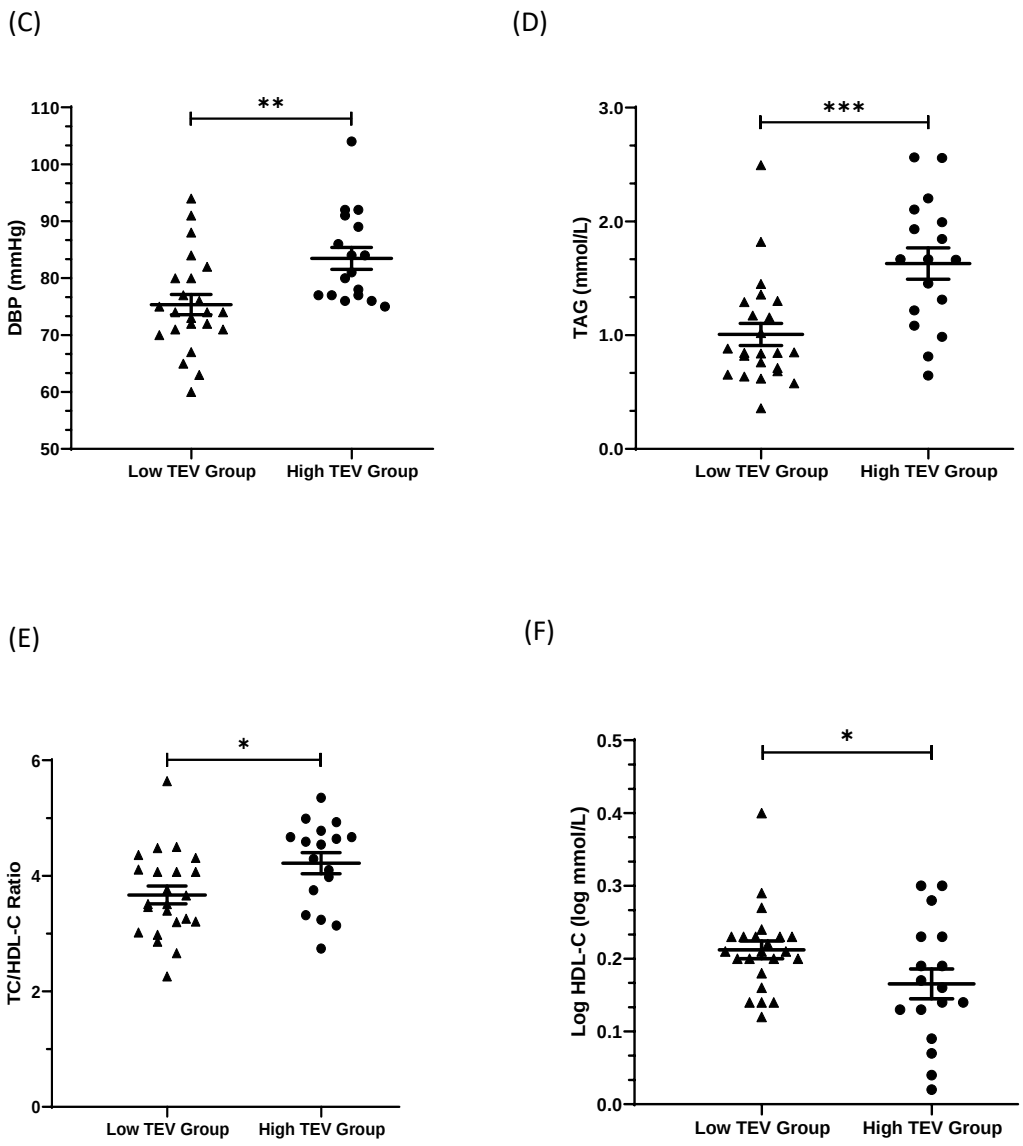
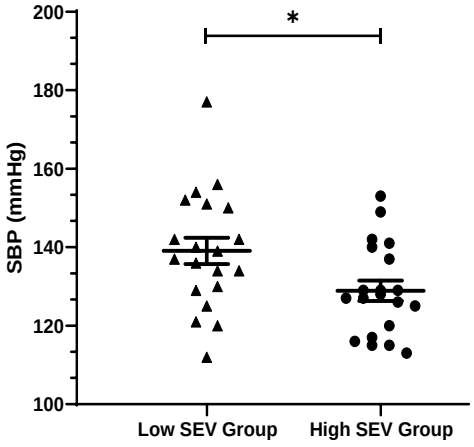


Figure 4.2. High numbers of TEVs are associated with traditional cardiovascular risk markers. Data are mean ± SEM, triangles represented the risk factors parameters of each individual in low TEV group and dots represented the risk factors parameters of each individual in high TEV group. Statistically significant differences between the low and high TEV groups (categorised by the median of TEV numbers) were observed for BMI (**A**), SBP (**B**), DBP (**C**), TAG concentration (**D**), TC/HDL-C ratio (**E**) and log HDL-C concentration (**F**). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. *BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; TAG, triacylglycerol; TC, total cholesterol; TEVs, extracellular vesicles.*

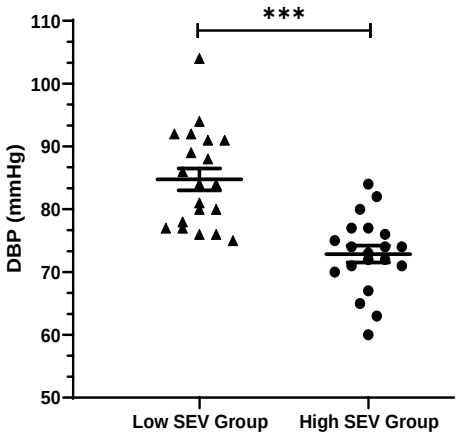
4.3.3.2 Comparison of CVD risk markers in groups categorised for high and low numbers of SEVs, LEVs and PS+EVs

Subjects with high numbers of SEVs had higher SBP (139±3.4 mmHg vs 129±2.6mmHg, *p*=0.022), DBP (85±1.7mmHg vs 73±1.3mmHg, *p*<0.001) and plasma TAG (1.5±0.1mmol/L vs 1.0±0.1mmol/L, *p*=0.004) than those with low numbers of SEVs (**Figure 4.3A~C**). Subjects with high numbers of LEVs had a significantly higher BMI (26.8±0.7kg/m^2 vs 24.1±0.5kg/m^2, *p*=0.002) and a significantly lower log HDL-C concentration (0.17±0.02mmol/L vs 0.22±0.01mmol/L, *p*=0.04) than those with low numbers of LEVs, but there were no other differences in other CVD risk factors (data not shown). When comparing between high and low PS+EV group, the high PS+EV group had a significantly higher TC concentration than the low PS+EV group (6.3±0.9mmol/L vs 5.7±1.1mmol/L, *p*=0.05) (**Figure 4.3D**). However, there was no significant difference in any other risk markers between the high and low PS+ EV groups (data not shown).

(A)



(B)



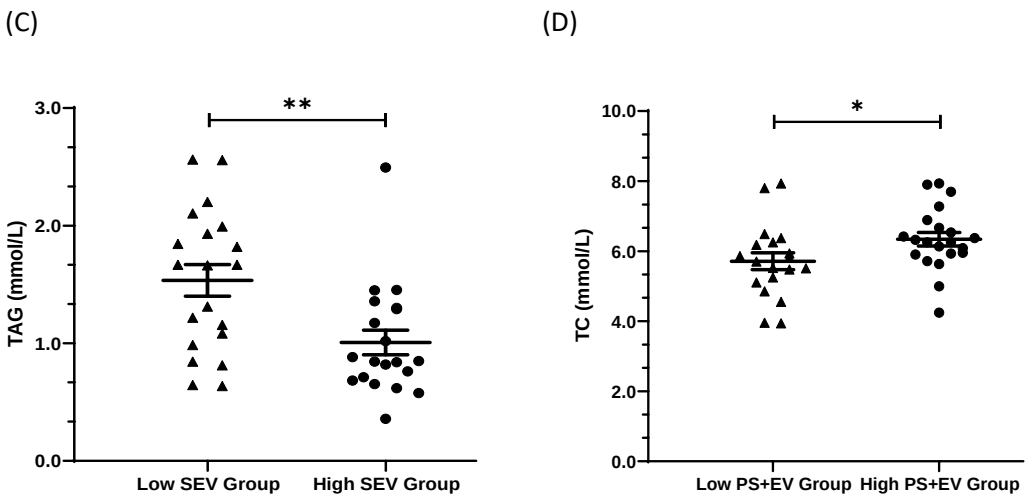


Figure 4.3. High numbers of SEVs or PS+EVs are associated with traditional cardiovascular risk markers. Data are mean ± SEM, triangles represented the risk factors parameters of each individual in low SEV/PS+EV group and dots represented the risk factors parameters of each individual in high SEV/PS+EV group. Statistically significant differences are found in SBP (**A**), DBP (**B**) and TAG concentration (**C**) between high and low SEV groups, and in TC concentration between high and low PS+ EV groups (**D**). **p*<0.05, ***p*<0.01 and ****p*<0.001. *BMI, body mass index; DBP, diastolic blood pressure; PS+EVs, phosphatidylserine positive extracellular vesicles; SBP, systolic blood pressure; SEVs, small total extracellular vesicles; TAG, triacylglycerol; TC, total cholesterol.*

4.3.4 Association between circulating EV numbers and 10-yr CVD risk score predicted by QRISK2

The quartile ranges of the log TEV numbers against 10-yr CVD risk, calculated by QRISK2, are shown in **Table 4.6**. Subjects in the highest quartiles of EV numbers had significantly higher 10-yr CVD risk scores without adjustment (**Table 4.6**). This association remained after adjusting for age; for age and TAG; for age, TAG and log HDL-C (all of them were associated with 10-yr CVD risk score, therefore were regarded as covariates) (**Table 4.6 and Figure 4.4A**). The similar results were found for SEV numbers, where subjects in the highest quartile had significantly higher 10-yr CVD risk, both before and after adjustment for age, but it became non-significant after adjustment for TAG and log HDL-C (**Figure 4.4B**). In contrast, there was no significant difference in risk score across quartiles of LEV numbers (**Figure 4.4C**).

Table 4.6. Association between TEV numbers and 10-yr CVD risk score predicted

TEVs	Q1 (10-10.36) n= 10	Q2 (10.37-10.56) n= 13	Q3 (10.37-10.56) n= 13	Q4 (10.37-10.56) n= 13	
Unadjusted	11.3% (10.3-12.4)	11.5% (10.6-12.4)	14.2% (11.4-17.1)	16.1% (12.4-19.8)	*p*=0.002
Adjusted for age	10.9% (9.3-12.5)	11.6% (10.2-13.0)	14.7% (13.1-16.3)	15.9% (14.0-17.8)	*p*<0.001
Adjusted for age and TAG	11.2% (9.3-13.0)	11.7% (10.2-13.1)	14.7% (13.0-16.3)	15.4% (12.9-17.9)	*p*<0.001
Adjusted for age, TAG and logHDL-C	11.3% (9.5-13.2)	11.7% (10.3-13.1)	14.6% (12.9-16.2)	15.2% (12.7-17.7)	*p*<0.001

The quartile range of log TEV numbers (n represents the number of subjects in each range) and the mean with 95% confidence interval of the 10-yr cardiovascular diseases risk after conducting analysis of covariance for unadjusted or adjusted for variables are shown. *HDL-C, high-density lipoprotein cholesterol; TAG, triacylglycerol; TEVs, total extracellular vesicles.*

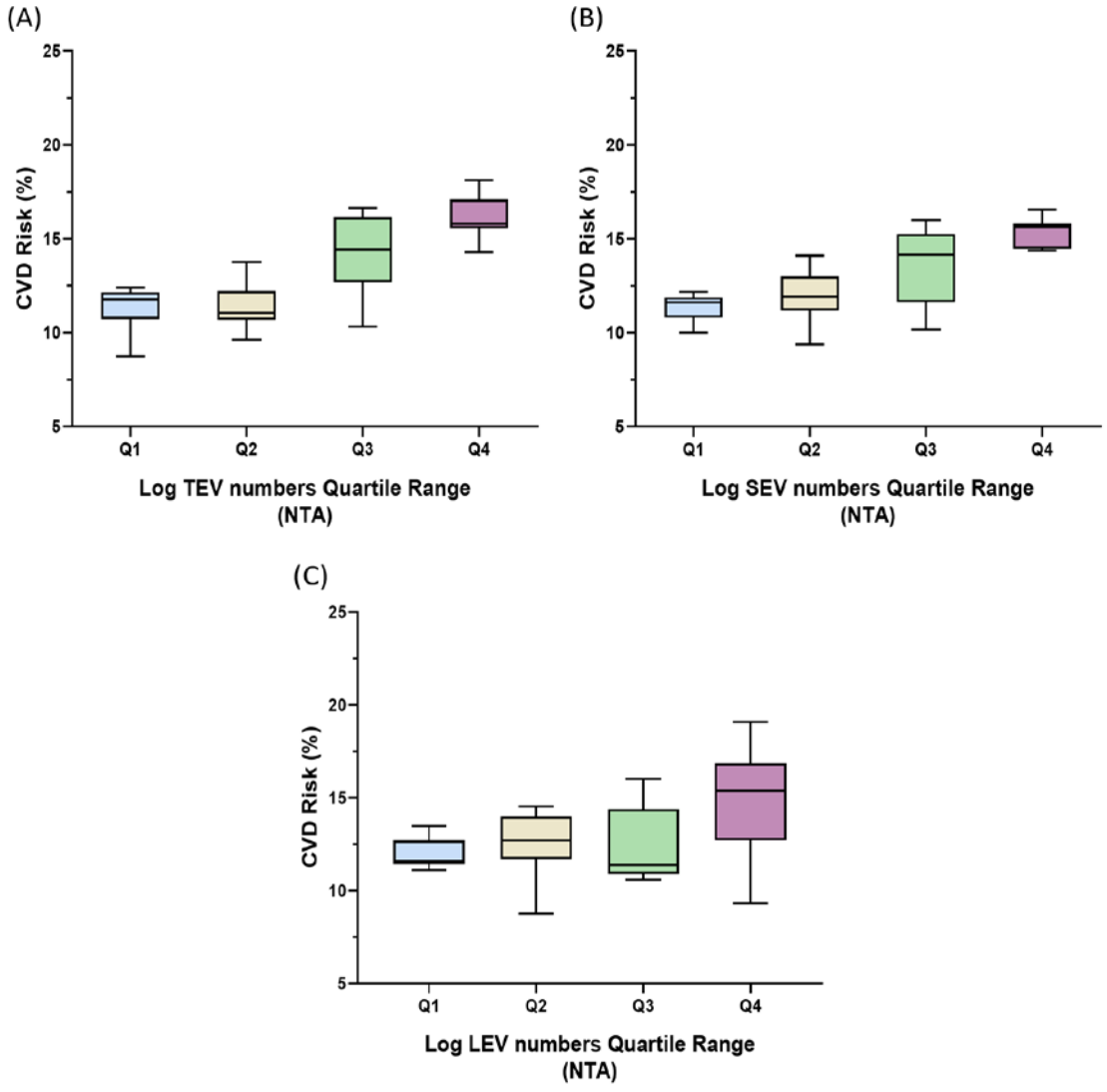


Figure 4.4. The adjusted 10-yr CVD risk in the quartile range of log EV numbers. (A) Elevated log TEV numbers were associated with high 10-yr CVD risk adjusted for age, TAG and logHDL-C (p<0.001); **(B)** Elevated log SEV numbers were associated with high 10-yr CVD risk adjusted for age (p=0.009); **(C)** Elevated log LEV numbers were not significantly associated with high 10-yr CVD risk adjusted for age, TAG and log HDL-C (p=0.200). The line within box indicates the median values; the bottom and top lines of the box indicate the 25th and 75th percentiles, respectively; and the bottom and top vertical lines outsides the boxes indicate 25th minus 1.5 inter-quartile distance (IQR) and 75th percentiles plus 1.5 IQR. *CVD, cardiovascular diseases; LEVs, large extracellular vesicles; NTA, nanoparticle tracking analysis; SEVs, small total extracellular vesicles; TEVs, total extracellular vesicles.*

4.4 Discussion

The current study demonstrated a strong association between circulating EV numbers and conventional cardiovascular risk markers such as BMI, BP and plasma lipid profile in subjects with moderate risk of CVDs. Compared to previous studies, which have only reported on large EVs as they used FCM only, this study applied NTA for the enumeration of circulating EVs and therefore provides novel insight into the influence of smaller EVs on CVD risk. Moreover, plasma TAG and DBP were found to independently predict levels of circulating TEVs and SEVs, but not LEVs. With the exception of LEVs, subjects in the highest quartiles of both TEV and SEV numbers had significantly greater 10-year CVD risk independent of other conventional cardiovascular risk markers, indicating that elevated numbers of TEVs and SEVs, but not LEVs, were independently associated with 10-yr CVD risk.

It is important to note that LEVs followed the same general patterns of association of TEVs and SEVs with CVD markers, but these failed to reach significance except for BMI. This is perhaps not surprising since LEVs only accounted for 4% of TEVs; with SEVs representing the majority of the EV population, it is inevitable that they will be more strongly associated with CVD markers and risk. These findings suggest that circulating EVs are predominantly < 200nm, that these smaller EVs might play a more prominent role in the development of CVDs, and that studies which rely on FCM for EV characterisation are only reporting on a minor fraction of the total EV population.

EVs are nanosized membranous particles ranging from 30nm to 5µm and their generation takes place in a variety of cells, including endothelial cells, monocytes and platelets under both physiological and pathological conditions. EVs can be classified into three major categories based on their size and current knowledge of biogenesis: exosomes (30nm~150nm), microvesicles (100nm~1µm) and apoptotic bodies (1µm~5µm) (Kalra *et al*., 2016; Devhare & Ray, 2018). In the current study, concentrations of total circulating EVs were assessed using NTA and PS+EVs (Annexin V+), PDEVs (Annexin V+/CD41+), EDEVs (Annexin V+/CD105+) were assessed using

fluorescence FCM. Since the isolation method applied in the current study did not specifically separate exosomes and microvesicles, and because vesicles were not labelled with exosome-specific markers, the term “SEVs” (smaller than 200nm) rather than “exosomes” was used to describe a particle population characterised purely on the basis of size. The mean size of EVs was 97.9±11.4nm and SEVs accounted for 96% of total EVs detected by NTA, indicating that the majority of circulating EVs are exosomes and small microvesicles with sizes < 200nm, thus FCM is significantly limited in its ability to detect EVs as the majority are below its limit for detection.

Higher BMI is known to be associated with an increased risk for the development of CVDs and other CVD-related diseases (Lavie *et al*., 2008). In the current study, BMI was strongly correlated with TEVs, SEVs and LEVs, but there was no significant relationship between BMI and numbers of PS+EVs, PDEVs or EDEVs. Moreover, individuals with higher than median numbers of TEVs and SEVs had a significantly higher BMI than those with lower than median numbers of TEVs and SEVs. The association between BMI and EV numbers reported by previous studies is summarised in **Table 4.7**. In agreement with the current findings partly, Amosse *et al.* (2018) reported that BMI was positively correlated with numbers of EVs detected by NTA. However, they also indicated a similar correlation between BMI and numbers of EV detected by FCM, which is consistent with most of the other studies only applying FCM. Only two studies indicated no significant association between BMI and numbers of PDEVs and EDEVs, which is consistent with the current findings (Preston *et al*., 2003; Esposito *et al*., 2006). One of the potential reasons for this discrepancy is that different antibodies were used for the phenotyping of EVs. Furthermore, some studies were conducted in obese subjects, while the mean of BMI of subjects in the current study was 25.4±0.5kg/m^2. Overweight or obesity is characterised as a status of excessive adipose tissue and increased oxidative stress (Marseglia *et al*., 2014). Oxidative stress will impair NO bioavailability and/or signalling and then affect normal endothelial function. Adipose tissue has been reported to produce proinflammatory cytokines including TNF-α, IL-1β, and IL-6 (Fonseca-Alaniz *et al*., 2007) and those cytokines can

promote PDEV and EDEV generation both *in vivo* (Nomura *et al*., 1999) and *in vitro* (Takahashi *et al*., 2013).

Table 4.7 Association between circulating EV numbers and BMI

Study	Subjects	Measured markers and techniques	Statistical analysis	Outcomes
Preston *et al*. (2003)	Subjects with severe hypertension (DBP ≥120mmHg) (n= 24); Subjects with mild hypertension (DBP ≥95 and ≤100mmHg) (n= 19); healthy women controls (DBP <90mmHg) (n=16)	**PDEVs**: labelled with anti-CD41-FITC **EDEVs**: labelled with anti-CD31-PE and anti-CD42-FITC (negative) *Flow cytometry*	2-sample t-test and multiple regression analysis	**PDEVs**: not associated with BMI (p=0.67) **EDEVs**: not associated with BMI (p=0.89)
Esposito *et al*. (2006)	Obese women (mean BMI: 41.2±5.3 kg/m^2) (n= 41); healthy women controls (mean BMI: 24.1±0.7 kg/m^2) (n=40)	**PDEVs**: labelled with anti-CD31-FITC and anti-CD42b-PE **EDEVs**: labelled with anti-CD31-FITC and anti-CD42b-PE (negative) *Flow cytometry*	Pearson's correlation coefficient and multiple linear regression analysis	**PDEVs**: not associated with BMI (r=−0.07, p=0.645) **EDEVs**: not associated with BMI (r=0.02, p=0.9)
Goichot *et al*. (2006)	Obese women (mean BMI: 42.4±0.9 kg/m^2) (n= 58); healthy women controls (mean BMI: 20.9±1.6 kg/m^2) (n=45)	**EVs**: labelled with Annexin V on strepavidin-coated microtitration plates *Prothrombinase assay*	Student t-test and multiple regression analysis	**EVs**: significantly increased in obese group (p<0.001); positively associated with BMI in all subjects of both obese and healthy group (r=0.572, p<0.01), but negatively associated with BMI in obese group (r =0.265, p<0.05)
Murakami *et al*. (2007)	Obese women (mean BMI: 27.4±0.3 kg/m^2) (n= 49); healthy women controls (mean BMI: 22.8±0.2 kg/m^2) (n=37)	**PDEVs**: labelled with anti-CD41-FITC *Flow cytometry*	Pearson's correlation coefficient or Spearman's rank correlation	**PDEVs**: positively associated with BMI in all subjects of both obese and healthy group (r=0.536, p<0.001)
Ueba *et al*. (2010)	Healthy men free of CVDs (n=190)	**PDEVs**: labelled with anti-CD42b and anti-CD42a *ELISA*	Univariate and multivariate regression analysis	**PDEVs**: positively associated with BMI (univariate regression: β=0.208, p=0.004; multivariate regression: β=0.015, p=0.840)

Table 4.7 Association between circulating EV numbers and BMI (continued)

Study	Subjects	Measured markers and techniques	Statistical analysis	Outcomes
Stepanian *et al*. (2013)	Obese women (mean BMI: 41.8 kg/m^2) with MS (n= 62)or without MS (n=89); healthy women controls (mean BMI: 22.0 kg/m^2) (n=60)	**PS+EVs**: labelled with anti-Annexin V-FITC; **PDEVs**: labelled with anti-Annexin V-FITC and anti-CD41-PE; **EDEVs**: labelled with anti-CD31-FITC and anti-CD41-PE (negative) *Flow cytometry*	Bivariate and multivariate regression analysis	**PS+EVs**: significantly increased in obese group (p=0.01); positively associated with obesity (p<0.01) **PDEVs**: significantly increased in obese group (p<0.01); positively associated with obesity (p<0.01) **EDEVs**: significantly increased in obese group (p<0.01)
Amabile *et al*. (2014)	Healthy subjects free of CVDs from Framingham Heart Study (n=844)	**EDEVs**: labelled with anti-CD144-PE; anti-CD31-PE and anti-CD41-PC7 (negative) *Flow cytometry*	Linear mixed effects regression; univariate and multivariate regression analysis	**EDEVs**: positively associated with BMI (CD144+EDEVs: β=0.07, p=0.020; CD31+/CD41-EDEVs: β=0.06, p=0.043)
Campello *et al*.(2015)	Overweight subjects (BMI: 25-29.9kg/m^2); subjects with I (BMI: 30-34.9kg/m^2), II (BMI: 35-39.9kg/m^2) and III (BMI: ≥40kg/m^2)degree obesity (n=20/group); healthy controls (n=40)	**PS+EVs**: labelled with anti-Annexin V-FITC *Flow cytometry*	Pearson's correlation coefficient	**PS+EVs**: significantly increased in overweight and obesity group; positively associated with BMI
Landers-Ramos *et al*.(2018)	Sedentary older subjects (50~75yrs): chronic stroke, T2DM or older healthy; younger healthy controls (18~39yrs) (n=17/group)	**EVs**: labelled with anti-CD62E-PE; anti-CD34-FITC; anti-CD31-PE and anti-CD42b-FITC (negative) *Flow cytometry*	Pearson's correlation coefficient	**EVs**: CD62E+EVs: positively associated with BMI (p<0.001); CD31+/CD41-EVs: positively associated with BMI (p<0.001) CD34+EVs: not significantly correlated with BMI (p>0.05)

Amosse *et al*. (2018)	Subjects with MS (n=34) and healthy controls (n=22) divided into obese group (mean BMI: 36.1±4.9kg/m^2) (n= 22); overweight group (mean BMI: 28.7±1.2kg/m^2) (n= 12); healthy controls (mean BMI: 23.6±2.1kg/m^2) (n=22)	**TEVs:** exosomes and microvesicles *NTA* **PS+EVs**: labelled with anti-Annexin V-FITC **PDEVs**: labelled with anti-CD41 **EDEVs**: labelled with anti-CD146 **ErDEVs**: labelled with anti-CD235a **LDEVs**: labelled with anti-CD45 **MDEVs**: labelled with anti-CD11b *Flow cytometry*	Pearson's correlation coefficient	**Exosomes (NTA)**: significantly increased in overweight and obesity group; positively associated with BMI (r= 0.339, p=0.026) **Microvesicles (NTA and FCM):** significantly increased in overweight and obesity group; positively associated with BMI (r=0.353, p=0.010)
Zahran *et al*. (2019)	Subjects with an early stage of MS (n=40); healthy controls (n=30)	**PS+EVs**: labelled with anti-AnnexinV **PDEVs**: labelled with anti-CD41-PerCP **EDEVs**: labelled with anti-CD144-PE and anti-CD45-APC (negative) *Flow cytometry*	Pearson's correlation coefficient and multiple linear regressions	**PS+EVs**: positively associated with BMI (r=0.46, p=0.003) **PDEVs**: positively associated with BMI (r=0.46, p=0.003) **EDEVs**: positively associated with BMI (r=0.40, p=0.011)

APC, Allophycocyanin; BMI, body mass index; CVDs, cardiovascular diseases; EDEVs, endothelial-derived extracellular vesicles; ErDEVs, erythrocytes-derived extracellular vesicles; FITC, fluorescein isothiocyanat; LDEVs, leukocytes-derived extracellular vesicles; MS, metabolic syndrome; MDEVs, monocytes-derived extracellular vesicles; PC7, phycoerythrin cyanine 7; PDEV, platelet-derived extracellular vesicles; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PS+EVs, phosphatidylserine positive extracellular vesicles; T2DM, type 2 diabetes.

A strong and independent relationship between BP and CVDs has been well characterised over the decades. A large number of observational studies and clinical trials have demonstrated that elevated BP is associated with elevated morbidity and mortality of cardiovascular events across a wide age, gender and region range (He & Whelton, 1999; Psaty *et al*., 2001; Lawes *et al*., 2003; Rapsomaniki *et al*., 2014; Stevens *et al*., 2016). Moreover, hypertension, defined as a SBP of 140mmHg or more, and/or a DBP of 90mmHg or more, has been acknowledged as a significant risk factor of CVDs. The current study demonstrated that there were significant correlations between SBP/DBP and TEVs and SEVs, but only SBP was positively correlated with LEVs. Also, subjects with higher than median TEV and SEV numbers had a higher SBP and DBP than those with lower than median TEV and SEV numbers. However, neither SBP nor DBP was positively associated with PS+EVs, PDEVs or EDEVs, which were congruent with three previous studies (Esposito *et al*., 2006; Murakami *et al*., 2007; Amosse *et al.*, 2018), although other studies demonstrated a direct association between BP and EV levels detected by FCM (**Table 4.8**). This discrepancy may again arise from differences in antibody labelling. Also, the lack of an association between DBP and numbers of PS+EVs, PDEVs and EDEVs is in fact specifically a lack of an association with large microvesicles assessed using FCM, which is in line with the finding that LEVs were also not significantly associated with DBP as they are > 200nm. However, LEVs only represented 4% of the total EV population, so it is perhaps not surprising that SEVs were more strongly associated with BP and that it is difficult to demonstrate relationships between FCM-characterised EVs and BP. Regarding SEVs such as exosome, *in vitro* work indicates that cells derived from pulmonary arterial hypertension (PAH) patients released greater numbers of exosomes compared to those from healthy controls (Ferrer *et al*., 2018). Also, numerous studies have indicated that exosomes act as efficient vehicles for the intracellular transmission of RNA and proteins, which, in turn, play an important role in the development of hypertension (Su *et al*., 2017). Exosomes isolated from spontaneously hypertensive rats have been found to increase SBP of normotensive rats (Otani *et al*., 2018). Furthermore, exosomes rather than microvesicles from rats with PAH modulated pulmonary vascular responses and contributed to the development of PAH when they

were injected into healthy rats (Aliotta *et al*., 2016). However, the mechanism of the effect of BP on the release of SEVs remains to be elucidated.

Table 4.8 Association between circulating EV numbers and BP

Study	Subjects	Measured markers and techniques	Statistical analysis	Outcomes
Preston *et al*. (2003)	Subjects with severe hypertension (mean BP: 195/127mmHg) (n= 24); Subjects with mild hypertension (mean BP: 142/96mmHg) (n= 19); healthy controls (mean BP: 122/80mmHg) (n=16)	**PDEVs**: labelled with anti-CD41-FITC **EDEVs**: labelled with anti-CD31-PE and anti-CD42-FITC (negative) *Flow cytometry*	2-sample t-test and multiple regression analysis	**PDEVs**: highest in severe hypertension compared to both mild hypertension and healthy controls (p=0.01); positively associated with SBP (r=0.36, p=0.005) and DBP (r=0.40, p=0.002). **EDEVs**: highest in severe hypertension compared to healthy controls (p=0.002); positively associated with SBP (r=0.36, p=0.006) and DBP (r=0.42, p=0.001)
Esposito *et al*. (2006)	Obese women (BMI > 30kg/m^2) (n= 41); healthy women controls (BMI: BMI < 25kg/m^2) (n=40)	**PDEVs**: labelled with anti-CD31-FITC and anti-CD42b-PE **EDEVs**: labelled with anti-CD31-FITC and anti-CD42b-PE (negative) *Flow cytometry*	Pearson's correlation coefficient and multiple linear regression analysis	**PDEVs**: not significantly associated with either SBP or DBP **EDEVs**: not significantly associated with either SBP or DBP
Murakami *et al*. (2007)	Obese women (BMI ≥ 25 kg/m^2) (n= 49); healthy women controls (BMI: 19.1~24.9 kg/m^2) (n=37)	**PDEVs**: labelled with anti-CD41- FITC *Flow cytometry*	Pearson's correlation coefficient or Spearman's rank correlation	**PDEVs**: not associated with SBP (r=−0.016, p=0.887) or DBP (r=0.037, p=0.745) in all subjects of both obese and healthy group

Table 4.8 Association between circulating EV numbers and BP (continued)

Study	Subjects	Measured markers and techniques	Statistical analysis	Outcomes
Bakouboula *et al*. (2008)	Subjects with PAH (n=20); healthy controls (n=23)	**Annexin V+EVs** *Prothrombinase assay* **TF+EVs** *TF activity assay* **PDEV**s: labelled with anti-GP Ib **EDEVs**: labelled with anti-CD31; anti-CD62E;anti-CD105; **LDEVs**: labelled with anti-CD11a *Flow cytometry*	Wilcoxon matched-pairs test; Spearman's rank coefficient test and multiple linear regression analysis	**Annexin V+EVs**: no significant difference between PAH and healthy controls (p=0.365), but significantly higher in pulmonary artery blood compared with jugular vein blood in PAH group(p=0.002) **TF+ EVs**: significantly increased in PAH group compared to healthy controls (p<0.001); Procoagulant EVs: positively associated with mean pulmonary artery pressure (r = 0.631, p=0.01); **PDEVs**: no significant difference between PAH and healthy controls (p=0.344) **EDEVs**: CD105+ EVs: significantly increased in PAH group compared to healthy controls (p<0.001) and significantly higher in pulmonary artery blood compared with jugular vein blood in PAH group (p=0.038); CD62E+EDEVs and CD31+EDEVs: no significant difference between PAH and healthy controls (p=0.444 and p=0.480) LDEVs: no significant difference between PAH and healthy controls (p=0.221)
Ueba *et al*. (2010)	Healthy men free of CVDs (n=190)	**PDEVs**: labelled with anti-CD42b and anti-CD42a *ELISA*	Univariate and multivariate regression analysis	**PDEVs**: positively associated with SBP (univariate regression: β=0.282, p<0.001; multivariate regression: β=-0.053, p=0.606) and DBP (univariate regression: β=0.373, p<0.001; multivariate regression: β=0.296, p=0.004)
Diehl *et al*. (2011)	Subjects with PAH (n= 19); healthy controls (n=16)	**PDEVs**: labelled with anti- CD31-PE and anti-CD61-FITC **EDEVs**: labelled with anti-CD62E-PE **LDEVs**: labelled with anti-CD11bPE *Flow cytometry*	Unpaired t-test and Spearman's rank correlation coefficient	**PDEVs**: significantly increased in PAH group compared to healthy controls (p=0.032) **EDEVs**: significantly increased in PAH group compared to healthy controls (p=0.001) **LDEVs**: significantly increased in PAH group compared to healthy controls (p=0.001)

Table 4.8 Association between circulating EV numbers and BP (continued)

Study	Subjects	Measured markers and techniques	Statistical analysis	Outcomes
Amabile *et al*. (2014)	Healthy subjects free of CVDs from Framingham Heart Study (n=844)	**EDEVs**: labelled with anti-CD144-PE; anti-CD31-PE and anti-CD41-PC7 (negative) *Flow cytometry*	Linear mixed effects regression; univariate and multivariate regression analysis	**EDEVs**: not significantly associated with SBP (CD144+EDEVs: β=0.04, *p*=0.15; CD31+/CD41- EDEVs: β=0.05, *p*=0.13); positively associated with DBP (CD144+EDEVs: β=0.07, *p*=0.024; CD31+/CD41-EDEVs: β=0.06, *p*=0.050)
Amosse et al. (2018)	Subjects with MS (n=34) and healthy controls (n=22) divided into obese group (BMI > 30kg/m^2) (n= 22); overweight group (BMI: 27~30kg/m^2) (n= 12); healthy controls (BMI <27kg/m^2) (n=22)	**TEVs:** exosomes and microvesicles *NTA* **PS+EVs**: labelled with anti-Annexin V-FITC **PDEVs**: labelled with anti-CD41 **EDEVs**: labelled with anti-CD146 **ErDEVs**: labelled with anti-CD235a **LDEVs**: labelled with anti-CD45 **MDEVs**: labelled with anti-CD11b *Flow cytometry*	Pearson's correlation coefficient	**Exosomes (NTA)**: not significantly associated with SBP (*r*=−0.084, *p*=0.602) or DBP (*r*=0.066, *p*=0.679) **Microvesicles (NTA and FCM)**: not significantly associated with SBP (*r*=−0.006, *p*=0.968) or DBP (*r*=0.104, *p*=0.466)
Zahran *et al*. (2019)	Subjects with an early stage of MS (n=40); healthy controls (n=30)	**PS+EVs**: labelled with anti-AnnexinV **PDEVs**: labelled with anti-CD41-PerCP **EDEVs**: labelled with anti-CD144-PE and anti-CD45-APC (negative) *Flow cytometry*	Pearson's correlation coefficient and multiple linear regressions	**PS+EVs**: positively associated with SBP (*r*=0.96, *p*<0.001) and DBP (*r*=0.54, *p*<0.001) **PDEVs**: positively associated with SBP (*r*=0.94, *p*<0.001) and DBP (*r*=0.62, *p*<0.001) **EDEVs**: positively associated with SBP (*r*=0.94, *p*<0.001) and DBP (*r*=0.42, *p*<0.001)

APC, Allophycocyanin; BMI, body mass index; BP, blood pressure; CVDs, cardiovascular diseases; DBP, diastolic blood pressure; EDEVs, endothelial-derived extracellular vesicles; ErDEVs, erythrocytes-derived extracellular vesicles; FITC, fluorescein isothiocyanat; GP, glycoprotein; LDEVs, leukocytes-derived extracellular vesicles; MDEVs, monocytes-derived extracellular vesicles; MS, metabolic syndrome; PAH, pulmonary arterial hypertension; PC7, phycoerythrin cyanine 7; PDEV, platelet-derived extracellular vesicles; PE, phycoerythrin; PH, pulmonary arterial hypertension; PerCP, peridinin chlorophyll protein; PS+EVs, phosphatidylserine positive extracellular vesicles; SBP, systolic blood pressure; TEVs, total extracellular vesicles; TF, tissue factor.

Hyperlipidaemia, characterised as elevated concentrations of any or all of fasting TC, TAG or lipoproteins, is another significant factor to accelerate atherosclerosis and CVDs (Nelson, 2013). Many studies have explored the association between the levels of EVs and plasma lipid profile; these are summarised in **Table 4.9** and in the current study, TAG, TC, HDL-C and TC/HDL-C ratio were measured to reflect the plasma lipid profile.

The results presented in this chapter suggested that plasma TAG concentration was strongly associated with the numbers of TEVs and SEVs, and plasma TC concentration was positively correlated with numbers of PDEVs. Also, subjects with higher than median numbers of TEVs had significantly higher plasma TAG concentrations, higher TC/HDL-C ratio and lower HDL-C concentrations than those with lower than median numbers of TEVs. Three previous studies have investigated associations of TAG and TC concentrations with EV numbers (detected by NTA) and in line with the current study, reported that fasting TAG concentrations, but not fasting TC concentrations, of healthy subjects were positively correlated with TEV numbers detected by NTA (Mørk *et al*., 2017; Jamaly *et al*., 2018; Amosse *et al*., 2018). Moreover, Jamaly and co-workers (2018) also explored the effect of postprandial TAG on EV levels after subjects were administered lipid-rich meals and demonstrated that TEV numbers and postprandial TAG concentrations were significantly associated three hours after consuming a high-fat meal. However, most other studies only employed FCM and while some reported associations between plasma lipids and EV subpopulations detected by FCM (Ferreira *et al*., 2004; Koga *et al*., 2006; Mikirova *et al*., 2011; Amabile *et al*., 2014; Landers-Ramos *et al*., 2018; Zahran *et al*., 2019), others did not (Preston *et al*., 2003; Esposito *et al*., 2006; Murakami *et al*., 2007) (**Table 4.9**).

The inconsistency may result from variation in the study population, the type of antibody used for EV enumeration and gating strategies when employing FCM. For example, Landers-Ramo *et al.* (2018) used polystyrene particle beads to set the upper limit gate, which as explained in **Chapter 2**, scatter more light compared to similar-

sized EVs and therefore appear smaller than they actually are. It is likely, therefore, that the EV population in that study contained larger particles, such as platelets and apoptotic debris as well as EVs. Also, as with BP, the current study demonstrated that plasma lipid levels, particularly plasma TAG concentration, is more closely related to SEVs than LEVs, but this may again be related to the fact that 96% of the EV population was < 200nm.

The release of EVs is reported to be associated with markers of endothelial activation/injury (e.g. soluble VCAM-1) and impaired flow-mediated vasodilation and platelet activation (e.g. P-selectin) (Preston *et al*., 2003; Esposito *et al*., 2006). It is therefore possible that the relationship between plasma TAG, HDL-C and TC/HDL-C ratio and TEV number is connected to endothelial dysfunction and platelet hyperreactivity. An increased concentration of TAG has been found to be related with a decreased FMD and subjects with TAG concentrations more than 1.2 mmol/L were presented with impaired endothelial function (Kajikawa *et al*., 2016). An elevated *in vivo* platelet activation exerted by the increased expression of P-selectin was also reported in hypertriglyceridemia subjects (de Man *et al*., 2000). Conversely, HDL-C has been reported to protect endothelial function by limiting endothelial cell apoptosis (Sugano *et al*., 2000) and promoting eNOS activity (Yuhanna *et al*., 2001). HDL-C could also prevent platelet activation by reducing the build-up of intraplatelet cholesterol and modulating platelet signalling pathways (van der Stoep *et al*., 2014). However, the exact mechanism of the effect of TAG, TC and HDL-C concentrations on the generation of TEVs still needs exploration.

The current study demonstrated that subjects with higher than median numbers of PS+EVs had higher concentrations of plasma TC than those with lower than median numbers and plasma TC concentration was also positively associated with numbers of PDEVs. These findings are consistent with several other studies (Koga *et al*., 2006; Ueba *et al*., 2010; Zahran *et al*., 2019), although some studies showed no such effect (Esposito *et al*., 2006; Murakami *et al*., 2007). The generation of EVs during cell

activation involves an increase in intracellular calcium concentration followed by the externalisation of PS to the outer leaflet. The outward exposure of PS then triggers the shedding of EVs from the plasma membrane (Bevers *et al*., 1983; Zwaal & Schroit, 1997). EVs release is reduced in patients with Scott syndrome, characterised by an inability to express PS, suggesting that PS exposure may be an essential process in EV formation (Sims *et al*., 1989). Cholesterol-enrichment of human monocytes/macrophages induces PS exposure and the release of PS-exposing EVs (Liu *et al*., 2007), which is consistent with the observation in the current study that subjects with higher plasma TC concentrations generated more PS+EVs. On the other hand, PDEVs are the most abundant circulating EVs and the mechanisms for their formation have been investigated for decades and the ability of activated platelet to form PDEVs is widely accepted. Lipid rafts are highly ordered membrane microdomains enriched in cholesterol and sphingolipid, which provide a platform for coordinating the signalling pathways of platelet adhesion and activation via various receptors (Sezgin *et al*., 2017). Depleting cholesterol with methyl-β-cyclodextrin blocked the production of PS+EVs derived from platelet, suggesting that cholesterol-rich lipid rafts may be required for the formation of PDEVs (Wei *et al*., 2018). Furthermore, the membranes of PDEVs tend to have a greater cholesterol concentration than those of unstimulated platelets (Biró *et al*., 2005).

Table 4.9 Association between circulating EV numbers and plasma lipid profile

Study	Subjects	Measured markers and techniques	Statistical analysis	Outcomes
Preston *et al*. (2003)	Subjects with severe hypertension (DBP ≥120mmHg) (n= 24); Subjects with mild hypertension (DBP ≥95 and ≤100mmHg) (n= 19); healthy women controls (DBP <90mmHg) (n=16)	**PDEVs**: labelled with anti-CD41-FITC **EDEVs**: labelled with anti-CD31-PE and anti-CD42-FITC (negative) *Flow cytometry*	2-sample t-test and multiple regression analysis	**PDEVs**: not associated with TAG (p=0.39) and TC/HDL-C ratio (p=0.17) **EDEVs**: not associated with TAG (p=0.54) and TC/HDL-C ratio (p=0.37)
Ferreira *et al*. (2004)	Healthy subjects free of CVDs (n=18)	**EDEVs**: labelled with anti-CD31-PE and anti-CD42-FITC(negative) *Flow cytometry*	Pearson's correlation coefficient	**EDEVs**: significantly increased companied by the significant increase in TAG 3 hours after ingestion of a high fat meal (p=0.001). The increase in EDEV numbers was significantly associated with the change in TAG at 3 hours (r=0.77, p=0.0004).
Esposito *et al*. (2006)	Obese women (BMI >30kg/m^2) (n= 41); healthy women controls (BMI: BMI < 25kg/m^2) (n=40)	**PDEVs**: labelled with anti-CD31-FITC and anti-CD42b-PE **EDEVs**: labelled with anti-CD31-FITC and anti-CD42b-PE (negative) *Flow cytometry*	Pearson's correlation coefficient and multiple linear regression analysis	**PDEVs**: not significantly associated with TAG, TC or HDL-C **EDEVs**: not significantly associated with TAG, TC or HDL-C
Koga *et al*. (2006)	Subjects with T2D group (n=105); healthy controls (n=92)	**PDEVs**: labelled with anti-CD42b-PE *Flow cytometry*	Unpaired two-sided t-test or Mann-Whitney U test univariate and multivariable linear regression analysis	**PDEVs**: significantly increased in diabetic group compared to healthy controls (p <0.001); positively associated with TAG (r=0.276, p=0.004) or TC (r=0.376, p <0.001), but not significantly associated with HDL-C (r=−0.140, p=0.2)

Table 4.9 Association between circulating EV numbers and plasma lipid profile (continued)

Study	Subjects	Measured markers and techniques	Statistical analysis	Outcomes
Murakami *et al*. (2007)	Obese women (BMI ≥ 25 kg/m^2) (n= 49); healthy women controls (BMI: 19.1~24.9 kg/m^2) (n=37)	**PDEVs**: labelled with anti-CD41-FITC *Flow cytometry*	Pearson's correlation coefficient or Spearman's rank correlation	**PDEVs**: not associated with TAG (r=−0.120, p=0.287) or TC (r=0.032, p=0.773) or HDL-C (r=−0.077, p=0.496) in all subjects of both obese and healthy group
Ueba *et al*. (2010)	Healthy men free of CVDs (n=190)	**PDEVs**: labelled with anti-CD42b and anti-CD42a *ELISA*	Univariate and multivariate regression analysis	**PDEVs**: positively associated with TAG (univariate regression: β=0.198, p=0.006) and TC (univariate regression: β=0.144, p=0.048; multivariate regression: β=0.029, p=0.713); negatively associated with HDL-C (univariate regression: β=−0.200, p=0.006; multivariate regression: β=−0.050, p=0.533)
Mikirova *et al*. (2011)	Healthy subjects free of CVDs (n=35)	**PDEVs**: labelled with anti-CD41-FITC **EDEVs**: labelled with anti-CD31-PE and anti-CD42b-FITC (negative); anti-CD62E; anti-CD51 *Flow cytometry*	Lack of details	**PDEVs**: positively associated with TC/HDL ratio (r=0.25, p=0.02) and negatively associated with HDL-C (r=-0.384, p=0.04) **EDEVs**: CD31+/CD42b-EDEVs: positively associated with TC/HDL ratio (r=0.46, p=0.002) and negatively associated with HDL-C (r=−0.42, p=0.02); CD62E+EDEVs: positively associated with TC/HDL ratio (r=0.43, p=0.01) but not significantly associated with HDL-C (r=0.25, p=0.09); CD51+EDEVs: not significantly associated with TC/HDL-C ratio (r=0.222, p=NS)
Amabile *et al*. (2014)	Healthy subjects free of CVDs from Framingham Heart Study (n=844)	**EDEVs**: labelled with anti-CD144-PE; anti-CD31-PE and anti-CD41-PC7 (negative) *Flow cytometry*	Linear mixed effects regression; univariate and multivariate regression analysis	**EDEVs**: positively associated with TAG (CD144+EDEVs: β=0.15, p<0.0001; CD31+/CD41-EDEVs: β=0.23, p<0.0001); not significantly associated with TC (CD144+EDEVs: β<0.01, p=0.16; CD31+/CD41-EDEVs: β<0.01, p=0.31); positively associated with TC/HDL-C ratio (CD144+EDEVs: β=0.11, p<0.0001; CD31+/CD41-EDEVs: β=0.18, p<0.0001); negatively associated with HDL-C (CD144+EDEVs: β=-0.0046, p=0.005; CD31+/CD41-EDEVs: β=−0.00892, p<0.0001)

Table 4.9 Association between circulating EV numbers and plasma lipid profile (continued)

Study	Subjects	Measured markers and techniques	Statistical analysis	Outcomes
Mørk *et al*. (2017)	Healthy subjects (n=10)	**TEVs** *NTA*	Spearman's rank correlation	**TEVs:** positively associated with TAG (r=0.64, p=0.05); not significantly associated with TC (r=0.47, p=0.18)
Jamaly *et al*. (2018)	Healthy subjects (n=40)	**TEVs** *NTA; Transmission EM; Immune EM; Scanning EM*	Pearson's correlation coefficient	**TEVs**: Fasting: positively associated with TAG (r=0.35, p=0.03) and VLDL (r=0.43, p=0.005); TAG and VLDL explained 13% and 19%, respectively, of the variation of EVs. Postprandial (4 hours after ingestion of a high fat meal): positively associated with TAG (r=0.77, p<0.0001) and VLDL (r=0.81, p<0.0001); TAG and VLDL explained 59% and 66%, respectively, of the variation of EVs.
Landers-Ramos *et al*.(2018)	Sedentary older subjects (50~75yrs): chronic stroke, T2DM or older healthy; younger healthy controls (18~39yrs) (n=17/group)	**EVs**: labelled with anti-CD62E-PE; anti-CD34-FITC; anti-CD31-PE and anti-CD42b-FITC (negative) *Flow cytometry*	Pearson's correlation coefficient	**EVs**: CD62E+EVs: positively associated with TAG (p<0.001); negatively associated with HDL-C (r = −0.32, p<0.001); CD31+/CD41-EVs: positively associated with TAG (p<0.001) and negatively associated with HDL-C (r = −0.35, p<0.001); CD34+EVs: not significantly correlated with TAG or HDL-C (p>0.05)

Amosse et al. (2018)	Subjects with MS (n=34) and healthy controls (n=22) divided into obese group (BMI > 30kg/m^2) (n= 22); overweight group (BMI: 27~30kg/m^2) (n= 12); healthy controls (BMI <27kg/m^2) (n=22)	**TEVs:** exosomes and microvesicles *NTA* **PS+EVs**: labelled with anti-AnnexinV-FITC **PDEVs**: labelled with anti-CD41 **EDEVs**: labelled with anti-CD146 **ErDEVs**: labelled with anti-CD235a **LDEVs**: labelled with anti-CD45 **MDEVs**: labelled with anti-CD11b *Flow cytometry*	Pearson's correlation coefficient	**Exosomes (NTA)**: positively associated with TAG (r=0.407, p=0.007); not significantly correlated with TC (r=0.201, p=0.196), HDL-C (r=−0.284, p=0.064) or LDL-C (r=0.205, p=0.188) **Microvesicles (NTA and FCM)**: positively associated with TAG (r=0.422, p=0.002); negatively associated with HDL-C (r=−0.279, p=0.045); not significantly correlated with TC (r=0.2010, p=0.943) or LDL-C (r=−0.024, p=0.865)
Zahran *et al*. (2019)	Subjects with an early stage of MS (n=40); healthy controls (n=30)	**PS+EVs**: labelled with anti-AnnexinV **PDEVs**: labelled with anti-CD41-PerCP **EDEVs**: labelled with anti-CD144-PE and anti-CD45-APC (negative) *Flow cytometry*	Pearson's correlation coefficient and multiple linear regressions	**PS+EVs**: positively associated with TAG (r=0.48, p=0.002) and TC (r=0.97, p<0.001); not associated with HDL-C (r=0.05, p=0.758) **PDEVs**: positively associated with TAG (r=0.44, p=0.005) and TC (r=0.96, p<0.001); not associated with HDL-C (r=−0.01, p=0.963) **EDEVs**: positively associated with TAG (r=0.50, p=0.001) and TC (r=0.95, p<0.001); not associated with HDL-C (r=−0.08, p=0.642)

APC, Allophycocyanin; BMI, body mass index; CVDs, cardiovascular diseases; EDEVs, endothelial-derived extracellular vesicles; EM, electron microscopy; ErDEVs, erythrocytes-derived extracellular vesicles; FITC, fluorescein isothiocyanat; HDL-C, high-density lipoprotein cholesterol; LDEVs, leukocytes-derived extracellular vesicles; LDL-C, low-density lipoprotein cholesterol; MDEVs, monocytes-derived extracellular vesicles; MS, metabolic syndrome; PC7, phycoerythrin cyanine 7; PDEV, platelet-derived extracellular vesicles; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PS+EVs, phosphatidylserine positive extracellular vesicles; TAG, triacylglycerol; TC, total cholesterol; T2DM, type2 diabetes; TEVs, total extracellular vesicles; VLDL, very-low-density lipoprotein.

Multivariate regression analysis demonstrated that TAG and DBP are independent predictors for both TEV and SEV numbers, but not for either LEVs or EV subpopulations detected by FCM. The association between higher TAG concentration and higher risk of CVDs have been confirmed over three decades, however, it still remains controversial about whether TAG is an independent risk marker of CVDs (Harchaoui *et al*., 2009; Miller *et al*., 2011). Some studies have reported an independent correlation of fasting TAG concentration with cardiovascular events even after adjustment for other risk markers (Hokanson *et al*., 1996; Sarwar *et al*., 2007; Klempfner *et al*., 2016), but the role of TAG in CVDs prediction may be attenuated with cholesterol and lipoprotein metabolism (Bansal *et al*., 2007; Morrison & Hokanson, 2009; Yang *et al*., 2017). Interestingly, the relationship between plasma TAG concentration and numbers of both TEVs and SEVs in the current study was independent of TC and HDL-C concentrations, which was in agreement with the finding observed from Amabile *et al*. (2014). However, since some TAG-rich lipoproteins (i.e. CM and VLDL) are of similar size with EVs and will be co-detected on NTA, some studies suggested that the correlation of EV numbers and TAG concentration maybe due to the possibility of NTA measuring TAG (Mørk *et al*., 2017; Jamaly *et al*., 2018). In order to minimise the interference of contaminants with the analysis of EVs by NTA, a series of solutions are applied, which includes applying SEC, collecting blood from fasting subjects and setting the lower threshold of 70nm in NTA software (details see **Chapter 2**). An in-house experiment has reported that most of CM were removed after SEC (unpublished data). Also, the median number of TEVs was $3.6*10^{10}$/ml blood ($1.7*10^{10}$/ml plasma) in the current study, which was close to the results obtained by detecting specific labelling EVs on fluorescence NTA ($1.2*10^{10}$/ml plasma) (Dragovic *et al*., 2011), suggesting that the NTA-based EV detection method employed excluded most contaminants after the above adjustments, and therefore the influence of NTA measuring TAG on the correlation between EVs and TAG was controlled. On the other hand, SBP is reported to be similar or superior to DBP in terms of predicting cardiovascular morbidity and mortality (Franklin *et al*., 2001; Lewington *et al*., 2002), and SBP becomes more powerful to predict CVD risk above 50 years of age (Franklin *et al*., 2001). In the current study, DBP rather than SBP independently predicted TEV and SEV numbers, even though the mean age of the subjects was 64 years of age. On

the other hand, EVs has been reported to impair endothelial function (Al-Nedawi *et al*., 2005; Liu *et al*., 2013) and confer proinflammatory status by promoting the exposure of adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin (Gidlöf *et al*., 2013; Hijmans *et al*., 2018), and the secretion of inflammatory cytokines, such as IL-1β, TNF-α and IL-6 (Distler *et al*., 2005a; Bretz *et al*., 2013). The procoagulatory properties of EVs may be due to the presence of anionic phospholipids, particularly PS, and the procoagulant protein TF on the EVs, in which PS provides a surface for the binding of many components involved in the coagulation cascade, while the binding of TF and Factor VIIa initiates the extrinsic pathway of coagulation cascade (Kapustin *et al*., 2017; Tripisciano *et al*., 2017). All of the above processes will accelerate the development of atherosclerosis and CVDs. Thus, the findings from the current study may provide potentially new insights about additional deleterious effects of TAG and DBP on CVDs through the increased generation of EVs. However, the mechanism of elevated levels of TAG and DBP independently increased the numbers of EVs should be further explored.

Finally, the current results suggested that higher TEV and SEV, but not LEV, numbers were significantly associated with higher 10-yr CVD risk score both with and without adjustment for covariates including age, TAG and HDL-C. There were no significant associations of PS+ EVs, PDEVs and EDEVs with 10-yr CVD risk. Previous studies have explored the association between cardiovascular risk and EV numbers based on FCM analysis only and are inconsistent, some demonstrating no relationship (Chironi *et al*., 2006) and others demonstrating greater numbers of CD31+/CD41-EDEVs related with higher 10-yr CVD risk using the FRS (Ueba *et al*., 2010; Amabile *et al*., 2014) or higher PS+EV or EDEVs numbers associated with the occurrence of major adverse cardiovascular and cerebral events and death (Sinning *et al*., 2011; Lee *et al*., 2012). Subjects with high CHD risk had a higher number of CD144+EDEVs compared to a low-risk group, and CD144+EDEVs in the high CHD risk group independently predicted future cardiovascular events (Nozaki *et al*., 2009). Different risk scoring systems for CVD risk can lead to different outcomes. The current study used the QRISK2 score rather than FRS and demonstrated that high numbers of TEVs and SEVs contributed

to the elevated 10-yr CVD risk score independent of other cardiovascular risk factors, which suggests the potential value of EVs as a diagnostic marker in the prediction for CVDs. If it is assumed that the majority of SEVs are exosomes, the underlying mechanisms for TEVs especially exosomes, to play a role in the pathogenesis of CVD could involve cellular intercommunication by transferring membrane and/or cytosolic components between cells, or by interacting with receptors on the surface of cells (Ridger *et al*., 2017), enhancing expression of plasminogen activator inhibitor type 1 (PAI-1) by endothelial cells, leading to the endothelial dysfunction and procoagulant states (Al-Nedawi *et al*., 2005), increasing adhesion of monocytes to endothelial cells (Zhan *et al*., 2009), or inducing cholesterol accumulation in monocytes and TNF-α generation through a PS receptor signalling pathway (Zakharova *et al*., 2007). These studies suggest the involvement of TEVs or exosomes in multiple pathological processes in atherosclerosis and therefore afford them great potential as a novel diagnostic and therapeutic marker of CVDs.

The current study suggested that EV numbers detected by NTA data were 10^3-fold higher than that obtained from FCM. Some previous studies have reported the similar findings. For instance, van der Pol *et al.* (2014) found NTA detected 300-fold higher EV numbers compared to FCM, while Yuana and co-workers (2015) also reported 10^4-fold higher numbers of EVs with detection by NTA compared with analysis by FCM. Moreover, NTA data indicated that 96% of TEVs are EVs with sizes < 200nm. In agreement with the current observation, Dragovic *et al.* (2011) have reported that 90% of EVs in PFP detected by NTA are less than 200nm. Aatonen *et al.* (2014) have detected circulating EVs by both NTA and transmission EM, and reported the main population of EVs were within 100~250nm. Another study also found EVs smaller than 200nm accounted for 94.4%~98.4% of circulating EVs measured by both NTA and scanning EM (Jamaly *et al*., 2018). In this case, FCM with its limited detection threshold lacks the ability to resolve the majority of circulating EVs compared to NTA, therefore, EV numbers determined by NTA were much higher than those determined by FCM. However, in the current study, numbers of PS+EVs (representing the closest possible FCM-based estimate of LEVs) were still much lower (~30-fold) than the numbers of

LEVs assessed using NTA. This may be due to underestimation of EV numbers by FCM because of their small size, resulting in limited expression of antigen and therefore low fluorescence intensity (Lacroix *et al*., 2010). Also, many studies have recently demonstrated that not all circulating EVs are systematically labelled with Annexin V, possibly because of insufficient binding of Annexin V with PS expressed on the surface of these EVs (Connor *et al*., 2010; Ayers *et al*., 2011; Arraud *et al*., 2014). Furthermore, the current protocol only measured EV derived from platelet and endothelial, and found that 63% of PS+EVs was PDEVs, while EDEVs accounted for 4% of PS+EVs. PDEVs have been found as the most abundant circulating EVs and the percentage of PDEVs varied among the studies applying different phenotype methods. Some studies found that PDEVs constitute approximately 70% to 80% of circulating EVs (Horstman & Ahn, 1999; Berckmans *et al*., 2001; Chandler *et al*., 2011), while Stepanian *et al*. (2013) reported that 68% PS+EVs originates from platelets and another study demonstrated that PDEVs represented 58% of the total EVs (Berckmans *et al*., 2019). Compared to PDEVs, EDEVs are much lower and it generally contribute to 5%~15% of circulating EVs (Berckmans *et al*., 2001; Hromada *et al*., 2017; Berckmans *et al*., 2019). However, EVs released from other cells such as erythrocytes or leukocytes are also the main source of circulating EVs and therefore the lack of those EV subpopulations measurement could explain that the current FCM protocol may underestimate EVs numbers as well. There may also be overestimation of EV numbers by NTA. Although the NTA protocol was refined to exclude contaminants such as soluble proteins and lipoproteins, the possibility that some contaminants remain and interfere with the EV detection cannot be entirely excluded. The true numbers of circulating EVs are therefore likely to be somewhere between those detected by NTA and those detected by FCM.

Several limitations need to be considered in the current chapter. Firstly, due to the nature of the study, it was unable to establish a causal relationship between cardiovascular risk factors and EV numbers. The follow-up of subjects developing further cardiovascular events was not conducted, so the independent predictive role of EVs in CVDs could not be identified by using Multivariate Cox Proportional hazards analysis. Also, the collection, isolation and characterisation of EVs are still undergoing

standardisation and most previous studies only applied FCM for EV detection, leading to difficulties in comparing the current findings with those of other studies due to the methodological variations. Although the protocol for the characterisation of EVs was refined by combining NTA and fluorescence FCM, it is still important to bear in mind that EVs measured in the current study did not represent all types of EVs as neither NTA nor FCM was able to provide a full picture of the EV population. The application of NTA allowed the detection of smaller vesicles, but there remained a possibility that some contaminants were present, despite SEC. Fluorescence FCM was able to phenotype EVs, but was unable to detect smaller EVs and to measure all of the main EV subpopulations, leading to the capture of only 4% of the total EV population.

In conclusion, the current study demonstrated that EVs are not only associated with traditional cardiovascular risk markers such as BMI, BP, plasma TAG, TC and HDL-C concentrations, but also directly correlated with CVD risk. Moreover, the current study indicated that circulating EVs are predominantly < 200nm and therefore exosomes and small microvesicles, and it is these particles which contribute to the association with cardiovascular risk markers and CVD risk rather than the larger particles captured by FCM. The mechanisms underlying the release of these EVs and their role in atherosclerosis still need to be elucidated. Moreover, some dietary fatty acids (e.g. n-3 PUFA) have been reported to play a role in reducing the risk of CVDs by modifying a wide range of risk factors, and there is therefore interest in exploring whether circulating EVs could be modulated by dietary fatty acids. The effects of n-3 PUFA on circulating EVs is described in the next chapters.

Chapter 5 Effects of fish oil-derived n-3 PUFA on numbers of circulating EVs in subjects with moderate risk of CVDs

5.1 Introduction

EVs are small membrane-bound vesicles released by almost all cells under both physiological and pathological conditions. Although the precise mechanisms underlying EVs generation are uncertain, the generated EVs featuring with lipid bilayer-enclosed structures are believed to assist the multiple pathological activities of CVDs such as endothelial dysfunction(Densmore *et al*., 2006; Liu *et al*., 2013), inflammation (Distler *et al*., 2005a; Bretz *et al*., 2013; Gidlöf *et al*., 2013; Hijmans *et al*., 2018) and thrombosis (Owens & Mackman, 2011) by mediating cellular intercommunication via lipid, protein and nucleic acids obtained from parent cells (for more details see **Chapter 1**). Moreover, elevated levels of circulating EVs have been reported in CVD patients (Simak *et al*., 2006; Sinning *et al*., 2011; Jansen *et al*., 201; Pawelczyk *et al*., 2017), and the previous chapter also reported that EV numbers were positively associated with traditional cardiovascular risk markers and that higher numbers of EVs were independently correlated with greater CVD risk; thus EVs are increasingly being appreciated as a novel potential marker of CVDs.

On the other hand, dietary modification of CVD risk factors could help to decrease the morbidity and mortality of CVDs. Dietary fats have been investigated for decades and over this time, the focus has shifted from the reduction of total and saturated fat intake to the relative benefits of unsaturated fat, particularly PUFA. The effect of n-3 PUFA on the prevention of CVDs has been suggested by various epidemiological and RCTs. However, recent studies have failed to support this, perhaps because of the widespread use of statins and the significant improvement in the medical management of CVDs, which may make any beneficial effects of n-3 PUFA harder to demonstrate (Bilato, 2013). Furthermore, while the most well-characterised cardio-protective action attributed to n-3 PUFA is the lowering of plasma TAG concentration (Roche & Gibney, 1999; Weber & Raederstorff, 2000), there have been concerns that

n-3 PUFA increases TC and LDL-C concentrations (Jeppesen *et al*., 2013; Tremblay *et al*., 2016). More recently, the effects of n-3 PUFA on other CVD-related risk markers including endothelial function, inflammation, platelet function and thrombosis, have attracted attention (Lavie *et al*., 2009; De Caterina, 2011; Balakumar & Taneja, 2012; DeFilippis *et al*., 2014) (details see **Chapter 1**). These effects may be dependent on the incorporation of n-3 PUFA into the phospholipid membranes of cells, so the question as to whether n-3 PUFA could also influence the generation of membrane-derived EVs, and therefore the number of circulating EVs, is of interest. Very few studies have investigated the effects of n-3 PUFA on numbers of circulating EVs, although most reported a significant decrease in the numbers of EVs after administering different doses of n-3 PUFA for different periods. However, most studies had limitations in study design and methodology for EV detection. For instance, some studies were uncontrolled parallel trials. Also, most applied only FCM to analyse EVs, and as described in previous chapters, this method on its own is limited as it is only able to detect larger EVs and there is no universal fluorescent marker for EVs.

The aim of this chapter is to examine the effects of fish oil-derived n-3 PUFA on the generation of circulating EVs in subjects with moderate risk of CVDs with refined protocols for EV isolation and characterisation, and to further understand the potential mechanisms behind the beneficial effects of n-3 PUFA on CVDs.

5.2 Subjects and methods

5.2.1 Subjects

A total of 40 subjects (aged 40~70yrs), with moderate risk of CVDs, were involved in the current study. Details about the recruitment and screening of participants are described in **Chapter 3**. Of the 416 subjects contacted, a total of 58 subjects were screened, 42 individuals were enrolled on the trial and 40 completed the study.

5.2.2 Study process and sample processing

The HI-FIVE study was a randomised, double-blind, placebo-controlled crossover intervention. Details about the study design are described in **Chapter 3**. Briefly, subjects were asked to consume six capsules per day of either fish oil providing a total daily intake of 1.8g n-3 PUFA, or control oil (high-oleic safflower oil) for 12 weeks. After 12 weeks of washout, subjects crossed over to the other intervention product for another 12 weeks. Compliance was monitored by capsule counts and changes in plasma phospholipid fatty acid composition. There were four visits in total and the processes of anthropometric measurement, blood collection, sample processing and sample analysis in each visit were described in **Chapter 2** and **3**. One of the prepared PFP (600µl) was immediately analysed for the enumeration and characterisation of circulating EVs by both NTA after the isolation by SEC (TEVs, SEVs and LEVs) and FCM (PS+EVs, PDEVs and EDEVs), while the remainder were stored at − 80°C for lipid profile analysis and fatty acid composition analysis.

a. Lipid profile analysis

A 250µl aliquot of frozen PFP was defrosted at room temperature using a roller mixer and centrifuged at 500xg for 5 minutes at room temperature (Eppendorf Centrifuge 5415 R, DJBlabcare, United Kingdom). Then the sample was analysed by a RANDOX clinical analyser (RANDOX Daytona+ Analyser, Randox Laboratories Ltd, United Kingdom) for the concentration of TC, TAG, HDL-C, LDL-C and TC/HDL-C ratio.

b. Fatty acid composition analysis

i. EV total lipid analysis

A 500µl aliquot of frozen PFP was defrosted at room temperature using a roller mixer and subjected to SEC for the isolation and purification of EVs (see details in **Chapter 3 Section 3.12.1**). The fractions 7~9 were pooled together, and 800µl of pooled fractions was prepared for total lipid extraction by adding 5ml chloroform: methanol (2:1) containing butylated hydroxytoluene antioxidant and 1ml NaCl, followed by

centrifugation at 1400xg for 10 minutes at room temperature. The lower phase was collected and dried under nitrogen at 40°C. Next, methyl esterification of the lipid extract was achieved by first adding 0.5ml dry toluene to the total lipid exact and then 1.0ml methanol containing 2% (v/v) H_2SO4. After heating at 50°C for 2 hours, 1.0ml neutralising solution (0.25M $KHCO_3$ (25.03g/l)), 0.5M K_2CO3 (69.10g/l)) and 1.0ml dry hexane were added, followed by centrifugation at 700xg for 2 minutes at room temperature. The upper phase, which included fatty acid methyl esters (FAMEs) was then collected and dried under nitrogen at 40°C. After being re-suspended in 100µl dry hexane, the EV total lipid methyl esters were ready for analysis by GC.

ii. Plasma total phospholipid analysis

A 400µl aliquot of frozen PFP was defrosted at room temperature using a roller mixer and centrifuged at 13,000xg for 5 minutes at room temperature to remove denatured protein. The 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 EVs (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 EVs (see above).

iii. GC analysis

GC analysis was performed on a Hewlett-Packard 6890 series GC (Hewlett-Packard, California, United States), equipped with flame ionisation detection (Agilent Technologies, Cheadle, United Kingdom). FAMEs were separated in a BPX-70 fused silica capillary column (30m×0.25mm×25µm; SGE Analytical Science, United Kingdom)

with the following protocol: split ratio was set as 30:1 for plasma and EV analysis. The injection volume was 1µl for plasma and 5µl for EVs, respectively. The temperature of both injector and detector were kept at 300°C and the temperature program was initial temperature 115°C for 2 minutes, increased at 10 °C/min to 200°C and hold at this temperature for 16 minutes, and finally increased at 60°C/min to 240°C for 2 minutes (total run time: 29.2 minutes). Helium was used as carrier gas (velocity: 29cm/s; pressure: 21.96psi and flow rate: 1.0ml/min), and detect gas flows were hydrogen flow: 40ml/min; air flow: 120ml/min and make-up flow: 45l/min. Samples were analysed by using ChemStation software (Agilent Technologies, Cheadle, United Kingdom) and Microsoft Excel (Microsoft Corporation, United States). Fatty acid composition data are expressed as g/100g total fatty acids (wt%) for EVs and as both g/100g total fatty acids (wt%) and as absolute concentrations (µg/ml plasma) for plasma.

This analysis was conducted in collaboration with researchers Helena Fisk and Christiaan Gelauf from the University of Southampton. I extracted and prepared the EV total lipids for analysis, while Helena and Christiaan performed the GC analysis and integration of peaks. Helena and Christiaan conducted the plasma total phospholipid analysis in full; this was unavoidable because of restricted access to the laboratory as a result of the COVID-19 pandemic.

5.2.3 Materials and reagents

All chemicals and reagents were from Sigma-Aldrich (Gillingham, United Kingdom) unless otherwise stated.

5.2.4 FFQ analysis

The FFQ used in this study was modified from EPIC-Norfolk FFQ and analysed by FETA software, which can provide general food group and nutrient intake data.

5.2.5 Statistical analysis

Data are expressed as mean ± SEM if normally distributed and as median with inter-quartile range when not. The Kolmogorov-Smirnov test was applied to assess the normal distribution of continuous variables. Numbers of TEVs, SEVs and LEVs were logarithmically transformed for analysis due to their positive-skewed distribution. A general linear model with fixed factors of treatment and period was conducted to determine the differences in the effect of two treatments and two periods on biological characteristics and EVs parameters. A paired t-test comparing the baseline data (before fish oil and before control oil) was performed to check potential carry-over effects. The effect of habitual dietary intake on the numbers of circulating EVs was checked by Pearson's correlation coefficient. The correlations of the potential changes in EVs parameters and biological characteristics were checked by Pearson's correlation coefficient or Spearman's rank correlation coefficient where appropriate. All statistical analyses were performed with SPSS Statistics version 25 and a p-value < 0.05 was considered statistically significant.

5.3 Results

Baseline characteristics of subjects (n=40) are presented in **Chapter 4 Section 4.3.1**.

5.3.1 Compliance

Compliance was monitored by providing subjects with a daily checklist, which they returned at the end of each arm, by capsule counts and by modification of the plasma 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 counted. Compliance was > 98% throughout the trial, as judged by capsule counts. Changes in the fatty acid composition of plasma total phospholipids also reflected good compliance (for details see **Section 5.3.8**).

5.3.2 Effect of fish oil supplementation on numbers and average size of TEVs

Fish oil supplementation significantly decreased numbers of TEVs (−54.3%, $p<0.001$), with no period effect ($p=0.552$) and no treatment x period interaction ($p=0.622$) (**Figure 5.1**). There was no carry-over effect for TEV numbers (**Table 5.1**), indicating that the washout period was sufficient.

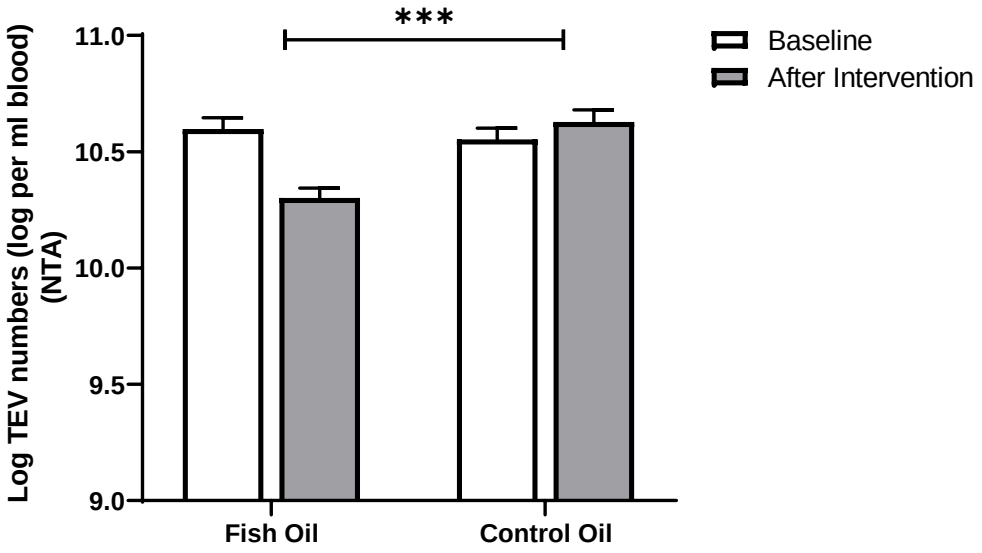


Figure 5.1 Effect of treatment on the numbers of TEVs. Data are mean ± SEM to indicate the differences in the numbers of TEVs from baseline after 12 weeks of fish oil or control oil. TEV numbers were significantly decreased in response to fish oil compared to control oil (treatment: $p<0.001$; period: $p=0.552$; treatment x period: $p=0.622$). *NTA, nanoparticle tracking analysis; TEV, total extracellular vesicles.*

Table 5.1. Numbers and sizes of EVs detected by NTA before each intervention

Intervention	Log TEV numbers (per ml blood)	Log SEV numbers (per ml blood)	Log LEV numbers (per ml blood)	Mean Size (nm)	Mode Size (nm)
Before Fish Oil	10.60±0.05	10.57±0.05	9.27±0.05	102±2.1	76±1.4
Before Control Oil	10.56±0.05	10.54±0.05	9.18±0.05	100±2.0	75±1.4
***p*-value**	*p*=0.103	*p*=0.135	*p*=0.059	*p*=0.320	*p*=0.655

Data are mean ± SEM. There were no significant differences in the numbers of TEVs and SEVs, mean and mode size of EVs between two baselines. *LEVs, large total extracellular vesicles; SEVs, small total extracellular vesicles; TEV, total extracellular vesicles.*

There was no effect of fish oil supplementation on either mean size (treatment: *p*=0.871; period: *p*=0.798; treatment x period: *p*=0.580) or mode size (treatment: *p*=0.917; period: *p*=0.309; treatment x period: *p*=0.674) of EVs (**Figure 5.2**). Also, there was no difference in mean/mode size of TEVs in fish oil and control oil groups at baseline (**Table 5.1**).

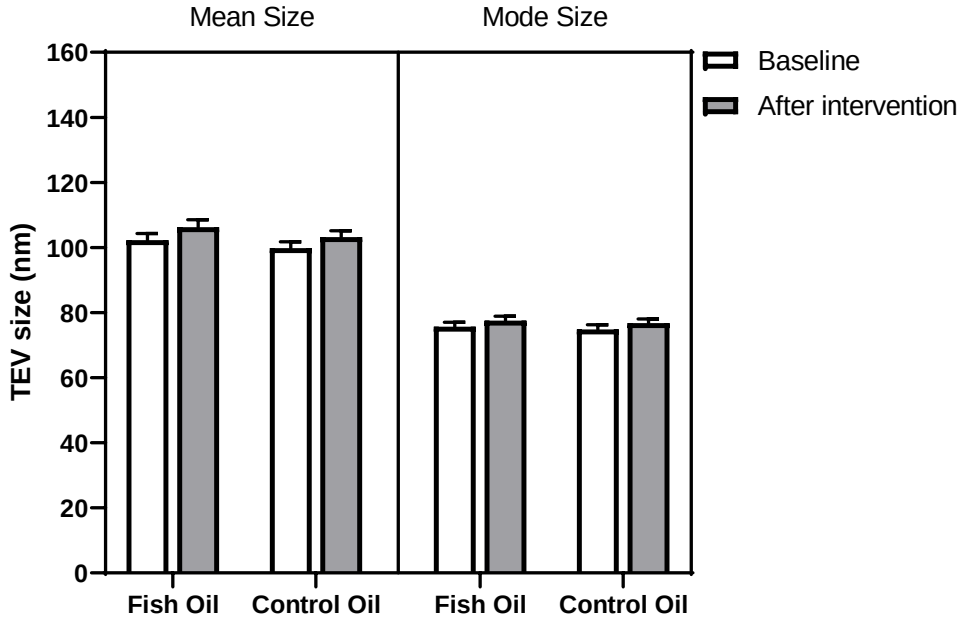
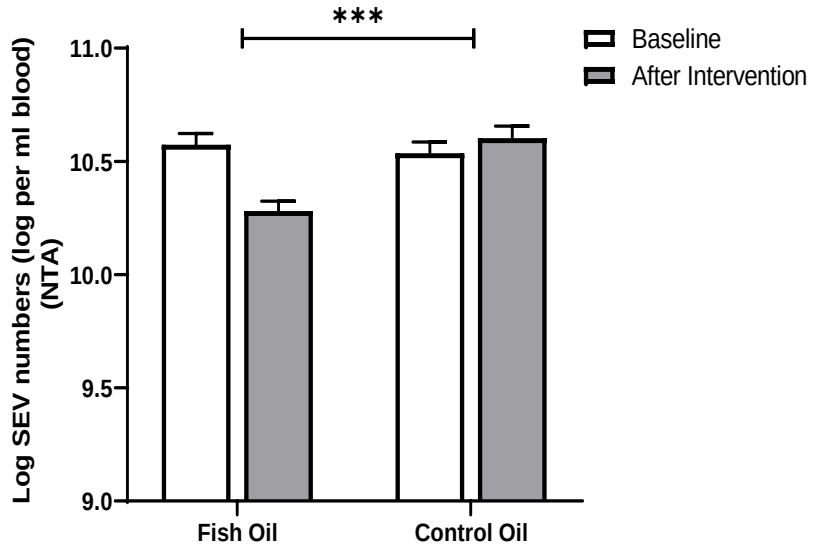


Figure 5.2 Effect of treatment on average size of TEVs. Data are mean ± SEM. There were no effects of treatment on either mean or mode size (mean size: treatment: *p*=0.871; period: *p*=0.798; treatment x period: *p*=0.580; mode size: treatment: *p*=0.917; period: *p*=0.309; treatment x period: *p*=0.674). *TEV, total extracellular vesicles.*

5.3.3 Effect of fish oil supplementation on numbers of SEVs and LEVs

Fish oil supplementation significantly lowered numbers of both SEVs (−54.9%, p<0.001) and LEVs (−32.5%, p<0.001) (**Figure 5.3**), with no significant effects of period or treatment x period interaction (SEV: period: p=0.477; treatment x period: p=0.652; LEVs: period: p=0.544; treatment x period: p=0.514). There was no carry-over effect for SEV and LEV numbers, indicating that the washout period was sufficient (**Table 5.1**).

(A)



(B)

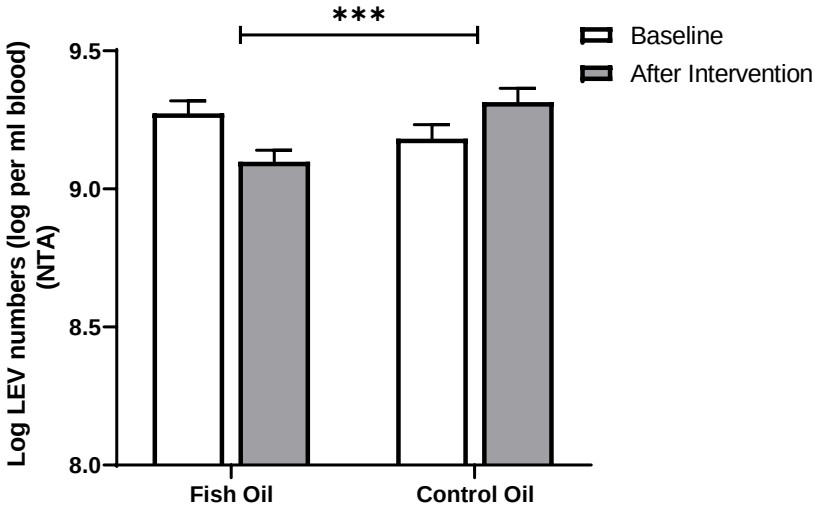


Figure 5.3 Effect of fish oil supplementation on numbers of SEVs and LEVs. Data are mean ± SEM. Fish oil significantly decreased both SEV and LEVs numbers compared to control oil (both: p<0.001). *LEVs, large total extracellular vesicles; NTA, nanoparticle tracking analysis; SEVs, small total extracellular vesicles.*

5.3.4 Effect of fish oil supplementation on numbers of PS+EVs

Fish oil supplementation significantly decreased the numbers of PS+EVs compared to the control oil (−34.2%, *p*=0.001), with no effects of period (*p*=0.646) or treatment x period interaction (*p*=0.267) (**Figure 5.4**). There was no carry-over effect for PS+EV numbers, indicating that the washout period was sufficient. (**Table 5.2**).

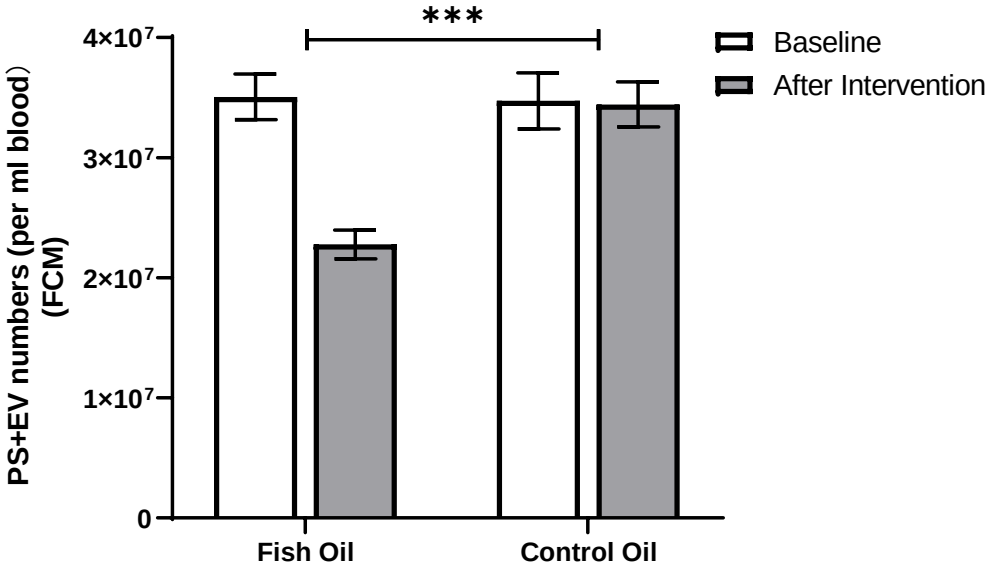


Figure 5.4 Effect of fish oil supplementation on numbers of PS+EVs. Data are mean ± SEM. PS+EV numbers were significantly decreased following fish oil supplementation (treatment: *p*=0.001; period: *p*=0.646; treatment x period: *p*=0.267). *FCM, flow cytometry; PS+EVs, phosphatidylserine positive extracellular vesicles.*

Table 5.2. Numbers of EV subpopulations detected by FCM before each intervention

Intervention	PS+EVs (per ml blood)	PDEVs (per ml blood)	EDEVs (per ml blood)
Before Fish Oil	$3.5*10^7 \pm 1.9*10^6$	$2.2*10^7 \pm 1.4*10^6$	$1.4*10^6 \pm 1.3*10^5$
Before Control Oil	$3.5*10^7 \pm 2.3*10^6$	$2.2*10^7 \pm 1.2*10^6$	$1.3*10^6 \pm 1.1*10^5$
***p*-value**	*p*=0.883	*p*=0.886	*p*=0.574

Data are mean ± SEM. There were no significant differences in the numbers of PS+EVs, PDEVs and EDEVs between two baselines. *EDEVs, endothelial-derived extracellular vesicles; PDEVs, platelet-derived extracellular vesicles; PS+EVs, phosphatidylserine positive extracellular vesicles.*

5.3.5 Effect of fish oil supplementation on numbers of PDEVs

Fish oil supplementation significantly decreased the numbers of PDEVs compared to the control oil (−27.3%, p=0.002), with no effects of period (p=0.350) or treatment x period interaction (p=0.572) (**Figure 5.5**). There was no carry-over effect for PDEV numbers, indicating that the washout period was sufficient (**Table 5.2**).

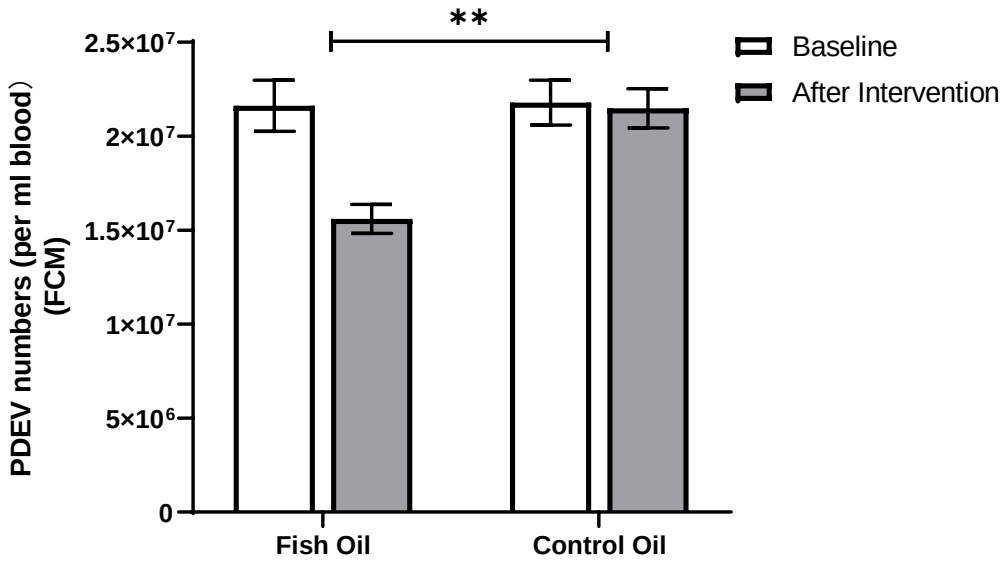


Figure 5.5 Effect of treatment on the numbers of PDEVs. Data are mean ± SEM. PDEV numbers were significantly decreased following fish oil supplementation (treatment: p=0.002; period: p=0.350; treatment x period: p=0.572). *FCM, flow cytometry; PDEVs, platelet-derived extracellular vesicles.*

5.3.6 Effect of fish oil supplementation on numbers of EDEVs

Fish oil supplementation significantly decreased the numbers of EDEVs compared to the control oil (−56.7%, p<0.001), with no effects of period (p=0.362) or treatment x period interaction (p=0.225) (**Figure 5.6**). There was no carry-over effect (**Table 5.2**), indicating that the washout period was sufficient.

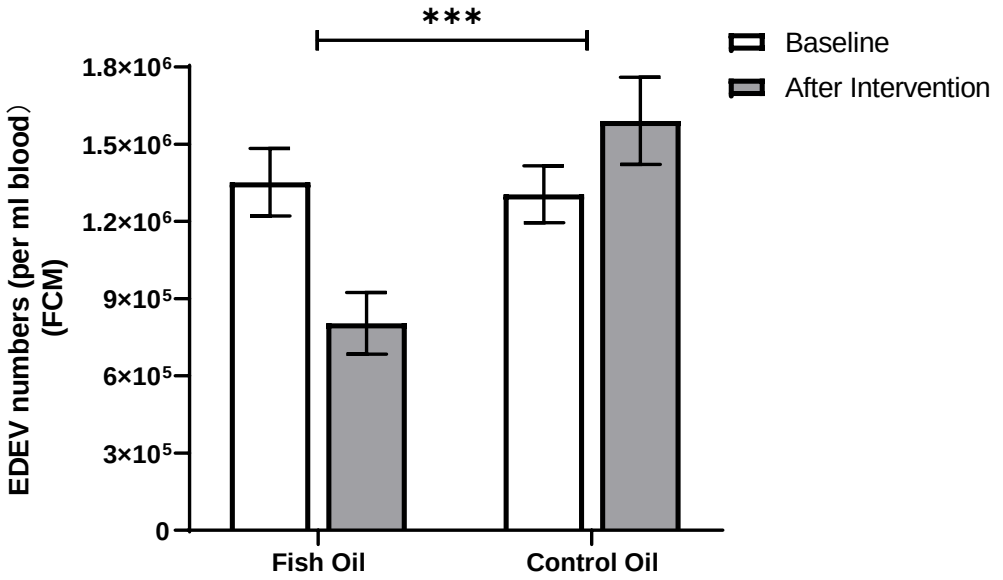


Figure 5.6 Effect of treatment on the numbers of EDEVs. Data are mean ± SEM. EDEV numbers were significantly decreased in response to fish oil supplementation (treatment: $p<0.001$; period: $p=0.362$; treatment x period: $p=0.225$). *EDEVs, endothelial-derived extracellular vesicles; FCM, flow cytometry.*

5.3.7 Effect of habitual dietary intake on the numbers and size of circulating EVs

Habitual dietary intake of subjects was analysed by using a modified EPIC-Norfolk FFQ and the FETA software. There were statistically significant negative correlations of daily n-3 PUFA intake with both PS+EVs ($r=-0.509$, $p=0.001$) and PDEVs ($r=-0.405$, $p=0.009$). Total PUFA was also negatively related with both PS+EVs ($r=-0.397$, $p=0.011$) and PDEVs ($r=-0.356$, $p=0.024$). Also, the mean size of TEVs was significantly negatively correlated with both total carbohydrate ($r=-0.335$, $p=0.035$) and total protein ($r=-0.328$, $p=0.039$). However, there were no other relationships between dietary constituents and EV numbers or size (**Table 5.3**).

Table 5.3. Correlations between EV numbers, EV sizes and dietary constituents

				Correlations									
				Energy (kcal/d)	Carbohydrate (g/d)	Protein (g/d)	Total fat (g/d)	N-3 PUFA (g/d)	Total PUFA (g/d)	Total MUFA (g/d)	Total SFA (g/d)	Alcohol (g/d)	Fibre (g/d)
	NTA	Log TEVs/ml blood	*r*	.141	.132	.183	.231	.109	.262	.195	.072	.030	.087
			p	.386	.417	.258	.152	.504	.103	.227	.657	.854	.591
		Log SEVs/ml blood	*r*	.154	.147	.194	.244	.118	.273	.207	.081	.036	.099
			p	.341	.365	.231	.129	.467	.088	.201	.620	.824	.543
		Log LEVs/ml blood	*r*	-.116	-.156	-.039	-.037	-.080	-.012	-.017	-.065	-.029	-.126
			p	.477	.337	.812	.822	.625	.957	.915	.691	.320	.547
	FCM	PS+EVs/ml blood	*r*	-.214	-.260	-.218	-.211	**-.509*** **	**-.397***	-.210	-.087	.138	-.247
			p	.184	.105	.176	.190	**.001**	**.011**	.193	.595	.396	.124
		PDEVs/ml blood	*r*	-.104	-.126	-.163	-.084	**-.405****	**-.356***	-.115	.142	.147	-.143
			p	.523	.437	.316	.608	**.009**	**.024**	.480	.383	.364	.380
		EDEVs/ml blood	*r*	-.045	.009	-.061	.023	-.158	-.047	.017	.011	.264	-.041
			p	.782	.956	.707	.890	.329	.774	.915	.947	.100	.800
	NTA	Mean size (nm)	*r*	-.278	**-.335***	**-.328***	-.262	-.263	-.290	-.210	-.140	-.095	-.238
			p	.082	**.035**	**.039**	.102	.102	.070	.194	.390	.560	.139
		Mode size (nm)	*r*	-.085	-.107	-.129	-.096	-.137	-.087	-.071	-.082	-.037	-.088
			p	.604	.511	.426	.555	.398	.593	.664	.613	.821	.589

Pearson's correlation coefficient was conducted to examine the correlation between the numbers and size of EVs and cardiovascular risk parameters. * Correlation is significant at the 0.05 level,** Correlation is significant at the 0.01 level, *** Correlation is significant at the 0.001 level (2-tailed). *EDEV, endothelial-derived extracellular vesicles; FCM, flow cytometry; LEVs, large total extracellular vesicles; MUFA, monounsaturated fatty acid; NTA, nanoparticle tracking analysis; PDEVs, platelet-derived extracellular vesicles; PS+EVs, phosphatidylserine positive extracellular vesicles; PUFA, polyunsaturated fatty acid; SEVs, small total extracellular vesicles; SFA, saturated fatty acid; TEVs, total extracellular vesicles*.

Also, dietary intake was assessed both before and after the intervention and results suggested that subjects did not alter their diets during the intervention (**Table 5.4**).

Table 5.4. Dietary intake of subjects before and during the intervention

	Fish Oil		Control Oil		*p*-value		
	Before	After	Before	After	Before-After Fish oil	Before-After 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±3.2	0.227	0.067	0.064
Fat (total, 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	21.1±1.1	0.472	0.068	0.117
Total SFA (g/d)	20.9±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 conducted using a modified EPIC-Norfolk FFQ and FETA software. Differences in dietary intake were compared by paired t-test. *MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.*

5.3.8 Effect of fish oil supplementation on the fatty acid composition of plasma phospholipids and circulating EV total lipids

There was a significant increase in the proportion of EPA, DHA and docosapentaenoic acid (DPA) in plasma total phospholipids after fish oil supplementation compared to control oil, as indicated by both wt% and absolute data, resulting in a substantial overall increase in total n-3 PUFA (**Tables 5.5**). Fish oil supplementation also decreased the proportions/amounts of linoleic acid, dihomo-γ-linolenic acid (DGLA) and AA, and total n-6 PUFA were reduced by > 13% in plasma total phospholipids (**Tables 5.5**). There was no period effect and no treatment x period interactions for either wt% data or absolute data (**Tables 5.5**). There was also no carry-over effect for plasma total

phospholipids, indicating that the washout period was sufficient (data not shown). There was a significant association between the extent of the increase in DHA in plasma total phospholipids and the decrease in the numbers of TEVs (r=−0.317, p=0.046) and SEVs (r=−0.329, p=0.038) after fish oil supplementation. The extent of the decrease in linoleic acid in plasma total phospholipids was also significantly correlated with the decrease in PDEV numbers (r=0.360, p=0.023), but there were no other associations between plasma total phospholipids and numbers of circulating total EVs and/or EV subpopulations (data not shown).

Fish oil supplementation more than doubled EPA and DHA in circulating EV total lipids (both p<0.001), and significantly increased the proportion of DPA (p=0.004), resulting in a substantial overall increase in total n-3 PUFA (p<0.001) (**Tables 5.5**). Supplementation also significantly decreased the proportion of oleic acid (p=0.011), AA (p<0.001) and total MUFA (−6.5%, p=0.013) in the total lipids of circulating EVs (**Tables 5.5**). The effects of fish oil on EPA, DHA, oleic acid, AA, total MUFA and total n-3 PUFA were not influenced by period and there were no treatment x period interactions (data not shown). However, there was a significant treatment x period interaction for DPA (p=0.017), suggesting that the effect of fish oil on DPA was influenced by the intervention sequence (**Tables 5.5**). There was also no carry-over effect for circulating EV total lipids, indicating that the washout period was sufficient (data not shown). There was a significant association between the extent of the increase in DHA in the total lipids of circulating EVs and the decrease in PS+EV numbers (r=−0.352, p=0.026) after fish oil supplementation, but there were no other associations between EV total lipids and numbers of circulating total EVs and/or EV subpopulations (data not shown).

Table 5.5. Effect of fish oil supplementation on the fatty acid composition of plasma phospholipids and circulating EV total lipids

	Plasma (Total phospholipids) (µg/ml)					**Plasma (Total phospholipids) (wt%)**					**EVs (Total lipids) (wt%)**				
	Fish Oil		Control Oil			Fish Oil		Control Oil			Fish Oil		Control Oil		
	Before	After	Before	After	*p*-value	Before	After	Before	After	*p*-value	Before	After	Before	After	*p*-value
Palmitic acid (C16:0)	465.3±13.2	461.2±14.8	469.9±12.7	470.6±11.2	NS	29.5±0.2	29.7±0.2	29.5±0.2	29.2±0.2	NS	26.9±0.5	26.8±0.4	27.0±0.6	26.6±0.5	NS
Stearic acid (C18:0)	216.9±7.4	212.2±7.2	217.9±7.6	221.4±6.8	NS	14.0±0.2	13.9±0.2	13.9±0.1	14.0±0.1	NS	10.9±0.6	11.4±0.7	10.3±0.6	10.3±0.6	NS
Oleic acid (C18:1 n-9)	179.4±6.4	164.6±7.0	185.8±6.3	186.9±7.0	NS	12.4±0.3	11.6±0.3	12.7±0.3	12.7±0.4	NS	29.4±0.7	27.5±0.7	29.9±0.7	30.4±0.8	**0.011**
Vaccenic acid (C18:1 n-7)	20.8±0.9	19.7±0.9	21.1±0.7	20.7±0.8	NS	1.3±0.03	1.3±0.02	1.4±0.04	1.3±0.04	NS	1.8±0.1	1.7±0.1	1.8±0.1	1.8±0.1	NS
Linoleic acid (C18:2 n-6)	335.6±11.1	299.5±11.1	337.2±10.3	346.8±9.1	**<0.001**	21.2±0.4	19.3±0.4	21.1±0.4	21.5±0.3	**<0.001**	17.1±0.5	17.0±0.6	17.7±0.5	17.6±0.5	NS
ALA (C18:3 n-3)	4.5±0.2	4.0±0.2	4.6±0.2	4.5±0.2	NS	0.3±0.01	0.3±0.02	0.3±0.01	0.3±0.01	NS	1.1±0.1	1.12±0.1	1.1±0.1	1.1±0.1	NS
DGLA (C20:3 n-6)	49.4±2.2	35.9±2.0	48.8±2.3	49.6±2.1	**<0.001**	3.1±0.1	2.2±0.1	3.0±0.1	3.0±0.1	**<0.001**	1.8±0.1	1.7±0.1	1.8±0.1	1.7±0.1	NS
AA (C20:4 n-6)	152.1±6.6	121.9±6.1	148.9±6.0	152.7±6.2	**<0.001**	9.9±0.3	8.1±0.2	9.6±0.3	9.8±0.3	**<0.001**	2.9±0.1	2.5±0.1	2.9±0.1	2.9±0.1	**<0.001**

ETA (C20:4 n-3)	3.0±0.2	3.2±0.2	3.2±0.2	3.1±0.2	NS	0.2±0.01	0.3±0.01	0.2±0.01	0.2±0.01	NS	1.7±0.2	2.0±0.2	1.4±0.2	1.5±0.2	NS
EPA (C20:5 n-3)	21.0±1.9	63.3±3.2	21.8±1.5	20.3±1.2	**<0.001**	1.3±0.1	4.6±0.2	1.4±0.1	1.3±0.7	**<0.001**	0.7±0.1	1.6±0.1	0.6±0.1	0.5±0.1	**<0.001**
DPA (C22:5 n-3)	16.9±0.8	20.4±0.8	17.4±0.8	16.4±0.7	**<0.001**	1.1±0.03	1.4±0.04	1.1±0.03	1.1±0.03	**<0.001**	0.4±0.03	0.6±0.03	0.4±0.02	0.4±0.03	**0.004**[#]
DHA (C22:6 n-3)	53.7±3.1	87.3±3.2	57.9±2.9	55.3±2.4	**<0.001**	3.6±0.2	6.0±0.1	3.9±0.2	3.7±0.1	**<0.001**	0.9±0.1	1.9±0.1	0.9±0.1	0.9±0.1	**<0.001**
Total SFA	682.3±20.0	673.4±21.2	687.9±19.8	692.0±17.5	NS	43.5±0.2	43.7±0.2	43.4±0.2	43.1±0.2	NS	37.8±0.9	38.2±0.9	37.3±0.9	36.9±0.9	NS
Total MUFA	215.9±7.4	198.1±8.5	223.2±7.5	223.3±8.3	NS	14.8±0.4	13.8±0.4	15.2±0.3	15.0±0.4	NS	34.3±0.8	32.1±0.8	34.9±0.8	35.1±0.9	**0.013**
Total n-3 PUFA	99.1±5.1	178.3±6.6	104.8±4.6	99.4±3.8	**<0.001**	6.6±0.2	12.2±0.3	6.8±0.2	6.6±0.7	**<0.001**	4.7±0.3	7.2±0.2	4.3±0.2	4.5±0.2	**<0.001**
Total n-6 PUFA	537.0±16.7	457.4±16.7	534.9±15.1	549.2±13.9	**<0.001**	34.2±0.3	29.6±0.5	33.7±0.3	34.3±0.3	**<0.001**	21.9±0.6	21.2±0.6	22.3±0.6	22.2±0.5	NS

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 acid (wt%) in either plasma phospholipids (left) or circulating EV total lipids (right), and the absolute values of each individual fatty acid (µg/ml) in plasma phospholipids (middle). Data are analysed by a general linear model with fixed factors of treatment and period. Only *p*-value of treatment is shown in table, which significant values indicate that fish oil significantly changed the fatty acid composition in plasma phospholipids or circulating EV total lipids compared to control oil, while NS indicate there is no significant difference between the effect of fish oil and control oil on the fatty acid composition in plasma phospholipids or circulating EV total lipids. There was no period effect and no treatment x period interactions except DPA in circulating EVs total lipids. [#] indicates there is a significant treatment x period interaction for DPA (*p*=0.017). *AA, arachidonic acid; ALA, α-linolenic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; ETA, eicosatetraenoic acid. MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.*

A key difference in the effects of fish oil supplementation on plasma phospholipids vs EV total lipids was that in plasma phospholipids, n-3 PUFA appeared to replace linoleic acid, AA and DGLA, whereas in EVs total lipids, they appeared to replace oleic acid and AA. As a result of this, total n-6 PUFA were significantly decreased by fish oil supplementation in plasma phospholipids, but not in EVs (**Tables 5.5**). The alterations in plasma total phospholipids after fish oil supplementation were also significantly correlated with those in circulating EV total lipids, indicating the changes in the proportions of fatty acids especially n-3 PUFA in EV total lipids were significantly influenced by the changes in the proportions of plasma phospholipid n-3 PUFA and n-6 PUFA (**Table 5.6**).

Table 5.6. Correlations of the alterations in fatty acids composition between plasma total phospholipid and circulating EV total lipids after fish oil supplementation

			Correlations							
			ΔPlasma (Total phospholipids)							
			ΔLinoleic acid (C18:2 n-6)	ΔDGLA (C20:3 n-6)	ΔAA (C20:4 n-6)	ΔEPA (C20:5 n-3)	ΔDPA (C22:5 n-3)	ΔDHA (C22:6 n-3)	ΔTotal n-6 PUFA	ΔTotal n-3 PUFA
ΔCirculating EVs (Total lipids)	ΔOleic acid (C18:1 n-9)	*r*	-.089	.242	.127	.069	.017	.022	-.006	.060
		p	.583	.132	.434	.671	.919	.895	.973	.714
	ΔAA (C20:4 n-6)	*r*	-.299	-.177	**.460****	.144	.249	.267	-.128	.245
		p	.061	.275	**.003**	.376	.121	.096	.432	.128
	ΔEPA (C20:5 n-3)	*r*	**-.338***	-.159	**-.351***	**.570*****	**.344***	**.380***	**-.471****	**.505*****
		p	**.033**	.327	**.026**	**.000**	**.030**	**.015**	**.002**	**.001**
	ΔDPA (C22:5 n-3)	*r*	**-.375***	.093	-.197	**.446****	**.383***	.227	**-.390***	**.352***
		p	**.017**	.568	.223	**.004**	**.015**	.159	**.013**	**.026**
	ΔDHA (C22:6 n-3)	*r*	**-.388***	-.199	**-.410****	**.400****	.263	**.484****	**.558*****	**.484****
		p	**.013**	.219	**.009**	**.010**	.101	**.002**	**.000**	**.002**
	ΔTotal MUFA	*r*	-.204	**.348***	.017	.119	.100	.038	-.111	.093
		p	.207	**.028**	.917	.464	.538	.818	.497	.567
	ΔTotal n-3 PUFA	*r*	-.210	-.116	**-.375***	.297	.232	**.459****	**-.373***	**.436****
		p	.193	.475	**.017**	.063	.149	**.003**	**.018**	**.005**

Spearman's rank correlation coefficient was conducted to examine the correlation of the alterations in fatty acids composition after fish oil supplementation between circulating EV total lipids and plasma total phospholipids. * Correlation is significant at the 0.05 level,** Correlation is significant at the 0.01 level, *** Correlation is significant at the 0.001 level (2-tailed). *AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.*

5.3.9 Effect of fish oil supplementation on traditional cardiovascular risk markers

BMI, BP and lipid profiles at baseline and after the intervention of fish oil or control oil are shown in **Table 5.7**. Fish oil significantly reduced SBP (−5.2%, $p<0.001$), DBP (−3.7%, $p=0.002$), TAG concentration (−11.1%, $p=0.016$) and significantly increased LDL-C concentration (4.3%, $p=0.014$) compared to the control oil, but had no effect on TC, HDL-C or the TC/HDL-C ratio. The effects of fish oil on DBP, TAG and LDL-C levels were not influenced by period and there were no treatment x period interactions. However, there was a significant period effect and a treatment x period interaction for SBP ($p=0.011$ and $p=0.033$, respectively), suggesting that the effect of fish oil on SBP was influenced by the intervention sequence (**Table 5.7**). The extent of change in BP, TAG and LDL-C levels as a result of fish oil supplementation was not significantly associated with the decrease in EV numbers (data not shown). There was no carry-over effect for the above traditional cardiovascular risk markers, indicating that the washout period was sufficient (data not shown).

Table 5.7. Effect of treatment and period on traditional cardiovascular risk parameters

	Fish Oil		Control Oil		*p*-value		
	Before	After	Before	After	treatment	period	treatment x period
BMI (kg/m^2)	25.6±0.5	25.6±0.5	25.5±0.5	25.5±0.5	0.675	0.076	0.675
SBP (mmHg)	134.0±2.2	127.4±2.2	131.9±2.2	135.2±2.1	**<0.001**	**0.011**	**0.033**
DBP (mmHg)	79.0±1.4	76.1±1.4	78.1±1.4	79.0±1.6	**0.002**	0.952	0.114
TC (mmol/L)	4.9±0.1	5.0±0.1	4.9±0.1	4.9±0.1	0.077	0.921	0.793
TAG (mmol/L)	1.0±0.1	0.9±0.1	1.0±0.1	1.0±0.1	**0.016**	0.566	0.659
HDL-C (mmol/L)	1.4±0.1	1.5±0.1	1.4±0.1	1.4±0.1	0.379	0.938	0.341
LDL-C (mmol/L)	3.0±0.1	3.2±0.1	3.1±0.1	3.0±0.1	**0.014**	0.842	0.943
TC/HDL-C ratio	3.6±0.1	3.6±0.1	3.6±0.1	3.5±0.1	0.285	0.954	0.528

Data are mean ± SEM and analysed by a general linear model with fixed factors of treatment and period. *BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TAG, triacylglycerol; TC, total cholesterol.*

5.4 Discussion

The current study investigated the chronic effects of fish oil-derived n-3 PUFA on circulating EVs in subjects with moderate risk of CVDs compared to high-oleic safflower oil. Fish oil significantly decreased numbers of circulating TEVs compared to the control oil, but did not affect the size of TEVs. Fish oil also significantly decreased numbers of PS+EVs, PDEVs and EDEVs, indicating that even this very small population of EVs was altered by dietary n-3 PUFA intervention. The intervention resulted in a significant alteration in the fatty acid composition of circulating EV total lipids, chiefly a more than doubling of the proportions of EPA and DHA in EV total lipids at the expense of oleic acid and AA; these are highly novel results, reported for the first time. Fish oil also increased the proportions of EPA and DHA in plasma phospholipids by replacing a range of n-6 PUFA including linoleic acid, AA and DGLA. The intervention reproduced some of the well-established effects of n-3 PUFA on traditional cardiovascular risk markers as well; a significant reduction in plasma TAG (Balk *et al*., 2006; Eslick *et al*., 2009; Leslie *et al*., 2015), a significant increase in LDL-C (Balk *et al*., 2006; Eslick *et al*., 2009), a significant decrease in DBP and a significant but intervention sequence-influenced reduction in SBP (Geleijnse *et al*., 2002; Campbell *et al*., 2013; Miller *et al*., 2014).

N-3 PUFA were efficacious in significantly reducing TAG and BP level (Balk *et al*., 2006; Eslick *et al*., 2009; Campbell *et al*., 2013; Miller *et al*., 2014). The roles of n-3 PUFA on other lipid indices such as TC, LDL-C, HDL-C and TC/HDL-C ratio are not always consistent. A study conducted in 80 hyperlipidaemic subjects showed that a supplement of 4g/d fish oil for three months significantly decreased fasting TC and LDL-C concentrations, but there was no significant change in HDL-C concentration (Qin *et al*., 2015). Another study demonstrated that the only benefit of 1.8g/d DHA+EPA on cholesterol parameters in 312 healthy subjects was the significant increase in the concentration of HDL-C, but there were no changes in TC, LDL-C and TC/HDL-C ratio (Sanders *et al*., 2011). However, a meta-analysis involving 47 studies reported that the average daily intake of 3.25g of n-3 PUFA did not alter concentrations of TC and LDL-C significantly (Eslick *et al*., 2009). The current findings reported that the consumption

of fish oil supplement only resulted in a significant increase in LDL-C concentration, although a slight increase in TC concentration and a slight increase in HDL-C concentration (neither were significant) were also reported. The discrepancies may be due to the different doses and composition of n-3 PUFA provided in those studies (Zibaeenezhad *et al*., 2017). For instance, Wei & Jacobson (2011) compared the effects of EPA and DHA on lipid profile and suggested that DHA increased LDL-C level more than EPA.

There are ten human studies that have investigated the effects of n-3 PUFA on the numbers of circulating EVs to date and a table summarising these studies is presented in **Chapter 1 Table 1.3**. Five of these studies involved healthy subjects, while others provided intervention to patients with various disease status, such as hyperlipidaemia, T2D and post-MI. In line with the current findings, most of the previous studies have indicated the favourable effects of n-3 PUFA on numbers of circulating total EVs and/or EVs subpopulations.

Nomura and co-works conducted four trials, of which two provided hyperlipidaemic patients (T2D and non-T2D) with EPA at a dose of 1.8g/d for four weeks (hyperlipidaemia with T2D: n=23; hyperlipidaemia without T2D: n=18; healthy controls: n=20) (Nomura *et al*., 2003) or six months (hyperlipidaemia with T2D: n=76; hyperlipidaemia without T2D: n=50) (Nomura *et al*., 2009a). The other two explored the effect of either EPA (1.8g/d) or pitavastatin (2mg/d) or a combination on numbers of PDEVs in 191 hyperlipidaemic patients with T2D for six months (Nomura *et al*., 2009b) or the effect of pitavastatin (2mg/d) with either EPA (1.8g/d) or sarpogrelate (300mg/d) in 84 patients with T2D for 12 months (Nomura *et al*., 2018). EPA reduced numbers of GPIX+PDEVs and CD14+MDEVs in patients, but not in healthy controls (Nomura *et al*., 2003). A significantly lower number of Annexin V+/CD51+EDEVs in T2D patients after EPA intervention was also reported in their later study (Nomura *et al*., 2009a), and they also demonstrated that EPA, either independently or combined with drugs, decreased numbers of PDEVs measured by ELISA (Nomura *et al*., 2009b;

Nomura *et al*., 2018). However, a major limitation is that the two parallel trials investigating EPA were uncontrolled and did not consider confounding factors such as age and BMI (Nomura *et al*., 2003; 2009a). Indeed, there were significant differences in age and BMI between diabetic and non-diabetic subjects at baseline ($p<0.01$ and $p<0.05$, respectively) (Nomura *et al*., 2009a). Another study, providing either 5.2g/d n-3 PUFA (n=21) or olive oil (n=21) to post-MI patients for 12 weeks, reported significantly lower numbers of CD61+PDEVs and CD14+MDEVs, but not CD62+EDEVs or TF+EVs after n-3 PUFA intervention compared to the control oil (Del Turco *et al*., 2008). The lack of effect may be due to the relatively small sample size of the study. Also, the fact that subjects had previously had MI may be another explanation as TF-antigen does not appear to be influenced by the long-term treatment with high-dose n-3 PUFA in MI patients (Grundt *et al*., 2003).

Five studies focussed on volunteers free of disease, including one involving young men only (Englyst *et al*., 2007) and another involving elderly men only (Marin *et al*., 2011). Englyst and colleagues (2007) reported a significant increase in CD61+PDEVs after providing 35 healthy young men with 6g/d n-3 PUFA for 12 weeks. This unexpected increase in PDEV numbers could be due to the fact that LDL was increased by 53% and collagen-induced platelet aggregation was increased by 7% after taking such a high dose of n-3 PUFA, since LDL and activated platelets have been found to promote the generation of PDEVs (Nomura *et al*., 2004). In the study by Marin *et al.* (2011), 20 healthy elderly men were randomised to receive three diets, including a Mediterranean diet enriched in MUFA with virgin olive oil (24% of energy (en) was provided by 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) and a low-fat, high-carbohydrate diet enriched with n−3 PUFAs (< 10% en SFA, 12% en MUFA, 8% en PUFA, of which 2% was ALA), each for four weeks. The n-3 PUFA enriched diet resulted in a significant decline in PS+EVs, activated Annexin V+/CD31+EDEVs and apoptotic CD144+/CD62E+EDEVs compared to the SFA-rich diet. Two studies conducted by Phang *et al*., one acute and one chronic, compared the effects of EPA and DHA on EVs. In the acute study, 30 healthy volunteers were blinded to receive EPA-rich (2g/d

capsules providing 1g EPA with an EPA/DHA ratio of 5:1), DHA-rich (2g/d capsules providing 1g DHA with an EPA/DHA ratio of 1:5) or placebo treatment for 24 hours with one-week washout period between treatments, and the results suggested that while the numbers of Annexin V+/CD41+PDEVs were not altered in either the EPA-rich or the DHA-rich group, the EPA-rich oil influenced the procoagulant activity of PDEVs in males only (Phang *et al*., 2012). This lack of acute effect may be because the alteration of PDEV numbers requires the incorporation of n-3 PUFA into the platelet membrane, which generally needs two to four weeks of n-3 PUFA administration for the changes of plasma phospholipids and cellular fatty acid composition reach a plateau (Yaqoob *et al*., 2000; Hodson *et al*., 2018). Also, the subjects involved in this study were healthy with normal numbers of EVs prior to intervention, which n-3 PUFA may have been unable to reduce further. The difference in the favourable effect of EPA on PDEV activity between genders may be linked to the possibility that EPA is more effectively incorporated into platelet membranes in males than females, while females have a greater ability to retain DHA than males (Childs *et al*., 2008); however, this is rather speculative. In the chronic study conducted by the same group, 94 healthy subjects were placed on the same interventions above for four weeks, but there were no effects of either EPA or DHA supplementation on circulating PS+EVs or EV subsets (CD36+EVs, CD41+EVs, CD36+/CD41+EVs, Annexin V+/CD41+PDEVs and Annexin V+/CD36+MDEVs), either in the whole population or within gender groups (Phang *et al*., 2016). This may again be due to the fact that the subjects were healthy, but it is also pertinent to note that the intervention employed a dose of 1.2g/d for only four weeks, whereas the current study employed a dose of 1.8g/d n-3 PUFA for 12 weeks. Finally, a double-blind, randomised crossover study examining the effects of either n-3 PUFA (1.8g/d) or placebo (corn oil) on EV numbers in 84 subjects at moderate risk of CVDs for eight weeks, including an eight-week washout, reported a significant reduction in numbers of CD31+/CD42-EDEVs, but not C31+/CD42+PDEVs and this effect was independent of eNOS genotype, although the reasons for different effects on EV subtypes were unclear (Wu *et al*., 2014). The methodology employed in this study was not optimal as the gating strategy relied on polystyrene beads (which underestimate particle size, as discussed below) and did not use fluorescence triggering. The current study represents a significant improvement on those methods

and may therefore have detected effects which were not evident in the study by Wu *et al*. (2014).

These studies highlight the fact that it is important to consider the influence of sample size, subject clinical characteristics, dose and duration of n-3 PUFA treatment in the interpretation of the results. Smaller studies and studies involving healthy subjects may be less likely to observe a favourable effect of n-3 PUFA on the numbers of circulating EVs. On the other hand, there are no standard protocols for the collection, isolation and characterisation of EVs; thus these contradictory findings may be the result of the variations in the methodologies used for EVs isolation and quantification.

It is notable that most previous studies applied FCM for the analysis of EVs. As discussed in this thesis, the detection limit of FCM is such that it can only detect larger EVs; smaller EVs account for 96% of the EV population and yet escape detection by FCM (details see in **Chapter 4**). Also, gating strategies of FCM employed by these studies were invariably different compared to the current study. Apart from two studies which did not provide sufficient details about the beads used for gating, the other five studies all used polystyrene beads with a different range of sizes, such as 1μm and 2μm (Nomura *et al*., 2003); 0.05μm, 0.8μm and 3μm (Del Turco *et al*., 2008; Nomura *et al*., 2009a); 0.5μm, 0.9μm and 3μm (Wu *et al*., 2014; Phang et al., 2016). Compared to the silica beads applied in the current study, polystyrene beads have a higher refractive index than EVs and are able to scatter more light compared to similar-sized biological particles (van der Pol *et al*., 2016). Therefore, it is highly likely that the EV gate in all previous studies captured not only EVs, but also platelets and apoptotic bodies. Regardless of the fact that EVs visible by FCM only represent 4% of the total population, the current study demonstrated that n-3 PUFA decreased PS+EV numbers detected by FCM. Within this PS+EV population, approximately 58%~69% were derived from platelets, while EDEVs only constituted 4%~5%. The numbers within these EV subpopulations were also decreased by n-3 PUFA. Furthermore, this corresponded with the novel results presented in this chapter, suggesting that

subjects with higher dietary total PUFA intake had significantly lower numbers of PS+EVs and PDEVs. In the current study, NTA was also applied to analyse the numbers of TEVs and EV size, and the results suggested that n-3 PUFA decreased the numbers of TEVs compared to the control oil. Moreover, NTA is able to detect SEVs, which are undetectable by FCM employed in this study. This is the first demonstration of the effect of n-3 PUFA specifically on numbers of SEVs within the defined range of 70~200nm. However, fish oil also decreased numbers of the smaller population of LEVs (> 200nm), which corresponds with the effects on the PS+EVs characterised by FCM. However, it is still important to bear in mind that EVs measured in the current study still did not represent all types of EVs since, as discussed before, neither NTA nor FCM was able to detect EVs smaller than 70 nm (due to the NTA and FCM threshold) or capture all larger EVs (due to lack of a universal fluorescent EV marker).

The current study provides new insight into the influence of fish oil supplementation on the fatty acid composition of EVs. There is very little comparative information about the fatty acid composition of different types of EVs, but the available data from four studies is summarised in **Table 5.8**, alongside baseline data from the current study. Both Connolly *et al*. (2014) and Willis *et al*. (2014) demonstrated that palmitic and oleic acids were the most abundant fatty acids in circulating EVs from patients with hyperlipidaemia or polycystic ovary syndrome, as well as from healthy controls, as was the case in the current study. The overall total lipid profiles were very similar to those reported in this chapter, with only minor differences, despite the fact that the EVs were isolated via differential ultracentrifugation rather than SEC and were derived from patients in some cases (Connolly *et al*., 2014; Willis *et al*., 2014). The fatty acid composition of EVs derived from specific cell types has also been reported, and examples are shown in **Table 5.8**. These suggested that the total lipids of adipocyte-derived EVs were broadly similar to those of circulating EVs, but adipocyte-derived EVs contain a higher proportion of stearic acid and vaccenic acid, and a lower proportion of oleic acid (Connolly *et al*., 2015). The total phospholipids of lower prostate cancer-derived EVs (PC3-EVs) and EVs derived from a metastatic site (left supraclavicular lymph node-derived EVs; LNCaP-EVs) is also similar compared to

circulating EVs, with the exception of a lower proportion of linoleic acid in both PC3-EVs and LNCaP-EVs, as well as a lower proportion of oleic acid and a higher proportion of DPA and DHA in LNCaP-EVs (Ferreri *et al*., 2020). Furthermore, both studies indicated that there were some differences in fatty acid composition between EVs and their parent cells (Connolly *et al*., 2015; Ferreri *et al*., 2020). Also, it was anticipated that the fatty acid composition of circulating EVs phospholipids might reflect that of platelets since PDEVs represent the most abundant EV population. However, this did not appear to be the case, since platelet phospholipids typically contain a lower proportion of palmitic, oleic and linoleic acid and a higher proportion of n-3 PUFA (Risé *et al*., 2007; Del Turco *et al*., 2008). Thus, it is argued that EVs may be 'packaged' with a unique fatty acid signature and may interact with other cells rather than merely reflecting of their cells of origin (Connolly *et al*., 2015; Ferreri *et al*., 2020; Willis *et al*., 2014).

Table 5.8. Fatty acid composition of different types of EVs in different studies

	Circulating EVs (Current study) (wt%)	Circulating EVs[a] (wt%)	Circulating EVs[b] (wt%)	Adipocyte EVs[c] (wt%)	PC3-EVs[d] (wt%)	LNCaPEVs[d] (wt%)
Palmitic acid (C16:0)	27.51±0.52	27.4	27.32±5.82	23.45 ± 2.08	33.32±0.74	35.62±0.96
Palmitoleic acid (C16:1 n-7)	2.51±0.16	1.1	1.59±2.15	2.16 ± 0.67	1.10±0.26	1.59±0.08
Stearic acid (C18:0)	10.82±0.62	14.2	12.95±4.07	26.12 ± 1.76	12.35±0.52	15.34±0.66
Oleic acid (C18:1 n-9)	29.70±0.71	31.5	30.69±5.58	11.63 ± 1.35	30.83±4.41	15.43±0.66
Vaccenic acid (C18:1 n-7)	1.82±0.06	0.1	0.36±1.73	3.66 ± 0.16	1.21±0.08	1.57±0.24
Linoleic acid (C18:2 n-6)	16.99±0.53	12.5	12.72±6.04	12.05 ± 4.44	3.43±0.50	2.91±0.18
ALA (C18:3 n-3)	1.04±0.05	N/A	0.51±0.43	1.09 ± 1.11	N/A	N/A
DGLA (C20:3 n-6)	1.74±0.12	N/A	N/A	N/A	0.49±0.08	0.98±0.16

AA (C20:4 n-6)	2.82±0.09	N/A	5.74±3.85	2.91 ± 0.52	0.70±0.15	2.05±0.13
ETA (C20:4 n-3)	1.40±0.17	N/A	N/A	N/A	N/A	N/A
EPA (C20:5 n-3)	0.61±0.08	0.30	0.31±0.25	N/A	0.29±0.05	0.55±0.09
DPA (C22:5 n-3)	0.38±0.03	0.40	0.34±0.32	0.63 ± 0.34	0.54±0.14	1.41±0.19
DHA (C22:6 n-3)	0.87±0.07	0.95	0.86±0.68	0.82 ± 0.18	1.68±0.24	3.22±0.23

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 (Current study) only involved baseline data (visit 1) of circulating EV total lipids in the current study, respectively. Circulating EVs[a] is estimated from figures of EVs from healthy subjects (Connolly *et al*., 2014); Circulating EVs[b] is mean ± SD and represent EV total lipids from across both polycystic ovary syndrome and healthy subjects (Willis *et al*., 2014); Adipocyte EVs[c] represent total lipids of adipocyte-derived EVs at cell-cultured day0 (Connolly *et al*., 2015); PC3-EVs[d] and LNCaPEVs[d] represent phospholipid fatty acid composition of PC3-EVs and LNCaPEVs, respectively (Ferreri *et al*., 2020). *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; LNCaP-EVs, left supraclavicular lymph node-derived extracellular vesicles; N/A, not applicable; PC3-EVs,lower prostate cancer-derived extracellular vesicles.*

The effect of fish oil supplementation on the fatty acid composition of plasma phospholipids and cellular lipids has been widely investigated. Most studies report that EPA and DHA in plasma phospholipids are enriched following fish oil supplementation and that these fatty acids tend to replace the n-6 PUFA, including linoleic acid, DGLA and AA, which is consistent with the findings of the current study (Lovegrove *et al*., 2004; Young *et al*., 2005; Cao *et al*., 2006; Harris *et al*., 2007; Walker *et al*., 2015; Arnold *et al*., 2017). However, to the best of our knowledge, this is the first study to explore the remodelling of EV total lipid fatty acid composition by n-3 PUFA and is therefore highly novel. The results demonstrated a doubling of EPA and DHA content in the total lipids of circulating EVs at the expense of oleic acid and AA, but not other n-6 PUFA. This pattern of fatty acid modification by n-3 PUFA resembles the impact of fish oil supplementation on platelets and cells, in which oleic acid tends to be displaced by EPA and DHA (Lovegrove *et al*., 2004), with decreases in linoleic acid and DGLA in platelets and erythrocytes being fairly limited (Walker *et al*., 2015). A potential explanation is simply that circulating EVs/platelets contain higher

proportions of oleic acid and lower proportions of linoleic acid and DGLA than plasma phospholipids (data from the current study; Connolly *et al*., 2014; Willis *et al*., 2014; Skeaff *et al*., 2006; Risé *et al*., 2007) and therefore replacement of oleic acid is more apparent. However, this is clearly not the case for AA, where replacement by n-3 PUFA appears to be targeted.

The exact mechanism by which n-3 PUFA treatment achieves a decreased number of circulating EVs remains elusive, but it is well appreciated that the incorporation of n-3 PUFA into cell membrane phospholipids may play a fundamental role in modulating lipid composition and function of platelets and many cell types, including endothelial cells (Felau *et al*., 2018). Modification of cell membrane lipids may alter the ability of the cell to produce EVs, as well as the composition of the EVs. The current study demonstrated that both the EPA and DHA content of circulating EVs was significantly increased after fish oil supplementation, and the increase in DHA was significantly associated with the decrease in PS+EV numbers. The externalisation of PS in the outer leaflet during cell activation, as a key step of EV generation, has been demonstrated to be inhibited by flaxseed oil-derived n-3 PUFA (Yang *et al*., 2012). Furthermore, EV shedding is highly dependent on cholesterol, which is abundant and essential in membrane lipid rafts (Pollet *et al*., 2018). DHA has been reported to alter the size and composition of lipid rafts and to form a unique DHA-rich, highly disordered, non-raft phase due to its strong aversion to cholesterol separation (Stillwell, 2006). Therefore, n-3 PUFA (and DHA in particular) may disrupt lipid rafts to the extent that EV shedding is reduced. An inhibitory effect of n-3 PUFA on platelet activation and aggregation may also result specifically in a reduction in PDEV numbers (Cohen *et al*., 2011). In general, AA can act as a substrate for TXA2 synthesis via COX-2 pathway, which can potently promote platelet activation and aggregation, while n-3 PUFA are metabolised to TXA3, a poor platelet agonist (von Schacky *et al*., 1985; Kim *et al*., 1995). In this regard, when n-3 PUFA are ingested in sufficient doses, they can compete with the metabolism of AA and inhibit the synthesis of TXA2, leading to inhibition of platelet activation and aggregation (von Schacky *et al*., 1985; Krämer *et al*., 1996; Yeung *et al*., 2014, 2017; Adili *et al*., 2018). The current study demonstrated a reduction in the proportion of AA

in the total lipids of circulating EVs after fish oil supplementation, which could lead to a reduction in platelet activation. Following on from this, a reduction in platelet activation may be associated with a reduction in EV generation, since platelets shed EVs when activated. In a similar way, reduced production and availability of NO have been reported to promote the generation of EDEVs by endothelial cells (Balakumar & Taneja, 2012). N-3 PUFA appear to activate eNOS and to reduce high level of oxidative stress, which directly and indirectly increase the generation and bioavailability of NO, and thus improve endothelial function, which may consequently reduce EV shedding (Omura *et al*., 2001; Gortan Cappellari *et al*., 2013; Zhang *et al*., 2013). It is also possible that n-3 PUFA reduce numbers of circulating EVs by influencing their clearance from the circulation; this depends on phagocytosis by macrophages in the liver or spleen (Augustine *et al*., 2014; Matsumoto *et al*., 2019) and has been demonstrated to be influenced by the fatty acid-derived maresins, which enhance phagocytosis in macrophage (Deng *et al*., 2014).

In the current study, n-3 PUFA resulted in a reduction in numbers of circulating EVs in conjunction with its effects on lowering of plasma TAG and DBP, although there was no significant relationship between the extent of reduction of plasma TAG and/or DBP and the decrease in EV numbers. Also, there is evidence that plasma LDL-C concentration and platelet aggregation affect circulating EV numbers (Nomura *et al*., 2004). It is therefore useful to consider the responsiveness of EVs to treatments such as lipid-lowering therapy, anti-hypertensive drugs and antiplatelet agents, and to compare the effects of n-3 PUFA with these treatment regimens. The reported findings are not consistent. Some studies demonstrate that statins reduce the numbers of EVs (Pawelczyk *et al*., 2015; Suades *et al*., 2013), while others do not (Camargo *et al*., 2014). However, fibrates, which lower plasma TAG concentrations more consistently lower EV numbers, suggest a stronger association between TAG lowering (which is a hallmark of n-3 PUFA supplementation) and EV numbers decrease. Kagawa and co-workers (2001) indicated that six months of treatment with 200mg/d bezafibrate resulted in a significant 22% reduction in PDEV numbers in patients with connective tissue diseases and hyperlipidaemia. Similarly, the numbers of

CD42b+PDEVs were significantly decreased by 53% after providing 400mg/d bezafibrate to diabetic patients for six months (Koga *et al*., 2006). Beneficial effects of anti-hypertensive drugs on EV numbers have also been reported. Numbers of GPIX+PDEVs, Annexin V+/CD51+ EDEVs and Annexin V+/CD14+MDEVs were reduced by 24%, 21% and 18% respectively after hypertensive patients with diabetes were treated with 20mg/d nifedipine for six months (Nomura *et al*., 2007). The numbers of CD42b+PDEV also significantly decreased by 35% after treatment of hypertensive patients with 600mg/d eprosartan (Labiós *et al*., 2004). The effects of antiplatelet drugs on EV numbers, on the other hand, are extremely contradictory (Bulut *et al*., 2011; França *et al*., 2012; Camargo *et al*., 2014; Duarte *et al*., 2015; Rosinska *et al*., 2020). In the current study, n-3 PUFA resulted in significant 34.2%, 27.3% and 56.7% reductions in the numbers of PS+EVs, PDEVs and EDEVs, respectively, which was comparable with the effects of fibrates and anti-hypertensive drugs on EV numbers. Moreover, the independent roles of plasma TAG concentration and DBP level in predicting EV numbers were demonstrated in **Chapter 4**. This therefore raises questions about whether the effects of n-3 PUFA on EV numbers might be partly exerted through its effects on plasma TAG and DBP. Further *in vitro* studies examining whether incubating endothelial cells or platelets obtained from patients with hypertriglyceridaemia or hypertension with n-3 PUFA alters their cell membrane composition and bioactive lipid mediator content, as well as the numbers of EVs they generate, could provide more mechanistic information.

One of the limitations that need to be considered in this chapter is the lack of a control investigating the effect of n-3 PUFA added directly to blood on EV analysis. Also, as mentioned before, only PDEVs and EDEVs were detected in the current study, while the effect of n-3 PUFA on other main EV subpopulations was still concerned.

In conclusion, the current study demonstrated that n-3 PUFA decreased numbers of both total circulating EVs and EV subpopulations in subjects with moderate risk of CVDs, along with the significant remodelling of their total lipid fatty acid composition,

and with the expected reduction in plasma TAG concentration and DBP, a significant increase in LDL-C concentration and a significant but intervention sequence-influenced decrease in SBP. A higher habitual dietary intake of n-3 PUFA was associated with lower numbers of EVs. The effects of n-3 PUFA on EV numbers were comparable to those of fibrates and anti-hypertensive drugs. The mechanisms by which n-3 PUFA influence the numbers of circulating EVs is as yet unclear, but they may affect both their generation and clearance directly or indirectly. Since EVs reportedly play a role in the pathology of CVDs, the modulation of EV generation may become a novel therapeutic target in various cardiovascular protective treatments. Indeed, these data provide exciting new evidence and insight into the effects of n-3 PUFA on circulating EV numbers, their comparative effects with treatment regimens targeting blood lipids and BP, and ultimately exert their potential clinical relevance. EVs are capable of triggering procoagulant activity, are associated with thrombotic diseases or atherosclerotic plaque burden in patients at high cardiovascular risk (Davizon & López, 2009; Suades *et al*., 2015; Mooberry & Key, 2016); thus it is important to consider the impact of n-3 PUFA on the functional activities of circulating EVs as well. The next chapter therefore focuses on the functional activities of circulating EVs, in particular, their influence on EV-mediated procoagulatory pathways.

Chapter 6 Influence of cardiovascular risk markers and n-3 PUFA on the procoagulatory activity of circulating EVs in subjects with moderate risk of CVDs

6.1 Introduction

Under normal physiological conditions, activated platelets triggering the coagulation cascade assist in arresting blood loss after blood vessel damage. However, pathological coagulation and thrombosis contribute to atherosclerosis and CVDs, which are described in detail in **Chapter 1**. Briefly, activated platelets and TF initiate the intrinsic and extrinsic pathway of the coagulation cascade, respectively. Thrombin is produced and cleaves fibrinogen to fibrin, resulting in platelet activation. If this occurs following the rupture of an atherosclerotic plaque, the resulting thrombus can partially or completely occlude the arterial lumen, leading to a cardiovascular event (Müller *et al*., 2009; Badimón *et al*., 2009; Persson & Olsen, 2010; Libby *et al*., 2011).

EVs are increased in patients after cardiovascular events (Sinning *et al*., 2011; Jansen *et al*., 2014; Pawelczyk *et al*., 2017) and in subjects with traditional cardiovascular risk markers (Esposito *et al*., 2006; Ueba *et al*., 2010; Amabile *et al*., 2014), and are thus increasingly being investigated as a novel marker of CVDs. Moreover, the procoagulatory properties of EVs attributed to the presence of PS and TF on their surface may contribute to the development of CVDs (Davizon & López, 2009; Mooberry & Key, 2016; Antonova *et al*., 2019) (details see **Chapter 1**). There are various approaches available to evaluate the procoagulatory activity of EVs, for example, measuring the presence and activity of PS and TF on EVs, changes in the clotting time of plasma in the presence or absence of EVs, or the generation of thrombin and the formation of thrombus after the addition of EVs (Aleman *et al*., 2011; Tripisciano *et al*., 2017; Shustova *et al*., 2017). Studies have reported the elevated numbers and/or procoagulatory properties of EVs in patients with acute coronary syndromes (Mallat *et al*., 2000; Bernal-Mizrachi *et al*., 2004), PAH (Bakouboula *et al*., 2008) and thrombosis (Bidot *et al*., 2008). However, there is little information about

the relationship between the procoagulatory activity of circulating EVs and cardiovascular risk markers in relatively healthy subjects (e.g. subjects with moderate risk of CVDs). Furthermore, n-3 PUFA has been extensively investigated for their potential benefits with respect to CVDs. Some studies have indicated favourable effects of n-3 PUFA on blood coagulation and thrombosis (Schmidt, 1997; Kristensen *et al*., 2001; Leray *et al*., 2001; Vanschoonbeek *et al*., 2004), although this has been challenged by some recent studies (Poreba *et al*., 2017; Bagge *et al*., 2018) (details see **Chapter 1**). Furthermore, the biochemical mechanisms facilitating these effects are yet to be fully elucidated. Since numbers of circulating EVs are significantly decreased following supplementation with n-3 PUFA (**Chapter 5**), the question as to whether EVs modified by n-3 PUFA could also influence their procoagulatory activity is of interest. However, to the best of our knowledge, only two previous studies have explored the alteration of the procoagulatory activity of EVs after treatment with n-3 PUFA. Although both suggested that n-3 PUFA decreased the procoagulant activity of EVs, they either focused on post-MI patients or subjects were only administered fish oil supplementation for 24 hours and the techniques employed had significant limitations (Del Turco *et al*., 2008; Phang *et al*., 2012). Thus, information about the chronic effect of n-3 PUFA on the procoagulatory activity of EVs in subjects with moderate risk of CVDs is still limited.

The aim of this chapter is i) to confirm that circulating EVs are procoagulatory by examining their *in vitro* thrombogenic potential in activating TF-dependent thrombin generation in baseline samples from the HI-FIVE study; ii) to explore the relationship between conventional cardiovascular risk markers and the thrombogenic potential of circulating EVs in activating TF-dependent thrombin generation in subjects with moderate risk of CVDs; iii) to examine the effects of fish oil-derived n-3 PUFA on the thrombogenic potential of circulating EVs in activating TF-dependent thrombin generation in subjects with moderate risk of CVDs.

6.2 Subjects and methods

6.2.1 Subjects

A total of 40 subjects (aged 40-70yrs), with moderate risk of CVDs, were involved in the current study. Details about the recruitment and screening of participants are described in **Chapter 3**. Of the 416 subjects contacted, a total of 58 subjects were screened, 42 individuals were enrolled on the trial and 40 completed the study.

6.2.2 Study process and sample processing

The HI-FIVE study was a randomised, double-blind, placebo-controlled crossover intervention. Details about the study design, study process, sample processing and analysis are described in previous chapters. Besides the enumeration and characterisation of circulating EVs, conventional cardiovascular markers evaluation and fatty acid composition analysis, an aliquot of frozen PFP (600µl) was analysed for the TF-dependent thrombin generation assay.

a. Preparation of EV suspension

An aliquot of frozen PFP was defrosted at room temperature using a roller mixer and 500µl of thawed PFP was subjected to SEC for the isolation and purification of EVs (see details in **Chapter 2**). Fractions 7~9 were pooled together and concentrated using a centrifugal concentrator (Fisher Scientific, Leicestershire, UK) at 1000xg for 10 minutes at room temperature. The protein concentration of EV suspensions was measured by Nanodrop-1000 spectrophotometer upon protein A280 measurements (Thermo Scientific, Loughborough, UK) and adjusted to 50µg/ml in at least 25µl.

b. Thrombin generation assay

A commercially available, plate-based thrombin generation assay was used to measure thrombin generation in either a standard, pooled vesicle and platelet-free plasma (termed vesicle-free plasma or VFP) or in the same VFP but with added

circulating EVs from subjects in the intervention study. This enabled the assessment of TF-dependent thrombin generation specifically attributed to circulating EVs in samples from the intervention study. A stock of VFP was prepared from healthy volunteers before the assay as recommended (Tripisciano *et al*., 2017) and stored frozen in aliquots until use. Blood samples were collected into vacutainer tubes containing 3.2% sodium citrate (Greiner Bio-One, Gloucestershire, UK) from three healthy volunteers and centrifuged twice at 2500xg for 15 minutes to remove blood cells and platelets and generate a pooled plasma sample (Heraeus Labofuge 400R Centrifuge, Thermo Scientific, United Kingdom). Large vesicles were first removed from the plasma by ultracentrifugation at 20,000xg for 1 hour at 4 °C, following which the supernatant was collected and the remaining pellets containing large vesicles was discarded. The vesicle-poor plasma was ultracentrifuged at 100,000xg for 1 hour at 4°C and the pellet containing small vesicles was discarded (Optima L-90K Ultracentrifuge, Beckman Coulter Life Sciences, United Kingdom). The supernatant was then filtered four times using a 0.1µm syringe filter (Merck Life Science, Dorset, UK), resulting in VFP. This process removed 81% and 89% of vesicles, as verified by NTA and FCM, respectively. The VFP was stored in aliquots of 1ml at − 80°C until use.

The thrombin generation assay quantifies thrombin generation against a thrombin calibration curve, constructed from dilutions of lyophilised Hepes-NaCl-buffer containing 0.5 % bovine serum albumin (BSA) and ~1000nM thrombin in buffer with BSA. A kinetic fluorescence intensity reading of a plate was initiated by addition of 50µl fluorogenic substrate 1mM Z-G-G-R-AMC and 15mM $CaCl^2$ (TECHNOTHROMBIN®TGA kit, Pathway Diagnostics Limited, Surrey, UK). Calibration curves were recorded at 37°C for 10 minutes with 30 seconds intervals using the fluorescence plate reader FlexStation at 360nm for excitation and at 460nm for emission (FlexStation 3 Multi-Mode Microplate Reader, Molecular Devices Limited, Wokingham, UK). After calibration, the following samples were added to separate wells of a 96 well microplate (Greiner Bio-One, Gloucestershire, UK) for each subject:

- 10µl EV suspension (50µg protein/ml) with 30µl of VFP stock

- 10µl PBS with 30µl of VFP stock (negative control)

This was followed by the addition of 10µl of a low concentration of phospholipid micelles containing recombinant human TF in Tris-Hepes-NaCl buffer (RCL) (TECHNOTHROMBIN®TGA kit, Pathway Diagnostics Limited, Surrey, UK). The plate was then immediately read at 37 °C for 1 hour at 1 minute intervals using FlexStation to detect their fluorescent intensities at excitation and emission wavelengths of 360nm and 460nm, respectively. All samples were analysed in duplicate and data were then analysed by the TECHNOTHROMBIN® TGA Evaluation Software manually to convert the unit of thrombin generation from RFU to nM. Results were presented as five variables: (i) lag-phase for initiation of thrombin generation after addition of the trigger (time to 1/6 of the peak height) (min); (ii) peak thrombin concentration (nM); (iii) time to reach the peak (min); (iv) velocity index, defined as = [peak height/(time to peak – lag time)] and (v) area under the curve, defined as endogenous thrombin potential (ETP) (expressed as nM thrombin × min) (Tripodi, 2016) (**Figure 6.1**). The thrombogenicity specifically supported by circulating EVs was calculated as TF-dependent thrombin generation resulting from purified circulating EVs plus VFP minus that resulting from VFP alone.

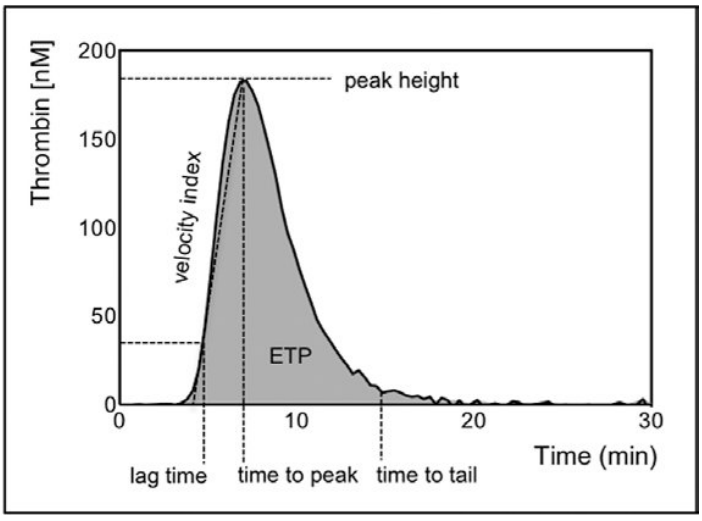


Figure 6.1. Thrombin generation curve and parameters. (Loeffen *et al*., 2015)

This analysis was conducted in collaboration with another PhD student, Esra Bozbas. I prepared the EV suspension, we prepared the VFP together, and Esra ran the thrombin generation assay.

6.2.3 Statistical analysis

Data are expressed as mean ± SEM if normally distributed and as median with inter-quartile range when not. The Kolmogorov-Smirnov test was applied to assess the normal distribution of continuous variables and parameters without normal distribution were logarithmically transformed for analysis. The differences in thrombin generation assay parameters between EVs plus VFP and VFP alone in baseline samples were determined by a paired t-test. The strength of the correlations between EV thrombin generation assay parameters and cardiovascular risk parameters/EV parameters were calculated by Pearson's correlation coefficient. In the categorical analysis, the median values for parameters of thrombin generation were used as a cut-off and means between two categories were compared with the use of a two-tailed unpaired Student's t-test. A general linear model with fixed factors of treatment and period was conducted to determine the differences in the effect of two treatments and two periods on thrombin generation assay parameters. A paired t-test comparing thrombin generation assay parameters before fish oil and before control oil was performed to check potential carry-over effects. The changes in biological characteristics and EVs parameters was checked by Pearson's correlation coefficient or Spearman's rank correlation coefficient where appropriate. All statistical analyses were performed with SPSS Statistics version 25 and a p-value < 0.05 was considered statistically significant.

6.3 Results

6.3.1 Characteristics of the study population

Subject baseline characteristics (n=40) are shown in **Table 6.1**. The mean of the lag time for TF-dependent thrombin generation, peak thrombin concentration, the time to reach peak thrombin generation, velocity index and ETP in VFP alone were 25.9±1.5min, 28.5±5.3nM, 42.9±2.3min, 2.1±0.5 and 445.9±100.5nM thrombin × min, respectively. The mean size of EVs was 97.9±11.4nm and the mode size of EVs was 73.7±8.0 nm.

Table 6.1. Characteristics of the Study Subjects

Sample Characteristic	All Subjects (n=40)
Age (years)	64 (5)
Male: Female ratio	24:16
BMI (kg/m^2)	25.4±0.5
SBP (mmHg)	134±2.2
DBP (mmHg)	79±1.4
TC (mmol/L)	6.0±0.2
TAG (mmol/L)	1.3±0.1
HDL-C(mmol/L)	1.6(0.3)
TC/HDL-C ratio	3.9±0.1
Glucose (mmol/L)	5.8±0.1
10-yr CVD risk score (%) (QRISK2)	12.9±0.01
Lag time for thrombin generation (min)	20.9±0.7
Peak thrombin concentration (nM)	73.6±6.8
Time to peak thrombin concentration(min)	36.6±1.3
Velocity index	5.8±0.7
ETP (nM thrombin × min)	1581.5±140.0

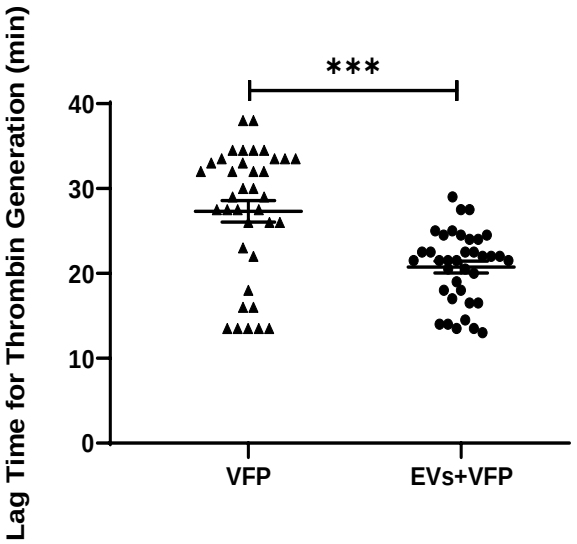
Values in normal distribution are shown as mean ± SEM, and the values in non-normal distribution as the median (inter-quartile range). *BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; ETP, endogenous thrombin potential; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; TAG, triacylglycerol; TC, total cholesterol.*

6.3.2 Influence of circulating EVs on TF-dependent thrombin generation

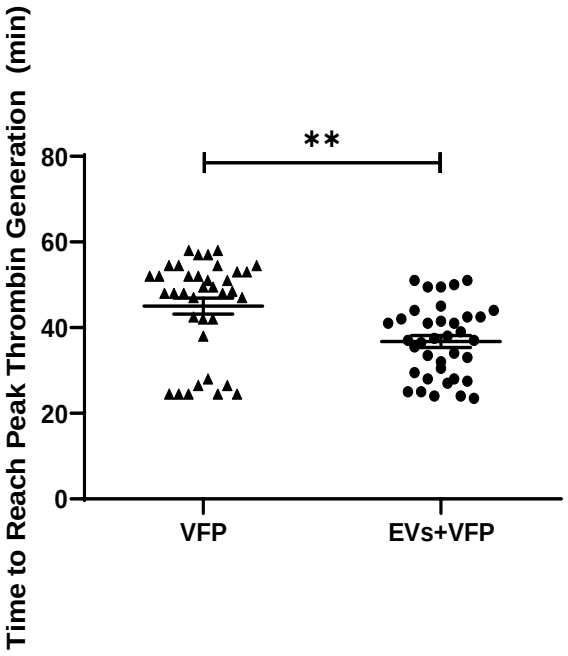
Addition of circulating EVs to VFP significantly shortened the lag time for TF-dependent thrombin generation (−23.9%, p=0.001) and the time to reach peak thrombin generation (−16.7%, p=0.002), as well as significantly increasing peak thrombin concentration (61.4%, p<0.001), velocity index (63.8%, p<0.001) and ETP

(71.9%, p<0.001) compared to VFP alone, indicating the TF-dependent thrombin generation was promoted by circulating EVs (**Figure 6.1**).

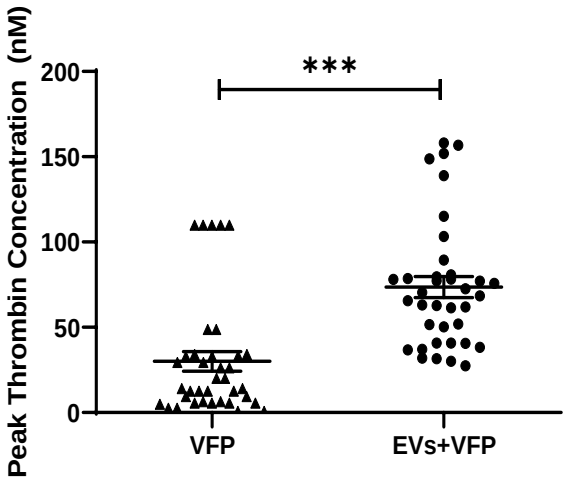
(A)



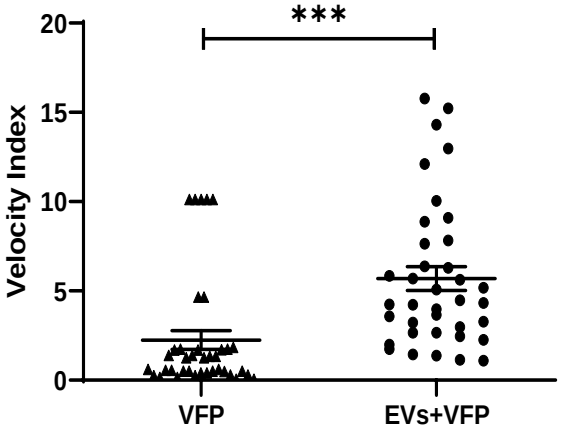
(B)



(C)


Peak Thrombin Concentration (nM)
200
150
100
50
0
***
VFP
EVs+VFP

(D)


Velocity Index
20
15
10
5
0
***
VFP
EVs+VFP

(E)

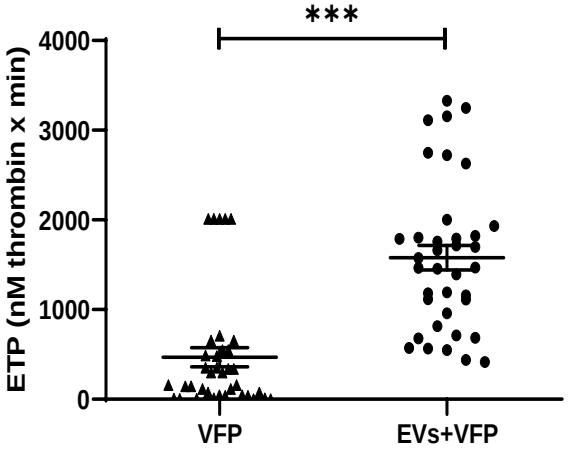

ETP (nM thrombin x min)
4000
3000
2000
1000
0
***
VFP
EVs+VFP

Figure 6.1. Thrombogenicity of circulating EVs in activating TF-dependent thrombin generation (baseline data). Data are mean ± SEM, triangles represent the TF-dependent thrombin generation exhibited in VFP of each individual and dots represent the TF-dependent thrombin generation exhibited in VFP with the addition of circulating EVs of each individual. Addition of circulating EVs into VFP significantly shortened the lag time for thrombin generation (**A**) and the time to reach peak thrombin generation (**B**), and significantly increased peak thrombin concentration (**C**), velocity index (**D**) and ETP (**E**) compared to VFP alone. $^{**}p<0.01$ and $^{***}p<0.001$. *ETP, endogenous thrombin potential; EVs, extracellular vesicles; VFP, vesicle-free plasma.*

6.3.3 Is the thrombogenicity of circulating EVs in activating TF-dependent thrombin generation associated with their size or with CVD risk markers?

The thrombogenic potential of circulating EVs in activating TF-dependent thrombin generation was not associated with any of the cardiovascular risk markers evaluated or with 10-yr CVD risk (**Table 6.2**). However, mode size of EVs was inversely associated with peak thrombin concentration, velocity index and ETP, and positively associated with the time to reach peak thrombin concentration, suggesting that smaller EVs had greater thrombogenicity in activating TF-dependent thrombin generation (**Table 6.2**). Subjects were also categorised into EV 'high' and 'low' thrombogenicity groups based on the median of EV-related thrombin generation parameters, but there was no significant difference in any CVD risk markers between high and low EV thrombogenicity groups (data not shown).

Table 6.2. Correlations between EV thrombogenicity, EV size and cardiovascular risk markers

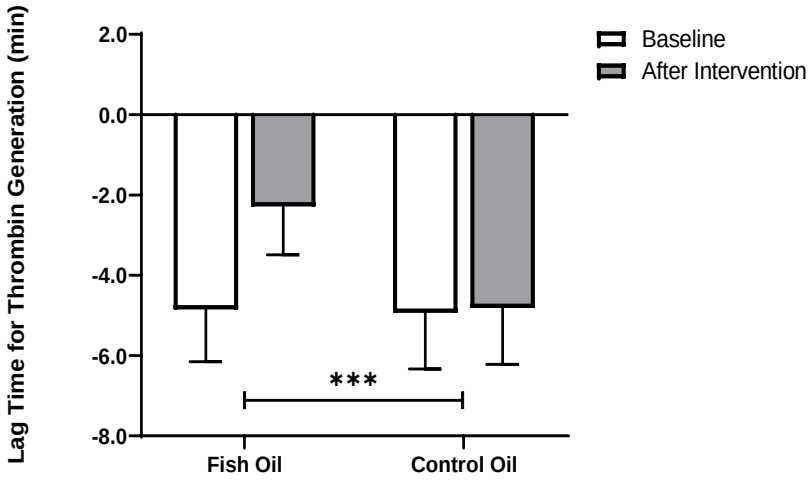
Correlations													
Thrombin generation assay		**Mean size (nm)**	**Mode size (nm)**	**Age**	**BMI (kg/m²)**	**SBP (mmHg)**	**DBP (mmHg)**	**TC (mmol/L)**	**TAG (mmol/L)**	**Log HDL-C (Log mmol/L)**	**TC/HDL-C ratio**	**Glucose (mmol/L)**	**Risk (%)**
Lag time for thrombin generation (min)	*r*	.145	.279	-.082	.043	.127	.041	.030	.238	.011	.014	.259	.247
	p	.380	.085	.618	.794	.441	.806	.858	.144	.945	.931	.112	.129
Peak thrombin concentration (nM)	*r*	-.226	**-.358***	-.058	-.016	-.180	-.138	.099	-.213	.023	.066	-.105	-.298
	p	.166	**.025**	.727	.924	.273	.403	.548	.192	.888	.688	.524	.065
Time to peak thrombin concentration(min)	*r*	.221	**.335***	-.035	.051	.041	.037	.023	.236	-.091	.094	.222	.281
	p	.176	**.037**	.835	.759	.806	.821	.889	.149	.583	.571	.174	.084
Velocity index	*r*	-.233	**-.366***	-.041	.009	-.149	-.102	.075	-.189	.134	-.047	-.089	-.309
	p	.154	**.022**	.804	.955	.366	.538	.650	.249	.416	.777	.589	.056
ETP (nM thrombin × min)	*r*	-.286	**-.413****	-.081	-.053	-.170	-.152	.120	-.239	-.013	.114	-.042	-.308
	p	.078	**.009**	.626	.749	.301	.356	.465	.143	.940	.489	.801	.057

Pearson's correlation coefficient was conducted to examine the correlation between the thrombogenicity of EVs in activating TF-dependent thrombin generation and the size of EVs and cardiovascular risk parameters. *. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed). *BMI, body mass index; DBP, diastolic blood pressure; ETP, endogenous thrombin potential; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; TAG, triacylglycerol; TC, total cholesterol.*

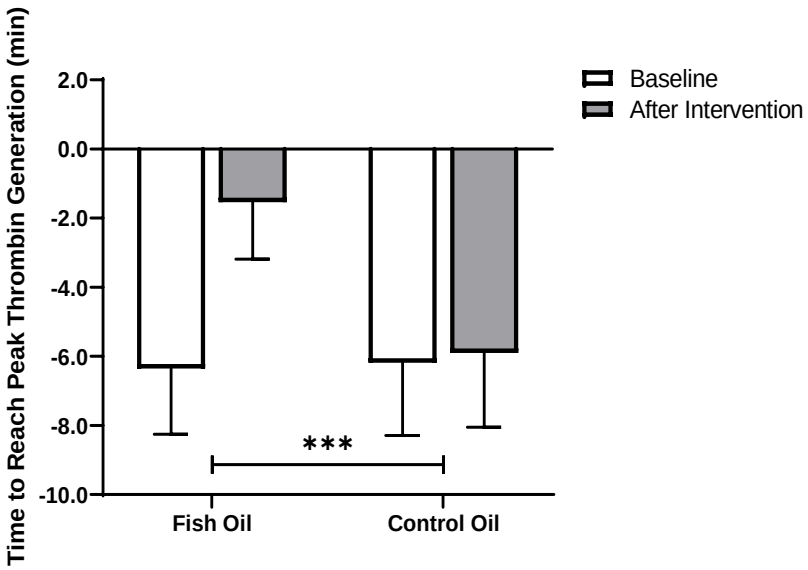
6.3.4 Effect of fish oil supplementation on the thrombogenicity of circulating EVs in activating TF-dependent thrombin generation

The thrombogenic potential of circulating EVs in activating TF-dependent thrombin generation was expressed as the thrombin generation parameters in EV plus VFP samples minus those in the VFP control. As described in **Section 6.3.2**, the presence of EVs shortened the lag time and the time to reach peak thrombin concentration relative to VFP alone; thus these parameters were presented as negative values after subtraction, whereas peak thrombin concentration, velocity index and ETP were positive values as they were increased by EVs relative to VFP alone (**Figure 6.2**). The shortening of lag time for thrombin generation by EVs was significantly less dramatic following fish oil supplementation compared to the control oil (p<0.001), as was the time to reach peak thrombin concentration (p<0.001). EVs from subjects supplemented with fish oil resulted in lower peak thrombin concentration (p<0.001), velocity index (p=0.015) and ETP (p<0.001) compared to those from subjects supplemented with control oil (**Figure 6.2**). There was no carry-over effect for thrombin generation assay parameters of circulating EVs, indicating that the washout period was sufficient (**Table 6.3**). Overall, EVs from subjects supplemented with fish oil were less thrombogenic in terms of promoting TF-dependent thrombin generation according to every parameter of the thrombin generation assay.

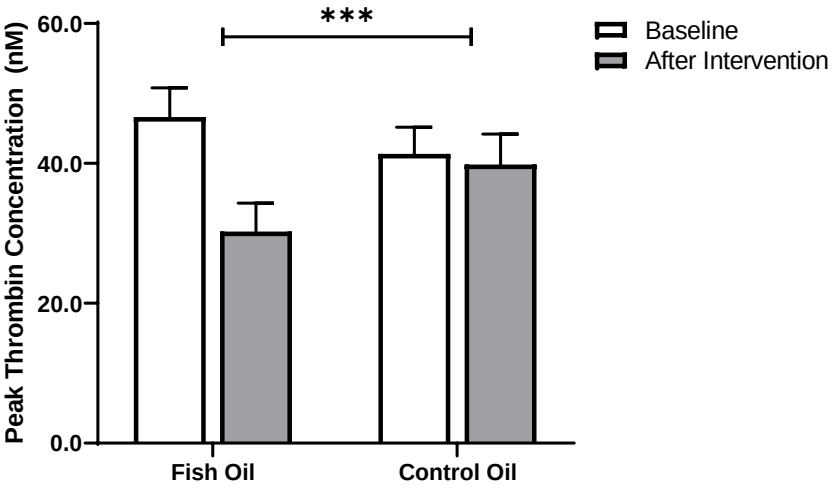
(A)



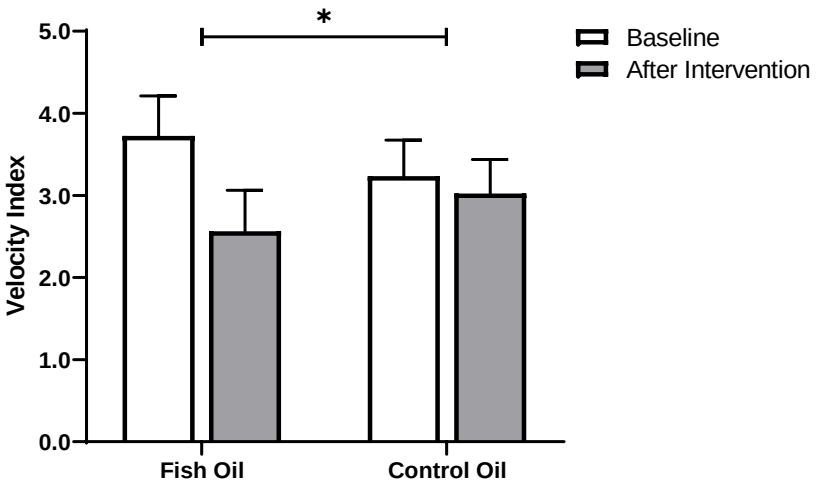
(B)


Time to Reach Peak Thrombin Generation (min)
2.0
0.0
-2.0
-4.0
-6.0
-8.0
-10.0
***
Fish Oil
Control Oil
Baseline
After Intervention

(C)


Peak Thrombin Concentration (nM)
60.0
40.0
20.0
0.0
***
Fish Oil
Control Oil
Baseline
After Intervention

(D)



(E)

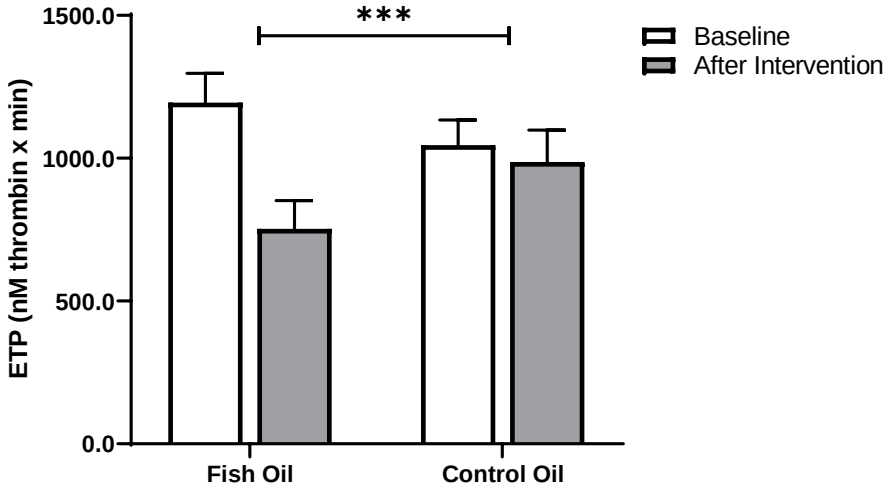


Figure 6.2 Effect of fish oil supplementation on the thrombogenicity of EVs in activating TF-dependent thrombin generation. Data are mean ± SEM to indicate the differences in TF-dependent thrombin generation parameters specifically supported by circulating EVs from baseline after 12 weeks of fish oil or control oil. The EVs-mediated shortened lag time for thrombin generation (**A**) (treatment: $p<0.001$; period: $p=0.010$; treatment x period: $p=0.608$) and the time to reach peak thrombin concentration (**B**) (treatment: $p<0.001$; period: $p=0.138$; treatment x period: $p=0.872$) were significantly prolonged, as well as the EVs-mediated increased thrombin peak concentration (**C**) (treatment: $p<0.001$; period: $p=0.073$; treatment x period: $p=0.667$), velocity index (**D**) (treatment: $p=0.015$; period: $p=0.059$; treatment x period: $p=0.220$) and ETP (**E**) (treatment: $p<0.001$; period: $p=0.118$; treatment x period: $p=0.802$) were significantly reduced in response to fish oil compared to control oil. *$p<0.05$ and ***$p<0.001$. *ETP, endogenous thrombin potential.*

Table 6.3. The thrombogenicity of EVs in activating TF-dependent thrombin generation before each intervention

Intervention	Lag time for thrombin generation (min)	Thrombin peak concentration (nM)	Time to reach peak thrombin concentration (min)	Velocity index	ETP (nM thrombin × min)
Before Fish Oil	−4.9±1.3	47.0±4.3	−5.1±1.9	3.7±0.4	1202.0±104.7
Before Control Oil	−4.4±1.3	43.2±3.4	−6.2±1.9	3.4±0.5	1085.4±80.6
***p*-value**	*p*=0.368	*p*=0.197	*p*=0.238	*p*=0.285	*p*=0.144

Data are mean ± SEM. There were no significant differences in the TF-dependent thrombin generation parameters specifically supported by circulating EVs between two baselines*. ETP, endogenous thrombin potential.*

Furthermore, the extent of the reduction in EV-mediated increased thrombin peak concentration significantly correlated with the decrease in TAG concentration after fish oil supplement (r=0.349, p=0.027), while there were no other associations of other thrombin generation assay parameters with EV numbers, size, fatty acid composition and other cardiovascular risk markers (data not shown).

6.4 Discussion

The current study explored the thrombogenic activity of circulating EVs to promote TF-dependent thrombin generation, the association between the thrombogenic potential of circulating EVs in activating TF-dependent thrombin generation and conventional cardiovascular risk markers, and the effects of fish oil-derived n-3 PUFA on the TF-dependent thrombin generation supported by circulating EVs compared to high-oleic safflower oil in subjects with moderate risk of CVDs. The addition of circulating EVs to VFP significantly promoted TF-dependent thrombin generation in plasma and this thrombogenicity of circulating EVs appeared to be greater for small EVs, but was not significantly correlated with any cardiovascular risk markers or CVD risk. Fish oil significantly decreased but did not eliminate TF-dependent thrombin generation promoted by circulating EVs, suggesting that EVs modified by n-3 PUFA have reduced their thrombogenicity and this may contribute to protective effects on thrombosis and atherosclerosis.

Procoagulatory features of EVs can be assessed using several assays, and one of the commonly used methods is to apply a thrombin generation assay, which measures a change in fluorescence as a result of cleavage of a fluorogenic substrate by thrombin over time upon the activation of the clotting cascade by TF (Baglin, 2005; Aleman *et al*., 2011; Van Der Meijden *et al*., 2012). In the current study, the thrombogenic potential of circulating EVs was examined by comparing TF-dependent thrombin generation in purified circulating EVs plus VFP with that in VFP alone. Addition of circulating EVs significantly shortened the lag time and the time to the peak, as well as increasing the thrombin peak, velocity index and ETP, which indicated that circulating EVs augmented TF-dependent thrombin generation. In agreement with the current findings, many previous studies, both *in vivo* and *in vitro*, have reported thrombogenic activity of EVs in either healthy subjects or patients with a hypercoagulable state (Berckmans *et al*., 2001; Bidot *et al*., 2008; van Beers *et al*., 2009; Aleman *et al*., 2011; Van Der Meijden *et al*., 2012; Marchetti *et al*., 2014; Tripisciano *et al*., 2017; Shustova *et al*., 2017; Durrieu *et al*., 2018). Although the precise mechanisms by which EVs facilitate thrombin generation are still a matter of debate,

it is believed that both PS and TF exposure on the surface of circulating EVs are likely to play an important role. EV-associated PS provides a surface for the binding of the coagulation Factors VII, IX and X, and the assembly of the coagulation complexes, contributing to the formation of thrombin (Owens & Mackman, 2011), while TF binding with Factor VIIa can activate Factor X and then initiate the extrinsic pathway of the coagulation cascade (Persson & Olsen, 2010). However, previous observations also suggested that the presence of PS and TF varies amongst EVs derived from different cells (Persson & Olsen, 2010; Morel *et al*., 2011; Owens & Mackman, 2011), but whether this translates to the differential thrombogenic potential of different EV subpopulations is not clear. Moreover, thrombin generation promoted by PDEVs appears to depend predominantly on the exposure of PS, whereas that promoted by MDEVs appears to depend on TF (Aleman *et al*., 2011; Tripisciano *et al*., 2017). It was not possible to investigate the effects of different EV subpopulations on their thrombogenic potential in the current study, but smaller EVs were observed to have a greater capacity of promoting TF-dependent thrombin generation, implying that exosomes may be more thrombogenic than larger EVs. Exosomes have certainly been shown to be thrombogenic (Kapustin & Shanahan, 2016; Dyer *et al*., 2019), although two studies demonstrated that microvesicles are more prothrombotic than exosomes (Tripisciano *et al*., 2017; Durrieu *et al*., 2018). It was not within the scope of the current study to separate EVs of different sizes and examine their thrombogenic potential, but this would be a promising area for future research.

Perhaps surprisingly, there appeared to be no relationship between cardiovascular risk markers and thrombogenicity of circulating EVs in activating TF-dependent thrombin generation, despite the fact that a hypercoagulable and prothrombotic state in obesity, hypertension and hyperlipidaemia is well described and has been extensively reviewed (Darvall *et al*., 2007; Kornblith *et al*., 2015; Lip& Blann, 2000; Bazan & Fares, 2018; Kim *et al*., 2015; Salvagno *et al*., 2019). Biró *et al.* (2003) demonstrated that pericardial blood collected from patients after cardiac surgery was rich in procoagulant TF-bearing EVs and Bidot *et al.* (2008) reported that circulating EVs from patients with thrombosis had greater thrombin-generating capacity than

healthy subjects. To the best of our knowledge, the current study was the first study to explore the relationship between cardiovascular risk markers and thrombogenic potential of EVs in relatively healthy subjects. Both Goichot *et al.* (2006) and Bakouboula *et al.* (2008) reported a significant association between numbers of 'procoagulant' EVs and BMI and BP; however, they defined procoagulant EVs as those expressing PS or TF rather than by thrombogenic activity, and it is clear that PS and TF exposure is not necessarily a direct determinant of procoagulatory activity (Persson & Olsen, 2010; Owens & Mackman, 2011). Interestingly, **Chapter 4** demonstrated that BMI, BP and TAG were positively associated with numbers of total circulating EVs, but not with numbers of PS+EVs or PDEVs. It is possible that subjects in the 'moderate risk' category do not provide a reference range sufficient to demonstrate a relationship with thrombogenicity of EVs. Furthermore, the current study only evaluated TF-dependent thrombin generation as an indicator of procoagulatory activity, and therefore other aspects of procoagulatory activity may provide a fuller picture.

The current study demonstrated that EVs modified by n-3 PUFA were significantly less thrombogenic than those from subjects supplemented by control oil, as indicated by effects on lag time, time to the peak thrombin generation, peak thrombin concentration, velocity index and ETP. To the best of our knowledge, only two previous studies have explored the alteration of the procoagulatory activity of EVs after treatment with n-3 PUFA. Del Turco *et al.* (2008) provided either 5.2g/d n-3 PUFA (n=21) or olive oil (n=21) to post-MI patients for 12 weeks and reported that a significant increase in the fibrin generation time was caused by fish oil treatment rather than control intervention compared with pre-treatment values, suggesting that only n-3 PUFA supplementation overall reduced thrombogenicity of circulating EVs. They also incubated EVs isolated after fish oil treatment with Factors VII-deficient plasma and Factors XII-deficient plasma and reported that the time to fibrin generation in both was prolonged compared with pre-treatment, indicating that the anticoagulant effect of n-3 PUFA on circulating EVs was dependent on both a TF-dependent and TF-independent coagulation pathway (Del Turco *et al*., 2008). In another study, 30 healthy volunteers were randomised to receive EPA-rich (2g/d

capsules providing 1g EPA with an EPA/DHA ratio of 5:1), DHA-rich (2g/d capsules providing 1g DHA with an EPA/DHA ratio of 1:5) or placebo treatment for 24 hours. The results suggested that only the EPA-rich oil significantly prolonged the lag time for thrombin generation and reduced EV activity, although this effect was only observed in males when the data were separated into gender groups (Phang *et al*., 2012). The current study is the first to provide robust data demonstrating the chronic effect of n-3 PUFA on the thrombogenic activity of EVs in subjects with moderate CVD risk, accompanied by more than a doubling of the proportions of both EPA and DHA in circulating EVs (details see **Chapter 5 Section 5.3.8**), although the extent of incorporation of n-3 PUFA into EVs did not appear to be associated with their thrombogenicity.

The effects of n-3 PUFA on thrombosis has been widely explored (Vanschoonbeek *et al*., 2004; Larson *et al*., 2013; McEwen *et al*., 2015; Adili *et al*., 2018); however, the exact mechanisms by which n-3 PUFA decrease the thrombogenic potential of circulating EVs are uncertain, although they could be speculated to be linked to the incorporation of n-3 PUFA into cell membrane phospholipids. AA, as a major component of membrane phospholipids, can be oxidised to TXA2 via the COX-2 pathway, and this eicosanoid potently promotes platelet activation and thrombin generation (Kim *et al*., 1995; Altman *et al*., 2000). Circulating EVs containing AA have also been found to generate TXA2 (Duchez *et al*., 2015; Boilard, 2018). **Chapter 5** reported the replacement of AA by EPA and DHA in both EV total lipids and plasma phospholipids, raising the possibility that n-3 PUFA not only inhibit the synthesis of TXA2, but also generate eicosanoid metabolites with lower thrombogenic potency, leading to the reduction of thrombin generation (Krämer *et al*., 1996; Altman *et al*., 2000; Adili *et al*., 2018). Moreover, the externalisation of PS in the outer cell membrane is a key contributor to the thrombin-generating capacity of EVs. Larson *et al*. (2011) reported that PS exposure on platelet was reduced by approximately 50% after healthy subjects receiving n-3 PUFA supplementation for 28 days. Another study also demonstrated that flaxseed oil-derived n-3 PUFA significantly prevented PS externalisation (Yang *et al*., 2012). However, in the current study, there was no

significant relationship between the extent of the reduction in thrombogenicity of EVs and the decrease in PS+EV numbers (data not shown), indicating that the thrombin-generating capacity of EVs might be exhibited via other pathways, such as TF. Some previous studies have suggested that the expression and/or activity of TF in several cell types was significantly inhibited by n-3 PUFA (Tremoli *et al*., 1994; Del Turco *et al*., 2007; Holy *et al*., 2011). However, both Grundt *et al*. (2003) and Del Turco *et al*. (2008) reported that the administration of n-3 PUFA did not affect TF expression in either plasma or on circulating EVs in post-MI patients, although they did not exclude the possibility that n-3 PUFA decreased the activity of TF expressed on EVs (Del Turco *et al*., 2008). Another *in vitro* study reported that incubation of n-3 PUFA with human umbilical vein endothelial cells decreased the expression of TF on endothelial cells, but induced the release of TF+EDEVs (Del Turco *et al*., 2007). Also, none of the above studies has checked the possibility of EVs possessing the same amount of TF but exhibiting different TF activity, therefore, more studies about the effect of n-3 PUFA on both expression and activity of TF on EVs is warranted. Finally, the current study demonstrated that the extent of the decrease in TF-dependent thrombin generation supported by EVs was modestly associated with the decrease in TAG concentration after fish oil treatment. It is not yet clear whether the effects of n-3 PUFA on thrombogenicity of EVs might be partly exerted through effects on plasma TAG, which deserves further exploration.

There are some limitations need to be considered. Firstly, EV suspensions were adjusted only based on their protein concentrations before thrombin generation assay due to the limitation of time. However, it may lead to the different numbers of EVs loaded into thrombin generation assay since it is possible that the same numbers of EV have quite different protein concentrations. The results suggesting small EVs have greater potential to promote TF-dependent thrombin generation may also due to the load of more EVs of a small size but with equalling amount of protein. Also, as mentioned above, the current study only measured *in vitro* thrombogenic potential in activating TF-dependent thrombin generation to examine the procoagulatory activity of circulating EVs, while other coagulation related assays such as TF-independent

coagulation, prothrombin time and activated partial thromboplastin time, the rate and amount of thrombus formation, fibrin network structure and resistance to fibrinolysis, can also be analysed.

In conclusion, the current study demonstrated that circulating EVs promoted TF-dependent thrombin generation. The thrombogenicity of EVs in activating TF-dependent thrombin generation was negatively correlated with EV size, but was not affected by conventional cardiovascular risk markers in subjects with moderate risk of CVDs. N-3 PUFA substantially reduced but did not eliminate the TF-dependent thrombin-generating capacity of circulating EVs. This work highlights a potential role of circulating EVs in thrombosis and potential beneficial effects of n-3 PUFA, which not only lower EV numbers, but also reduce their procoagulatory activity, supporting their preventative application in CVDs.

Chapter 7 General discussion and future perspectives

7.1 Introduction

As the leading cause of global death, CVDs are a multifactorial disease, whose morbidity and mortality can be significantly decreased with the efforts to control or treat a wide range of modifiable risk markers (Cannon, 2008). EVs, as small membrane-derived vesicles released from various cell types under both physiological and some pathological conditions, including endothelial dysfunction and platelet activation, are increased in patients with CVDs (Ferreira *et al*., 2004; Koga *et al*., 2006; Jansen *et al*., 2014; Pawelczyk *et al*., 2017). They can act as mediators of cell-to-cell communication in various pathological processes relating to CVDs; thus the procoagulatory and proinflammatory properties of EVs involved in the development of CVDs have been investigated (Jansen *et al*., 2017; Antonova *et al*., 2019). However, prior to this project, there was little information about the relationship between numbers and function of circulating EV and conventional cardiovascular risk markers in relatively healthy subjects. Also, although a set of guidelines and criteria has been established for the standardisation in techniques for EVs analysis, the ambiguity in appropriate methods had limited the reliability and reproducibility of studies investigating the predictive and therapeutic potential of EVs for CVDs (Sáenz-Cuesta *et al*., 2015; Ridger *et al*., 2017).

N-3 PUFA have been suggested to exhibit cardioprotective effects and to reduce mortality from CVDs (Burr *et al*., 1989; Oh *et al*., 2005; Saravanan *et al*., 2010). However, due to the widespread use of statins and the significant improvement in the medical management of CVDs, studies conducted in the last decade have not been supportive of earlier data, making the benefits of n-3 PUFA more difficult to demonstrate (Rizos *et al*., 2012; Bilato, 2013; Balk *et al*., 2016; Walz *et al*., 2016). Also, although n-3 PUFA have been well-characterised to lower blood TAG concentration (Roche & Gibney, 1999; Weber & Raederstorff, 2000), their potential effects on other lipid parameters and other CVD-related risk markers are still under debate (Lavie *et*

al., 2009; De Caterina, 2011; Jeppesen *et al*., 2013; Tremblay *et al*., 2016). Since n-3 PUFA have been reported to improve endothelial dysfunction and inhibit platelet activation by incorporating into the phospholipid membranes of cells, the question as to whether n-3 PUFA could also influence the generation and properties of membrane-derived EVs is of interest. However, very few studies have investigated whether n-3 PUFA influence numbers and functional activity of circulating EVs. The current study investigated the influence of conventional cardiovascular risk markers and the effects of a fish oil-derived n-3 PUFA supplement on the numbers and procoagulatory activity of circulating EVs in subjects with moderate CVD risk using novel techniques and refined protocols for EVs analysis. The key findings are summarised below:

- A combination of NTA after SEC isolation and fluorescence FCM enabled the reliable characterisation of EVs, with neither alone being able to provide a full picture of the EV population due to either detection limitation or ability to phenotype all of main EV subpopulations. It was confirmed that circulating EVs ideally need to be analysed immediately in fresh samples to minimise alterations in EVs numbers and characteristics.

- In subjects with moderate CVD risk, circulating EVs and EV subpopulations were not only strongly associated with conventional cardiovascular risk markers, such as BMI, BP and plasma lipid profile, but also directly correlated with overall CVD risk. Moreover, the application of NTA, with its ability to differentiate between small and large EVs, suggested that circulating EVs were predominantly < 200nm and that EVs with diameters of < 200nm were more strongly associated with cardiovascular risk markers and CVD risk than larger particles. Circulating EVs promoted TF-dependent thrombin generation, and this thrombogenicity of circulating EVs was inversely associated with their mode size, indicating that smaller EVs also exhibited greater thrombogenic ability than larger particles.

- Consumption of fish oil-derived n-3 PUFA supplement (1.8g/d) for 12 weeks in subjects with moderate CVD risk significantly decreased numbers of both circulating EVs and EV subpopulations. Fish oil supplementation resulted in the remodelling of the fatty acid composition of EVs and the decreased TF-dependent thrombin generation promoted by these modified EVs.

7.2 Development of methodologies for EVs analysis

Although various methods for EV analysis have been developed within the last decades, there is still inconsistency in the methods applied, and this has posed a major barrier for EV research (Witwer *et al*., 2013; Yuana *et al*., 2015; Sáenz-Cuesta *et al*., 2015; Coumans *et al*., 2017). The work described in **Chapters 2** and **3** in this thesis, therefore set out to establish competence with refined protocols for the isolation, characterisation and storage of EVs.

Chapter 2 compared two widely used EV isolation methods: SEC (employing qEV columns or sepharose columns) and UC (individually or combined with DCG). SEC using qEV columns was selected as the method of choice due to its ability to produce a higher yield of EVs and better separation of EVs from contaminants compared to UC. Although UC is still the most commonly used method to isolate EVs to date, the starting material tends to be conditioned cell culture media rather than plasma (Gardiner *et al*., 2016), and it appears that SEC is a better option for plasma (Ismail *et al*., 2013; Yuana *et al*., 2015; Taylor & Shah, 2015; Takov *et al*., 2018). Most of the published research on EVs employed FCM for enumeration and characterisation (Gardiner *et al*., 2016). However, FCM is unable to detect very small EVs due to its detection threshold ranging from about 200~500nm (Orozco & Lewis, 2010). Furthermore, a recently identified limitation indicates that the polystyrene beads which have been widely employed for EV gating, actually have a higher refractive index than EVs and scatter more light compared to similar-sized biological particles, leading to the likelihood that the gating inadvertently captures platelets and apoptotic bodies as well as EVs (Harrison & Gardiner, 2012; van der Pol *et al*., 2016), and

therefore previous reports have overestimated numbers of circulating EVs (Wu *et al*., 2014; Phang *et al*., 2016; Landers-Ramos *et al*., 2018). The use of silica beads in the current research, which scatter light in a manner more closely resembling similar-sized particles, therefore overcame this limitation and improved the accuracy of EV detection. The application of fluorescence FCM can improve the ability to detect EVs because it avoids the background noise associated with debris on side scatter mode, but more importantly, it enables phenotyping of EVs so that cellular origins can be ascertained. However, the fact that there is no generic ligand which can label all types of EVs remains a significant challenge. Both lactadherin and Annexin V are commonly used markers which bind PS and which were used in the experiments described in **Chapters 2 and 3** to detect circulating PS+EVs above the FCM instrument threshold. **Chapter 3** described the development of the protocol to a three-antibody panel consisting of Annexin V, anti-CD41 and anti-CD105 in order to classify PS+EVs derived from either platelets or endothelial cells, with due consideration of negative controls. However, it was still limited in phenotyping other main EV subpopulations, such as ErDEVs or LDEVs. Furthermore, the detection limit of FCM remains an issue, and indeed **Chapter 4** reported that it only captures approximately 4% of the total EV population compared to NTA. This must be considered in the context of the fact that studies which have employed only FCM to detect EVs are actually only reporting on a minor fraction of the EVs in the circulation. Therefore, the adoption of NTA, with its 70nm detection threshold, enables the capture of smaller vesicles that could not be detected by FCM. Although NTA does have fluorescence detecting capacity, issues with fluorescence intensity and quenching mean that the technology is not yet adequately able to discriminate EVs from contaminants and from different cellular sources (Dragovic *et al*., 2011, 2015). The work described in this thesis established competence and improved confidence in the detection of EVs by refining existing methods and by combining FCM and NTA methods in order to gain a fuller picture of the circulating EV population. However, even combined, the methods do not allow capture of all types of EVs over the entire size range; thus new solutions and new techniques will be essential in the future.

7.3 Do conventional cardiovascular risk markers influence numbers and procoagulatory activity of circulating EVs?

Chapter 4 demonstrated that conventional cardiovascular risk markers, including BMI BP, and plasma TAG, were positively associated with the numbers of TEVs and SEVs (70nm~200nm) detected by NTA in subjects with moderate CVD risk. When subjects were categorised into 'high' and 'low' TEV groups based on the median of TEV numbers, subjects in the high TEVs group had a significantly higher TC/HDL-C ratio and lower HDL-C concentration than those in the low TEV group. Furthermore, stepwise regression analysis indicated that plasma TAG and DBP independently predicted numbers of TEVs and SEVs. Plasma TC concentration was positively associated with PDEV numbers, and higher TC concentrations were exhibited in the high PS+EV group when subjects were categorised into 'high' and 'low' PS+EV groups based on the median of PS+EV numbers. However, there were no other associations between risk markers and numbers of EV subpopulations, and the thrombogenicity of circulating EVs in promoting TF-dependent thrombin generation was not affected by any risk markers (**Chapter 6**). Some previous studies have investigated the relationships between CVD risk markers and EV numbers and/or procoagulatory activity of EVs in healthy subjects or patients with different diseases, and as described in **Chapter 4** and **Chapter 6**, they have largely reported inconsistent results (Preston *et al*., 2003; Goichot *et al*., 2006; Murakami *et al*., 2007; Bakouboula *et al*., 2008; Ueba *et al*., 2010; Aleman *et al*., 2011; Van Der Meijden *et al*., 2012; Amabile *et al*., 2014; Tripisciano *et al*., 2017). This may be due to wide variations in subject characteristics and methodology for EV characterisation. Growing evidence suggests that higher BMI, BP and plasma lipid levels are associated with the increased oxidative stress, which impairs NO production and bioavailability (Marseglia *et al*., 2014; Taddei *et al*., 2001; Katsuki *et al*., 2004). The secretion of proinflammatory mediators, such as C-reactive protein, leptin and TNF-α is exacerbated by excessive adipose tissue, hypertension or hyperlipidaemia (Vilahur *et al*., 2017; Siasos *et al*., 2011), all of which promote endothelial dysfunction and/or platelet activation, leading to the formation of EVs, and further procoagulatory activity may be a result of this (Heitzer *et al*., 2001; Siasos *et al*., 2011; Persson & Olsen, 2010; Owens & Mackman, 2011; Ghimire et al., 2016).

This scenario is supported by an animal study, which reported that increased numbers of circulating EVs in hypertensive rats were accompanied by an increase in oxidative stress and endothelial dysfunction (López Andrés *et al*., 2012). Furthermore, increased numbers of EVs in obesity were associated with reduced endothelial flow-mediated dilation and increased C-reactive protein concentration, while a decrease in EV numbers after weight loss was positively correlated with the decrease in BMI, C-reactive protein and leptin (Esposito *et al*., 2006; Campello *et al*., 2016). Significantly greater numbers of procoagulant PS+EVs or TF+EVs have also been reported in obesity and hypertension respectively (Goichot *et al*., 2006; Bakouboula *et al*., 2008), and TF-bearing EVs were not only increased in numbers in patients after cardiac surgery, but were also strongly procoagulant *in vitro* and highly thrombogenic in a rat model (Biró *et al*., 2003).

The current study indicated that there was no significant relationship between glucose concentration and EV numbers, or between CVD risk markers and EV-associated thrombogenicity. Increased numbers of PDEVs and/or EDEVs have been reported elsewhere in T2D patients (Koga *et al*., 2006; Tramontano *et al*., 2010). However, Sabatier *et al.* (2002) suggested that the number of EDEVs was only increased in Type 1 diabetics rather than T2D compared to healthy controls, which also appears to be the case in the Framingham Heart Study (Amabile *et al*., 2014), and it is not clear whether there were confounding effects of cardiovascular markers (Koga *et al*., 2006) and/or active CADs (Tramontano *et al*., 2010). Subjects in the current study were at 'moderate risk' of CVDs, with most risk markers being relatively close to the normal reference range; for example, mean fasting glucose concentration at baseline was 5.8mmol/L (105mg/dL), which is classified as prediabetes. Endothelial function is not likely to be affected at this level (Rodriguez *et al*., 2005), and it is perhaps unlikely to be sufficient to alter the thrombogenic activity of EVs. However, it is worth noting that the mean DBP and plasma TAG concentrations of the subjects in the current study were within normal reference ranges, and yet they independently predicted circulating EV numbers, which suggested a particularly strong relationship between these risk markers and EV numbers. Ferreira and co-workers (2004) reported a

significant association between EV numbers and plasma TAG concentration, but not TC and HDL-C concentrations three hours after consumption of a high-fat meal and a relationship between DBP and PDEV numbers has also been reported elsewhere (Ueba *et al*., 2010). The fact that TAG-lowering and anti-hypertensive drugs reduce numbers and procoagulatory activity of EV subpopulations supports the idea that plasma TAG concentration and BP are strongly linked with EVs (Kagawa *et al*., 2001; Labiós *et al*., 2004; Koga *et al*., 2006; Nomura *et al*., 2007; Mobarrez *et al*., 2011; Owens *et al*., 2012); however, whether this is a causal relationship is not clear and mechanisms remain to be elucidated.

Chapter 4 indicated that higher numbers of circulating TEVs and SEVs were directly related to greater overall 10-year CVD risk, as assessed by the QRISK2 scoring system, independent of other risk markers. There are inconsistent reports relating to this relationship in the literature, with some demonstrating no relationship (Chironi *et al*., 2006) and others demonstrating greater numbers of EVs related to higher 10-year CVD risk (Ueba *et al*., 2010; Amabile *et al*., 2014) or with the higher occurrence of cardiovascular events and death (Sinning *et al*., 2011). **Chapter 6** demonstrated that circulating EVs had the potential to promote TF-dependent thrombin generation, which highlighted a potential role in thrombosis in addition to their ability to impair endothelial function (Densmore *et al*., 2006) and to confer proinflammatory activities by increasing the secretion of inflammatory cytokines and the expression of adhesion molecules (Distler et al., 2005a; Bretz et al., 2013; Gidlöf *et al*., 2013; Hijmans *et al*., 2018). These effects of EVs could contribute to the development of CVDs, but the ability of EVs to independently predict the risk of CVDs deserves further evaluation.

7.4 Are small or large EVs more strongly related to CVD risk?

Chapter 4 suggested that SEVs (mainly exosomes) accounted for 96% of total EVs and were more strongly associated with cardiovascular risk markers and overall CVD risk than LEVs (mainly microvesicles). **Chapter 6** further found that smaller EVs appeared to have a greater thrombogenic ability. Based on these findings, it could be speculated

that exosomes play a more important role in CVD risk than microvesicles. Although both exosomes and microvesicles are small membrane-derived particles with similar structures, they differ in size, density and biogenesis, and they have specific signatures in composition, cellular origin and functional properties (Ståhl *et al*., 2019). Cell culture-based and animal-based studies have compared the differences in the secretion and activity of exosomes and microvesicles. Durcin *et al.* (2017) reported that 3T3-L1 adipocytes secreted about 100-fold more exosomes than microvesicles, and they presented different lipid and protein characteristics, with microvesicles expressing more PS. Another study demonstrated that exosomes, but not microvesicles, from rats with PAH modulated pulmonary vascular responses and induced the development of PAH when they were injected into healthy rats (Aliotta *et al*., 2016). Exosomes can be generated from practically all cells in the cardiovascular system, and the cargos that they carry could contribute to CVDs (Gupta & Knowlton, 2007; Davidson *et al*., 2017). For instance, the expression of microRNA-208a in exosomes was significantly elevated in subjects with acute coronary syndromes (Bi *et al*., 2015) and there were elevated levels of three specific p53-responsive microRNAs enriched in exosomes in patients with heart failure (Matsumoto *et al*., 2013). Exosomes derived from mature dendritic cells increased endothelial inflammation and atherosclerosis through a TNF-α-mediated nuclear factor kappa B pathway (Gao *et al*., 2016) and exosomes isolated from spontaneously hypertensive rats increased SBP when delivered to normotensive rats (Otani *et al*., 2018). Similarly, platelet-derived exosomes from trauma patients and mice increased thrombin generation and platelet aggregation (Dyer *et al*., 2019) and the addition of vascular smooth muscle cells derived exosomes to normal plasma also stimulated thrombin generation (Kapustin & Shanahan, 2016). However, Durrieu *et al.* (2018) demonstrated that microvesicles from several cancer cell lines possessed more procoagulant activity than exosomes and Tripisciano *et al.* (2017) reported that platelet-derived and monocyte-derived exosomes did not induce thrombin generation. It is also important to note that a small number of studies reported that exosomes appear to have some cardioprotective roles. For example, platelet-derived exosomes suppressed platelet aggregation and thrombosis in a murine model of carotid artery injury (Srikanthan *et al*., 2014). Exosomes derived from human umbilical vein endothelial cells reduced cardiomyocyte

death in an *in vitro* ischaemia/reperfusion model (Davidson *et al*., 2018). In the current study, LEVs were not as strongly associated with CVD risk as SEVs, but it should be remembered that they only accounted for 4% of TEVs. Only one study separated small and large EVs from PFP from obese subjects with MS by differential centrifugations and attempted to compare the relationship between numbers of small and large EVs and cardiovascular risk markers using a combination of NTA and FCM. Partly in agreement with the current study, both small and large EVs were associated with increased CVD risk markers, although different isolation methods and detection thresholds were applied (Amosse *et al*., 2018). There are clearly a number of outstanding questions regarding the influence of EV size on their activities and the potentially different roles of exosomes and microvesicles in CVDs.

7.5 Effects of n-3 PUFA on numbers, fatty acid composition and procoagulatory activity of circulating EVs

A previous study conducted in our lab reported a significant decrease in EDEV numbers after fish oil supplementation in subjects with moderate CVD risk (Wu *et al*., 2014). Favourable effects of n-3 PUFA on numbers and/or procoagulatory activity of circulating total EVs and/or EVs subpopulations in healthy subjects or patients with hyperlipidaemia, T2D or post-MI have also been reported elsewhere (Nomura *et al*., 2003; Del Turco *et al*., 2008; Nomura *et al*., 2009a; Nomura *et al*., 2009b; Marin *et al*., 2011; Nomura *et al*., 2018). However, two studies suggested that there was no effect of n-3 PUFA on numbers of either circulating total EVs or EV subpopulations (Phang *et al*., 2012; Phang *et al*., 2016) and one study reported a significant increase in PDEV numbers after n-3 PUFA supplementation (Englyst *et al*., 2007). This inconsistency may be due to differences in sample size, subject clinical characteristics, dose and duration of n-3 PUFA treatment, methodologies used for EV isolation and quantification. Most studies, including the one conducted in our lab, relied solely on FCM for EV detection, limiting the analysis of larger EVs, which are now understood to comprise only 4% of the total EV population (according to the results in this thesis). They also suffered limitations in gating techniques, as described earlier (Nomura *et al*., 2003; Del Turco

et al., 2008; Nomura *et al*., 2009a; Wu *et al*., 2014; Phang *et al*., 2016). With refined protocols for EVs analysis and more reliable analytical techniques, the current study demonstrated that the consumption of 1.8g/d n-3 PUFA for 12 weeks by subjects with moderate CVD risk significantly decreased numbers of both circulating total EVs detected by NTA, and PS+EVs, PDEVs and EDEVs characterised by fluorescence FCM compared to high-oleic safflower oil (**Chapter 5**). Furthermore, circulating EVs modified by n-3 PUFA had a significantly lower capacity to promote TF-dependent thrombin generation. (**Chapter 6**). Only two previous studies to date have explored alterations of procoagulatory activity of EVs after treatment with n-3 PUFA, and both suggested that n-3 PUFA decreased the procoagulant activity of EVs (Del Turco *et al*., 2008; Phang *et al*., 2012). The n-3 PUFA intervention also resulted in more than a doubling of the proportions of EPA and DHA in EV total lipids at the expense of oleic acid and AA. The proportions of EPA and DHA in plasma phospholipids were also increased following fish oil supplementation, largely by replacing n-6 PUFA, including linoleic acid, AA and DGLA, rather than oleic acid and AA, as in EVs (**Chapter 5**). Few studies have reported the fatty acid composition of circulating EV lipids, and those that did reported only minor differences following an intervention (Connolly *et al*., 2014; Willis *et al*., 2014). Also, there is very little comparative information about the remodelling of EV total lipid fatty acid composition by n-3 PUFA, but the pattern of fatty acid modification in EVs seems to resemble the impact of fish oil supplementation on platelets and cells (Lovegrove et al., 2004; Walker *et al*., 2015).

To the best of our knowledge, the current study is the first to report the incorporation of n-3 PUFA into EVs linked with alterations in both their numbers and function. The exact mechanisms by which n-3 PUFA decrease EV numbers and thrombogenicity are still uncertain, but at least are due in part to the n-3 PUFA mediated modification of cell membrane lipids. AA is a major component of membrane phospholipids, which can act as a substrate for a wide range of bioactive fatty acids metabolites by different oxygenases (Yeung *et al*., 2017). AA is converted to TXA2 under COX-2 pathway, leading to platelet activation and thrombin generation (Kim et al., 1995; Altman *et al*., 2000; Yeung *et al*., 2014, 2017; Adili *et al*., 2018). The effect of 12-

hydroxyeicosatetraenoic acid (12-HETE) oxidised from AA via 12-LOX is controversial, but some studies have reported its ability to promote thrombin generation and coagulation (Setty, *et al*., 1992; Yeung *et al*., 2014; Slatter, *et al*., 2018). Circulating EVs containing AA have also been found to generate TXA2 and 12-HETE (Duchez *et al*., 2015; Boilard, 2018). Thus, the incorporation of EPA and DHA into cell and EV membranes not only reduces the proportion of AA and inhibits AA metabolism, but also produces some anti-platelet and/or anti-thrombotic metabolites from EPA and DHA, including TXA3 via COX-1, and 12-hydroxyeicosapentaenoic acid (12-HEPE) and 11-hydroxydocosahexaenoic acids (11-HDHA) via 12-LOX (Takenaga *et al*., 1986; Yeung *et al*., 2017; Adili *et al*., 2018). Moreover, n-3 PUFA have been reported to inhibit the externalisation of PS and TF in the outer cell membrane, both of which significantly contribute to the formation and thrombogenicity of EVs (Larson *et al*., 2011; Yang *et al*., 2012; Del Turco *et al*., 2007; Holy *et al*., 2011). Other effects of n-3 PUFA on endothelial function and inflammatory response, including increasing the generation and bioavailability of NO, and decreasing proinflammatory cytokines, could also contribute to their influence on EVs (Omura *et al*., 2001; Gortan Cappellari *et al*., 2013; von Schacky *et al*., 2007; Calder, 2006, 2017).

Taken together, this research advanced the understanding of the nature and behaviour of circulating EVs with respect to CVDs. It also demonstrated that the numbers, composition and function of EVs, as well as some conventional CVD risk markers, could be modified by dietary n-3 PUFA in a manner which suggests a protective role of n-3 PUFA in cardiovascular health.

7.6 Future perspectives

This research described in this thesis highlights some key issues relating to the analytical methods for EV characterisation and addresses some important questions about the role of EVs in CVDs and the influence of dietary n-3 PUFA on them. Plasma TAG concentration and DBP independently predicted numbers of circulating EVs, but it remains to be determined whether the relationship is causal and whether EVs have

potential as an independent and reliable predictor for CVDs. Detailed prospective studies investigating whether alterations in plasma TAG concentration or DBP are accompanied by alterations in EV numbers, and whether subjects with higher EV numbers are more likely to develop CVDs are warranted.

However, given current limitations in analytical techniques, a priority is to establish more reliable and standardised methods for EV isolation and characterisation in both laboratory and clinical environment, and such developments may require a multi-disciplinary approach. The current study suggested that small EVs may be critical regarding their role in CVDs. It would be important, therefore, to compare the properties and functions of exosomes and microvesicles, which is dependent on methods, such as applying a filter at 0.22µm, to fractionate EVs into exosomes and microvesicles effectively. The characterisation of EVs needs a more complete labelling panel on current FCM to include the majority of circulating EVs from different cells, and requires further development of fluorescence NTA or other novel techniques, such as high-resolution FCM in order to better understand the roles of exosomes and microvesicles in CVDs. Moreover, exosomes and microvesicles should be compared with respect to their properties by the application of western blotting and/or EM, and their functional activity, including their thrombogenic ability and other aspects of procoagulatory activity. This would verify whether there is a more prominent role of exosomes in CVDs compared to microvesicles and whether this is due to their composition and/or functional activity. If exosomes do indeed play a more prominent role in cardiovascular pathology, further research would focus on the mechanisms.

There is significant scope for *in vitro* experiments which generate EVs from endothelial cells and/or platelets obtained from patients with hypertriglyceridaemia or hypertension and then examine their size, composition and function. EVs or exosomes could be pre-incubated with Annexin V or with blocking anti-TF antibody in order to block PS or TF, and to examine the potential contribution of PS or TF on thrombin generation. Finally, EVs or exosomes could be injected into an animal model followed

by observation of the development of thrombosis, which could provide mechanistic information about their contributions in coagulation and thrombosis.

Modification by n-3 PUFA of the numbers, fatty acid composition and thrombogenic ability of circulating EVs provided valuable insight into the potential beneficial effects of these fatty acids with respect to CVDs. Future work should focus on clinically relevant assays such as TF numbers/activity, thrombus formation or fibrinolysis, as well as examining whether the effects on exosomes and microvesicles differ. *In vitro* studies could examine whether incubating endothelial cells or platelets with n-3 PUFA alters their cell membrane composition and bioactive lipid mediator content, and numbers and function of EVs they generate. The EVs modified could also be introduced into an animal model to explore their effects on coagulation and thrombosis. These experiments would reveal the details of underlying mechanisms for the influence of n-3 PUFA on EV function and advance the knowledge about potential pathways for n-3 PUFA to improve cardiovascular health.

7.7 Concluding remarks

This thesis demonstrated a strong relationship between circulating EVs and CVDs, as well as a significant modification of numbers, composition and function of circulating EVs by n-3 PUFA in subjects with moderate risk of CVDs. It emphasised the fact that small EVs may play a more prominent role in CVD risk, and this highlighted a gap in knowledge regarding potential differential effects of exosomes and microvesicles in CVDs. These findings afford EVs great potential to serve as a novel predictive biomarker and a new therapeutic target for CVDs, and also provide new insight into the beneficial effects of n-3 PUFA on cardiovascular protection and treatment. Research in this area is just emerging, and future efforts should be devoted to moving these academic discoveries towards clinical applications.

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Appendix: Ethics 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:

(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
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Name: Professor Jon Gibbins (Staff) Institution/Department: Biomedical Sciences
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Name: Dr Chris Jones (Staff) Institution/Department: Biomedical Sciences
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Name: Ruihan Zhou (Student) Institution/Department: Food and Nutritional Sciences
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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: 28/2/17

Signed. Head of Department) Date: 24/2/17

Signed.. …… (SCFP Ethics Administrator) Date: 01/03/17

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 (40-70y) at moderate CVD risk will be supplemented with either fish oil (1.8 g/d n-3 PUFA) or placebo (safflower 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

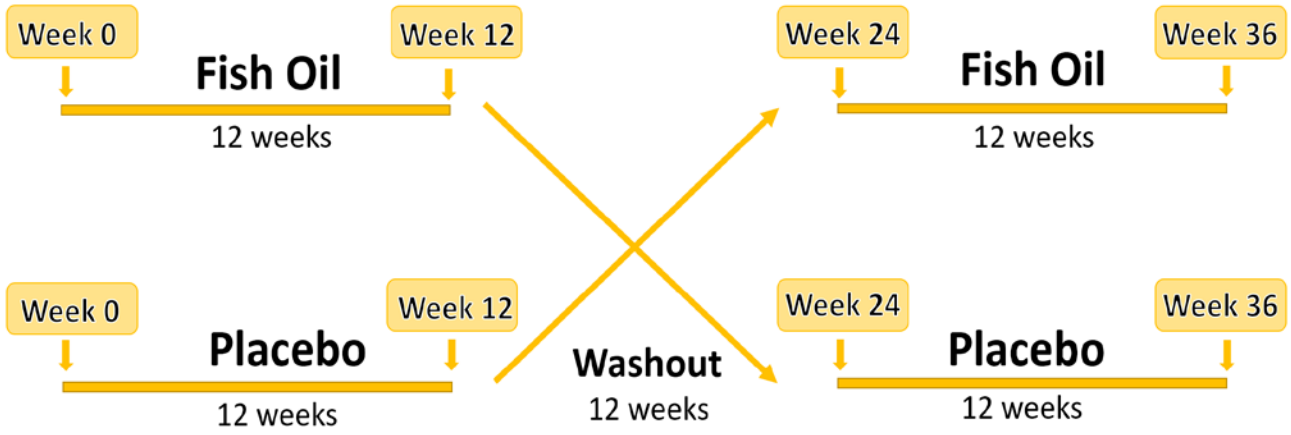
Study Day Procedures

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 (safflower 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 safflower 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: 22/2/17

.. (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

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…24/2/17

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

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

   OR

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

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

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

   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:
Email: p.yaqoob@reading.ac.uk

School of Chemistry, Food & Nutritional Sciences and Pharmacy

Whiteknights
PO Box 266, Reading RG6 6AP,
UK
phone
fax

Appendix C Consent Form

Consent Form for HI-FIVE STUDY

UREC 17/18 (10th April 2017)

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 others. ☐ 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, 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 safflower 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 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 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 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 safflower 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

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: 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*)

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*)

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

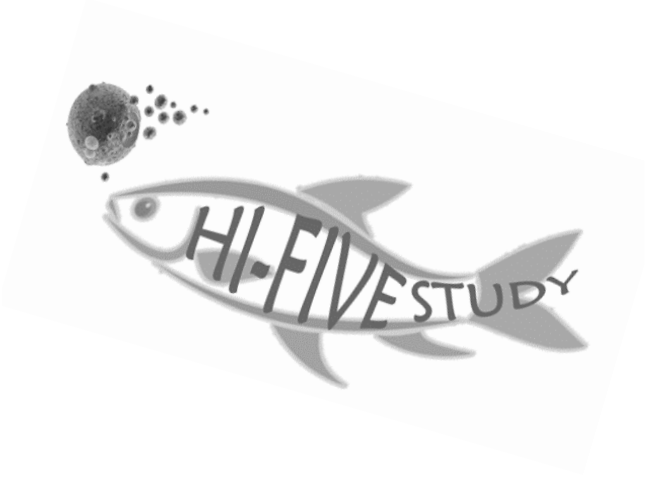
List holiday period:

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.

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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 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								

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 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								
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

4

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 required 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**.

Kind Regards,
Ruihan Zhou

PhD Candidate
Hugh Sinclair Unit of Human Nutrition
Department of Food and Nutritional Sciences
University of Reading

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 required 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**.

Kind Regards,
Ruihan Zhou

PhD Candidate
Hugh Sinclair Unit of Human Nutrition
Department of Food and Nutritional Sciences
University of Reading

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 <u>5x 30 min visits</u> 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**

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

Appendix J leaflet

Volunteers Needed Aged 40-70yrs

University of Reading

HUGH SINCLAIR Unit of Human Nutrition

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 University of Reading

To find out more:

- Contact **Ruihan Zhou**
- Email: **hi-five@reading.ac.uk**

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 to 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**