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Do neutrophil microvesicles affect atherosclerotic plaque erosion?

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Thesis summary

Atherosclerosis is a major cause of death and imparts a significant burden on health services globally. It is characterised by the development of lesions in the artery called plaques. As these plaques develop, they can impede blood flow to the heart resulting in symptoms of angina and in some cases trigger rapid blood clotting, called thrombosis, which can partially or fully occlude the artery lumen, manifesting in myocardial infarction. Historically research has focused primarily on atherosclerosis caused by lipid-rich and highly inflammatory plaques with a thin fibrous cap which is prone to rupture. However, more recently the importance of a second form of plaque, characterised by a lower lipid content, fewer inflammatory cells and a thick fibrous cap, has garnered more interest. These plaques do not cause thrombosis via plaque rupture but rather the endothelium of the plaque progressively erodes until thrombosis occurs. This process is referred to as plaque erosion. As the proportion of major cardiac events accounted for by erosion increases, largely as a result of improved treatments targeting the cause of plaque rupture whilst being ineffective in the management of erosion research interest has intensified. Neutrophils have recently been implicated in plaque erosion through the induction of endothelial cell dysfunction. Neutrophils produce 0.1-1µm microvesicles from their cell membrane and these neutrophil microvesicles have been linked with atherosclerotic plaque progression. In this study I investigated the hypothesis that NMVs affect plaque erosion through driving endothelial cell dysfunction.

Several techniques were used to investigate the effect of neutrophil microvesicles on primary human coronary artery endothelial cells and platelet function. Neutrophils and platelets were isolated from whole blood from healthy volunteers and neutrophils stimulated to generate neutrophil microvesicles. Human coronary artery endothelial cells were cultured under static and atheroprone flow conditions on varying extracellular matrix components found within eroded plaques to model the environment of an eroded plaque.

Key findings

1. Neutrophil microvesicles are generated *in vitro* in response to native low-density lipoprotein stimulation.
2. Neutrophil microvesicles contain active neutrophil elastase and matrix metalloproteinase-9 at similar levels regardless of whether they are generated by unstimulated or native low-density lipoprotein stimulated neutrophils.

3. Neutrophil microvesicles degrade analogues of the extracellular matrix in a dose-dependent and matrix metalloproteinase-dependent manner.
4. Neutrophil microvesicles increase human coronary artery endothelial cell apoptosis and detachment whilst reducing proliferation and wound healing responses.
5. Neutrophil microvesicles increase platelet P-selectin expression and platelet adhesion to human coronary artery endothelial cells under static conditions but did not increase platelet recruitment under atheroprone flow conditions.

This study demonstrates the propensity of neutrophil microvesicles to promote functions within human coronary artery endothelial cells that may predispose the endothelium to erosion and thrombosis and identifies a potential link between a known risk factor for atherosclerosis, elevated low-density lipoprotein cholesterol, and the production of neutrophil microvesicles. These findings highlight the need for further research to better understand the effects of neutrophil microvesicles in atherosclerosis.

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List of abbreviations

ACS – Acute coronary syndrome

ApoE - Apolipoprotein E

CHD – Coronary heart disease

CKD – Chronic kidney disease

CVD – Cardiovascular disease

DM – Diabetes mellitus

ECG – Electrocardiogram

ECM – Extracellular matrix

EV – extracellular vesicles

fMLP - N-Formylmethionine-leucyl-phenylalanine

GP – Glycoprotein

HCAEC – Human coronary artery endothelial cell

HMW-HA – High molecular weight hyaluronan

ICAM-1 – Intracellular adhesion molecule 1

LAD – Left anterior descending artery

LDLR – Low density lipoprotein receptor

LMW-HA – Low molecular weight hyaluronan

LOSS – Low oscillatory shear stress

MI – Myocardial infarction

Mir-155 – Micro RNA 155

MMP – Matrix metalloproteinase

MPO – Myeloperoxidase

MV – microvesicle

NE – Neutrophil elastase

NF-κB – Nuclear factor-kappa-B

nLDL – Native low-density lipoprotein

NMV – Neutrophil microvesicle

NSTEMI – Non-ST-segment myocardial infarction

OCT – Optical coherence tomography

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate buffered saline

PPP – Platelet-poor plasma

PRP – Platelet-rich plasma

ROS – Reactive oxygen species

SMC – Smooth muscle cell

STEMI – ST-segment elevated myocardial infarction

TCFA – Thin capped fibroatheroma

TF – Tissue factor

THBD – Thrombomodulin

TLR2 – Toll-like receptor 2

VCAM-1 – Vascular endothelial cell adhesion molecule 1

VWF – Von Willebrand factor

1. Introduction

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is the broad term referring to a group of diseases affecting the heart and circulation. In most instances, blockages in blood vessels restrict blood flow to the heart, brain and limbs resulting in coronary, cerebrovascular and peripheral artery diseases. CVD is the leading cause of death globally causing 17.9 million deaths in 2016, equating to 31% of all deaths (World Health Organisation; 2017). In the UK, CVD accounts for 27% of all deaths, 25% of which are below the age of 75 (British Heart Foundation; 2020). A further 7.9 million people are living with CVD in the UK costing the NHS £9 billion a year. Wider implications of CVD contribute towards a combined cost to the UK economy of £19 billion per year (British Heart Foundation; 2020). Symptoms arise when fatty deposits accumulate in the blood vessels and impede blood from reaching the heart to varying degrees depending on the severity of the blood flow restriction. This can cause symptoms including angina for milder, partial restriction of blood flow resulting from progressive narrowing of the arterial lumen or myocardial infarction (MI) for more severe restrictions caused by a rapid thrombotic response that blocks blood flow (Libby, 2021; Libby *et al.*, 2019; Soehnlein and Libby, 2021).

1.2 Atherosclerosis

Atherosclerosis is the most common cause of CHD, although it can also occur in blood vessels that do not directly supply the heart. Characterised by the formation of plaques in the tunica intima of vessels, atherosclerosis causes the progressive narrowing of the vessel lumen and restriction of blood flow. The most severe symptoms occur due to the formation of thrombi following disruption of atherosclerotic plaques, leading to partial or total blockage of the artery lumen. When this happens in a coronary artery, blood flow to the heart is restricted resulting in myocardial infarction (MI) (Libby, 2021). MI can take two forms; ST-elevation MI (STEMI) and non-ST-elevation MI (NSTEMI) as defined by electrocardiogram (ECG) (figure 1.1). Whilst in most cases of MI severe cardiac events occur because of this thrombotic response, the processes that precipitate thrombosis can be quite distinct and are determined by the type of atherosclerotic plaque. There are broadly two distinct types of atherosclerotic plaques that result in thrombosis: vulnerable plaques and stable fibrous plaques (Fahed and Jang, 2021; Partida *et al.*, 2018). Vulnerable plaques are highly inflammatory lesions prone to rupture in a process that exposes highly pro-thrombotic materials to the blood and

forms a vessel occluding thrombus. Intact fibrous plaques are less well understood. They are significantly less inflammatory than ruptured plaques and often exhibit a smaller lipid core (Yamamoto *et al.*, 2019b). Thrombosis associated with stable plaques is believed to occur through some, yet to be fully described mechanism that causes the erosion of the plaque endothelium and the exposure of pro-thrombotic components of the ECM. The lack of understanding of plaque erosion makes it an increasingly prominent cause of acute coronary syndrome (ACS) (Higuma *et al.*, 2015; Jia *et al.*, 2013; Yamamoto *et al.*, 2019b). There are few effective non-invasive treatments for plaque erosion in contrast to vulnerable plaque rupture. Widespread statin use, alongside preventative therapies have improved outcomes for patients with or at high risk of suffering from ACS resulting from plaque rupture. However, the risk of cardiovascular events following use of these treatments remains unacceptably high. Residual inflammatory risk was identified in patients treated with both statins and a PCSK9 inhibitor (Pradhan *et al.*, 2018). Part of this residual risk for cardiovascular events may be contributed by superficial erosion of intact fibrous plaques. Furthermore, as treatments for plaque rupture continually improve, the proportion of cardiovascular events resulting from erosion is increasing (Pasterkamp *et al.*, 2017).

1.3 Vulnerable plaque rupture vs superficial erosion

Vulnerable plaques are highly inflammatory and are characterised by high lipid accumulation in the tunica intima, infiltration of pro-inflammatory immune cells, such as monocytes and the presence of a thin fibrous cap (Hansson *et al.*, 2015). This thin fibrous cap separates the lipid-rich necrotic core from the blood. The necrotic core is highly pro-thrombotic inducing rapid thrombosis upon exposure to the blood. Autopsy analyses of vulnerable plaques found that 95% of thin fibrous caps were less than 65μm thick with a mean of only 23 μm (Burke *et al.*, 1997). These types of plaque are referred to as thin-cap fibroatheroma (TCFA) and are subject to continual thinning by proteases expressed by infiltrating immune cells, which reduce fibrous cap integrity (Olejarz *et al.*, 2020). Overexpression of active MMP-9 by macrophages in advanced lesions present in $ApoE^{-/-}$ mice resulted in greater elastin degradation and enhanced plaque disruption (Gough *et al.*, 2006). Overexpression of MMP-9 driven by intraluminal incubation with an adenovirus expressing human MMP-9 increased signs of a vulnerable plaque morphology in intermediate and advanced plaques as well as increasing intraplaque haemorrhage (de Nooijer *et al.*, 2006). Progressive degradation of the thin fibrous cap can cause the exposure of the necrotic core beneath to the blood, a process known as plaque rupture. This results in thrombosis.

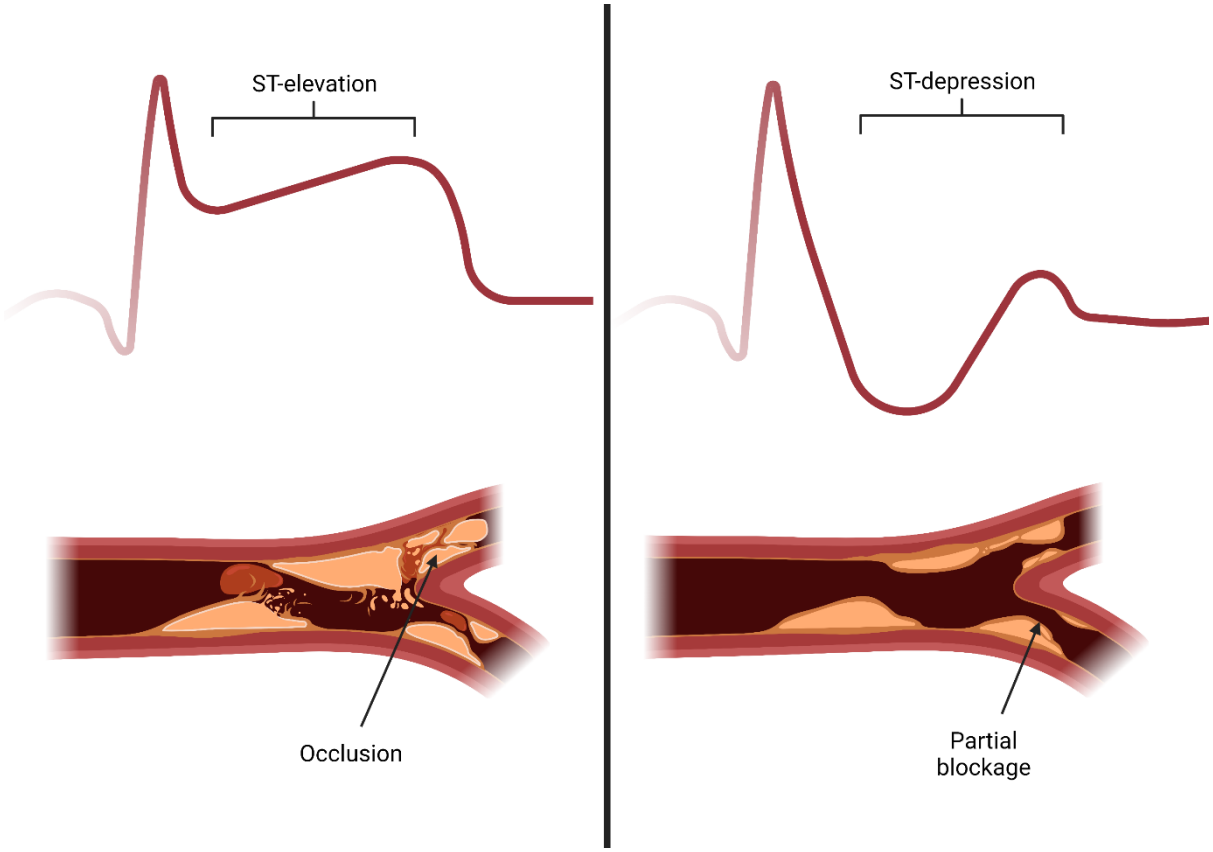


Figure 1. 1. ST-elevation vs non-ST elevation myocardial infarction.

Myocardial infarction is defined by the shape of the ST-segment of the electrocardiogram (ECG). ST-elevation more commonly arises from a complete occlusion of the vessel whilst non-ST-elevation is the result of an incomplete blockage of the vessel. Created with Biorender.com

The formation of rupture-prone plaques is dependent on a variety of well-defined risk factors. Many large-scale epidemiological studies have identified dyslipidaemia, smoking, diabetes mellitus (DM), hypertension and obesity as key risk factors (Burke *et al.*, 1997; Yusuf *et al.*, 2004). Identification of these key risk factors has informed the development of successful interventions such as drugs to reduce serum low-density lipoprotein (LDL) levels such as statins, anti-platelet drugs such as aspirin and lifestyle alterations including improving diet and increasing physical exercise. Despite these effective interventions, the risk of cardiovascular events remains high for over a year in patients discharged following MI (Jernberg *et al.*, 2015).

Autopsy analyses identified thrombi overlying intact fibrous caps in between 25-40% of patients who died from coronary artery thrombosis (Arbustini *et al.*, 1999; Farb *et al.*, 1996). These studies gave insight into fatal plaque erosion however, the contribution of erosion to non-fatal ACS was unknown. More recently, advanced imaging techniques such as optical coherence tomography (OCT) have been used to expand on our knowledge of non-fatal erosion. OCT is an invasive intravascular imaging method that uses infrared light waves to discern the morphology of atherosclerotic plaques with a resolution of 20-30 μm (Jia *et al.*, 2013). Using OCT, studies have reported a similar prevalence of erosion with figures ranging between approximately 25-31% of patients presenting with acute coronary syndrome (ACS) having culprit lesions classified as eroded (Dai *et al.*, 2018; Higuma *et al.*, 2015; Jia *et al.*, 2013). The key criterion that separates a classification of plaque rupture from erosion is the presence of an intact fibrous cap in the latter (Jia *et al.*, 2013). Plaque erosion is often defined in clinical studies as a thrombi associated with a plaque displaying no evidence of fibrous cap rupture or communication of an exposed lipid core with the blood (Jia *et al.*, 2013). In addition, a classification of probable erosion is made when a culprit lesion site displays endothelium irregularity in the absence of thrombus.

Recently, two large scale studies have given the greatest insight into the structural differences separating plaque erosion and plaque rupture. In 2018 Dai et al published a large scale OCT study consisting of 822 ST-segment elevated myocardial infarction (STEMI) patients of which, 209 had plaque erosion (Dai *et al.*, 2018). Then in 2019 Yamamoto et al conducted statistical analysis using data collated from several smaller studies to create a larger sample size. They collated data from 1,241 STEMI and NSTEMI patients from 11 different institutions around the world making their analysis the most comprehensive to date. Many of the most strongly supported assertions to follow are derived from their studies (Yamamoto *et al.*, 2019b).

Eroded plaques display structural characteristics that differentiate them from ruptured plaques (Table 1) (figure 1.2). Eroded plaques are rich in smooth muscle cells (SMCs) and typically have thick fibrous caps in contrast to the thin fibrous cap of ruptured plaques (Braganza and Bennett, 2001; Kolodgie *et al.*, 2002). A thinner fibrous cap does not necessarily preclude a classification of erosion however, eroded plaques are significantly less likely than ruptured plaques to have a thin fibrous cap (Dai *et al.*, 2018). Indeed, the mean minimum fibrous cap thickness in eroded plaques

has been reported as almost twice that in ruptured plaques (Yamamoto *et al.*, 2019b) (Yamamoto et al., 2019b). Another key difference is in the composition of the fibrous caps. Eroded plaques are rich in collagens III and IV, proteoglycans such as versican and polysaccharides such as hyaluronan (HA) (Kolodgie et al., 2002; Kolodgie et al., 2004) whereas, ruptured plaques are typically rich in collagen type I and show progressive reductions in collagen content as they progress from intact caps to ruptured caps (Kolodgie *et al.*, 2001).

In ruptured plaques the fibrous cap separates a lipid-rich necrotic core from the blood. However, eroded plaques are significantly less likely to be lipid-rich and when present, lipid pools in the artery intima are smaller and do not communicate with the thrombus marking a significant divergence from the pathology of plaque rupture (Dai et al., 2018; Yamamoto et al., 2019b). In a further departure from what is found in ruptured plaques, eroded plaques are significantly less inflammatory containing fewer infiltrating macrophages and T-cells (Kim et al., 2019; Yamamoto et al., 2019b). In addition to reduced inflammatory cell infiltration, eroded plaques have reduced concentrations of pro-inflammatory molecules including matrix metalloproteinases (MMP) MMP-1 and MMP-9 as well as NGAL, CXCL11, IL-18 and complement factor D (Campbell et al., 2014; Chandran et al., 2017; Yamamoto et al., 2019b).

Eroded plaques also present differently in patients. Typically, eroded plaques are shorter in length and induce a less severe stenosis than ruptured plaques (Yamamoto *et al.*, 2019b). Erosion is also more commonly associated with NSTEMI (59.3%) rather than STEMI (40.5%) whilst plaque rupture showed the opposite, more commonly resulting in STEMI (63.4%) rather than NSTEMI (36.6%). Interestingly, eroded plaques are less likely to be found in multiple vessels and show a preference for the left anterior descending artery (LAD) compared with the right coronary artery (RCA) (Dai *et al.*, 2018; Yamamoto *et al.*, 2019b). Yamamoto reported 56% of eroded plaques were observed in the LAD compared to 28% in the RCA. This contrasts sharply with ruptured plaques which were distributed more evenly (45.8% LAD; 40.5 RCA) (Yamamoto et al., 2019b). The increased number of bifurcations in the LAD have led some to speculate that disturbed flow caused by these bifurcations is more prominently involved in plaque erosion (Kim *et al.*, 2019; Thondapu *et al.*, 2021; Yamamoto *et al.*, 2019b). Whilst the mechanism behind this is not known one potential avenue through which disturbed flow may induce plaque erosion is through disrupting the endothelium. Immunohistochemical studies of plaques from patients who died from MI observed the discontinuity of the endothelium beneath the resulting thrombi (Farb *et al.*, 1996). ECs were hypothesised to be eroded away by some unknown mechanism, which causes thrombosis. This led to the term ‘plaque erosion’. This removal or ‘denudation’ of the endothelium coupled with the concomitant exposure of the underlying ECM in broad terms forms the central hypothesis explaining plaque erosion.

Together, these studies demonstrate that eroded plaques are significantly different in morphology from ruptured plaques (figure 1.2). The absence of a necrotic lipid core and a thicker fibrous cap in eroded plaques suggests that plaque erosion does not precipitate thrombosis via the same

mechanisms as plaque rupture. Instead, a loss of the endothelium overlying the fibrous plaque may be the crucial event that triggers platelet recruitment. However, the factors that contribute to this process are not well defined.

Erosion	Rupture
Lipid-poor plaque	Lipid-rich plaque
Smaller lipid core length	Larger lipid core length
Thick fibrous plaque	Thin fibrous cap
Proteoglycan-rich	Proteoglycan-poor
More common in LAD	Evenly distributed between LAD and LCX
More often found close to bifurcation	Less often found close to bifurcation
Larger luminal area	Smaller luminal area
Less calcified	More calcified
Macrophage poor	Macrophage rich
More likely to present as NSTEMI	More likely to present as STEMI

Table 1.1. Characteristics differentiating eroded plaques from ruptured plaques. *LAD (left anterior descending), LCX (left circumflex), STEMI (ST-elevation myocardial infarction), NSTEMI (non-ST-elevation myocardial infarction).*

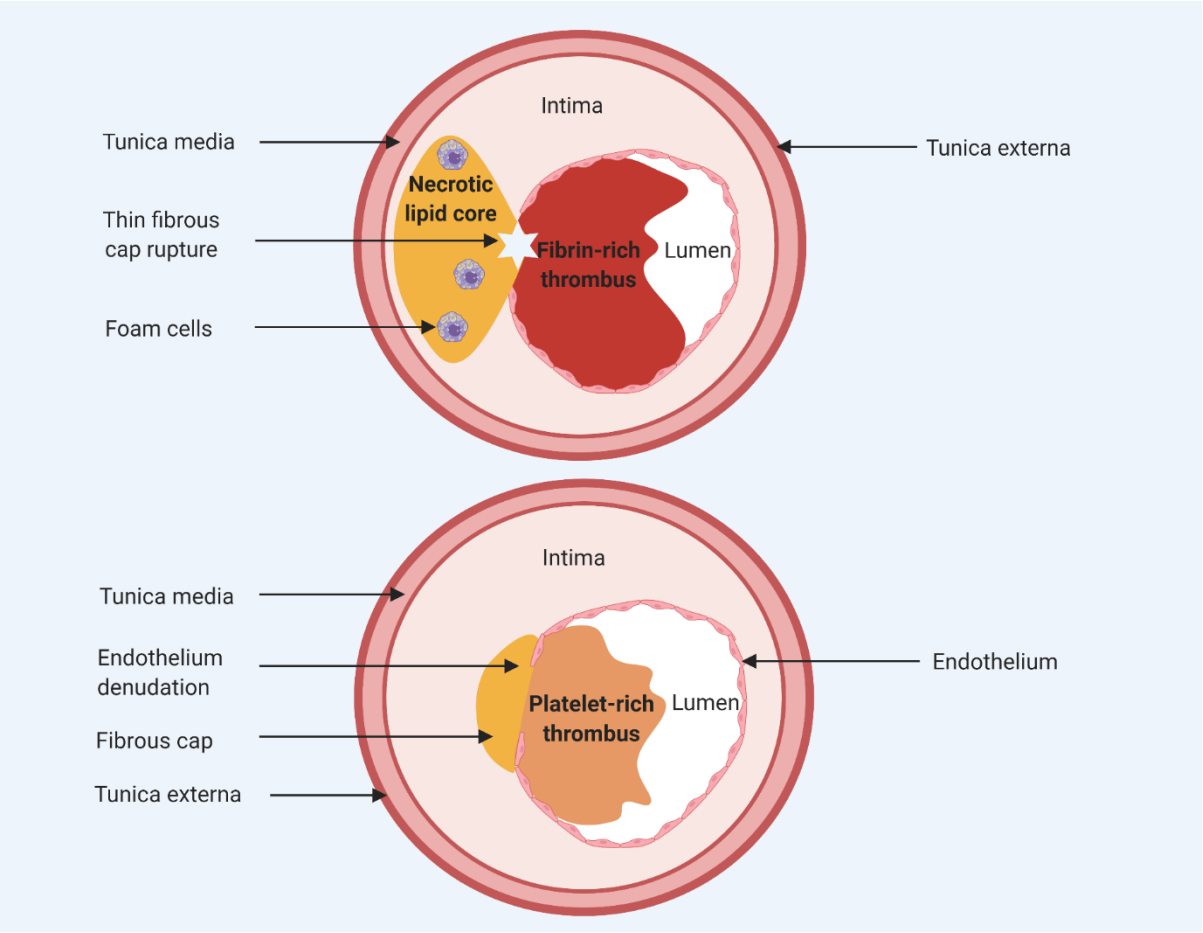


***Figure 1. 2 Ruptured vs eroded plaque structure**. **(Top)** Ruptured plaques are highly inflammatory lesions and contain a necrotic lipid core covered with a thin fibrous cap. Following rupture this necrotic material initiates rapid thrombosis that culminates in most cases in a fibrin-rich thrombus and severe lumen occlusion. **(Bottom)** Eroded plaques are significantly less inflammatory proteoglycan and collagen-rich lesions. These ECM proteins become exposed following EC denudation. This precipitates thrombosis resulting in a platelet-rich thrombus that partially occludes the vessel lumen. Created with Biorender.com.*

1.4 Risk factors

Identification of risk factors for plaque rupture led to pharmaceutical treatments, such as the use of statins to lower blood cholesterol levels and the ability to lower risk through managing factors such as smoking, diet and activity levels. Together these interventions have contributed to a > 75% decline in CVD related deaths since 1961 (British Heart Foundation; 2020). Whilst this statistic is encouraging, these interventions have little impact on mitigating the risk of plaque erosion. This is likely because many of the traditional risk factors associated with plaque rupture used to inform treatments do not correlate with an increased risk of plaque erosion. As discussed above, two large scale publications investigating plaque erosion have recently been published, Dai et al 2018 and Yamamoto et al 2019b. Alongside characterising plaque morphology the authors investigated potential risk factors that could be predictive of future plaque erosion. These studies combined provide the largest scale and most up to date analysis of risk factors for plaque erosion. Identifying co-morbidities with plaque erosion is important as it could help researchers understand what mechanisms are involved in erosion. For instance, a co-morbidity with dyslipidaemia and obesity in plaque rupture patients indicates a role for excess cholesterol and inflammation in the pathogenesis of rupture. This information has informed effective treatments to reduce cholesterol with statins and increases in physical exercise. Unfortunately, clear associations with plaque erosion are not evident. ACS patients with plaque erosion have fewer comorbidities compared with plaque rupture patients. Incidences of hypertension, dyslipidaemia, diabetes mellitus (DM) and chronic kidney disease are all significantly lower in patients with erosion compared with rupture (Dai *et al.*, 2018; Yamamoto *et al.*, 2019b). Yamamoto et al identified five risk factors that were strongly associated with plaque erosion: age <68 years, haemoglobin concentrations above 15 g/dL, normal kidney function, absence of DM and the presence of ischemia in the LAD (Yamamoto *et al.*, 2019b).

Patients with plaque erosion are significantly younger than patients with rupture. In studies comparing cohorts of patients with erosion vs patients with rupture a higher percentage of patients <50 years of age was reported in the erosion group (Dai *et al.*, 2018). Interestingly, Dai *et al* found the incidence of plaque erosion was highest among women <50 years of age and significantly higher than men <50 years of age. The incidence of plaque erosion reduced in both men and women >50 years of age, however the decrease was greatest in women (Dai *et al.*, 2018). The influence of sex in plaque erosion has been reported previously. Virmani et al 2006 showed that whilst 79.7% of patients with eroded plaques are male, erosion disproportionately affects women, accounting for over 80% of coronary thrombotic events (Virmani *et al.*, 2006). These results indicate that both age and sex are involved in plaque erosion, although how they are involved is not currently known.

Haemoglobin concentrations greater than 15 g/dL were found to be related to plaque erosion (Yamamoto *et al.*, 2019b). Greater concentrations of haemoglobin occur due to haemoconcentration.

This is where a decrease in plasma volume increases the concentration of RBCs. This can raise the viscosity of the blood and consequently raise the shear stress ECs are exposed to in the vessel. Shear stress is the frictional force imparted on the endothelium and is determined by vessel geometry, blood flow and fluid viscosity (Cunningham and Gotlieb, 2005; Grégory, 2021). Elevated shear stress can stimulate activation of platelets and promote their adherence to exposed components of the ECM. Therefore, haemoglobin is unlikely to be involved directly in the mechanisms of erosion but its elevated concentrations in patients with plaque erosion may point towards an important role for shear stress either in the physical erosion of the endothelium or the propagation of thrombosis.

Chonic kidney disease (CKD) is a known risk factor for atherosclerosis (Kon *et al.*, 2011; Valdivielso *et al.*, 2019). However, notably patients with plaque erosion had a significantly lower prevalence of CKD (Dai *et al.*, 2018). In fact, the analysis by Yamamoto et al went further finding a lack of CKD in non-ST elevation myocardial infarction (NSTEMI) patients as predictive of erosion compared to rupture (Yamamoto *et al.*, 2019b). DM is similarly associated with an increased risk of atherosclerosis (Beckman *et al.*, 2002). However, DM prevalence is also reduced in patients with plaque erosion (Yamamoto *et al.*, 2019b). In addition, hypertension was significantly less prevalent in patients with erosion compared to those with rupture. These data do not suggest a lack of CKD, DM or hypertension increase a patient's risk of suffering plaque erosion, rather they suggest that amongst a cohort of patients who suffered from ACS that an absence of CKD, DM and hypertension meant their ACS was more likely to occur due to plaque erosion than rupture. Whilst interesting, this fact contributes little to our understanding of erosion other than to demonstrate traditional risk factors for atherosclerosis do not always apply to erosion.

The factors are not completely comprehensive. For instance, cigarette smoking is a risk factor for atherosclerosis. Yamamoto et al found little difference in the percentage of current smokers between plaque rupture and plaque erosion however, when smoking history was taken into account 63.9% of patients with erosion were currently or had a history of smoking compared to 59.5% of patients with plaque rupture (Yamamoto *et al.*, 2019b). Dai and colleagues found 63.6% of patients with erosion were current smokers compared to 52.1% of patients with plaque rupture (Dai *et al.*, 2018). However, the study by Dai et al focused exclusively on patients with STEMI whilst Yamamoto et al included both STEMI and NSTEMI patients. This key difference in study design could explain subtle differences in reported conclusions.

Blood samples from patients with plaque erosion and rupture have been analysed to investigate differences that may predict erosion. These data show that patients with eroded plaques had lower WBC counts, creatinine concentrations, LDL cholesterol levels and total triglyceride levels compared to those with ruptured plaques (Dai *et al.*, 2018; Yamamoto *et al.*, 2019b). Additionally, a recent study found increased collagen type VI α-2 chain (COL6A2) and insulin-like growth factor 1 (IGF1)

and decreased fermitin family homolog 3 (FERMT3) correlated with plaque erosion in STEMI patients (Liu *et al.*, 2020). These factors may provide useful biomarkers for erosion.

In summary, the most significant predictors of plaque erosion are age <68 years, haemoglobin concentration elevated above 15 g/dL, normal kidney function and location of ischemia in the LAD (Table 2) however, additional risk factors may also contribute (Table 3) (Yamamoto *et al.*, 2019b). These results do little to identify an underlying cause of erosion. Instead, they merely suggest many of the traditional risk factors involved in atherosclerosis do not appear to be relevant to plaque erosion. This is likely why treatments developed to combat these traditional risks are not effective in mitigating the risk of erosion. However, it is precisely this lack of effective treatments and preventative techniques for plaque erosion that places even greater importance on understanding the pathological mechanisms of erosion in order to tackle an increasingly common disease.

Erosion
Age <68
Anterior ischaemia
Haemoglobin >15 g/dL
Absence of DM
Absence of CKD
More common in women (both <50 years and >50 years)
Lower hypertension prevalence
More likely to be a current or past smoker
Lower WBC count
Lower creatine levels
Lower LDL cholesterol levels
Lower total triglyceride levels

Table 1. 2 Factors associated with plaque erosion. *Table summarising the five factors (identified in bold) identified by Yamamoto et al that most strongly predicted plaque erosion in a cohort of ACS patients. When all five factors were present in a patient who presented with NSTEMI the chance that patient suffered from plaque erosion rose to 73.1%. Several further factors are displayed that were associated with plaque erosion compared with plaque rupture but were not predictive. Table adapted from (Yamamoto et al., 2019b). DM (diabetes mellitus), CKD (chronic kidney disease), WBC (white blood cell), LDL (low-density lipoprotein).*

1.5 Mechanisms involved in erosion

The lack of many strong risk factors for superficial erosion has shifted more importance onto the identification of structural aspects of eroded plaques that may indicate a pathological mechanism. As discussed earlier there are a multitude of structural differences between eroded plaques and ruptured plaques but the majority of these serve to rule out potential mechanisms of erosion rather than identify them. Despite this, several key processes have been identified that are thought to be involved in plaque disruption and thrombosis. These features will be discussed in detail below but in broad terms they are the detachment of ECs directly beneath the thrombus, accumulation of specific ECM components, the accumulation of platelets to sites of injury and disturbed blood flow.

1.5.1 Endothelial cell removal

Eroded plaques frequently lack ECs beneath the thrombus. This observation led to the hypothesis that this loss of endothelium propagates the thrombotic response or perhaps primes the plaque for thrombosis by exposing underlying collagen to which platelets adhere. Farb et al first linked removal of ECs with thrombosis. Immunohistochemical analysis of plaques obtained after sudden death from coronary thrombosis found a lack of ECs directly beneath the thrombi, implicating their detachment in thrombosis (Farb *et al.*, 1996). Subsequently, Durand et al also found that apoptosis of ECs was sufficient to induce thrombosis in rabbits. In this study, femoral artery sections were isolated with ligatures before incubation for 30 min with either staurosporine, a non-specific inhibitor of protein kinases widely used to induce apoptosis, or saline. Ligatures were removed and the treated artery segments exposed to normal blood flow for a further 3 days. An approximately 7-fold increase in thrombosis was observed in staurosporine treated arteries compared to saline treated. This effect was abolished following pre-incubation of isolated arteries with the caspase inhibitor ZVAD-fmk, directly implicating apoptosis in thrombosis (Durand *et al.*, 2004). A similar *in vivo* study investigated apoptosis specifically in sections of rabbit aorta containing plaques. Rabbits were fed a high-fat diet for 3 months and plaque formation induced through balloon injury of the aorta. Following plaque formation, the aorta was clamped, and a small region exposed to either saline or two different concentrations of staurosporine. EC apoptosis was determined using TUNEL staining and thrombotic scores were assigned based on aorta angiography. Rabbits treated with staurosporine showed greater levels of EC apoptosis and importantly this correlated with higher thrombotic scores compared to controls (Xu *et al.*, 2009). Further to this, Sumi et al showed disturbed flow downstream of stenosis in femoral arteries was sufficient to increase EC apoptosis and detachment resulting in

greater platelet adhesion in these areas (Sumi *et al.*, 2010). This agrees with immunohistochemical analysis of human carotid atherosclerotic plaques that show regions downstream of the plaque exposed to LOSS are significantly more likely to display apoptotic ECs than upstream regions exposed to HSS (Tricot *et al.*, 2000). As discussed earlier, a preponderance for eroded plaques occurring in the highly bifurcated LAD suggests a role for disturbed flow. Together, these data suggest disturbed flow can induce apoptosis and that this disruption of the endothelium contributes to thrombosis.

1.5.2 Extracellular matrix effect on endothelial cells

ECs like all cells are intrinsically linked to their surroundings and modulate their cellular activities based on interactions with their microenvironment. It is now recognised that a crucial component of a cell's microenvironment is the ECM. Previously considered an inert scaffold for cells to adhere, the ECM is now known to modulate several key EC functions including proliferation, angiogenesis and apoptosis as well as determining expression of surface adhesion molecules and pro-inflammatory cytokines.

ECs bind to the ECM through interactions between a variety of cell surface integrins and specific regions of ECM proteins. Eroded plaques are specifically enriched in hyaluronan (HA), a glycosaminoglycan and toll-like receptor 2 (TLR2) agonist linked with EC dysfunction and plaque erosion (Kolodgie *et al.*, 2002; Kolodgie *et al.*, 2004; Quillard *et al.*, 2015). The enrichment of HA in eroded plaques is interesting as other TLR2 agonists such as lipoteichoic acid (LTA) and Pam3 have been shown to increase EC expression of IL-8, E-selectin and ICAM-1 as well as augmenting apoptosis, endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) production (Quillard *et al.*, 2015). In addition, TLR2 is overexpressed in ECs exposed to LOSS linking it to the pathology of atherosclerosis (Mullick *et al.*, 2008; Mullick *et al.*, 2005). Recent studies into TLR2 and HA have provided evidence of their combined roles in plaque erosion. EC stimulation with low-molecular weight HA (LMW-HA) resulted in increased expression of VCAM-1, E-selectin and IL-8 alongside increased EC apoptosis. Interestingly, these effects were only observed when ECs were cultured on a LMW-HA-supplemented coating and not when stimulated with soluble LMW-HA. TLR2 stimulation resulted in smaller increases in inflammatory gene expression compared with TNF-α stimulation. This is consistent with the hypothesis that TLR2 ligation triggers EC priming, potentially leaving ECs more vulnerable to further damage.

Further studies have found increased expression of hyaluronidase 2 (HYAL2), the enzyme responsible for catalysing the conversion of high molecular weight HA (HMW-HA) into pro-

inflammatory LMW-HA, and the CD44v6 splicing variant of the HA receptor in peripheral blood mononuclear cells (PBMCs) from patients with plaque erosion compared with plaque rupture (Pedicino *et al.*, 2018). Furthermore, HA does not bind integrins on the EC surface responsible for binding components of the ECM and therefore does not contribute towards EC adherence. It is therefore reasonable to speculate that a HA-rich eroded plaque may constitute an environment where ECs are predisposed to detaching from the ECM. Together, these studies indicate a role for LMW-HA in the pathology of erosion.

If these interactions are disrupted and cells detach, apoptotic pathways are activated, and ECs can undergo a form of programmed cell death known as anoikis. For instance, EC attachment to the ECM has been shown to influence Fas-mediated apoptosis in HUVECs (Aoudjit and Vuori, 2001). HUVEC attachment to the ECM protects against anoikis by reducing expression of Fas and upregulating expression of the anti-apoptotic regulator protein c-Flip, which inhibits caspase-8 (Aoudjit and Vuori, 2001). This confers protection against apoptosis. Furthermore, laminin a key component of the ECM, binds to α3β1 integrins expressed on EC surfaces. Following binding of α3β1 integrin with laminin the complexed integrin-linked kinase (IlK) is activated and stabilises a larger complex including adenomatous polyposis coli-α3 (APC), a tumour-suppressive β-catenin. Together this complex phosphorylates AKT, inhibiting pro-apoptotic signalling through Bad/Bax and promoting anti-apoptotic signalling through Bcl2 (Suhr and Bloch, 2012). Disrupted laminin binding altered APC binding and inhibited phosphorylation of AKT, inducing pro-apoptotic signalling. Furthermore, specifically inhibiting human brain microvascular endothelial cell (HBMEC) integrins αvβ3/αvβ5 increased EC apoptosis and induced HBMEC detachment from the glycoprotein vitronectin (Erdreich-Epstein *et al.*, 2005).

These studies demonstrate the importance of EC contact with the ECM in maintaining EC survival. Given the significance of the endothelium in plaque erosion it is important to understand how the ECM of eroded plaques may influence their function. Indeed, studies have shown eroded plaques are enriched in specific ECM components that are not found or are found in significantly less quantity in ruptured plaques. Examining these differences and the effects of these different ECM components on EC function could lead to interesting insights into plaque erosion. In particular, the ability of HA to stimulate EC inflammation through TLR2 signalling (Franck *et al.*, 2017; Quillard *et al.*, 2015) and its upregulation under atheroprone conditions make it an interesting ECM component to study further (figure 1.3.)

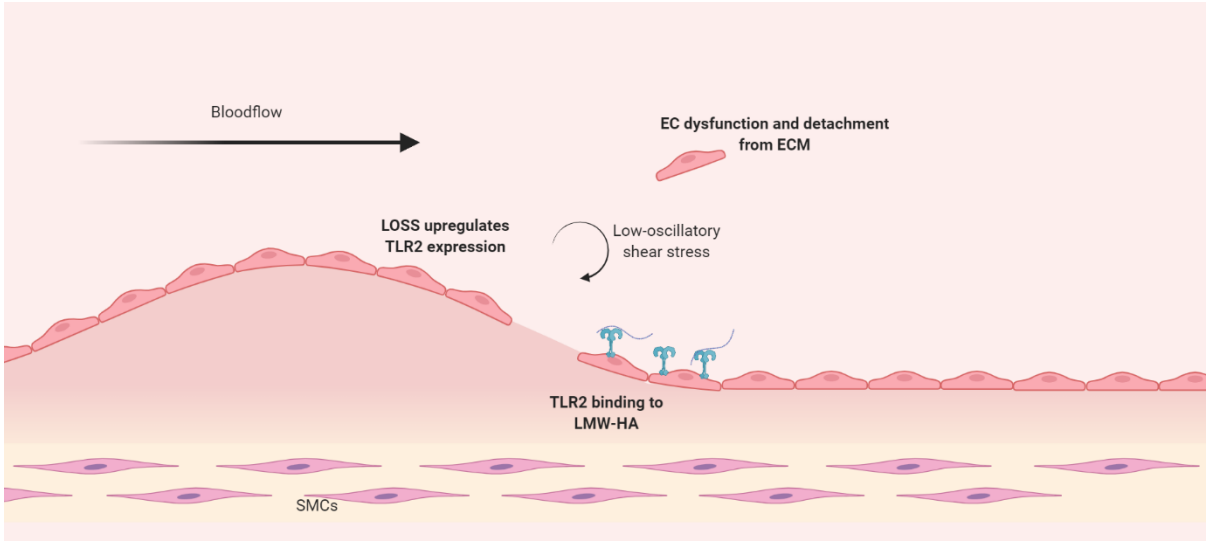


Figure 1. 3. TLR2 signalling in plaque erosion. *Low oscillatory shear stress (LOSS) upregulates TLR2 expression in endothelial cells. TLR2 binds to low molecular weight hyaluronan (LMW-HA) and promotes endothelial dysfuction. This induces endothelial cell detachment from the extracellular matrix (ECM). Figure adapted from (Pedicino et al., 2018). Created with Biorender.com.*

1.5.3 Platelet recruitment

Whilst EC dysfunction may be central to the process of plaque erosion, life threatening complications associated with plaque erosion result from thrombosis at the site of vessel injury. A thrombus consists of an aggregated mass of activated platelets cross-linked by fibrin which restricts blood flow by occluding the artery lumen. In plaque erosion, relatively slow forming platelet-rich thrombi occur blocking blood flow to the heart most commonly resulting in NSTEMI. There are broadly two relevant processes that cause thrombosis. The first is the generation of thrombin. Thrombin is a crucial enzyme in regulating the blood coagulation cascade. Thrombin propagates thrombosis by converting fibrinogen into fibrin and activating procoagulant factors, but also has roles in inhibiting coagulation by activating the anticoagulant protein, protein C when complexed with thrombomodulin. Tissue factor (TF) is the enzyme that begins the coagulation cascade which results in the production of many procoagulant factors. This cascade of reactions involves the conversion of the zymogen prothrombin to the active thrombin enzyme. Thrombin can then catalyse the conversion of fibrinogen to fibrin. The second relevant process is the activation of platelets. Platelets circulate in the blood stream under normal physiological conditions only becoming procoagulant following stimulation by relevant signals. Platelets can be activated by interactions between glycoprotein (GP) VI on their surface and exposed collagen of the ECM resulting in their degranulation. Platelets are also receptive to secreted factors including ADP released by damaged endothelial cells (Yun *et al.*, 2016). Amongst these soluble factors thrombin is the most potent platelet activator. Thrombin activates protease activated receptor 1 (PAR1) on the platelet surface by cleaving the peptide. This generates a new amino acid sequence that subsequently binds to and activates the G-protein coupled receptor (GPCR) region of its structure inducing intracellular signalling (Xu *et al.*, 2009). These distinct mechanisms of platelet activation have a common outcome which is the activation of GP IIb/IIIa on the platelet surface. This glycoprotein is crucial to the formation and stability of the thrombus as it binds fibrin to link platelets together in a mesh structure.

Following injury, the endothelium shifts towards a pro-thrombotic environment, inducing platelet activation and aggregation. Platelets can adhere directly to inflammatory endothelium through interactions between platelet GPIb-IX-V or GPIIb/IIIa and EC Von-Willebrand factor (vWF), and between GPIbα and EC intercellular adhesion molecule 1 (ICAM-1) (Bombeli *et al.*, 1998; Manon-Jensen *et al.*, 2016).

In healthy arteries ECs provide a tight barrier between the blood and the ECM within the artery intima. This helps maintain an anti-thrombotic environment. However, EC loss is typical in eroded plaques therefore the endothelium is no longer functioning to separate platelets from the subendothelial matrix. Nor is the endothelium present to facilitate direct platelet adhesion. Therefore, platelet adhesion to the exposed subendothelial matrix is likely to be the most relevant process

causing thrombosis above eroded plaques. The interactions between proteins making up the ECM, especially collagens and receptors on the surface of activated platelets, mediate recruitment and adherence of platelets in the first stages of thrombosis (Bergmeier and Hynes, 2012). Collagens are potently pro-thrombotic, and platelets possess a multitude of receptors to detect them. Broadly, there are three major receptors involved in direct platelet adhesion to exposed collagen: glycoprotein VI (GPVI), alpha 1 beta 1 ($\alpha_1\beta_1$) and alpha 2 beta 1 (α2β1) integrin (Emsley *et al.*, 2000; Knight *et al.*, 2000). More recently, type III collagen binding protein (TIIICBP) has also been identified (Maurice *et al.*, 2006) and there are further receptors such as p65 and GPIV (Ruggeri and Mendolicchio, 2007). Platelets also express a large repertoire of receptors for other components of the ECM. For example, $\alpha_6\beta_1$ binds laminin, $\alpha_5\beta_1$ binds fibronectin, $\alpha_V\beta_3$ binds vitronectin and $\alpha_{IIb}\beta3$ binds fibrinogen (Manon-Jensen *et al.*, 2016). $\alpha_2\beta_1$ predominantly bind collagens I and III, $\alpha_1\beta_1$ binds collagen IV and GPVI binds most strongly to collagen III (figure 1.4). These collagens are all present within the thick fibrous caps of eroded plaques and become exposed following EC erosion.

Platelets can also bind collagen indirectly through vWF. vWF is secreted by ECs and becomes extended under HSS, exposing the A2 domain for cleavage by the protease ADAMTS-13 (Zhang *et al.*, 2009). The resulting smaller vWF multimers bind collagens via their A3 domain. The A1 domain can then bind to platelet GPIbα receptors, tethering platelets to collagens. The affinities of both the interactions between vWF-A3 and collagen and vWF-A1 and GPIbα increase under shear stress, making vWF crucial to the initial adherence of platelets to the exposed ECM and may mediate a crucial first step which slows platelets allowing lower affinity integrin binding to secure platelets later in the process (Kim *et al.*, 2010). Interestingly, vWF may also affect binding by other platelet receptors. GPVI only binds collagen when in its dimerised form (Loyau *et al.*, 2012). In resting platelets GPVI is present in the monomeric form but evidence suggests that stimulation with vWF among other agonists, induces a conformational shift towards the high affinity dimerised form (Loyau *et al.*, 2012).

Following initial platelet adherence mediated by vWF, GPVI and α2β1 are likely to be the most important factors involved in direct platelet adhesion to collagen. GPVI and α2β1 bind the Gly-Pro-Hyp and GFOGER motifs, respectively (Knight *et al.*, 1999; Knight *et al.*, 2000). Specific deletion of GPVI gene in mice produced platelets unable to aggregate on type I collagen (Kato *et al.*, 2003). These findings were attributed to impaired post-adhesion processes as platelets did adhere to collagen but failed to aggregate. Further to this, murine platelets lacking GPVI no longer adhere to collagen whilst platelets lacking α2β1 still demonstrate adherence, but at a slower rate (Nieswandt *et al.*, 2001). The authors suggest that GPVI signalling may be involved in activating integrins that further contribute to platelet adhesion. In support of this, Sarratt et al demonstrated blocking α2β1 and /or GPVI on human platelets reduced platelet adhesion to collagen *in vitro.* Blockage of GPVI alone had a greater effect on reducing platelet adhesion than blocking α2β1 alone. However

simultaneously blockage had the greatest effect (Sarratt *et al.*, 2005). These data suggest GPVI and α2β1 likely contribute towards platelet adhesion to collagen through independent mechanisms with GPVI having the more influential effect. The important role of GPVI in platelet recruitment is supported by clinical studies that correlate GPVI platelet surface expression with ACS (Bigalke *et al.*, 2006). However, GPVI deficiency in mice and humans only results in mild bleeding disorders, suggesting there may compensatory mechanisms present (Bynagari-Settipalli *et al.*, 2014). More recent studies have found that dimeric GPVI binds fibrinogen although this binding was inhibited by collagen III, suggesting GPVI dimers interact with both substrates through the same binding site or the binding sites overlap (Induruwa *et al.*, 2018). A larger repertoire of substrates may help explain the disproportionate contribution of GPVI relative to other platelet collagen receptors. Whilst the exposure of ECM resulting from EC desquamation likely contributes the most to platelet recruitment at sites of erosion there is new evidence to suggest ECs in atherosclerotic lesions may be particularly prone to platelet recruitment. Researchers found a receptor for platelet CLEC-2 called podoplanin is expressed in advanced atherosclerotic lesions (Hatakeyama *et al.*, 2012). Podoplanin is expressed by macrophages and lymphatic ECs under normal conditions, however recent research has demonstrated that podoplanin is also expressed by vascular ECs and contributes to thrombosis. In an *in vivo* rat model of superficial erosion, overexpression of podoplanin in carotid artery ECs using recombinant adenovirus transfection increased thrombosis after 4 days compared with controls (Furukoji *et al.*, 2019). The mechanisms underlying this result were investigated using human aortic endothelial cells (HAECs) cultured *in vitro.* Podoplanin was first shown to be expressed in HAECs in response to physiological stimuli. HAECs stimulated with vascular endothelial growth factor A (VEGF-A) increased podoplanin protein expression after 24 h. Researchers then showed VEGF-A transfected HAECs increased ADP-stimulated platelet aggregation compared to controls. This effect was abolished following treatment with an anti-podoplanin antibody (Furukoji *et al.*, 2019). It is worth noting this study used a VEGF-A concentration far exceeding normal physiological levels. It is therefore not certain that VEGF-A would induce such enhanced podoplanin expression *in vivo*. Nor is it clear how relevant platelet adherence directly to the endothelium is in the context of plaque erosion as a key characteristic of erosion is the absence of ECs directly beneath the thrombus. However, it is possible that following initiation of platelet adhesion, the accumulation of platelets to a pro-thrombotic endothelium may cause sufficient disruption to induce EC detachment. Despite these concerns, these results are interesting as they imply a role for vascular EC podoplanin in thrombosis and suggest complex inflammatory signalling may create an endothelium prone to thrombosis.

In summary, it is well established that platelets can specifically adhere to components of the ECM, especially collagens as well as directly to the endothelium (figure 1.4). Given the enrichment of collagens in eroded plaques and the evidence that this ECM may be exposed due to EC desquamation, understanding the specific interactions between platelets and the ECM and the

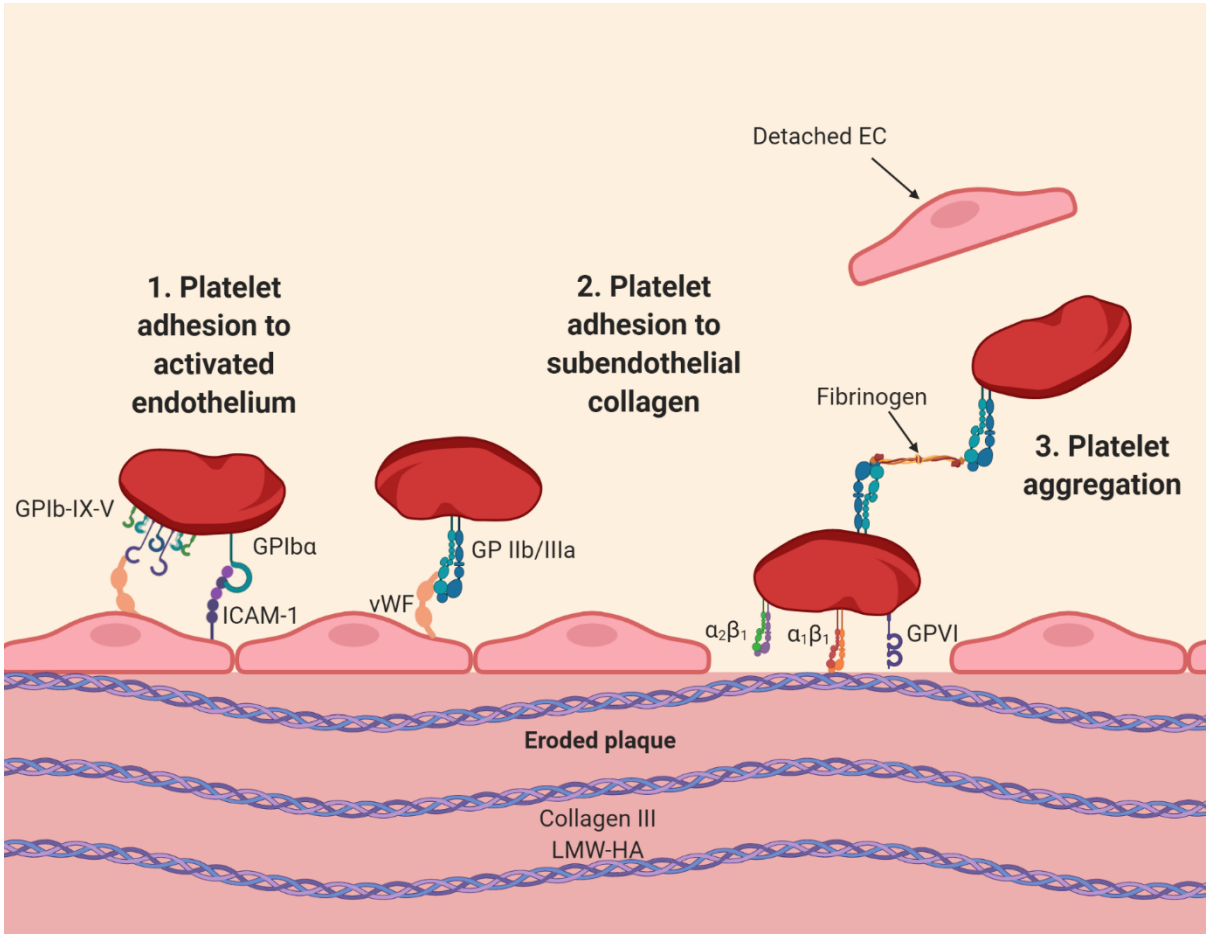


Figure 1. 4. Platelet adhesion and aggregation. *Activated platelets can adhere to the endothelium via interactions between GPIb-IX-V complex or GPIIb/IIIa and endothelial von-Willebrand factor (vWF). Alternatively, platelets can adhere via GPIba binding endothelial intercellular adhesion molecule 1 (ICAM-1). Following EC desquamation platelets can adhere to exposed subendothelial collagen through integrins a2b1 and a1b1 or glycoprotein VI (GPVI). GPIIb/IIIa contributes to platelet aggregation through binding fibrinogen eventually culminating in thrombus formation. LMW-HA (low molecular weight hyaluronan). Created with Biorender.com.*

factors that influence these interactions is potentially vital to understanding the pathology of plaque erosion.

1.5.4 Role of shear stress

The vascular architecture creates varied shear stress environments that influence atherosclerotic plaques development, progression, and complication. Artery bifurcations induce low and oscillatory shear stress (LOSS) conditions, known to predispose to atherosclerotic plaque development (Souilhol *et al.*, 2020) These regions are hence referred to as 'atheroprone'. Researchers have used physical models of human arteries to simulate blood flow dynamics and compared these measurements with actual measurements of arterial intima thickness. Correlations appeared between intimal thickness and areas of the artery predicted to experience LOSS, giving the first indications that altered blood flow impart pathogenic effects (Friedman *et al.*, 1987; Ku *et al.*, 1985). More recent studies using advance computer modelling have confirmed these early findings. Suo et al found significant correlations between the location of atherosclerotic plaques and areas of the vasculature predicted to experience LOSS by computer tomography (Suo *et al.*, 2008). Additionally, Stone et al demonstrated regions of arteries exposed to LSS had thicker plaques than regions exposed to normal physiological shear stress in a 6-month follow up of patients with atherosclerosis (Stone *et al.*, 2003). The importance of disturbed flow may be particularly relevant to plaque erosion as discussed earlier. Differences in plaque location were identified by Yamamoto et al. Eroded plaques are most commonly found in the left anterior descending (LAD) artery with 56% being found in this area, whilst ruptured plaques are evenly distributed between the LAD and right carotid artery (RCA) (Dai *et al.*, 2018; Yamamoto *et al.*, 2019b). The reason for this is not currently clear however, some hypothesise that the greater number of bifurcations in the LAD and the disturbed flow dynamics these bifurcations create may contribute specifically to plaque erosion. The preponderance of eroded plaques near these bifurcations further underlines the importance of considering these flow dynamics in plaque erosion. There are several well-established mechanisms through which disturbed flow can contribute to atherosclerosis. These mechanisms are often discussed in relation to plaque rupture, but many may also apply to plaque erosion. There is substantial evidence to indicate disturbed flow induces a pro-inflammatory phenotype in ECs that accounts for its pro-atherogenic effects (figure 1.5) (Davies *et al.*, 2013; Hsiai *et al.*, 2003; Jersmann *et al.*, 2001; Sheikh *et al.*, 2003). Studies have shown reduced expression of protective factors, endothelial nitric oxide synthase (eNOS) and Kruppel-like factor (KLF) 2 in ECs exposed to disturbed flow (DePaola *et al.*, 1999; Nam *et al.*, 2009; Won *et al.*, 2007). eNOS promotes vascular protection through reducing superoxide concentration, thrombosis, and leukocyte recruitment. Disturbed flow also induces sustained upregulation of low-

density lipoprotein receptors (LDLR) through inducing nuclear translocation of sterol regulatory element-binding protein 1 (SREBP1) controlling its transcription (Liu *et al.*, 2002). LDL is an important risk factor for atherosclerosis and although it is believed to be less prominently involved in the pathogenesis of plaque erosion, studies have reported LDL levels in patients with erosion above healthy levels (Dai *et al.*, 2018; Yamamoto *et al.*, 2019b). Disturbed flow also induces upregulation of ICAM-1 and VCAM-1 transcription which are crucial in the recruitment and infiltration of circulating leukocytes (Nam *et al.*, 2009). Despite data showing disturbed flow is important in plaque erosion there are very few studies investigating inflammation specifically in the context of plaque erosion. Chandran et al conducted a cytokine array of plasma taken from arteries with either a ruptured or intact fibrous plaque from patients with STEMI. They concluded from analysis of 102 cytokines that whilst ruptured plaques demonstrated elevated expression of 10 key cytokines, only endothelial growth factor (EGF) and thrombospondin-1 (TSP-1) expression was significantly elevated in intact fibrous caps (IFC) compared with ruptured fibrous caps (RFC) (Chandran *et al.*, 2017). It is not clear whether these inflammatory differences between eroded and ruptured plaques are the result of differing flow dynamics or some other factor.

However, whilst a plaque may form in an atheroprone region, specific areas of the plaque experience highly localised shear stress patterns (figure 1.5). A plaque developing in size and shape will impart an increasingly strong effect on blood flow dynamics. As stenosis progresses and luminal area decreases shear rate and shear stress increase. Physiological arterial shear stress is approximately 16 dyn/cm^2 (Stone *et al.*, 2003; Wentzel *et al.*, 2003) but as shear stress is related to the third power of luminal radius, a 75% stenosis causes a 64-fold increase in shear stress (White *et al.*, 2011). Computer modelling of stenosis shows this localised region of ultra-high shear stress (UHSS), far higher than physiological shear stress, occurs at the apex of the developing plaque with low and oscillatory shear stress downstream of the plaque (White *et al.*, 2016; Yamamoto *et al.*, 2019a). Recent work has pointed towards a role for pathologically high shear stress at the plaque apex. Whilst widely acknowledged to be atheroprotective with anti-inflammatory and anti-apoptotic effects, studies have suggested HSS with positive wall stress gradients can increase EC apoptosis (Dolan *et al.*, 2011; Macario *et al.*, 2008). In addition, White et al found UHSS led to increased p38 and ATF signalling in ECs which have been linked to EC dysfunction and atherosclerosis (White *et al.*, 2011). However, Chin et al also reported upregulation of protective heat shock protein 70 (HSP70) and reduced IL-6 and ERK1/2 signalling (Chin, 2008).

Bark et al found pathologically high shear rate (>1,000 S^{-1}) induced a two to four-fold increase in thrombus growth rate compared to physiological arterial shear rate (<400 S^{-1}). Thrombi continued to grow at shear rates up to 100,000 S^{-1} (Bark *et al.*, 2012). Furthermore, Yamamoto et al specifically considered ACS patients with plaque erosion. OCT informed computer modelling of diseased arteries and allowed for calculation of shear stress, shear stress gradient and oscillatory flow index. They reported 77.8% of thrombi propagated from the apex of the plaque, correlating with the highest

shear stress, highest shear stress gradient and lowest oscillatory flow measurements (Yamamoto *et al.*, 2019a). Additionally, they found whilst the thrombi originated from the UHSS region it extended into the distal portion of the plaque, the region exposed to LOSS suggesting thrombi originate from regions of UHSS and propagate along the direction of blood flow into the area downstream of the plaque and may account for contradictory reporting of thrombi location.

Recently the influence of UHSS on plaque erosion has been investigated further and greater emphasis has been placed on the importance of magnitude of endothelial shear stress gradients (ESSG) experienced by the endothelium over the magnitude of endothelial shear stress (ESS) (Thondapu *et al.*, 2021). ESSG was independently associated with both plaque erosion and plaque rupture whilst ESS was similar in both. Plaque erosion was independently associated with high oscillatory shear index (OSI) and OSI was higher at sites of erosion than sites of plaque rupture. Regions downstream of plaques typically experience OSS and, in support of this, erosion occurred more frequently at the distal plaque shoulder (Thondapu *et al.*, 2021). These data indicate regions of high OSI, as predicted in regions downstream of plaques, are associated with plaque erosion. This is supported by research linking high OSI with TLR2 activation and EC desquamation in investigations into plaque erosion (Franck *et al.*, 2017; Quillard *et al.*, 2015).

The prevailing hypothesis currently is that endothelial denudation occurs at the plaque apex exposed to UHSS and initiates thrombosis. This thrombus then propagates downstream into the region of LOSS where endothelial dysfunction may contribute to its progression.

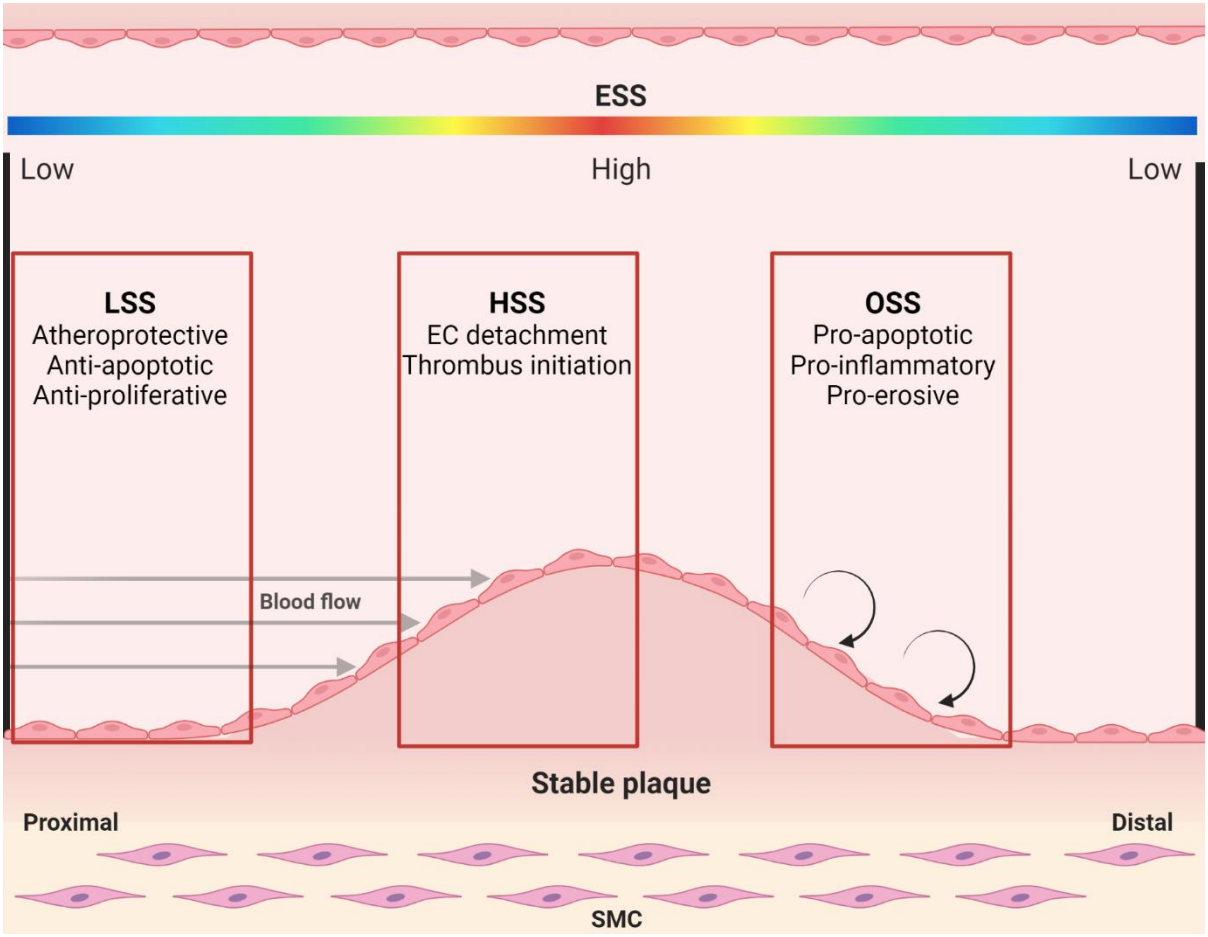


Figure 1. 5. Effects on endothelial cells of localised shear stress conditions. *Plaques in the vessel wall disrupt blood flow resulting in different shear stress conditions in different regions of the plaque. The proximal face of the plaque experiences laminar shear stress (LSS) which is atheroprotective, reducing apoptosis, inflammation and proliferation. Shear stress increases towards the plaque apex which experiences uhigh shear stress (HSS). HSS has been linked with physical removal of ECs and platelet activation. The distal regions of the plaque experiences oscillatory shear stress (OSS) which is linked with atherogenic processes such as apoptosis, inflammation and proliferation. Created with Biorender.com.*

1.6 Neutrophils in atherosclerosis

Neutrophils are the first responders in the innate immune system. Despite rarely being detected in atherosclerotic plaques there is substantial evidence suggesting they may play a crucial role in atherosclerosis. Elevated levels of circulating neutrophils are associated with greater plaque vulnerability with neutrophil depletion reducing both the formation of new plaques and the exacerbation of existing plaques (Zernecke *et al.*, 2008). Systematic review of 21 independent studies measuring on-admission neutrophil levels in ACS patients found elevated circulating neutrophils levels were predictive of cardiovascular events. Additionally, circulating neutrophil levels positively correlated with increased risk of future clinical events and total mortality (Guasti *et al.*, 2011). Studies also suggest neutrophils identified from culprit lesions in STEMI patients are more activated than circulating neutrophils (Mangold *et al.*, 2015).

Neutrophils are recruited to sites of inflammation by binding to adhesion molecules expressed on the surface of ECs (Nourshargh and Alon, 2014). Alterations in shear stress induce differential EC gene expression (Hsiai *et al.*, 2003; Jersmann *et al.*, 2001; Sheikh *et al.*, 2003). Some of the most important genes affected by changes in shear stress are those coding for adhesion molecules. *In vitro* studies have shown that the expression of adhesion molecules E-selectin, P-selectin and ICAM-1 increases under atheroprone LOSS compared with atheroprotective HSS conditions (Hsiai *et al.*, 2003; Sheikh *et al.*, 2003). This upregulation in adhesion molecule expression is linked to increased neutrophil recruitment under LOSS (Sheikh *et al.*, 2003). This hypothesis is supported by *in vivo* studies. For example, Nam *et al* induced LOSS in mice through ligation of three branches of the left carotid artery (LCA). ICAM-1 and VCAM-1 expression was elevated in mice with ligated LCA compared to those with unaltered right carotid arteries (RCA) or mice that underwent a sham procedure (Nam *et al.*, 2009). Furthermore, Franck *et al* observed elevated E-selectin and VCAM-1 protein expression in regions of disturbed flow *in vivo* compared to controls. Consequently, these regions displayed the greatest number of adherent neutrophils (Franck *et al.*, 2017).

Neutrophils can induce EC dysfunction through release of a wide variety of cytotoxic granule factors. Studies have shown that the products released during neutrophil degranulation can activate key apoptotic proteins, namely caspase 3, 7 and 8 in HUVECs through mitogen-activated protein kinase (MAPK) signalling (Koshio *et al.*, 2012). These degranulation products include reactive oxygen species (ROS), matrix metalloproteinases (MMPs), neutrophil elastase (NE), myeloperoxidase (MPO) and various pro-inflammatory cytokines (Herman *et al.*, 2001; Jacobi *et al.*, 2006; Tecchio *et al.*, 2014). Elevated NE and MPO levels have been reported at culprit lesion sites in STEMI patients (Mangold *et al.*, 2015). NE has been shown to induce apoptosis in ECs through activating proteins involved in the unfolder protein response and directly degrading vascular endothelial growth factor (VEGF) (Grechowa *et al.*, 2017; Lee *et al.*, 2015). Additionally, NE and cathepsin G may contribute

to atherothrombosis by degrading TF inhibitors (Massberg *et al.*, 2010). Studies have demonstrated that NETosis, the process through which neutrophils extrude DNA, can also cause endothelium injury through inducing EC apoptosis (Villanueva *et al.*, 2011). NET burden was found to correlate with infarct size (Mangold *et al.*, 2015).

Recent research suggests neutrophils are involved in the mechanisms of plaque erosion. Quillard et al showed neutrophils and NETs accumulate at sites of apoptotic ECs present in SMC-rich plaques. Here they exacerbate inflammation by potentiating the effects of TLR2 stimulation by ECM proteins, specifically enriched in eroded plaques (Quillard *et al.*, 2015). Inhibition of neutrophil recruitment under disturbed flow resulted in less endothelium permeability, greater continuity and reduced apoptosis compared with controls (Franck *et al.*, 2017). Taken together, these data strongly suggest neutrophils have a role in plaque rupture and erosion, although the precise mechanisms underpinning this are currently unclear. As well as release of NETs neutrophils are known to release microvesicles (MVs) and these have recently been shown to play a role in plaque development (Gomez *et al.*, 2020). However, their role in plaque erosion has not been investigated.

1.7 Neutrophil microvesicles

MVs are 0.1-1 μm vesicles generated by a variety of cells and have roles in cell-to-cell communication. Activated neutrophils generate MVs in response to stimulation by exogenous or host-derived factors or because of apoptosis. The effects of neutrophil microvesicles (NMVs) have been largely overlooked until recently when improved techniques have allowed for more accurate identification and characterisation prompting more studies into their roles in disease (Finkielsztein *et al.*, 2018).

NMVs are found in healthy individuals and therefore their presence is not indicative of disease, however elevated numbers of NMVs have been associated with a wide spectrum of inflammatory diseases including sepsis, acute respiratory distress syndrome (ARDS) and atherosclerosis (Chironi *et al.*, 2006; Guervilly *et al.*, 2011; Prakash *et al.*, 2012) and have also been found to be elevated by HFD (Gomez *et al.*, 2020). Atherosclerotic plaques from patients display significantly elevated levels of MVs compared to sections of arterial wall adjacent to plaques. MVs were identified as originating from lymphocytes, macrophages, neutrophils, and ECs (Leroyer *et al.*, 2007). Of note, leukocyte MVs correlate with the number of atherosclerotic plaques as assessed by ultrasound and are predictive of plaque instability, whilst no correlation is observed with MVs derived from other cell types, including ECs or platelets (Chironi *et al.*, 2006; Sarlon-Bartoli *et al.*, 2013). Furthermore, leukocyte MVs have also been positively associated with an increased Framingham risk score which

predicts the likelihood that an individual will develop heart disease in the future using risk factors such as age, sex, cholesterol levels and smoking history (Chironi *et al.*, 2006). Despite this association and their presence in atherosclerotic plaques their role in plaque erosion has not been investigated.

NMVs 'bud' from neutrophil membranes giving a right-side-out morphology the consequence of which is the mirroring of neutrophil cell surface proteins on the surface of NMVs (figure 1.6). Given the role of neutrophils in responding to infection and inflammation it is unsurprising that NMVs are generated in response to a number of bacterial products including LPS (Watanabe *et al.*, 2003) and fMLP (Dalli *et al.*, 2008; Eken *et al.*, 2010) as well as host pro-inflammatory cytokines TNF-α (Hong *et al.*, 2012), IL-8 (Mesri and Altieri, 1998) and complement factors (Gasser and Schifferli, 2004). Whilst neutrophils respond strongly to presence of bacterial infection NMVs may also be generated in response endogenous factors, including cholesterol. Previous studies have demonstrated NMV production is elevated in response to a high-fat diet (Gomez *et al.*, 2020). The precise mechanisms responsible for NMV generation are not fully understood however, alterations in plasma membrane lipid composition and associated changes in membrane curvature, alongside changes in cytoskeletal organisation occur following stimulation (Morel *et al.*, 2011; Verderio *et al.*, 2018). Calcium ions (Ca^{2+}) are known to be involved in MV biogenesis with levels positively correlating with MV biogenesis (Taylor *et al.*, 2020). Additionally, raising Ca^{2+} levels through calcium ionophore treatment increases MV biogenesis (Roseblade *et al.*, 2015). Ca^{2+} activates the calcium ion-dependent enzyme calpain. Calpain can degrade components of the cytoskeleton, allowing for structural alterations that facilitate NMV generation and inhibition of calpain reduces MV formation (Giannella *et al.*, 2021; Miyoshi *et al.*, 1996; Nolan *et al.*, 2008; Välimäki *et al.*, 2016). Ca^{2+} also correlates with reduced activity of lipid translocating enzymes called flippases. Flippases retain phosphatidylserine (PS) within the inner leaflet of the membrane. Reductions in flippase activity lead to increased PS exposure, commonly identified on MV membranes (Kou *et al.*, 2019; Wang *et al.*, 2016; Wang *et al.*, 2018a) and alterations in the membrane curvature that can contribute to increased MV biogenesis (Yu *et al.*, 2018a) (figure 1.6).

Different neutrophil stimuli methods produce NMVs with different properties, suggesting that NMVs are packaged with specific factors according to the method of stimulation (Dalli *et al.*, 2013). There are suggestions that enrichment of specific areas of the plasma membrane with proteins facilitates MV formation through exerting pressure on the membrane (Stachowiak *et al.*, 2012). This would link active packaging of NMVs with their biogenesis.

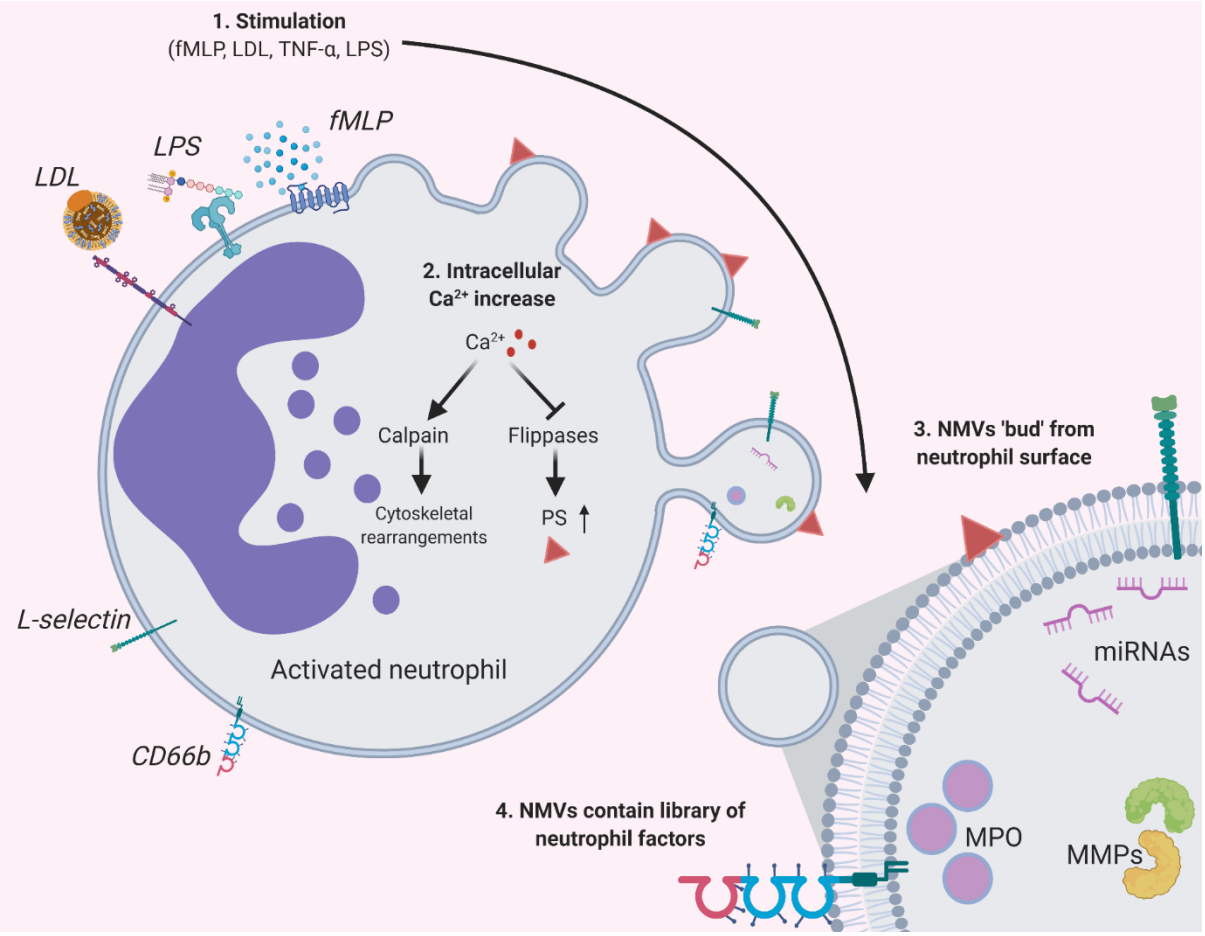


Figure 1. 6. Neutrophil microvesicle formation and contents. *Neutrophil microvesicles (NMVs) are generated by neutrophils stimulated by a variety of factors including N-Formylmethionine-leucyl-phenylalanine (fMLP) and low-density lipoprotein (LDL). Although the precise mechanisms of NMV formation is unclear it is believed to involve calcium influx, calpain activation, reorganisation of the cytoskeleton and protein accumulation at the site of NMV formation. Active packaging of neutrophil factors including micro RNAs, matrix metalloproteinases (MMPs), neutrophil gelatinase-associated lipocalin (NGAL), neutrophil elastase (NE) and reactive oxygen species (ROS). Created with Biorender.com.*

Recent evidence has shown that NMVs preferentially adhere to ECs under LOSS both *in vitro* and *in vivo* (Gomez *et al.*, 2020). Adherence occurs through binding of CD18 to EC ICAM-1 (Gomez *et al.*, 2020; Hong *et al.*, 2012). These NMVs were found to augment expression of IL-6, IL-8, VCAM-1, ICAM-1 and EC reactive oxygen species (ROS) (Gomez *et al.*, 2020; Hong *et al.*, 2012). Treatment of ECs with NMVs enhanced monocyte recruitment suggesting NMVs may play a role in plaque progression (Gomez *et al.*, 2020).

1.7.1 Neutrophil microvesicles and pro-inflammatory mediators

NMVs contain a large library of molecules derived from parent neutrophils including ROS, MMPs and cytokines (Gasser and Schifferli, 2004). Following NMV adhesion to and internalisation by ECs many of these molecules may contribute to EC dysfunction and the creation of a vascular environment conducive to plaque erosion. Hong et al demonstrated that NMVs induced elevated expression of IL-6 and IL-8 in addition to increased ROS and ICAM-1 expression in ECs (Hong *et al.*, 2012). The combination of enhanced pro-inflammatory cytokine secretion and adhesion molecule expression enhances neutrophil recruitment. In support of this, previous work has suggested NMVs preferentially adhere to regions predisposed to plaque formation, are internalised by human coronary artery ECs (HCAECs) and deliver microRNA-155 (miR-155) to HCAECs (Gomez *et al.*, 2020). Mir-155 increases expression of the RelA NF-κB subunit in HCAECs through reducing the expression of its negative regulator BCL6. This resulted in greater expression of pro-inflammatory genes CCL2, VCAM-1 and ICAM-1 and the effect was abolished in the presence of an antagomir, which inhibits miRNA function through irreversible binding.

NMVs have also been shown to express active transmembrane ADAM-10 (a disintegrin and metalloproteinase) on their surface. ADAM proteins are membrane-bound structures, many of which display catalytic activity. NMVs were identified using electron microscopy and ADAM-10 /17-specific antibodies linked to gold particles. Gold atoms scatter electrons allowing ADAM-10 and 17 positive regions to be observed using transmission electron microscopy (TEM). Flow cytometric analysis found a significant number of ADAM-10 and 17-postive microvesicles also co-labelled for CD66, indicating neutrophil origin (Folkesson *et al.*, 2015). ADAMs perform roles in catalysing the activation of other endogenous factors. For instance, ADAM-17 is known to activate TNF-α from its pro-form (Black *et al.*, 1997). TNF-α has potent pro-inflammatory effects and its activation by NMV-bound ADAM-17 may propagate an inflammatory environment conducive to plaque erosion.

1.7.2 Neutrophil microvesicles and protease-mediated extracellular matrix degradation

The search for mechanisms that contribute to EC desquamation is central to understanding the wider process of plaque erosion. One mechanism thought to be involved is the degradation of the collagen and proteoglycan-rich subendothelial matrix. This is thought to lead to reduced EC attachment and an increased propensity to detach (figure 1.7). NMVs contain a variety of proteases capable of ECM degradation among which are MMPs. MMPs are zinc-dependent proteolytic enzymes involved in the degradation of almost every component of the ECM. This family of enzymes is closely linked with the process of ECM turnover as part of a normal healthy vasculature. However, in the context of erosion their proteolytic functions may instead manifest in the denudation of the endothelium. Active MMP-9 has been identified in NMVs through western blot analysis, gelatin zymography and flow cytometry (Butin-Israeli *et al.*, 2016; Slater *et al.*, 2017). It is not clear from these results whether active MMP-9 is contained within NMVs or expressed on their surface. The probability is that MMP-9 is expressed in both locations. Resting neutrophils have been shown to express MMP-9 on their surface (Owen *et al.*, 2003). It follows that NMVs would express similarly low levels of surface MMP-9 due to their method of formation from the neutrophil membrane. Conversely, NMVs may specifically incorporate MMP-9 into their membranes. Interestingly, research suggests membrane-associated MMP-9 is more resistant to inhibition by TIMP-1 and TIMP-2 with IC50 values 21-fold and 68-fold greater, respectively compared to soluble MMP-9 (Owen *et al.*, 2003). MMP-9 is able to degrade interstitial collagens I and III in their native conformations (Bigg *et al.*, 2007). This indicates NMVs may be able to degrade subendothelial matrix in eroded plaques. In addition to the ECM degrading properties of NMVs, Butin-Israeli et al also showed MMP-9 confers the ability of NMVs to cleave Dsg-2 protein in epithelial cells. This effect was inhibited following treatment with MMP-9 inhibitors, but not MMP-2 inhibitors. Disruption of Dsg-2 reduces the integrity of the epithelium and NMV incubation resulted in increased monolayer fragmentation compared to negative controls (Butin-Israeli *et al.*, 2016). This study was conducted in epithelial cells so the results may not be representative of the effects in ECs. However, it is plausible that MMP-9 contributes to the erosion of endothelial monolayers. NMVs also contain the serine protease neutrophil elastase (NE) which can degrade collagens I and III as well as laminin (Gadek *et al.*, 1980; Gasser *et al.*, 2003; Kafienah *et al.*, 1998; Mydel *et al.*, 2008).

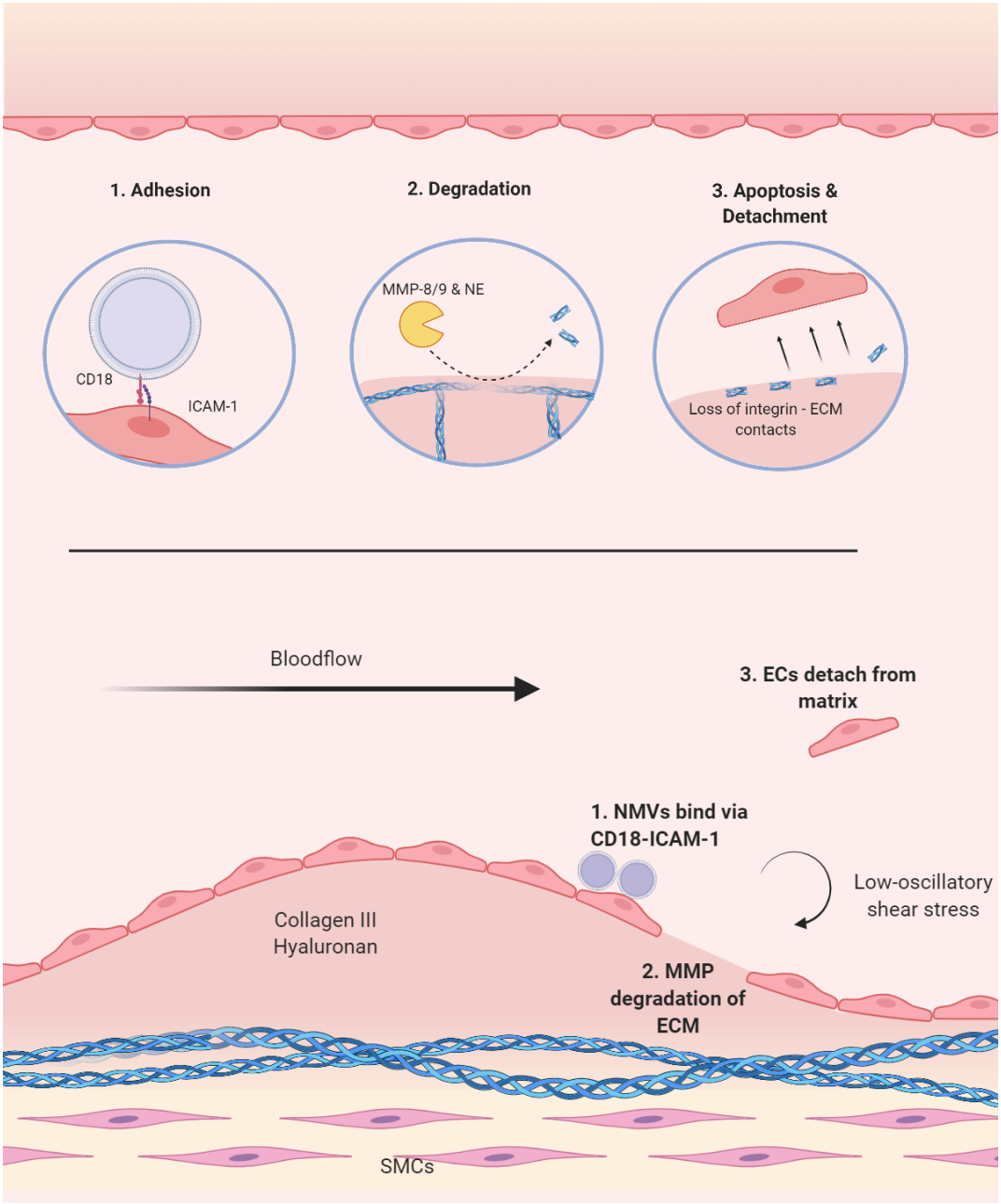


Figure 1. 7. Role of neutrophil microvesicles in extracellular matrix degradation and endothelial cell detachment. 1) *NMVs adhere to regions of the endothelium exposed to disturbed flow via CD18 binding intracellular adhesion molecules 1 (ICAM-1).* ***2)*** *NMV associated proteases such as matrix metalloproteases (MMPs) and neutrophil elastase (NE) degrade ECM proteins.* ***3)*** *ECM degradation weakens contact with ECs and in combination with disturbed blood flow causes the detachment of ECs. Created with Biorender.com.*

1.7.3 Neutrophil microvesicles and apoptosis

The degradation of ECM components specifically enriched in eroded plaques may induce EC anoikis, the process of apoptosis resulting from insufficient contacts between cells and the ECM. Studies into neuronal cell anoikis have established evidence of MMP-mediated degradation of the ECM as a cause of anoikis. For example, Guo *et al* found that following subarachnoid haemorrhage in rats MMP-9 activity increased significantly within the hippocampus and correlated with increased apoptosis of hippocampal neurons. Interestingly, levels of laminin, which is a key component of the ECM, decreased to its minimum measured concentration at the same time point that MMP-9 activity peaked (Guo *et al.*, 2010). Furthermore, MMP-2 and MMP-9 have been shown to degrade fibronectin and disrupt human cerebral EC integrin signalling *in vitro* resulting in greater cell death (Lee and Lo, 2004). Concomitantly, broad spectrum inhibitors of MMPs reduced activation of caspase-3 and reduced human cerebral EC death (Lee and Lo, 2004). In studies of epithelial cells, MMP-9 was found to disrupt the localisation of junctional proteins claudin-1 and occludin, responsible for ensuring intercellular integrity, and increased apoptosis through anoikis (Vermeer *et al.*, 2009). Whilst being cautious not to generalise the results observed in rat neurons, human cerebral ECs and epithelial cells to human vascular ECs, it is not unreasonable to speculate that MMP-9-mediated degradation of the ECM may evoke a similar process of anoikis in human vascular ECs given that I have already considered evidence to show ECs depend on correct interactions with the ECM for cell survival.

When cultured *in vitro,* HUVECs treated with MMP-9 displayed increased apoptosis compared to controls, as determined by measuring cleaved caspase-3 expression (Florence *et al.*, 2017). Furthermore, MMP-9 associated with human plaque ECs but not with ECs from normal tissue. These plaques displayed higher concentrations of caspase-3 compared to normal tissue, potentially implicating MMP-9 in apoptosis (Florence *et al.*, 2017). Dorweiler *et al* identified increased MMP-8 expression in neutrophils activated with low-density lipoprotein (LDL). High levels of LDL are a known risk factor in atherosclerosis. The authors reported a significant correlation between MMP-8 concentration and increased SMC and EC apoptosis (Dorweiler *et al.*, 2008). Further to this, Spallarossa *et al* investigated MMP-9 and myeloperoxidase (MPO) levels in the serum of healthy adults following a high-fat meal challenge. They reported enhanced activity of MMP-9 and MPO in the serum of volunteers exposed to a high-fat meal compared to normal controls. Serum was collected 1 or 2 h later and incubated with HUVECs *in vitro.* Increased MMP-9 and MPO activity positively correlated with increased HUVEC annexin V and cleaved caspase-3 positivity (Spallarossa *et al.*, 2008). Interestingly, a recent study identified increased MPO plasma concentrations in STEMI patients with erosion compared to those with rupture. Mean MPO concentration in patients with erosion was more than double that in patients with rupture (Tan *et al.*, 2020).

1.7.4 Neutrophil microvesicles and immune cell recruitment

In addition to the direct effect of MMPs on matrix degradation, MMPs may also contribute to the enhanced recruitment of neutrophils to sites of erosion through the generation of matrix-derived chemokines sometimes referred to as ‘matrikines’. Breakdown of the ECM by MMP-8 and MMP-9 has been shown to liberate the matrix fragment proline-glycine-proline (PGP), which is a potent neutrophil chemoattractant by Gaggar et al (Gaggar *et al.*, 2008). This study collected sputum samples from cystic fibrosis (CF) patients and found it sufficient to generate PGP from purified collagen. Inhibition of MMP-8 and MMP-9 abolished the ability to form PGP thus identifying these proteolytic enzymes as specifically responsible for PGP generation. The generation of matrikines may establish a positive-feedback loop in which neutrophil-derived MMP activity promotes neutrophil recruitment. Furthermore, NE-mediated degradation of laminin produces neutrophil chemotactic peptide fragments (Mydel *et al.*, 2008).

Together, these studies suggest proteases are involved in anoikis and classical apoptosis of ECs through proteolytic degradation of the ECM, disruption of intercellular interactions and the potential establishment of a pro-inflammatory feedback loop exacerbating immune cell recruitment. Considering the importance of endothelium disruption in plaque erosion the contribution of these proteases must be considered. Further to this, the presence of these proteases in NMVs which have been shown to be specifically recruited to atheroprone ECs makes NMVs an interesting area to consider in the context of plaque erosion. However, much more research is needed to confirm this hypothesis.

1.7.5 Neutrophil microvesicles in thrombosis

MVs from many cell types are present in inflammatory disease and there is evidence MVs from multiple cellular origins are involved in thrombosis. Platelet and EC MVs have been positively associated with ACS (Bernal-Mizrachi *et al.*, 2003; Mallat *et al.*, 2000). Additionally, leukocyte, platelet and neutrophil-derived MVs were found to be elevated in patients with cardiovascular events and positively correlated with mortality (Suades *et al.*, 2019). Furthermore, platelet, EC and leukocyte-derived MVs have been shown to increase deposition of platelets on human atherosclerotic plaques (Suades *et al.*, 2012). Many of the pro-thrombotic effects MVs are reported to have derive from their expression of pro-coagulant factors such as phosphatidylserine (PS) and tissue factor (TF), or factors involved in platelet adhesion such as P-selectin glycoprotein ligand 1 (PSGL-1) and CD11b/CD18. There is limited research into NMVs specifically, however studies of

broader leukocyte and granulocyte-derived MV populations can shed light on the potential pro-thrombotic effects of NMVs.

There is evidence that MVs may be involved in cardiovascular events through raising the risk of thrombosis by contributing to a pro-coagulant environment. Shustova et al who found that EC, monocyte, THP-1 and granulocyte-derived MVs decreased serum coagulation time significantly (Shustova *et al.*, 2017). One mechanism by which this could occur is through generation of thrombin. In support of this, granulocyte MVs isolated from patients with meningococcal sepsis have been shown to induce thrombin generation in normal serum (Nieuwland *et al.*, 2000). The mechanisms involved in this process are not fully understood but PS is hypothesised to be involved. PS is an anionic phospholipid localised to the inner cell membrane of various cell types, including neutrophils (Zwaal and Schroit, 1997). Following cell activation PS is redistributed to the cell surface. PS is relevant to thrombosis as it provides sites for clotting factors and prothrombinase complexes to adhere which catalyse the conversion of pro-thrombin into thrombin which in turn catalyses the conversion of fibrinogen to fibrin, a key component of thrombi. NMVs are known to express PS on their surface (Zhang *et al.*, 2016) in fact, the expression of PS may be involved in the biogenesis of MVs (Yu *et al.*, 2018a) (figure 1.6) and have been associated with commonly observed thrombotic complications following carotid artery stenting (CAS) (Zhao *et al.*, 2017). This association has been attributed to PS exposure. Blood samples taken post-CAS and isolated MVs were analysed. PS expression increased on the surface of NMVs as well as MVs from platelets, erythrocytes, monocytes and ECs 2 h post procedure, reaching their highest level after 48 h and then persisting at heightened levels for the following 7 days (Zhao *et al.*, 2017). PS^+ MVs correlated with increased thrombin and fibrin formation, and decreased coagulation times. This is supported by data from stenting in NSTEMI patients (Wang *et al.*, 2016). In this study PS^+ MVs were significantly higher at baseline in NSTEMI patients compared to healthy controls. PS^+ MVs also increased substantially 18 h post-stenting with MVs from leukocytes, platelets, erythrocytes, and ECs correlating with increased thrombin and fibrin generation and reduced coagulation time. Inhibition of PS with lactadherin reduced the procoagulant activity of MVs, confirming the involvement of PS (Wang *et al.*, 2016). Furthermore, NMVs have recently been reported to form complexes with NETs and these complexes enhance thrombin production (Wang *et al.*, 2018a). In this study, NMVs adhered to NETs through PS binding to histones. Thrombin production was almost completely abolished in Factor XII-deficient plasma and interestingly NMVs were shown to specifically bind Factor XII. It is not clear exactly how NMVs contribute to thrombin generation in this situation but none-the-less the association of NMVs with coagulation factors and known pro-thrombotic NETs is interesting and further suggests a role for NMVs in thrombosis.

Tissue factor (TF) is also involved in the blood coagulation response. It is expressed by vascular cells including ECs but also some leukocytes. TF forms a complex with plasma coagulation factor VIIa. This complex catalyses the conversion of factor X to Xa which is involved in the conversion of

prothrombin into thrombin (Rao *et al.*, 2012). The importance of TF is demonstrated by unsuccessful attempts to generate viable total TF knockout (KO) mice. Instead, low TF mice have been bred which show longer bleeding times in models of vessel injury, display reduced fibrin generation and reduced thrombosis (Darbousset *et al.*, 2012; Getz *et al.*, 2015). Studies investigating TF found anti-TF antibodies significantly reduced thrombus growth both in a model involving perfusion of *ex vivo* human blood and in a rabbit model (Himber *et al.*, 2003). In fact, inhibition of TF had comparable anti-thrombotic effects to inhibition of thrombin. It is therefore well established that TF has a significant effect on blood coagulation and thrombosis.

The highly pro-thrombotic effects of TF mean that whilst it is expressed by vascular cells it is separated from the blood under normal conditions. Following injury to the vessel wall TF can become exposed and initiate blood clotting. TF also associates with circulating cells, including leukocytes and leukocyte-derived MVs (Chou *et al.*, 2004). The accumulation of this circulating TF to the vessel wall is an important secondary mechanism involved in thrombosis. Neutrophils express TF and blockage of neutrophil recruitment to injured endothelium via ICAM-1 reduces TF expression, fibrin formation and platelet accumulation in these regions, indicating a role for leuckocyte-associated TF in atherothrombosis (Darbousset *et al.*, 2012). However, there are conflicting reports of TF expression in NMVs. Multiple studies have demonstrated that whilst granulocyte MVs generated in response to LPS stimulation induce a coagulant response in human plasma, they do not express active TF (Khaspekova *et al.*, 2016; Shustova *et al.*, 2017). This is interesting as although no TF was detected granulocyte MVs did contribute to coagulation, potentially indicating another pro-thrombotic mechanism is present. Conversely, Kambas et al reported TF-positive NMVs in patients with active anti-neutrophil cytoplasmic antibody associated vasculitis and patients in remission. TF expression on NMVs was confirmed through co-staining for CD66b. The possibility of TF being detected on contaminating platelet-derived MVs was ruled out as CD62P-positive MVs were excluded from analysis (Kambas *et al.*, 2014). Active incorporation of factors into NMVs in response to different stimuli may explain the disparate results, however it is not clear what these stimuli are nor is it clear if different stimuli activate separate pathways for NMV generation and packaging. Additionally, leukocyte MVs have been shown to increase tissue factor expression on ECs and this correlated with an elevated coagulation response (Mesri and Altieri, 1999). This indicates that NMVs may also have an indirect effect on thrombosis through increasing the procoagulant properties of ECs. NMVs may also contribute towards a more pro-thrombotic environment through protease-mediated degradation of subendothelial matrix. Partial degradation of monomeric collagen type I by MMP-13 was found to result in increased platelet deposition under arteriolar flow conditions (Howes *et al.*, 2015). Exposure of motifs normally inaccessible in the collagens native form could explain the increased platelet deposition.

NMVs may also impart direct effects on circulating platelets. The role of cross-talk between innate immune cells and platelets is becoming increasingly important to understanding mechanisms of

thrombo-inflammation. It is well established that platelets and neutrophils bind through P-selectin/PSGL-1 and GPIbα/CD11b/CD18-dependent interactions and these interactions can activate neutrophils, inducing ROS production and NETosis (Moore *et al.*, 1995; Pircher *et al.*, 2018; Simon *et al.*, 2000; Sreeramkumar *et al.*, 2014). NMVs express the same surface receptors and can also directly adhere to and activate circulating platelets. NMVs express PSGL-1 and CD11b/CD18 that facilitate adhesion to platelets through binding P-selectin and GPIbα, respectively (Falati *et al.*, 2003; Pluskota *et al.*, 2008). Ligation of GPIbα with CD11b/CD18 induces intracellular signalling and upregulation of platelet integrins involved in platelet aggregation, such as $\alpha_{IIb}\beta_3$, as well as P-selectin expression. Neutrophils activated with phorbol 12-myristate 13-acetate (PMA), platelet activating factor (PAF) and lipopolysaccharide (LPS) all generated NMVs with elevated CD11b/CD18 expression compared with 'resting' neutrophils (Pluskota *et al.*, 2008). NMVs from PMA-activated neutrophils compared to resting neutrophils expressed greater levels of active CD11b/CD18 and induced greater activation of platelets, assessed by P-selectin expression and activation of $\alpha_{IIb}\beta_3$ (Pluskota *et al.*, 2008). Platelet P-selectin is strongly linked to atherosclerosis. For example, soluble P-selectin levels were found to be significantly higher in healthy women who later suffered cardiovascular events compared to those who did not (Ridker *et al.*, 2001). In addition, mice deficient in platelet P-selectin develop plaques 30% smaller than WT mice (Burger and Wagner, 2003). P-selectin is also involved in thrombus formation. Blocking platelet P-selectin was found to reduce early platelet aggregation (Théorêt *et al.*, 2006) and P-selectin deficient mice demonstrate reduced thrombus formation, have fewer leukocytes present in the thrombi and display fewer large aggregates in *ex vivo* blood coagulation experiments (Yokoyama *et al.*, 2005). Platelet P-selectin binding to its ligand PSGL-1 triggers Ca^{2+} increases and platelet activation (Sathish *et al.*, 2004). P-selectin residues can also become phosphorylated suggesting a role in intracellular signalling (Modderman *et al.*, 1994). P-selectin ligation with PSGL-1 increases $\alpha_{IIb}\beta_3$ activation and resulted in greater platelet adhesion to collagen surfaces *in vitro (Théorêt et al., 2006)*. These effects were abolished in the presence of an anti-P-selectin antibody. These studies indicate platelet P-selectin ligation plays a pivotal role in platelet activation and aggregation. However, the role of NMVs in activating platelets through this mechanism has not been researched in much depth. P-selectin ligation is unlikely to be the sole route NMVs may influence platelet function. For instance, NMVs produced in response to endotoxin contain PAF and were able to activate resting human platelets (Watanabe *et al.*, 2003). This PAF secretion via NMVs was only observed in neutrophils that were stimulated following adherence to a gelatin matrix, potentially indicating some intracellular signalling triggered by adherence is necessary for this process to occur.

Platelets and NMVs may interact through other less direct mechanisms. In a study by Tersteeg *et al* adherent platelets exposed to shear stress produced long membranous structures called flow-induced protrusions (FLIPRs) (Tersteeg *et al.*, 2014). These FLIPRs extend downstream from the adherent platelets and were shown to bind neutrophils in a P-selectin/PSGL-1-dependent manner.

Co-incubated neutrophils displayed fragments of FLIPRs on their surface and were activated, displaying greater expression of CD11b and L-selectin shedding. PSGL-1 has been shown to be expressed on NMVs and, therefore, NMVs have the capacity to interact with FLIPRs like their parent neutrophils (Nolan *et al.*, 2008). This proposed NMV-FLIPR interaction could represent a mechanism through which platelets facilitate NMV recruitment to sites of vessel injury.

Taken together, these studies suggest NMVs may have pro-thrombotic effects that cooperate to increase the risk of thrombosis (figure 1.8). Specifically, they contribute to thrombosis through expression of PS and TF which catalyse the production of pro-coagulant factors, the activation of platelets through direct binding and the generation of a more pro-thrombotic environment through degradation of the matrix. Following adhesion to the endothelium NMVs may provide an adhesive surface on which platelets can adhere. NMVs express PSGL-1 the ligand for P-selectin highly expressed on the surface of activated platelets. Whilst this is interesting, further investigation of these topics is required to uncover any potential role for NMVs in plaque erosion.

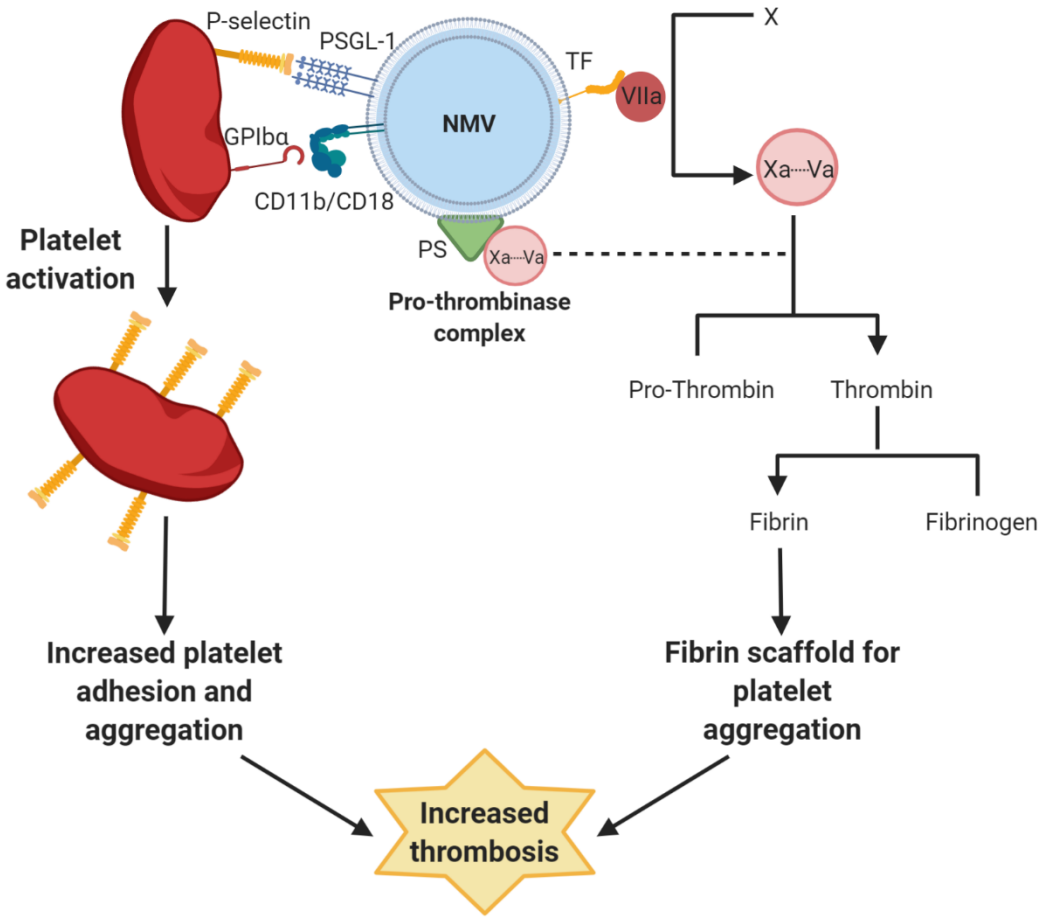


Figure 1.8. Pro-coagulant properties of neutrophil microvesicles. *Neutrophil microvesicles (NMVs) reportedly express TF which converts the proteinase factor X into factor Xa. Factor Xa forms a complex with co-factor Va to form the pro-thrombinase complex. The pro-thrombinase complex binds to exposed PS on the membrane of NMVs and other MVs, notably platelet MVs. This enhances the activity of pro-thrombinase and increases conversion of pro-thrombin to thrombin. Thrombin converts fibrinogen into fibrin which forms a major structural component of thrombi. NMVs also activate platelets through binding P-selectin and GPIbα. This enhances platelet integrin and P-selectin expression increasing platelet adhesion to collagen, platelets and further strengthening interactions with NMVs. Increased platelet activity in combination with enhanced fibrin production may contribute to thrombosis. TF (tissue factor), PS (phosphatidylserine), PSGL-1 (P-selectin glycoprotein ligand 1). Created with Biorender.com.*

1.8 – Summary

In the absence of strong predictors of risk factors for plaque erosion, identification of structural features specific to eroded plaques offers the best insight into understanding the underlying pathological mechanisms. In brief, observations that thrombi occur without communication of the plaque contents with the blood and the absence of necrotic lipid pools within the plaque fundamentally differentiates the process of plaque erosion from plaque rupture. A denuded endothelium directly beneath thrombi indicates EC dysfunction in the propagation of thrombosis. A suggestion supported by reports of specific accumulation of certain fibrous proteins and proteoglycans in the ECM which induce low-level EC inflammation and detachment through TLR2 signalling (Quillard *et al.*, 2015). Removal of the endothelium, a process likely initiated or exacerbated by disturbed blood flow exposes collagens and other ECM proteins to which activated platelets can adhere. Neutrophils exacerbate these effects leading to speculation of their role in the mechanism of plaque erosion (Quillard *et al.*, 2015). However, despite an association between circulating neutrophil levels and CVD (Silvestre-Roig *et al.*, 2020) neutrophils are not commonly observed in great number within atherosclerotic plaques. I therefore, hypothesise that their effects are mediated through generation of NMVs which have been shown previously to adhere to atheroprone regions (Gomez *et al.*, 2020). NMVs contain a specific library of neutrophil granule proteins determined by the stimulus by which MV release is induced. These include MMPs and cytotoxic molecules that may contribute to EC dysfunction and detachment. NMVs have also been implicated in platelet activation and express many of the surface receptors that allow neutrophils and platelets to interact. Additionally, NMVs can influence EC gene expression causing upregulation of adhesion molecules. NMVs impact on ECs suggests they may play a role in the mechanisms of plaque erosion by increasing EC denudation, exposure of the ECM and platelet adhesion.

1.9 Hypothesis and aims

There is a significant gap in knowledge regarding the process that induces plaque erosion. Additionally, there is little understood about how NMVs may influence this process despite the fact that neutrophils have been strongly associated with plaque erosion.

I hypothesise that NMVs produced in response to pathologically relevant stimuli contribute to plaque erosion by facilitating EC denudation through degradation of the ECM. I also hypothesise that NMVs increase platelet recruitment to sites of plaque erosion via activation of platelets, exposure of pro-

thrombotic ECM proteins and upregulation of platelet adhesion molecules in ECs (figure 1.9). These hypotheses were investigated using *in vitro* techniques.

The main aims of the project were to determine if:

- Neutrophils produce NMVs in response to pathologically relevant stimuli (nLDL)
- ECM proteins enriched in eroded plaques contribute to EC erosion and dysfunction
- NMVs facilitate ECM degradation
- NMVs induce detachment of ECs cultured *in vitro* on a matrix consisting of erosion-specific and pathologically relevant proteins
- NMVs form complexes with and activate platelets via specific cell surface receptor-ligand interactions
- NMVs increase platelet recruitment to intact endothelium
- NMVs increase platelet recruitment to exposed collagens

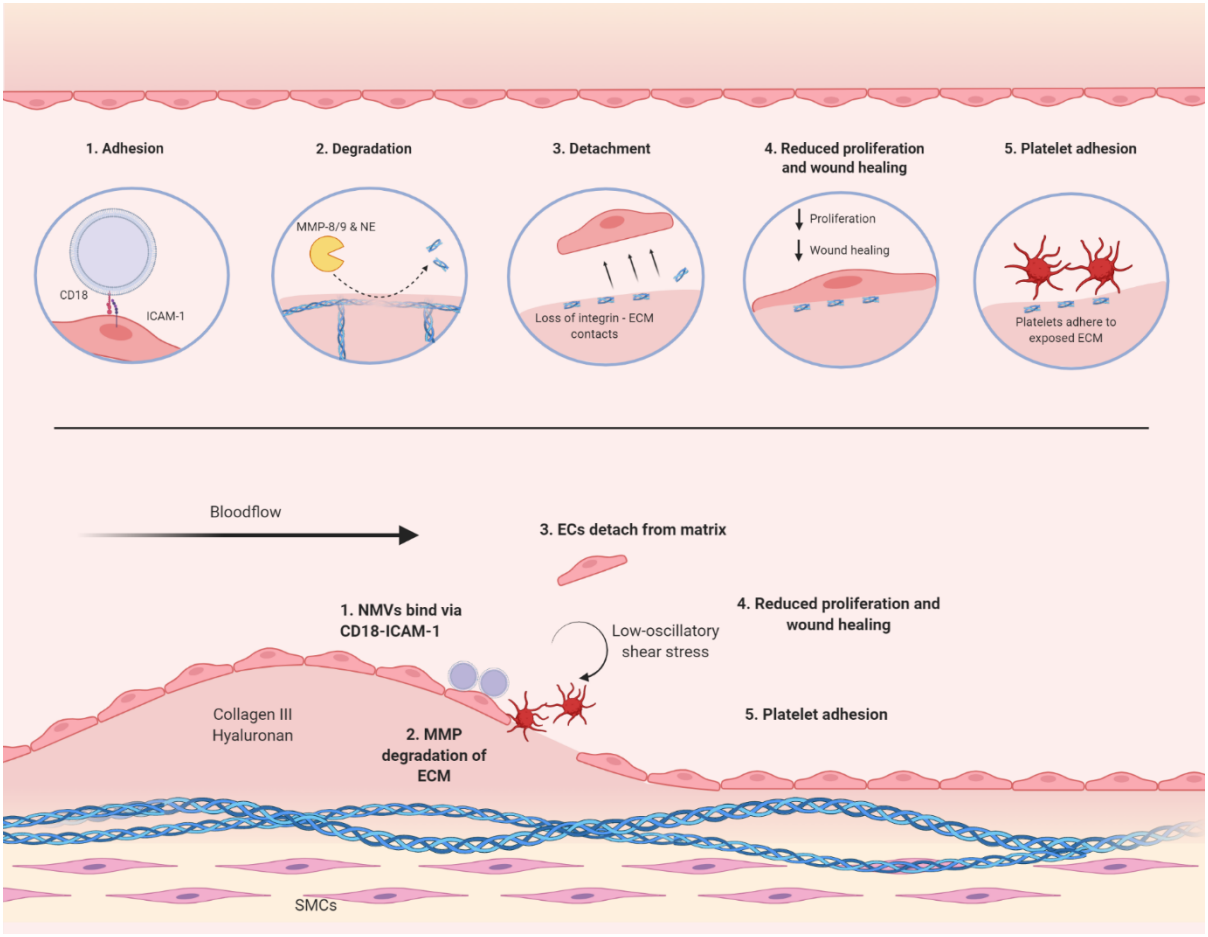


Figure 1.9. Hypothesis summary diagram. *1. NMVs adhere to endothelial cells (ECs) via CD18-ICAM-1 interactions. 2. NMV proteases including matrix metalloproteinases (MMPs) and neutrophil elastase (NE) degrade extracellular matrix (ECM) components of atherosclerotic plaques leading to 3. detachment of ECs. 4. NMVs reduce proliferation and wound healing responses exacerbating the erosion of ECs. 5. Exposure of ECM following EC erosion provide platforms for platelet adhesion and aggregation. Created with Biorender.com.*

2 Methods

2.1 Ethics and participant recruitment

The work undertaken in this project was approved by the University of Sheffield Research Ethics Committee (reference number: 031330). 80 mL blood samples from healthy volunteers over the age of 18 with no known medical conditions were obtained by venepuncture. Participants provided written informed consent and were informed that they could withdraw their consent at any time.

2.2 Neutrophil isolation from whole human blood

An equal ratio of healthy female to male volunteers were recruited for the study. Neutrophils were isolated following the methods outlined in Gomez *et al., 2020.* 80 mL of venous blood from healthy volunteers was collected using a 21 gauge butterfly needle and dispensed into a 50 mL falcon tube containing 4 mL 3.8% sodium citrate to prevent clotting. To separate plasma from the red blood cells, blood was centrifuged at 260 ×g for 20 mins at room temperature. This separated the blood into plasma (upper layer) and red blood cells (lower layer). Plasma was then removed by pipetting and discarded. 6 mL dextran (6% w/v) from *Leuconostoc spp (Sigma-Aldrich, USA)* was added to the remaining red blood cell-rich layer and the solution made to 50 mL using 0.9% sterile saline *(Baxter, Switzerland).* Dextran facilitates the removal of red blood cells from solution by inducing red blood cell cross-linkage causing red blood cells to clump and sink to the bottom. Clean lids were placed on tubes to prevent red blood cells contaminating the red blood cell-poor upper layer following sedimentation. Care was taken to remove bubbles as these can activate neutrophils. The red blood cell-poor upper layer was then removed by pipetting and made up to 35 mL with sterile saline in a new tube. This solution was then gently pipetted over 16 mL of histopaque 1077 (Sigma – USA) which was brought to room temperature. Tubes were centrifuged at 400 ×g for 25 mins. Components of the blood solution separated out according to their density. After centrifugation clear bands corresponding to the neutrophil-rich pellet (bottom layer), peripheral blood mononuclear cells (PBMCs) (middle layer) and plasma (upper layer) were visible. Plasma and PBMCs were removed and discarded leaving only the neutrophil-rich pellet. 25 mL of 0.2% NaCl was added and tubes were inverted for 30 secs to hypotonically lyse the remaining red blood cells. A further 25 mL of 1.6% NaCl was added to achieve isotonicity. Neutrophils were pelleted by centrifuging at 250 ×g for 7 mins. The

supernatant was discarded, and the neutrophil pellet resuspended in 10 mL of Roswell Park Memorial Institute (RPMI) 1640 media. 10 µL of this solution was added to 90 µL RPMI. 10 mL was then pipetted onto an AC1000 modified Neubauer hemocytometer *(Hawksley, UK)* in order to count the neutrophils. Neutrophils were counted in four quadrants of the hemocytometer to achieve a mean value. The following equation was then used to determine the total neutrophil number:

$$\frac{total\ cell\ number}{4} \times 1 \times 10^5 = no.of\ cells\ /mL$$

The remaining neutrophil solution was centrifuged again at 250 ×g for 7 mins. The supernatant was discarded and the neutrophil pellet resuspended in 1 mL PBS supplemented with Ca^{2+} and Mg^{2+} *(Sigma-Aldrich, USA)* per 10^6 neutrophils.

2.3 Stimulation of neutrophil microvesicle production and quantification

Neutrophils were stimulated to produce microvesicles firstly through resuspension in PBS containing calcium and magnesium ions. This resulted in a population of NMVs from unstimulated neutrophils. Although referred to as 'unstimulated', it is important to note that the process of isolating neutrophils likely results in some degree of activation. Following resuspension in PBS, a proportion of the neutrophils were incubated with 50 µg/mL of human native low-density lipoprotein (nLDL) (*ProSpecBio, Israel)* and incubated at 37˚C and 5% CO2 for 1 hr. This produced a population of NMVs. Neutrophils were pelleted by centrifugation at 500 ×g for 5 mins. The supernatant containing the NMVs was aspirated and centrifuged at 2,000 ×g for 5 mins at 4˚C. This step removes any remaining cellular debris present. The supernatant was transferred into new Eppendorf tubes and centrifuged at 20,000 ×g for 30 mins at 4˚C to pellet the NMVs. The supernatant was aspirated and replaced with 4˚C filtered PBS. NMVs were then centrifuged again at 20,000 ×g for 30 mins at 4˚C. The supernatant was aspirated and stored at -20˚C for use as a control in future experiments. NMV pellets were stored at -20˚C (figure 2.1).

NMVs were quantified by flow cytometry (*LSRII, BDbiosciences, USA*) with gates established using Megamix calibration beads according to the manufacturer's instructions. Following the identification of a region of interest spanning ~100-1,000 nm NMVs could be quantified (figure 2.2). To ensure the same volume of sample was processed in each run Sphero AccuCount Blank particles (ACBP-20-

10) were used. 10 µL of beads were added to 285 µL filtered PBS and 5 µL resuspended NMV. The LSRII stopping gate was set at 1,000 beads for every run and NMVs counted. Total numbers of NMVs in the original samples were calculated using the equation below:

$$\frac{no.of\ NMVs}{no.of\ beads} \times \frac{total\ no.of\ beads\ in\ sample}{final\ volume\ of\ undiluted\ sample}$$

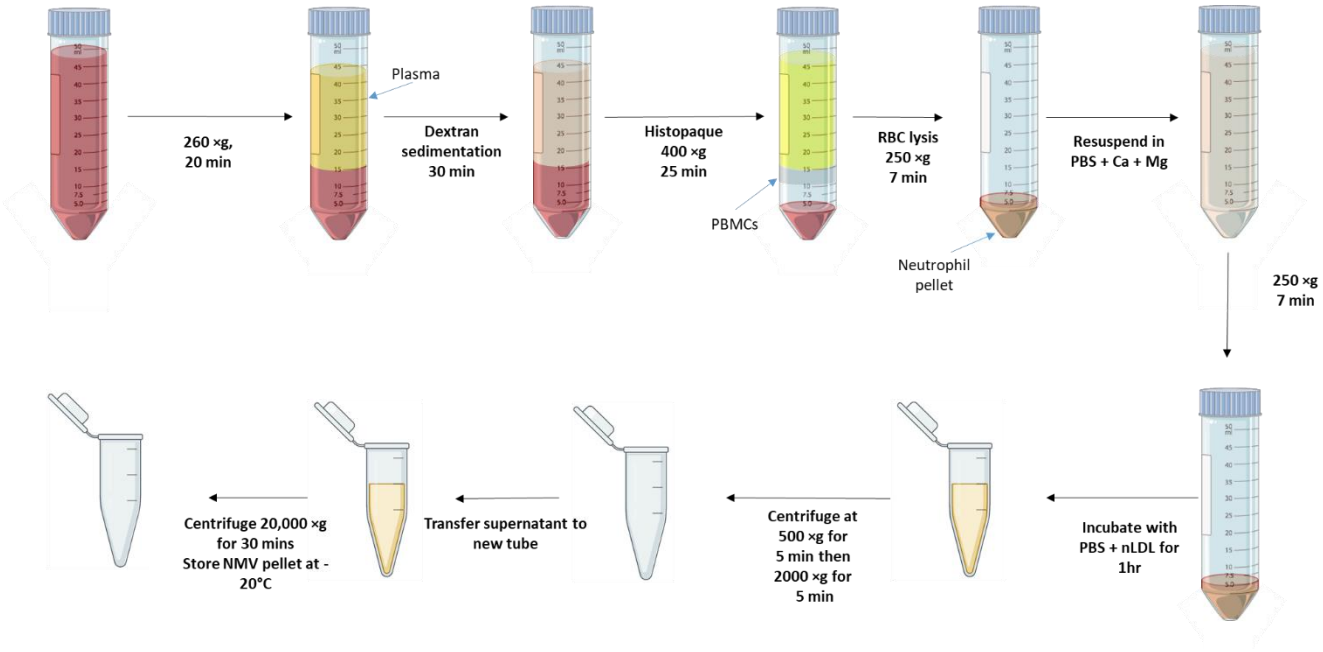


Figure 2. 1. Neutrophil microvesicle isolation protocol. *Whole blood was collected from healthy volunteers and neutrophils isolated through a series of centrifugation steps. Red blood cells (RBCs) were lysed and the isolated neutrophils treated with nLDL (native low-density lipoprotein) in PBS. Following treatment neutrophils were dispensed into Eppendorf tubes and centrifuged to remove contaminating cells before neutrophil microvesicles (NMVs) were isolated by ultracentrifugation. Created with Biorender.com.*

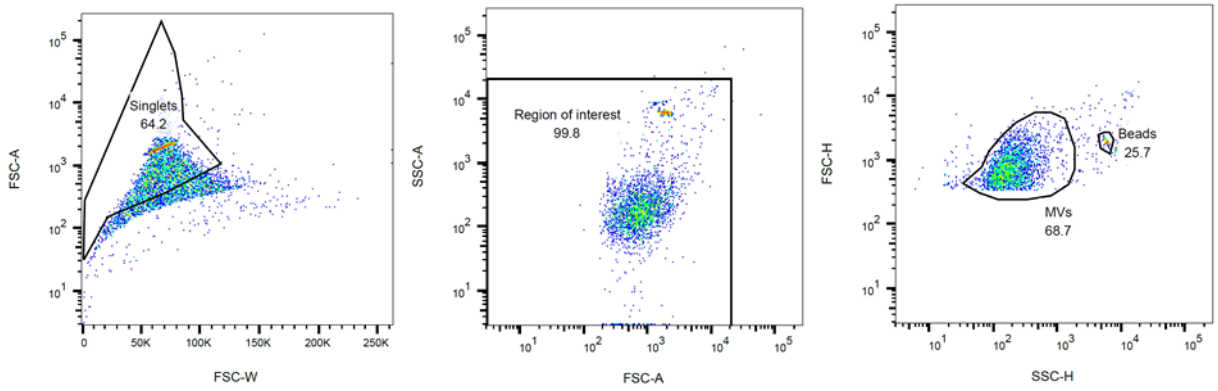


Figure 2. 2. Representative plots showing neutrophil microvesicle quantification. *Gates were positioned using Megamix calibration beads. Forward scatter (FSC) area (FSC-A) and FSC width (FSC-W) plots used to identify single events and exclude debris. A region of interest was identified using side scatter (SSC) area (SSC-A) and FSC-A and MVs were identified along with AccuCount beads using FSC height (FSC-H) and SSC height (SCC-H).*

2.4 Nanoparticle tracking analysis of neutrophil microvesicles

ZetaView (Particle Metrix, Germany) was used to analyse NMV size. The ZetaView system assesses particle size by analysing the movement of particles in a liquid under Brownian motion. A series of video clips are collected and analysed by applying the Einstein-Stokes equation to determine the hydrodynamic particle size. 15 mL of MilliQ water (Merck, Germany) was introduced into the cell to assess the cleanliness of the cell. A 1:250,000 dilution of 110 nm polystyrene beads was used to calibrate the machine. 2-3 mL of this solution was added with a 5 mL syringe. The sensitivity and shutter settings were set to 70. NMV samples and 200nm, 500nm and 760nm size calibration beads were diluted between 1:50 and 1:200 with MilliQ water. 2 mL of sample was introduced to the cell via syringe. The readout was set to show the frequency distribution of particle size. The machine was rinsed with 15 mL of MilliQ between reading of samples to ensure no residual particles remained to confound the results.

2.5 Surface antigen labelling of neutrophil microvesicles and analysis by flow cytometry

Flow cytometry allowed the analysis of NMV surface molecules derived from the parent neutrophil. NMVs produced in response to 50µg/mL nLDL from human serum (*ProSpec, Israel)* were resuspended to 1,000 NMV/µL in 100µL 4°C cell staining buffer (BioLegend, UK). To determine if contaminating nLDL in the NMV samples could retain CD66b-FITC antibody both NMVs and 50µg/mL nLDL were incubated with 5µg/mL CD66-FITC antibody and incubated on ice for 45 mins (BioLegend, UK). Samples were made up to 200µL with additional cell staining buffer. Samples were then centrifuged at 20,000 ×g for 30 minutes at 4°C to pellet the labelled NMVs. The resulting supernatant was removed with care taken to avoid disturbing the pellet. Samples were resuspended in 400µL 4°C cell staining buffer prior to analysis by flow cytometry. NMVs were measured by LSRII and data analysed using FlowJo software.

2.6 Quantification of neutrophil microvesicle protease contents using profiler array

Reagents provided with the Proteome profiler human protease array (*R&D Systems, USA*) were prepared according to the manufacturer's instructions. PBS and nLDL stimulated NMVs were pooled

from multiple donors and lysed via freeze/ thaw cycles prior to resuspension in 100 µL PBS. NMV samples were diluted in 1.5mL 1X array buffer.

Nitrocellulose membranes containing 35 separate protease capture antibodies were blocked with 2mL array buffer in a 4-well multi-dish for 1 hour at room temperature on a rocking platform. This limits non-specific antibody binding and improves signal to noise ratio. 15µL of reconstituted biotinylated protease detection antibody cocktail was added to 1.5mL NMV samples and incubated at room temperature for 1 hour. The blocking array buffer was aspirated from the 4-well multi-dish and membranes incubated with 1.5mL NMV samples incubated with protease detection antibody cocktail overnight at 4°C on a rocking platform. During this time NMV-derived proteases specifically bind to capture antibodies on the membrane.

Membranes were removed and washed with wash buffer for 10 minutes on a rocking platform. This process was repeated three times. Membranes were incubated with 2mL of streptavidin-HRP for 30 minutes at room temperature. Streptavidin binds to biotin on detection antibodies. Membranes were washed three times with wash buffer to remove unbound streptavidin-HRP. Membranes were placed within protective plastic and incubated with chemi reagent mix (provided by manufacturer) for 1 minute. During this step HRP catalyses a chemical reaction to produce a fluorescent product. This fluorescent signal was quantified through multiple exposure times using a BioRad ChemiDoc XP system. The mean pixel intensity for the negative control spots were quantified and subtracted from the mean pixel intensity of the pair of duplicate spots on the membrane corresponding to each protease. This analysis was performed using ImageJ software.

2.7 Quantification of neutrophil microvesicle protein concentration by ELISA

MMP-9 and NE DuoSet ELISA kits were used to measure protein concentration in isolated NMVs in accordance with the manufacturer's instructions. Capture antibody specific to a particular protein was allowed to adhere to a 96-well ELISA plate overnight. Sample containing the protein of interest was added to wells and adhered to the capture antibody. A biotinylated detection antibody specific to the protein of interest is added to wells and binds to streptavidin conjugated to a horse radish peroxidase (HRP) enzyme. HRP converts the substrate to produce a colour change the intensity of which can be quantified by using a plate reader. All wash steps were performed using a Geneflow ELx50 plate washer (Biotek Instruments, USA) and plates analysed using a VarioSkan microplate reader (*ThermoFisher, USA*).

NMV pellets were resuspended in 100 µL of sterile PBS, lysed via freeze/ thaw cycles and centrifuged at 10,000 ×g to pellet debris. The supernatant was removed and diluted 1:10 and 1:100 in 2% BSA/PBS. A range of dilutions were used in every human NE Duoset ELISA (R&D Systems) to account for the variation in NE concentration observed between donors.

Wells of a 96-well plate were incubated at room temperature overnight with 100µL of 250 ng/mL capture antibody. Plates were washed and blocked with 300 µL of 1% BSA and incubated for 1 hour at room temperature. 100 µL of diluted standards/samples was added to the appropriate wells. The plate was covered and incubated for 2 h at room temperature. 100 µL of 250 ng/ml detection antibody was added to each well. The plate was covered and incubated for a further 2 h at room temperature.

100 µL of a 40-fold dilution of streptavidin-HRP (horseradish peroxidase) was added to each well. The plate was incubated away from direct light for 20 min at room temperature. The aspiration wash step was repeated to remove all unbound streptavidin-HRP. A substrate solution was prepared from a 1:1 ratio of colour reagent A (hydrogen peroxide) and colour reagent B (tetramethylbenzidine). 100 µL of this was added to each well. Plates were incubated for 20 min at room temperature away from direct light. After 20 min 50 µL of stop solution (2N H2SO4) was added to each well to inhibit HRP and induce a colour change from blue to yellow. The optical density of each well was quantified at 450 nm with wavelength correction set at 540 nm and 570 nm. Wavelength correction prevents imperfections in the plate from effecting the result.

2.8 Identification of active proteases in neutrophil microvesicles using gelatin zymography

Gelatin zymography was used to identify active proteases. Proteins present in the sample are separated on a conventional SDS-PAGE gel supplemented with gelatin. Following separation the SDS is removed from the gel and the separated proteins are allowed to refold into their active conformations. The gels are incubated at 37°C in the presence of necessary magnesium and zinc co-factors. In their active form the proteases degrade the gelatin within the gel. The remaining protein in the gel is stained using coomasie blue dye. Regions of the gel that have been degraded appear as clear bands on a dark blue background. These regions can be quantified by densitometry. These regions correspond with the active proteases that separated in the gel with the proteases being identifiable in reference to a molecular weight marker. 7.5% acrylamide SDS-PAGE gels were prepared by combining 2 mL 1.5 M Tris pH 8.8, 2 mL 30% acrylamide, 2 mL dH_20, 2 mL 4 mg/mL gelatin, 80 µL 10% SDS and 80 µL 10% APS in a 50 mL falcon tube. 10 µL TEMED was added last to polymerise the acrylamide and form the gel. Separating gels were poured into 1 mm plates and

left to solidify for 5 min. Any bubbles formed on the gel surface were removed by adding 80% isopropanol.

Stacking gels were prepared by combining 1.25 mL 0.5 M Tris pH 6.8, 0.67 mL 30% acrylamide, 3.08 mL dH_2O, 50 µL 10% SDS and 50 µL 10% APS. 10 µL TEMED was added last to polymerise the acrylamide. Stacking gels were poured over solidified separating gels. A 1 mm 10 lane comb was inserted to form wells for the samples to be added. The stacking gel was left to set for 5 min.

Isolated NMVs were processed using a freeze/thaw cycle to lyse the membrane and release their proteolytic contents**.** NMVs were diluted in sterile PBS to 200 NMVs/µL, 500 NMVs/µL or 1,000 NMVs/µL concentrations. 8 µL of the resulting NMV samples was added to 12 µL 2× laemmli buffer to produce the final samples ready for loading. Laemmli buffer contains 2-mercaptoethanol to reduce protein disulphide bond interactions and SDS to denature proteins and assign an overall negative charge to allow separation according to size. 10 µL of sample was loaded per well alongside a protein molecular weight marker. The gel was run at 150V until good band separation was achieved. Gels were removed from their plates and washed 2× 30 min in fresh washing buffer at room temperature with agitation.

The washing buffer was replaced, and gels rinsed in incubation buffer for 5-10 min at 37˚C. The incubation buffer was then replaced with fresh incubation buffer and gels incubated at 37˚C for 24 h. The incubation buffer contains co-factors (calcium and zinc) required for optimum activity of proteases.

Incubation buffer was removed and replaced with enough staining buffer to completely cover the gel. Gels were incubated at room temperature for 30 min to 1 hour with agitation. The staining solution contains Coomassie blue which binds to proteins through interactions between its sulphonic acid groups and the protein's amine groups. As the gel contains gelatin the entire gel is stained blue.

Gels were then washed with dH_2O to remove unbound staining solution and incubated with de-staining solution. De-staining solution removes the Coomassie blue stain from all regions of the gel where the dye is not bound to protein. These regions are those where the denatured proteases separated by SDS-PAGE have re-folded and degraded the gelatin. These regions appear as white bands on a dark blue background. The specific protease responsible for a particular region of degradation can be identified by reference to the protein molecular weight marker.

Images of the gels were taken with a BioRad imager and gelatin degradation quantified by comparing the relative intensity of the regions of gelatin degradation.

2.9 Quantifying neutrophil elastase activity in neutrophil microvesicles

A NE ELISA was used to assess NE activity in NMVs. NE binds specifically to capture antibody adhered to the surface of a 96-well plate. NE substrate was added to wells which initiates a colour change that can be quantified using a plate reader. A concentration range of NE standards were included to serve as a reference. NMVs were resuspended to 12,000 NMV/µL in sterile PBS before being lysed via freeze thaw method. 10µL NMV supernatant was diluted by an equal amount to serve as control. NE standard was plated in duplicate in a 96-well plate to create a standard curve with concentrations of 0, 5, 10, 15, 20 and 25 ng/well according to the manufacturer's instructions. Wells were made up to 50µL with additional NE assay buffer.

20µL of NMV sample or supernatant control were plated per well and made up to 50µL with additional NE assay buffer. 50µL NE substrate mix was prepared from 48µL NE assay buffer and 2µL NE substrate. 50µL of NE substrate mix was added to 50µL of samples per well to make 100µL per well.

Fluorescence was measured over 20 minutes at 37°C (ex = 380nm/ em = 500nm). Two time points were chosen that fell within the linear range and the corresponding fluorescence values recorded. The fluorescence of the 0ng/well standard was subtracted and the corrected fluorescence obtained from the NMV samples compared to the standard curve to obtain the amount of NE in ng.

NE activity was calculated using the following equation:

$$\boldsymbol{NE\ Activity = \frac{B}{V} \times dilution\ factor = ng/mL}$$

B = NE amount from standard curve (ng)

V = Sample volume added into the reaction well (mL)

2.10 Assessing protease activity in neutrophil microvesicles using a microplate assay of gelatin degradation

DQ-gelatin was also used in a microplate assay to assess gelatin degradation by NMVs. DQ-gelatin is a FITC-labelled product used to detect gelatin degradation. The FITC signal is quenched when DQ-gelatin is not degraded. However, following degradation by proteases a fluorescent signal is

emitted proportional to the degree of degradation. This fluorescence can be quantified using a plate reader. 80 µL sterile filtered PBS+Ca+Mg was added per well of a black 96-well microplate. 250 µg/mL DQ-gelatin was prepared by diluting in PBS+Ca+Mg and 20 µL added per well. This yields a final concentration of 25 µg/mL. Solutions were pipetted up and down to mix. NMVs were freshly isolated and resuspended in phenol red-free media to an appropriate concentration. Phenol red-free media containing NMV supernatant was used as a negative control. 100 µL of NMV samples and negative control were added to appropriate wells. The microplate was incubated in the dark at room temperature and measurements recorded every 15 mins for 4 h. Fluorescence intensity was measured using a microplate reader set for excitation at 485 ± 10nm and emission detection at 530 ± 15nm. Fluorescence intensity values were corrected by subtracting the value of the negative control. Area under the curve analysis was performed using GraphPad Prism 9.

2.11 Optimisation of extracellular matrix components for *in vitro* plaque erosion model

96-well plates were coated with 100 µL collagen type III (60 – 100 µg/mL) for 2 hours at room temperature. Collagen type III was supplemented with low molecular weight hyaluronan (LMW-HA) between 5 – 20 µg/mL. HCAECs were seeded at 10,000 HCAECs/well and cultured statically for 24 hours prior to fixation with 4% paraformaldehyde and staining with 300 nM DAPI. HCAECs were imaged by fluorescence microscopy and the data analysed using ImageJ software.

2.12 Human coronary artery endothelial cell culture under shear stress

Human coronary artery endothelial cells (HCAECs) were purchased from Promocell. Lot numbers: 440Z021 (male, 52, Caucasian), 411Z027 (male, 30, Caucasian), 424Z011 (male, 14, Caucasian), and 439Z021 (male, 55, Caucasian).

µ-Slide I $^{0.4}$ luer channel slides (Ibidi – Germany) were coated with 100 µg/mL human collagen type III with or without 5 µg/mL supplementary low molecular weight hyaluronan (LMW-HA). Slides were incubated at room temperature in a laminar flow cell culture hood for 2 h before washing with 1mL sterile PBS. HCAECs between passages 3-6 were seeded at 1.5 x 10^6 cells/mL in MV2 media (Promocell, Germany) in pre-coated 0.4 luer µ-slides (Ibidi - Germany) and allowed to adhere for 24 h. Following firm adhesion to the slide HCAECs were cultured under low oscillatory shear stress (4 dyn/cm^2) using the Ibidi Pump System. After 72 h the conditioned media was removed and replaced with fresh media containing the appropriate concentration of NMVs or NMV supernatant controls

(see individual experiments for details of concentrations). HCAECs were incubated with NMVs under the same shear stress.

2.13 Identifying neutrophil microvesicle internalisation by endothelial cells

Glass coverslips were coated with 70 µg/mL collagen type III for 2 hours at room temperature in a 6 well plate. Coverslips were washed three times with sterile PBS and 3×10^5 HCAECs/ well seeded. HCAECs were cultured for 24 hours or until a confluent monolayer had formed. NMVs were added to HCAECs at a concentration of 400 NMV/µL and incubated at 37°C and 5% CO_2 for 2 hours. HCAECs were fixed in 4% paraformaldehyde for 10 minutes at room temperature before washing 3 times with sterile PBS. HCAECs were permeabilised with 1% triton X-100 for 5 minutes prior to washing again. HCAECs were then blocked for 30 minutes with 1% BSA solution. A 1:50 dilution of FITC-phalloidin was prepared in 1% BSA solution and incubated with HCAECs for 40 minutes at room temperature. FITC-phalloidin labelled actin within HCAECs. HCAECs were washed again, and the nuclei labelled with a 1:500 dilution of TO-PRO-3 for 1 minute at room temperature. Coverslips were removed and washed in sterile PBS and sealed to prevent evaporation. HCAECs were visualised with a Zeiss confocal microscope.

2.14 Endothelial cell-induced degradation of gelatin investigated using DQ-gelatin coated slides

DQ-gelatin was used in combination with HCAEC culture to determine the effect of HCAECs on ECM degradation following NMV treatment. 0.4 luer µ-slides (*Ibidi, Germany*) were coated with 100 µg/mL of DQ-gelatin substrate and incubated at room temperature for 2 hours protected from light. Excess DQ-gelatin was aspirated, and slides washed thoroughly with sterile PBS. HCAECs were seeded into coated slides at a concentration of 150,000 HCAECs/ slide in pre-warmed MV2 cell culture media and cultured statically for 24 hours in the presence of NMV supernatant, 240 NMV/µL or 240 NMV/µL + 1µg TIMP-1. Following treatment HCAECs were washed gently with sterile PBS and fixed in 4% paraformaldehyde. HCAEC nuclei were labelled with 300nM DAPI solution and imaged by widefield fluorescence microscopy (*Leica AF6000).* Fluorescence intensity was quantified using ImageJ software and adjusted to correct for cellular area.

2.15 Measuring endothelial cell detachment induced by neutrophil microvesicles

HCAEC was assessed using a detachment assay adapted from previous published studies (Quillard *et al.*, 2015). This assay uses low concentration trypsin-EDTA in combination with a treatment hypothesised to increase detachment to measure endothelial detachment. The principal behind this method is the treatment, in this case NMVs, promotes degradation of the integrins necessary for attachment of ECs but is not sufficient to induce detachment in isolation. Rather, NMvs make ECs more vulnerable to detachment in combination with other factors. This experiment assesses the EC's vulnerability to detachment following NMV treatment. Due to differences in application, it was necessary to optimise the concentrations of trypsin-EDTA for our purposes.

96-well microplates were coated with 70µg/mL collagen type III and incubated at room temperature for 2 hours. Slides were thoroughly washed with sterile PBS. HCAECs were seeded at 20,000 cells/well in MV2 cell culture media (*PromoCell, Germany)* and allowed to adhere for 24 hours. HCAECs were washed with sterile PBS to remove non-adherent cells and residual MV2 media and then treated with 3.125 µg/mL, 6.25 µg/mL, 12.5 µg/mL or 25 µg/mL concentrations of trypsin-EDTA for 3 minutes. HCAECs were gently washed with MV2 media to inhibit trypsin activity and imaged by widefield phase contrast microscopy (*Leica, AF600*). Detaching HCAECs were defined as cell appearing bright and displaying a rounded morphology. Detaching HCAECs were counted and expressed as a percentage of total HCAECs.

Having determined the optimum concentration of trypsin-EDTA to use in our experiment Iused this assay to determine how NMVs effected HCAEC detachment. HCAECs were cultured on 70 µg/mL collagen type III for 24 hours prior to treatment with 60 NMV/µL, 120 NMV/µL, 240 NMV/µL, 240 NMV/µL + 400ng TIMP-1 or 2.5 units/mL MMP-9 for 24 hours. HCAECs were washed with sterile PBS and incubated with 6.25µg/mL trypsin-EDTA for 3 minutes before being washed with fresh MV2 cell culture media and imaged.

2.16 Quantifying endothelial cell caspase-3/7 expression by flow cytometry in response t neutrophil microvesicles

HCAECs were cultured under LOSS according to methods detailed in section 2.13. HCAECs were incubated with 5:1 ratio of NMVs for 24 hours. Following treatment, HCAECs were detached with 25 µg/mL trypsin-EDTA and resuspended at a concentration of 20,000 HCAECs/mL in MV2 cell culture

media. 1µL CellEvent Caspase-3/7 Green Detection Reagent was added to 1 mL of HCAECs and incubated at 37°C for 30 minutes protected from light. HCAECs were analysed by flow cytometry without washing or fixing. Fluorescence was excited using a 488nm laser and emission collected at 530/30 nm. Unlabelled HCAECs were used to set gating strategy and median fluorescence intensity and percentage positivity values assessed by FlowJo software.

2.17 Quantifying endothelial cell proliferation in response to neutrophil microvesicles by fluorescence microscopy

96-well plates were coated with 70 µg/mL collagen type III for 2 hours prior to washing with PBS. HCAECs were seeded at a low concentration of 3,500 HCAECs/well to ensure confluency, which would inhibit the proliferative response, was not reached during the experiment. HCAECs were cultured statically for 24 hours to allow for adherence. HCAECs were treated with 240 NMV/µL, 480 NMV/µL or 960 NMV/µL for 24 hours. HCAECs were then washed with sterile PBS and fixed with 4% paraformaldehyde for 5 minutes. HCAECs were washed again and permeabilised with 0.2% triton-X100 for 15 minutes. The washing step was repeated and HCAECs were blocked with 5% FBS for 1 hour. HCAECs were washed again and incubated with 1µg/mL Alexa 700 conjugated Ki-67 monoclonal antibody (*ThermoFisher, USA*) for 2 hours at room temperature. HCAECs were washed and labelled with 300nM DAPI prior to imaging by widefield fluorescence microscopy (*Leica, AF6000*).

2.18 Measuring endothelial wound healing response in the presence of neutrophil microvesicles by time-lapse phase contrast microscopy

The wound healing response of HCAECs was assessed by time-lapse phase contrast microscopy to determine if NMVs could reduce wound recovery.

Ibidi 4-well cell culture inserts were placed in wells of a 6-well cell culture plate. 30,000 HCAECs were seeded within each quadrant of the cell culture inserts and allowed to adhere for 24 hours. The culture media in each quadrant was aspirated and replaced with media containing 240 NMV/µL or NMV supernatant and cultured for 2 hours. The cell culture inserts were then removed using sterilised tweezers to create a sterile and reproducible cell gap between confluent HCAEC

monolayers. HCAECs were washed with sterile PBS to remove non-adherent HCAECs. 300 µL MV2 media containing 240 NMV/ µL or NMV supernatant was added to relevant wells and HCAECs were imaged by time-lapse widefield phase contrast microscopy (*Leica, AF6000*) at 37°C and 5% CO_2. Images were taken every 30 minutes for 24 hours. Data was analysed using ImageJ software.

2.19 Platelet isolation from whole human blood

2.19.1 Blood collection

Whole blood was collected from healthy volunteers according to university ethics detailed in section 2.1 and dispensed into 3.8% sodium citrate. Care was taken to ensure donors had not taken medications in the past 2 weeks that could affect platelet function (e.g. aspirin, non-steroidal anti-inflammatory drugs and anti-histamines).

2.19.2 Platelet isolation from whole human blood

Platelet-rich plasma (PRP) was used for most platelet assays, however in the case of measuring platelet adhesion to HCAECs it was necessary to prepare isolated platelets.

Blood was centrifuged at 260×g for 20 minutes to separate PRP which was collected and diluted 1:1 with HEP buffer (140nM NaCl, 2.7 mM KCl, 3.8 mM HEPES, 5 mM EGTA) containing 2 µM prostaglandin E1 (PGE1). PRP was mixed gently and centrifuged at 100×g for 20 minutes to pellet contaminating red blood cells. The resulting supernatant was removed and centrifuged at 800 ×g for 20 minutes to pellet platelets. The platelet pellet was washed without resuspension in wash buffer (10 mM sodium citrate, 150 mM NaCl, 1mM EDTA, 1% w/v dextrose). The platelets were resuspended in Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES) containing 5 mM glucose and freshly added BSA (3 mg/mL) and 1 µM PGE1. Platelets were counted using a Neubauer modified haemocytometer (*Hawksley, UK*) and platelet concentration adjusted if necessary, using Tyrode's buffer. Platelets were subsequently centrifuged at 800 ×g for 20 minutes and resuspended to the appropriate concentration for experiments using MV2 cell culture media.

2.20 Platelet labelling for CD66b and analysis by flow cytometry

To ascertain whether NMVs interact with platelets *in vivo*, freshly isolated resting platelets were dual labelled with CD42a and the neutrophil specific marker CD66b in the absence of isolated NMVs, and expression compared to isotype-labelled controls. Median fluorescence intensity (MFI) was measured for isotype control and CD66b labelled platelets and normalised to MFI for the isotype control.

2.21 Platelet labelling for P-selectin and analysis by flow cytometry

Flow cytometry was used to quantify the effect of NMVs on platelet P-selectin expression and therefore platelet activation. Blood was collected according to the details in section 2.1 and processed at the Northern General Hospital, Sheffield. Flow cytometry tubes were prepared containing CD62P-PE (*BD Biosciences, USA*), CD42a-FITC (*BD Biosciences, USA*), 5 mM ADP and 1000 NMV/ µL or NMV supernatant. 5 µL whole blood was added to each tube and incubated at room temperature for 20 minutes. Platelets were then fixed in FACS buffer for 10 minutes and analysed by flow cytometry. Data was analysed using FlowJo software.

2.22 Assessing effect of neutrophil microvesicles on platelet aggregation by light transmission aggregometry

Light transmission aggregometry (LTA) was used to assess the effect of NMVs on platelet activation and their propensity to aggregate. LTA requires the establishment of a sample with a value corresponding to 100% of platelets having aggregated. For this, tubes were prepared containing platelet-poor plasma (PPP), 0.25 µg/mL HORM collagen (native equine tendon collagen I) and PBS. Additionally, LTA requires a sample with a value corresponding to 0% platelet aggregation. For this, tubes were prepared containing PRP and PBS only. The values from these samples represent the paraemeters of the assay. As a control PRP was added alongside 0.25 µg/mL HORM collagen and NMV supernantant. In our treated sample PRP was added alongside 0.25 µg/mL HORM collagen and 1000 NMV/ µL. Platelet aggregation was tracked over a period of 7 minutes resulting in a value for maximum aggregation.

2.23 Assessing effect of neutrophil microvesicles on platelet adhesion to endothelial cells by fluorescence microscopy

Widefield fluorescence microscopy was used to assess the effect of NMVs on the interaction between platelets and HCAECs.

Wells of a 48-well plate were coated with 70 µg/mL collagen type III and incubated at room temperature for 2 hours. Wells were washed with sterile PBS and HCAECs seeded at a concentration of 20,000 HCAECs/well in MV2 cell culture media and allowed to adhere for 24 hours. HCAECs were washed with sterile PBS and treated with 5:1 ratio of NMV:HCAECs for a further 24 hours. HCAECs were washed again with sterile PBS.

Isolated platelets (section 2.21) were labelled with the cell membrane dye PKH-26 (*ThermoFisher, USA*). Isolated platelets were resuspended in 2mL diluent C. A 4 µM dye solution was prepared by diluting PKH-26 in diluent C and added to this platelet solution to create a final dye concentration of 2 µM. Platelets were incubated at room temperature for 3 minutes with gentle agitation. The staining reaction was stopped by adding 4 mL of 1% BSA solution and incubating at room temperature for 1 minute. Platelets were centrifuged at 800 ×g for 20 minutes and washed in fresh MV2 cell culture media. This was repeated twice more.

HCAECs were treated with 1×10^6 PKH-26 labelled platelets/ well for 30 minutes under static conditions. HCAECs were washed gently with sterile PBS. This was repeated twice more. Fresh MV2 cell culture media was added to each well and HCAECs were immediately imaged by widefield fluorescence microscopy at 37°C and 5% CO_2 to assess platelet adhesion.

2.24 Reverse transcriptase quantitative polymerase chain reaction

2.24.1 Isolating RNA from endothelial cells for RT-qPCR analysis

RNA was isolated from treated HCAECs using the Direct-Zol RNA Microprep Kit (Zymo Research – USA) following the manufacturer's instructions. 100 µL TRI Reagent (Sigma, USA), a guanidine thiocyanate and phenol monophase solution was added to each channel slide to lyse HCAECs and inhibit RNase activity. An equal volume of room temperature 100% ethanol was added, and the solution transferred into a Zymo-Spin IC column and centrifuged at 16,000 ×g for 30 seconds. 0.75 unit/mL DNase I was added to the columns and incubated at room temperature for 15 minutes. RNA was washed through a series of centrifugation steps with RNA PreWash and RNA Wash Buffer provided in the kit. RNA was eluted from the column in 15mL RNase-free water. RNA concentration and purity was quantified using a NanoPhotometer N60 (Implen – Germany) prior to storage at -80°C. Immediately prior to use RNA was diluted to 10ng/µL with RNase-free water.

2.24.2 Reverse transcriptase quantitative polymerase chain reaction procedure

KiCqStart pre-designed primers (Sigma – USA) were used for RT-qPCR analysis. Primers were chosen to analyse expression of endothelial target genes involved in promoting or inhibiting thrombosis. Namely, F3 (tissue factor), SERPINE1 (plasminogen activator inhibitor-1), THBD (thrombomodulin), VWF (Von-Willebrand factor) and PROCR (protein C receptor). Additionally, primers were used to analyse expression of the reference gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase). Primers were resuspended in TE buffer (10mM Tris pH 7.5, 1mM EDTA) to 100mM concentration and stored at -20°C. Primer sequences are provided in table 1.

HCAEC gene expression was assessed using the QuantiNova SYBR Green RT-PCR Kit (Qiagen – Germany). This kit allows the reverse transcription and PCR reaction to occur in the same tube minimising risks of contamination and allowing the analysis to proceed in one step. Reaction mixtures were prepared according to table 2 using components provided with the kit.

10µL of reaction mixture was added per well of a 384 plate. Each gene of interest was run in triplicate. In addition, no template controls (NTC) were included to assess contamination. Plates were sealed with adhesive strips and analysed by CFX384 thermal cycler (Bio-Rad, USA) according to the procedure detailed in table 3.

qRT-PCR data was analysed using BIO-RAD CFX manager 3.0 software. For each gene of interest the threshold cycle (CT) was determined. Relative gene expression was calculated using the ΔΔCt method.

Gene	Forward	Reverse
F3	GGAGAAAACTACTGTTTCAGTG	TCTCTGAATTCCCCTTTCTC
SERPINE1	ATCCACAGCTGTCATAGTC	CACTTGGCCCATGAAAAG
THBD	AAATGCTATGAGATGCATGG	TTGAAAATCAGAGATGGTGC
VWF	TGTATCTAGAAACTGAGGCTG	CCTTCTTGGGTCATAAAGTC
PROCR	TTCTCTTTTCCCTAGACTGC	CATATGAAGTCTTTGGAGGC
GAPDH	TCGGAGTCAACGGATTTG	CAACAATATCCACTTTACCAGAG

Table 2. 1. KiCqStart primer sequences. *Nucleotide sequences of primers for genes F3 (tissue factor), SERPINE1 (plasminogen activator inhibitor-1), THBD (thrombomodulin), VWF (von-Willebrand factor) PROCR (protein-C receptor) and the reference gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase (GAPDH).*

Component	Volume / reaction	Final concentration
2x Quantinova SYBR Green RT-PCR Master Mix	5 µL	1×
QN SYBR Green RT-Mix	0.1 µL	1×
Primer Mix	1 µL	0.5 µM forward 0.5 µM reverse
Template RNA	1 µL	10ng/reaction
RNase-free water	2.9 µL	
Total Reaction volume	10 µL	

Table 2. 2. Reaction mix for QuantiNova SYBR Green RT-qPCR kit. *The reaction mix includes SYBR green RT-PCR master mix containing DNA polymerase, necessary for DNA amplification and RT-Mix consisting of reverse transcriptase necessary for converting sample RNA into DNA. The mix also contains relevant primers and template RNA.*

Step	Time	Temperature (°C)
Reverse transcription	10 minutes	50
PCR initial activation step	2 minutes	95
Two-step cycling		
Denaturation	5 seconds	95
Combined annealing / extension	10 seconds	60
Number of cycles	35-40	
Melt curve analysis		

Table 2. 3.Thermal cycling procedure. *Reverse transcriptase (RT) enzyme within the RT-Mix is complexed with an RT-blocker which keeps RT inactive at ambient temperature. Raising the temperature to 50°C removes the RT-blocker and reverse transcription of sample RNA begins. DNA polymerase is kept inactive at ambient temperature by a QuantiNova blocking antibody. DNA polymerase is activated at 95°C and RT inactivated. Two-step cycling amplifies DNA via denaturation and extension. This cycle is repeated prior to melt curve analysis. Melt curve analysis can identify non-specific PCR amplification products.*

2.25. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.0.0 (*GraphPad software, USA)*. Data presented in table and graph form as mean ± standard error of the mean (SEM). The comparison of data sets and the assessment of statistical significance was conducted using the appropriate parametric tests. In all experiments the 'n' number refers to biological repeats rather than technical replicates and the graph appearance was chosen to show each data point in a group for clarity.

3 Neutrophil microvesicle production by human neutrophils and analysis of protease content

3.1 Introduction

NMVs are produced constitutively, but also in response to a broad range of stimuli *in vitro* including fMLP (Hong *et al.*, 2012), TNF-α (Johnson *et al.*, 2013; Rhys *et al.*, 2018), PMA and LPS (Pluskota *et al.*, 2008). Neutrophils are important first responders in the innate immune system and therefore many of the factors that provoke their activation derive from bacteria. In the absence of infection, endogenous activators of neutrophils are more important. A key endogenous factor in the development of atherosclerosis is low-density lipoprotein (LDL). LDL is a known risk factor in atherosclerosis and, whilst LDL is not found in the same quantities in stable plaques as rupture prone plaques, a large-scale study of patients determined that those who suffered an acute coronary event as a result of plaque erosion had mean serum LDL levels above the healthy range (Yamamoto *et al.*, 2019b). In rupture prone plaques, lipids are present in oxidised form owing to the action of infiltrating macrophages (Rhoads and Major, 2018). Whilst modified and oxidised LDL activate neutrophils (Awasthi *et al.*, 2016; Obama *et al.*, 2019) and, in particular, induce NETs formation leading to increased risk of plaque erosion and thrombosis (Franck *et al.*, 2018; Stakos *et al.*, 2015), eroded plaques exhibit much less macrophage infiltration and are typically much lower in oxLDL content (Yamamoto *et al.*, 2019b). In its unmodified form LDL exists in the circulation as native LDL (nLDL) and is known to stimulate calcium flux in neutrophils (Palvinskaya *et al.*, 2013); this is interesting as NMV biogenesis relies on the rapid influx of calcium ions into the neutrophil cytoplasm and the action of the calcium-dependent enzyme calpain to reorganise the neutrophil cytoskeleton allowing microvesicles MV formation (section 1.7). This work indicated that nLDL, but not oxLDL, induces this rapid influx of calcium ions in neutrophils (Palvinskaya *et al.*, 2013).

Whilst specific research into NMV biogenesis is limited, the process of MV biogenesis is ubiquitous and unlikely to differ substantially between cells. Transient increases in intracellular calcium concentration, alterations in plasma membrane lipid composition and associated changes in membrane curvature, alongside changes in cytoskeletal organisation occur following stimulation and are central to MV biogenesis (Morel *et al.*, 2011; Verderio *et al.*, 2018). Cytosolic Ca^{2+} levels correlate with MV biogenesis in malignant breast cancer cells (Taylor *et al.*, 2020) and treatment with calcium ionophores, resulting in increased Ca^{2+} concentration, leads to MV production (Roseblade *et al.*, 2015). Structural rearrangements of the plasma membrane are calcium dependent since Ca^{2+} inhibits lipid transporting enzymes, flippases (Yu *et al.*, 2018a), but is required by calpain which is

linked to changes in cytoskeletal structure. Decreases in flippase activity correlate with increased cytoplasmic Ca^{2+} levels, increased PS exposure and MV production. These correlations were abolished following pre-treatment of ECs with a calcium channel blocker (Nagata *et al.*, 2016; Yu *et al.*, 2018a) and similarly, inhibition of calpain reduced MV production (Giannella *et al.*, 2021; Nolan *et al.*, 2008; Välimäki *et al.*, 2016).

Due to the mechanism through which MVs are generated, they retain and express on their surface the same molecules as their parent cells, making it possible to identify their cellular origin by analysing marker proteins expressed on their surface. Neutrophil markers CD66b, CD11b, L-selectin and CD18 are all found expressed on the surface of NMVs (Gomez *et al.*, 2020; Hong *et al.*, 2012; Nolan *et al.*, 2008) and NMVs contain many neutrophil derived factors including MPO (Slater *et al.*, 2017), MMPs (Butin-Israeli *et al.*, 2016) and micro-RNAs (Gomez *et al.*, 2020). It is worth noting that the environment at the time of NMV biogenesis likely dictates the capabilities of the NMVs, resulting in NMVs with disparate functions. For example, NMVs from unstimulated neutrophils may exert more anti-inflammatory effects, reducing cytokine and reactive oxygen species (ROS) production in neutrophils, whereas apoptotic neutrophils or neutrophils exposed to opsonised particles produce more pro-coagulant and pro-inflammatory NMVs, respectively (Kolonics *et al.*, 2021). This is likely due to different proteins being incorporated into NMVs under different circumstances. There is evidence to support this from Dalli et al who reported adherent neutrophils release NMVs with proteomes distinct from neutrophils in suspension (Dalli *et al.*, 2013). Whilst stimulation method is likely to effect protease content of NMVs the effect of nLDL stimulation on the protease content of NMVs has not been reported previously.

Neutrophils are known to contain a variety of potent proteases with roles in ECM degradation and cellular dysfunction that may be relevant to plaque erosion including MMP-9 (Florence *et al.*, 2017; Rosell *et al.*, 2008), neutrophil elastase (NE) (Grechowa *et al.*, 2017) and proteinase-3 (PR3) (Pendergraft *et al.*, 2004; Preston *et al.*, 2002). These proteases may contribute to the degradation of ECM in stable atherosclerotic plaques and promote the detachment of ECs through reducing EC viability and increasing apoptosis (Jerke *et al.*, 2015). MMP-9 is already linked with atherosclerotic plaque disruption (Ezhov *et al.*, 2019; Luttun *et al.*, 2004) and neutrophil elastase has been shown to enhance MMP activity (Jackson *et al.*, 2010). Both NE and PR3 have also been shown to promote tissue factor (TF) expression in ECs potentially inducing a more pro-thrombotic environment (Haubitz *et al.*, 2001; Steppich *et al.*, 2008) with PR3 also shown to degrade tissue factor pathway inhibitor (Steppich *et al.*, 2008).

The aim of this chapter is to determine if NMVs are produced in response to the known risk factor for CVD, nLDL, and determine the identity, concentration, and activity of their protease cargo and whether stimulation with nLDL alters these measurements compared to NMVs from unstimulated neutrophils.

3.2 Hypothesis and Aims

It was hypothesised that neutrophils produce MVs in response to stimulation with native low-density lipoprotein that differ in protease content compared to MVs from unstimulated neutrophils.

The work presented in this chapter addresses 2 aims:

Aim 1 - To determine if human native low-density lipoprotein (nLDL) induces NMV biogenesis.

Aim 2 - To determine whether the stimulus for NMV release alters i) the protease content and ii) the protease activity of the resulting NMVs.

3.3 Methods

Neutrophils isolated from human whole blood (section 2.2) were treated with PBS or 50 μg/mL human nLDL ± calpain inhibitor for 1 hour at 37^{o}C. The neutrophil supernatant containing the NMVs was removed and centrifuged at 2,000 ×g to pellet any contaminating neutrophils. The resulting supernatant was centrifuged at 20,000 xg for 30 minutes at 4^{o}C to pellet the NMVs. The supernatant was removed and retained for control samples, and the NMV pellet resuspended in sterile PBS before centrifuging again at 20,000 ×g for 30 minutes at 4^{o}C.

NMVs were quantified by flow cytometry (BD LSRII). MV biogenesis was selectively inhibited by a panel of inhibitors targeting calpain 1 and calpain 2. The size of isolated NMVs was assessed by nano particle tracking using ZetaView (*Particle Metrix, Germany*) (section 2.4).

To assess NMV protease content, NMVs were lysed via freeze thaw cycles and their contents analysed. A protease array was used to screen for 35 different human proteases in NMVs generated by unstimulated and nLDL-stimulated neutrophils (methods section 2.6). Once identified, specific proteases were quantified by ELISA and their activity assessed through ELISA (methods section 2.7) or gelatin zymography (methods section 2.8) (figure 3.1).

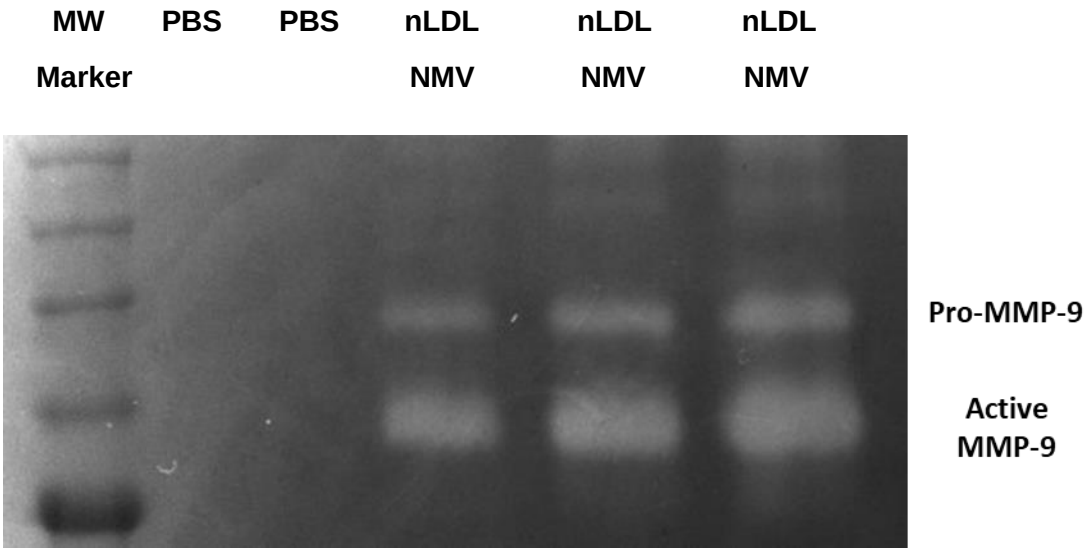


Figure 3. 1. Representative gelatin zymography blot. *Blot shows white regions resulting from gelatin degradation corresponding to pro-enzyme matrix metalloproteinase-9 (MMP-9) and active MMP-9 identified through comparison with a molecular weight marker.*

3.4 Results

3.4.1 Neutrophil microvesicle biogenesis stimulated by native low-density lipoprotein treatment of human neutrophils *in vitro*

NMVs are produced following neutrophil activation. Due to the link between high fat diet and elevated NMV levels in plasma (Gomez et al., 2020) I hypothesised that neutrophils treated with human nLDL would produce more NMVs compared with untreated neutrophils. NMVs were quantified by flow cytometry (BD LSRII). Aliquots of sterile PBS were run to establish a baseline number of events and compared with NMVs from unstimulated neutrophils. Analysis of nLDL particles alone was performed to determine if residual nLDL could be a confounding factor. I found nLDL particles present in the NMV gate (figure 3.2 A) and therefore included a nLDL control to control for contaminating nLDL particles not removed during the centrifugation steps. Significantly fewer events were detected in the nLDL control group compared to when NMVs from nLDL-stimulated neutrophils were analysed. Significantly more NMVs were produced by nLDL-stimulated neutrophils compared to unstimulated neutrophils (figure 3.2 C) (Mean 346% increase; SEM±59.7, n=30, p=0.0003). Biogenesis of MVs is reliant on the calcium-dependent enzyme calpain therefore, I included a calpain inhibitor alongside nLDL stimulation of neutrophils. ALLN, EST, PD 15606, calpain inhibitor III and calpeptin were incubated with neutrophils for 20 minutes at 37°C prior to stimulation with 50µg/mL nLDL to selectively inhibit calpain 1 and calpain 2. Calpain inhibition reduced NMV generation by neutrophils stimulated with nLDL by 41.9% compared to NMV generation by neutrophils stimulated with nLDL without calpain inhibition (Mean 41.9% decrease, SEM±14.81, n=7, p=0.0904) (figure 3.2 D).

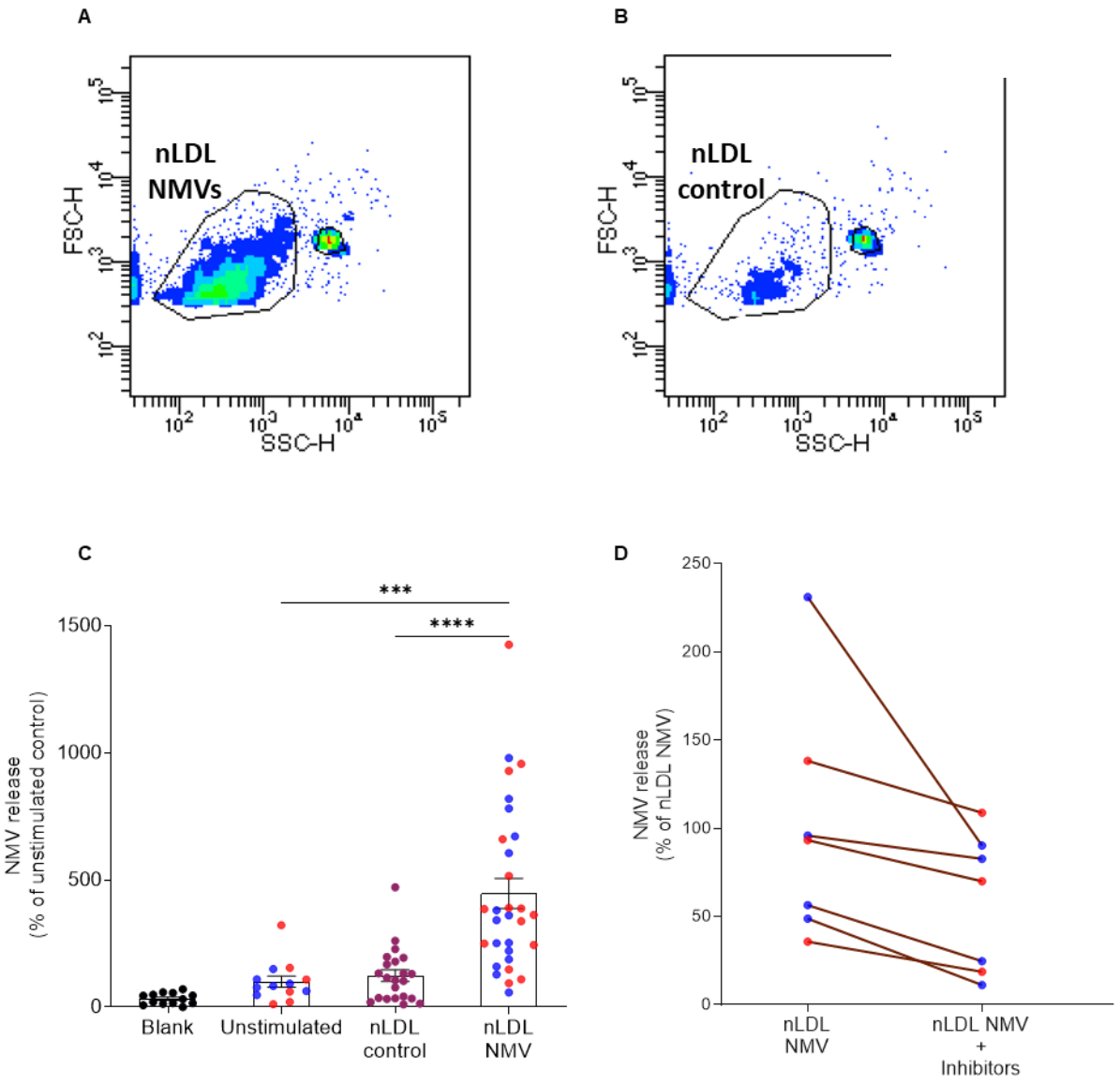


Figure 3.2. Neutrophil microvesicle biogenesis in response to native low-density lipoprotein stimulation. (A) *A representative flow cytometry plot of neutrophil microvesicles (NMVs) generated by native low-density lipoprotein (nLDL)-stimulated neutrophils and* ***(B)*** *nLDL particles only obtained using identical parameters and gates.* ***(C)*** *Human neutrophils were isolated from whole blood of healthy volunteers and resuspended in PBS containing calcium and magnesium with (nLDL NMV) or without (unstimulated) 50µg/mL nLDL for 1 hour at 37°C. The number of NMVs was quantified by flow cytometry and compared with the number of detected events in a blank PBS sample and a PBS sample spiked with 50µg/mL nLDL. Red and blue points denote female and male donors, respectively. Data is expressed as mean number of events per 1×10^7 neutrophils ±SEM. n=30. Significance assessed by ordinary one-way ANOVA test. **** P=<0.0001.* ***(D)*** *Isolated neutrophils stimulated with 50 µg/mL nLDL after pre-loading with a panel of cell permeable calpain 1 and calpain 2 inhibitors or PBS only for 20 min at 37°C. Data expressed as mean number of events per 1×10^7 neutrophils ±SEM. n=6. Significance assessed by unpaired t-test.*

3.4.2 Characterisation of size of microvesicles generated by native low-density lipoprotein stimulated neutrophils

NMV size was assessed by Zeta view nano particle tracking (*Particle Metrix, Germany*). Initial calibration with 110nm polystyrene beads was performed before the accuracy of the chosen parameters was confirmed by analysis of size calibration beads of 200nm (figure 3.3 A), 500nm (figure 3.3 B) and 760nm (figure 3.3 C) (*BangLabs, UK)*. Plotting the frequency distribution of NMVs identified a cluster of peaks between 105-255nm (figure 3.3 D). The modal peak for NMVs was 165nm. This size range is within the size range of MVs (100-1,000 nm). The low level of detected particles below 100 nm and above 1000 nm suggests there is low contamination from exosomes and cells, respectively (figure 3.3 D). Analysis of nLDL particles revealed a modal peak at 195nm (figure 3.3 E) confirming previous reports that LDL particles can be detected within the size ranges of MVs (Sódar et al., 2016) and supporting the flow cytometry data presented in figure 3.2. The ZetaView determines these values through tracking Brownian motion of particles via video. Representative frames from these videos showing nLDL particles (figure 3.3 F) and NMVs (figure 3.3 G) are included for reference.

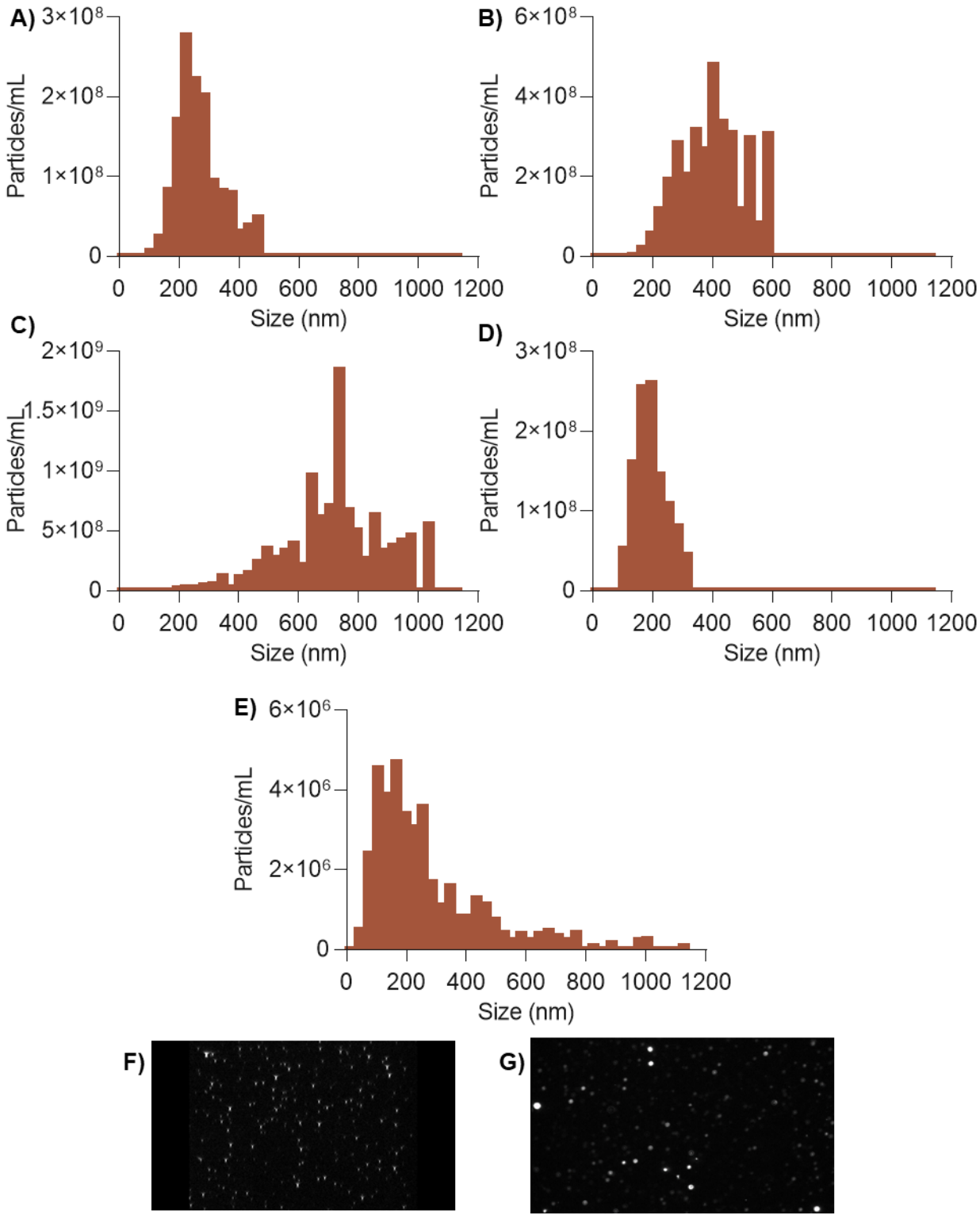


Figure 3.3. Size distribution of native low-density lipoprotein neutrophil microvesicles. *ZetaView particle tracking was calibrated with 110nm polystyrene beads prior to size distribution analysis of 200nm, 500nm and 760nm calibration beads* ***(A-C)****. Representative histograms of native low-density lipoprotein (nLDL) particles* ***(D)*** *and nLDL neutrophil microvesicles (NMVs)* ***(E),*** *n=3. Representative frames from videos used to track Brownian motion of nLDL particles* ***(F)*** *and NMVs* ***(G)****.*

3.4.3 Identification of neutrophil specific marker CD66b on microvesicles from native low-density lipoprotein stimulated neutrophils

The previous data suggested the size range of nLDL particles is similar to that of NMVs (figure 3.3), however contamination in the final NMV preparation is relatively low (see figure 3.2). Whilst earlier data suggested that few nLDL particles remained following NMV preparation it was important to confirm that the events detected by flow cytometry in the NMV gate were indeed NMVs and not contaminating nLDL particles. To confirm this, fluorescent staining for the neutrophil specific marker CD66b was performed. Quantification of the median fluorescence intensity (MFI) showed NMVs had a significantly higher MFI (mean =1798, SEM±590.5, n=3, p=0.049) compared with events in the supernatant control (mean =146, SEM±58.3, n=3) (figure 3.4 A). Significance was assessed using an unpaired t-test.

These data confirm that the events detected by flow cytometry were in fact MVs of neutrophil origin. Additionally, significantly greater percentages of NMVs were CD66b-positive (mean =53.5%, SEM±3.04, n=3, p=0.0001) compared to supernatant controls (mean =2.37%, SEM±0.99, n=3) (figure 3.4 C). Absence of CD66b-positive events in the nLDL supernatant samples suggests that CD66b-positive events in the NMV condition are the result of NMVs and not CD66b antibody adhering to free nLDL particles (figure 3.4 C).

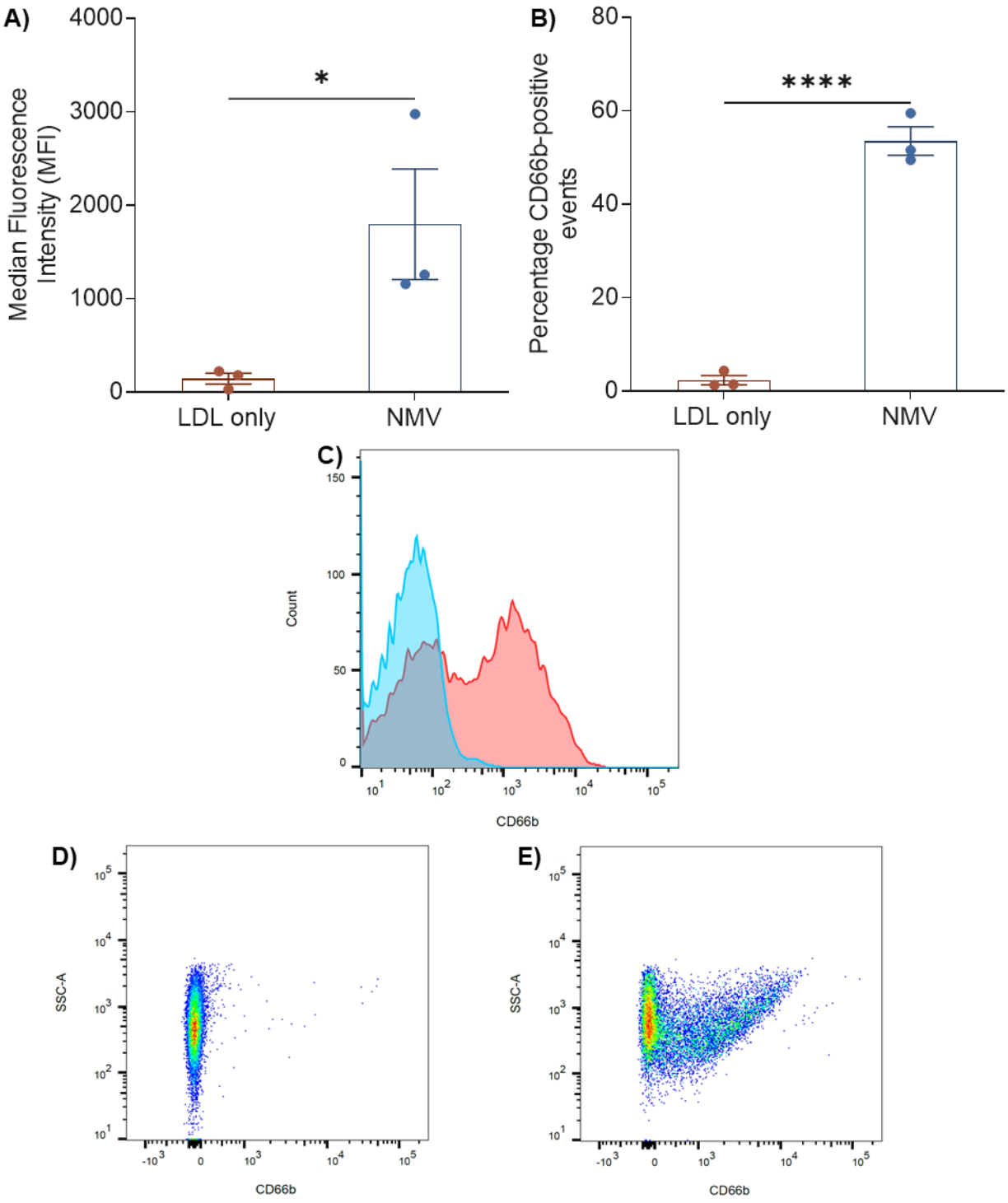


Figure 3.4. CD66b expression by neutrophil microvesicles from native low-density lipoprotein stimulated neutrophils. *Neutrophil microvesicles (NMVs) were generated in response to native low-density lipoprotein (nLDL) prior to labelling with anti-CD66b antibody and analysis by flow cytometry. Staining was compared with nLDL particles to assess for non-specific antibody binding.* ***(A)*** *Median fluorescence intensity (MFI) of CD66b staining of NMVs and nLDL particles. Data expressed as mean ±SEM p=0.019, n=3. Statistical significance assessed by unpaired t-test.* ***(B)*** *Percentage of CD66b-positive events in nLDL control and NMV groups. Data expressed as mean ±SEM, p=0.0001, n=3. Statistical significance assessed by unpaired t-test.* ***(C)*** *Representative histogram of events in the nLDL control (blue peak) and NMV (red peak) groups* ***(D)*** *Representative dot plot of nLDL control and* ***(E)*** *NMVs. *=p<0.05, ****=p<0.0001.*

3.4.4 Identification and comparison of proteases in neutrophil microvesicles from unstimulated and native low-density lipoprotein stimulated neutrophils

Having established that nLDL induces NMV formation, I next sought to determine whether these NMVs contained proteases, since neutrophils are known to contain several proteolytic factors that are involved in matrix degradation and inflammation (Pham, 2006). To determine which proteases became associated with NMVs during neutrophil stimulation, a protease array kit (R&D Systems, USA) was used to screen 35 human proteases for their relative abundance. From 35 screened proteases 9 were detected in NMVs from unstimulated neutrophils and 7 detected in NMVs from nLDL-stimulated neutrophils (figure 3.5).

Of the 10 different proteases detected 6 were detected in both NMV populations (table 3.1). These proteases were MMP-9, MMP-8, proteinase-3, cathepsin S, A and D were all detected in similar relative quantities in both NMV populations (figure 3.6). A difference was observed in relative quantities of MMPs between the NMV populations. MMP-9 was the most abundant protease detected in NMVs from nLDL-stimulated neutrophils followed by MMP-8, whereas this was reversed in NMVs from unstimulated neutrophils with MMP-8 being most abundant, followed by MMP-9.

Interestingly, neutrophil stimuli (unstimulated vs nLDL-stimulated) appeared to regulate the differential incorporation of 4 proteases in NMVs. Cathepsin V was detected in NMVs from nLDL-stimulated neutrophils whilst it was absent in NMVs from unstimulated neutrophils. Conversely, Cathepsin X/Z/P, ADAM8 and Urokinase were detected in NMVs from unstimulated neutrophils but were absent in NMVs from nLDL-stimulated neutrophils (table 3.1).

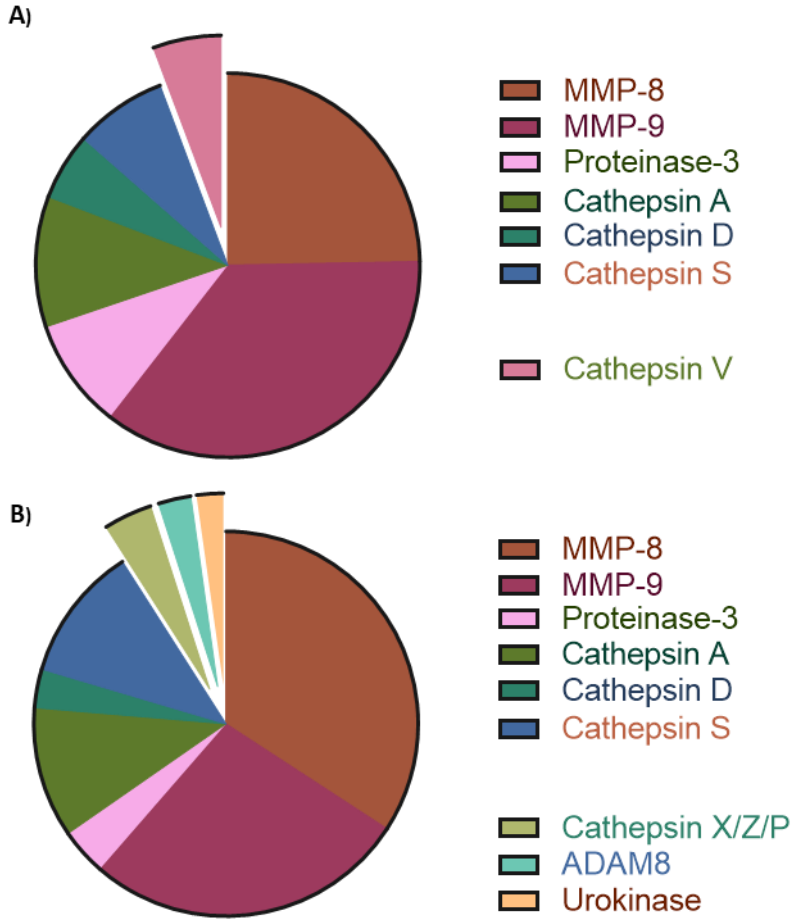


Figure 3. 5. Pie charts demonstrating relative protease contents of neutrophil microvesicles. *Neutrophil microvesicles (NMVs) from* ***(A)*** *native low-density lipoprotein (nLDL)-stimulated neutrophils and* ***(B)*** *unstimulated neutrophils. Detached segments represent proteases not conserved between the populations of NMVs*

Protease	NMVs (Unstimulated neutrophils)	NMVs (nLDL-stimulated neutrophils)
MMP-8	+	+
MMP-9	+	+
Proteinase-3	+	+
Cathepsin A	+	+
Cathepsin D	+	+
Cathepsin S	+	+
Cathepsin V	-	+
Cathepsin X/Z/P	+	-
ADAM8	+	-
Urokinase	+	-

Table 3.1 - Comparison of human proteases detected in neutrophil microvesicles from unstimulated and native low-density lipoprotein stimulated neutrophils. *Protease array was used to identify and compare proteases present within neutrophil microvesicles (NMVs) from unstimulated and native low-density lipoprotein (nLDL) stimulated neutrophils. Matrix metalloproteinase-8 (MMP-8), matrix metalloproteinase-9 (MMP-9), Proteinase-3, cathepsin S, cathepsin A and cathepsin D were detected in both NMV populations. Urokinase, cathepsin X/Z/P and A Disintegrin and Metalloproteinase domain 8 (ADAM8) were detected only in NMVs from unstimulated neutrophils. Cathepsin V was detected only in NMVs from nLDL-stimulated neutrophils.*

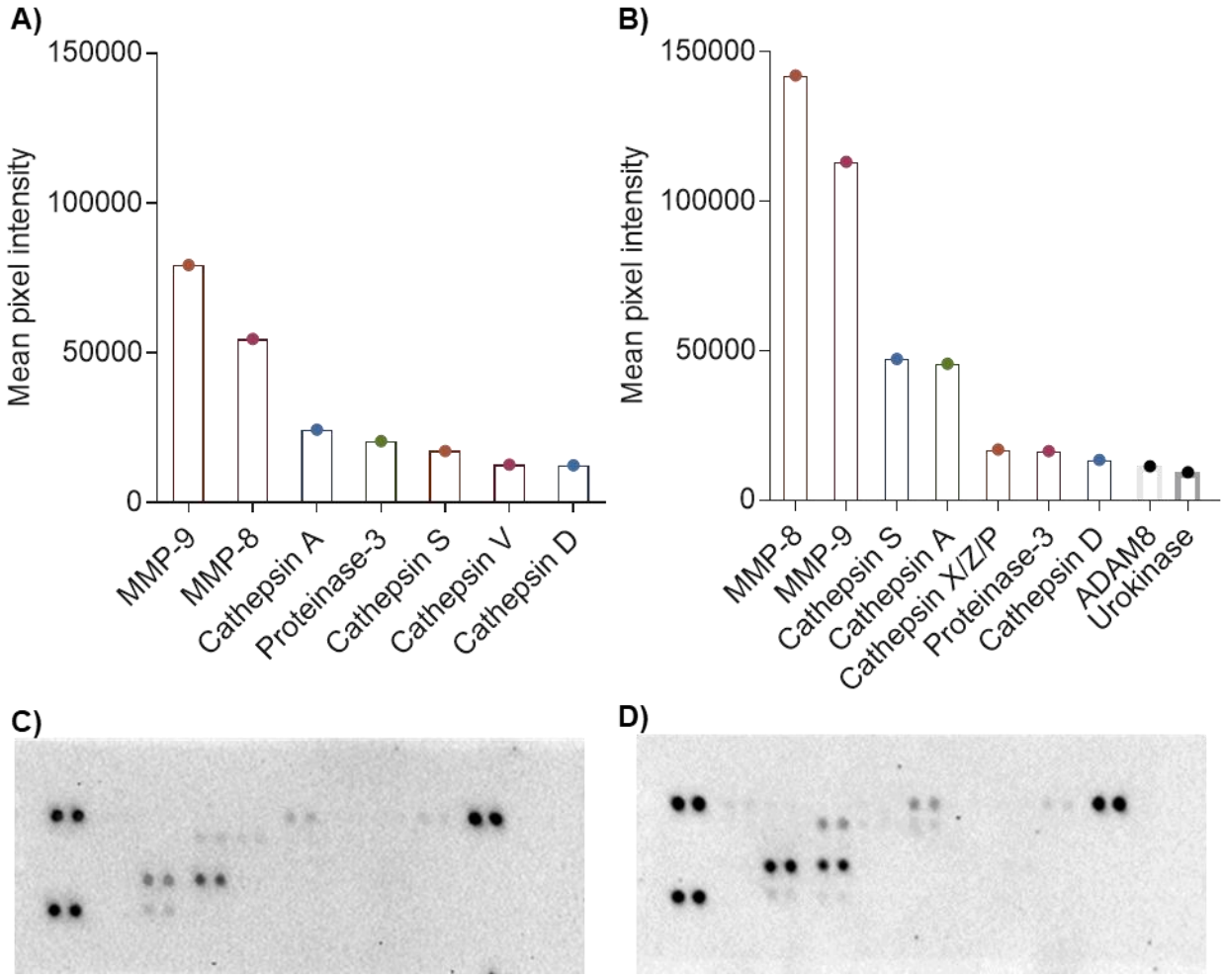


Figure 3.6. Protease content of NMVs from unstimulated and native low-density lipoprotein stimulated neutrophils. *Neutrophil microvesicles (NMVs) were lysed and the lysate analysed by protease profiler array. Antibodies to specific proteases were spotted in duplicate on the membrane. Following sample incubation and treatment with a detection reagent these regions can be detected by chemiluminescent analysis. The pixel intensity of spot corresponds to the concentration of protease in the sample.* ***(A)*** *Relative concentrations of detected proteases in NMVs from native low-density lipoprotein (nLDL)-stimulated and* ***(B)*** *unstimulated neutrophils. Representative membranes showing spots corresponding to specific proteases in NMVs from* ***(C)*** *nLDL-stimulated and* ***(D)*** *unstimulated neutrophils. Data expressed as mean pixel intensity.*

3.4.5 Quantification of neutrophil elastase concentration and enzymatic activity in neutrophil microvesicles released by unstimulated and native low-density lipoprotein stimulated neutrophils

NMVs from both unstimulated and nLDL-stimulated neutrophils contained the serine proteases, proteinase-3 and cathepsin A. Therefore I investigated whether NMVs would also contain another common neutrophil protease, neutrophil elastase (NE). NE is highly expressed in neutrophil granules and released by neutrophils during activation. As NE is already known to be released during degranulation following neutrophil activation, I assessed whether stimulation with nLDL significantly altered the quantity of NE packaged in NMVs. There was no significant difference in NE concentration per NMV between NMVs from unstimulated and nLDL-stimulated neutrophils (figure 3.7 A). However, there was significantly higher NE concentration in NMVs from nLDL-stimulated neutrophils (mean =7923pg/mL, SEM±326.8, n=4) compared to the supernatant control (mean =1407pg/mL, SEM±201.7, n=2, p=0.0039). When NE concentration was expressed per 1×10^7 neutrophils to account for the greater number of NMVs expressed in the nLDL condition, less NE was produced in the unstimulated condition (mean =3505pg/mL, SEM±1184, n=4) compared to nLDL condition (mean =16349pg/mL, SEM±6361, n=4) (figure 3.7 B). However, no statistically significant difference was observed between NMV populations due to large variability in the nLDL group (p=0.094). These data are summarised in figure 3.7 B. These data suggest that NE is present in NMVs from both unstimulated and nLDL-stimulated neutrophils and that, whilst a higher mean concentration of NE was observed in NMVs from nLDL-stimulated neutrophils, this did not amount to a statistically significant difference.

Having confirmed the presence of NE in NMVs, I next assessed NE activity in NMVs derived from unstimulated and nLDL-stimulated neutrophils using a Neutrophil Elastase Activity Assay Kit (Sigma, USA). As with total concentrations of NE, there was no significant difference in NE activity per NMV in either NMVs from unstimulated or nLDL-stimulated neutrophils (figure 3.7 C). Interestingly, analysis of NE activity showed a significant increase in the concentration of active NE in NMVs from nLDL-stimulated neutrophils (mean =5243pg/mL, SEM±1420, n=3) compared to NMVs from unstimulated neutrophils (mean =740pg/mL, SEM±385, n=3, p=0.0376) when NE concentrations were expressed per 1×10^7 neutrophils (figure 3.7 D).

These data suggest that NE is present in NMVs from both unstimulated and nLDL-stimulated neutrophils in its active form, and, whilst nLDL stimulation does not appear to enrich NMVs with NE, the fact that so many more NMVs are produced following nLDL stimulation means much greater levels of NMV-associated NE being released by neutrophils following nLDL stimulation.

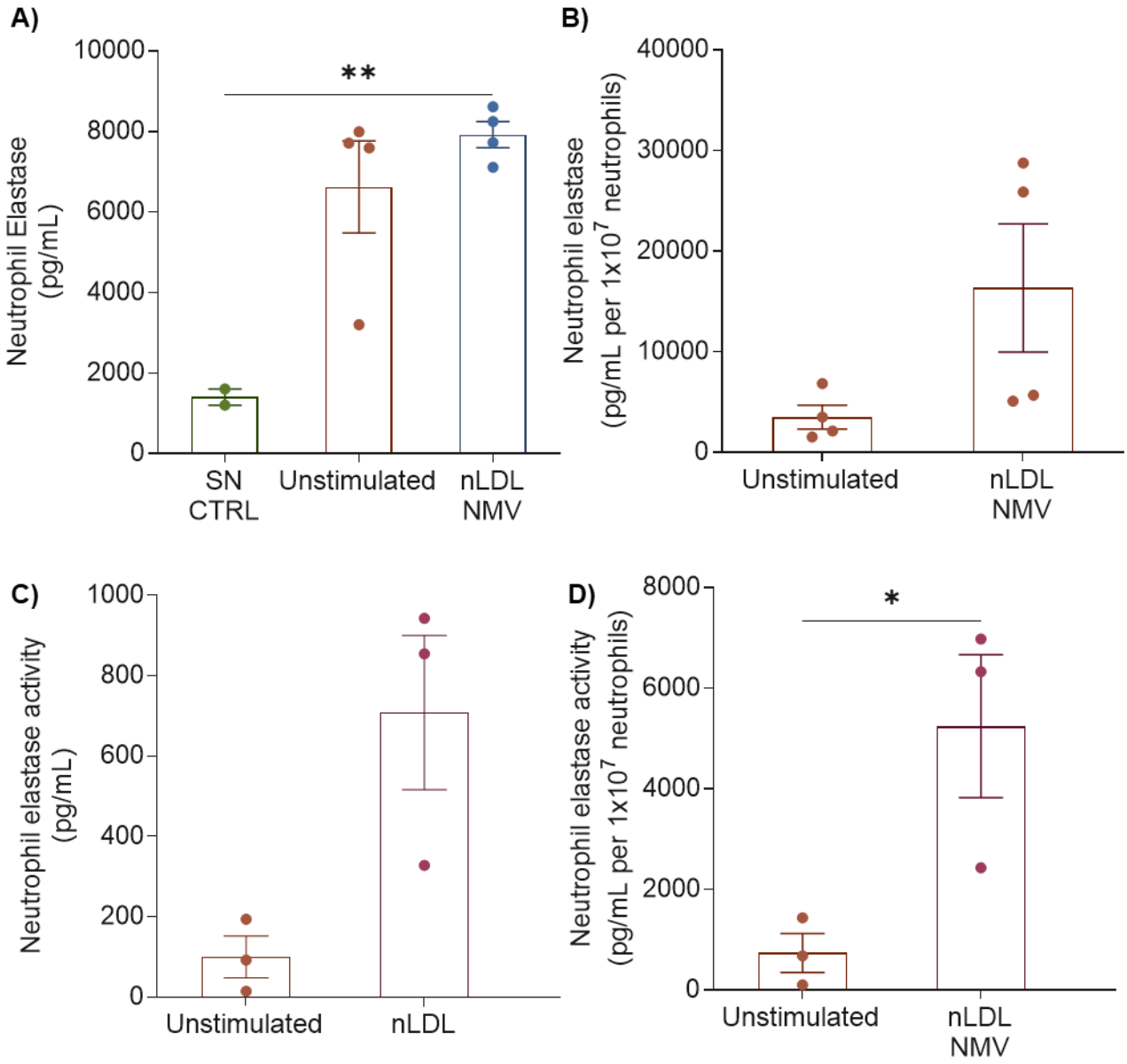


Figure 3.7. Comparison of the concentration and enzymatic activity of neutrophil elastase in neutrophil microvesicles from unstimulated and native low-density lipoprotein stimulated neutrophils. (A) *Absolute concentration of neutrophil elastase (NE) in supernatant (SN) control and equal concentrations of neutrophil microvesicles (NMVs) from unstimulated and native low-density lipoprotein (nLDL)-stimulated neutrophils detected by ELISA. Data expressed as mean ±SEM, n=2-4. Statistical significance assessed by one-way ANOVA with Tukey's multiple comparisons test.* ***(B)*** *NE concentration in NMVs from unstimulated and nLDL-stimulated neutrophils expressed per 1×10^7 neutrophils. Data expressed as mean ±SEM, n=4. Statistical significance assessed by unpaired t-test.* ***(C)*** *NE activity in the SN control and equal concentrations of NMVs from unstimulated and nLDL-stimulated neutrophils and* ***(D)*** *NE activity in NMVs from unstimulated and nLDL-stimulated neutrophils expressed per 1×10^7 neutrophils. Data expressed as mean ±SEM. Statistical significance assessed by unpaired t-test, n=3. *p=<0.05, **p=0.01.*

3.4.6 Concentration and activity of matrix metalloproteinase-9 in microvesicles from native low-density lipoprotein stimulated and unstimulated neutrophils

The protease array data showed MMP-9 to be the most abundant protease in NMVs from nLDL-stimulated neutrophils. MMP-9 is a potent protease involved in ECM turnover and has been linked to atherosclerotic plaque rupture (Sluijter et al., 2006)**.** To further investigate the levels of MMP-9 in NMVs, total MMP-9 concentrations (both active and pro-enzyme forms) in NMVs from unstimulated and nLDL-stimulated neutrophils were measured by ELISA (R&D Systems, USA). NMV pellets were resuspended in 0.2 µm filtered PBS to 1,000 NMV/µL and subjected to a cycle of sonication, vortexing, freezing and thawing. This process lysed the NMVs allowing the release of their contents. Lysed NMVs were centrifuged at 20,000 ×*g* to pellet debris. The resulting supernatant was removed and serial dilutions prepared in 0.2 µm filtered PBS.

Similar concentrations of MMP-9 were detected per NMV in nLDL-stimulated and unstimulated conditions (figure 3.8 A). A greater mean MMP-9 concentration was measured in NMVs from nLDL-stimulated NMVs (mean =32.56pg/mL, SEM±10.45, n=4) compared to NMVs from unstimulated neutrophils (mean =4.38pg/mL, SEM±0.21, n=3) when concentrations were expressed per 1×10^7 neutrophils (figure 3.8 B). However, there was still no statistically significant difference between NMV populations (p=0.071). There was considerable variation in MMP-9 concentration between individual donors. Despite all donors being healthy volunteers, genetic differences and recent infection history could potentially alter NMV contents and account for this variability. These data are summarised in figure 3.8.

Our chosen MMP-9 ELISA assay did not allow for the distinction between active MMP-9 and the pro-enzyme form. Since MMP-9 is a member of the gelatinase family of proteases it was possible to use gelatin zymography to assess enzymatic activity. Zymography gels allowed the identification of pro-MMP-9 and active MMP-9 (figure 3.9). A dose-dependent increase in the activity of pro-MMP-9 and active MMP-9 was measured in NMVs from nLDL-stimulated and unstimulated neutrophils. Gelatin degradation by pro-MMP-9 and active MMP-9 was increased in NMVs from nLDL-stimulated neutrophils compared to NMVs from unstimulated neutrophils (figure 3.9).

Neutrophils are known to secrete soluble MMP-9 and therefore the possibility remained that the MMP-9 I detected in our NMV samples could have been released directly by neutrophils instead of packaged in NMVs. To rule out this possibility I once again analysed the supernatant remaining after the NMVs were centrifuged. No active MMP-9 was detected in these samples confirming low contamination from soluble MMP-9 released by neutrophils and that the majority of detected MMP-9 was associated with NMVs.

These data, confirm the presence of MMP-9 in all tested NMV populations and indicate that the method of neutrophil stimulation increases MMP-9 activity in NMVs.

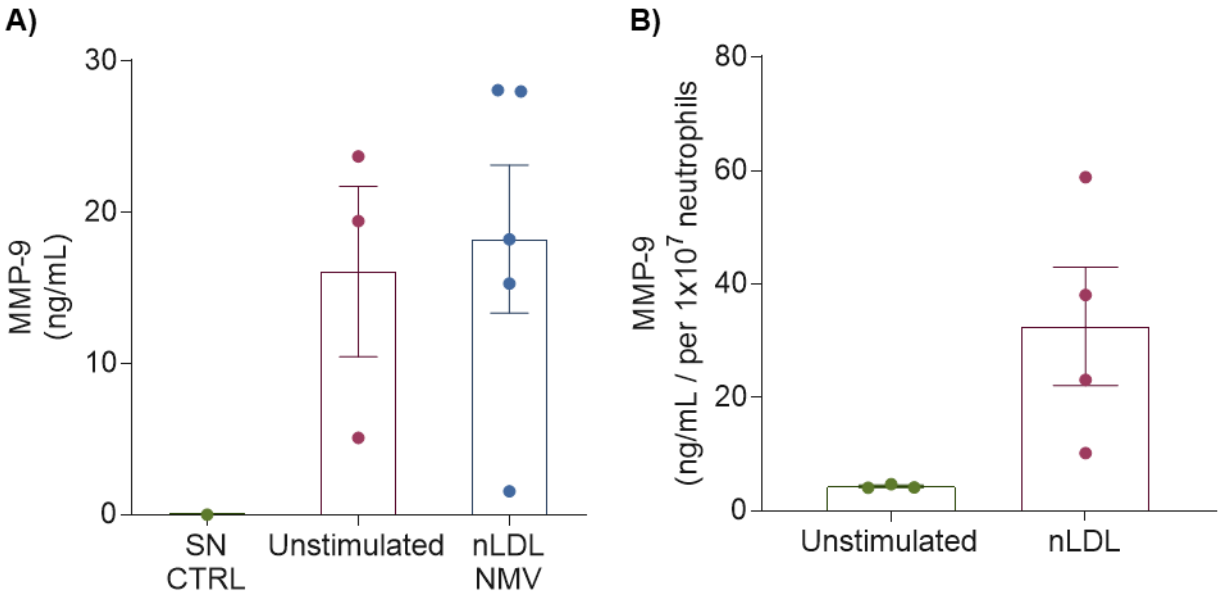


Figure 3.8. Matrix metalloproteinase-9 concentration in neutrophil microvesicles from unstimulated and native low-density lipoprotein-stimulated neutrophils. *Neutrophil microvesicle (NMV) lysate was collected and analysed for the presence of matrix metalloproteinase-9 (MMP-9) by ELISA.* ***(A)*** *MMP-9 expression in supernatant (SN) control and equal concentrations of NMVs from unstimulated and native low-density lipoprotein (nLDL)-stimulated neutrophils.* ***(B)*** *MMP-9 concentrations in NMVs from unstimulated and nLDL-stimulated neutrophils expressed per 1×10^7 neutrophils. Data expressed as mean ±SEM, n=3-5. Statistical significance assessed by unpaired t-test.*

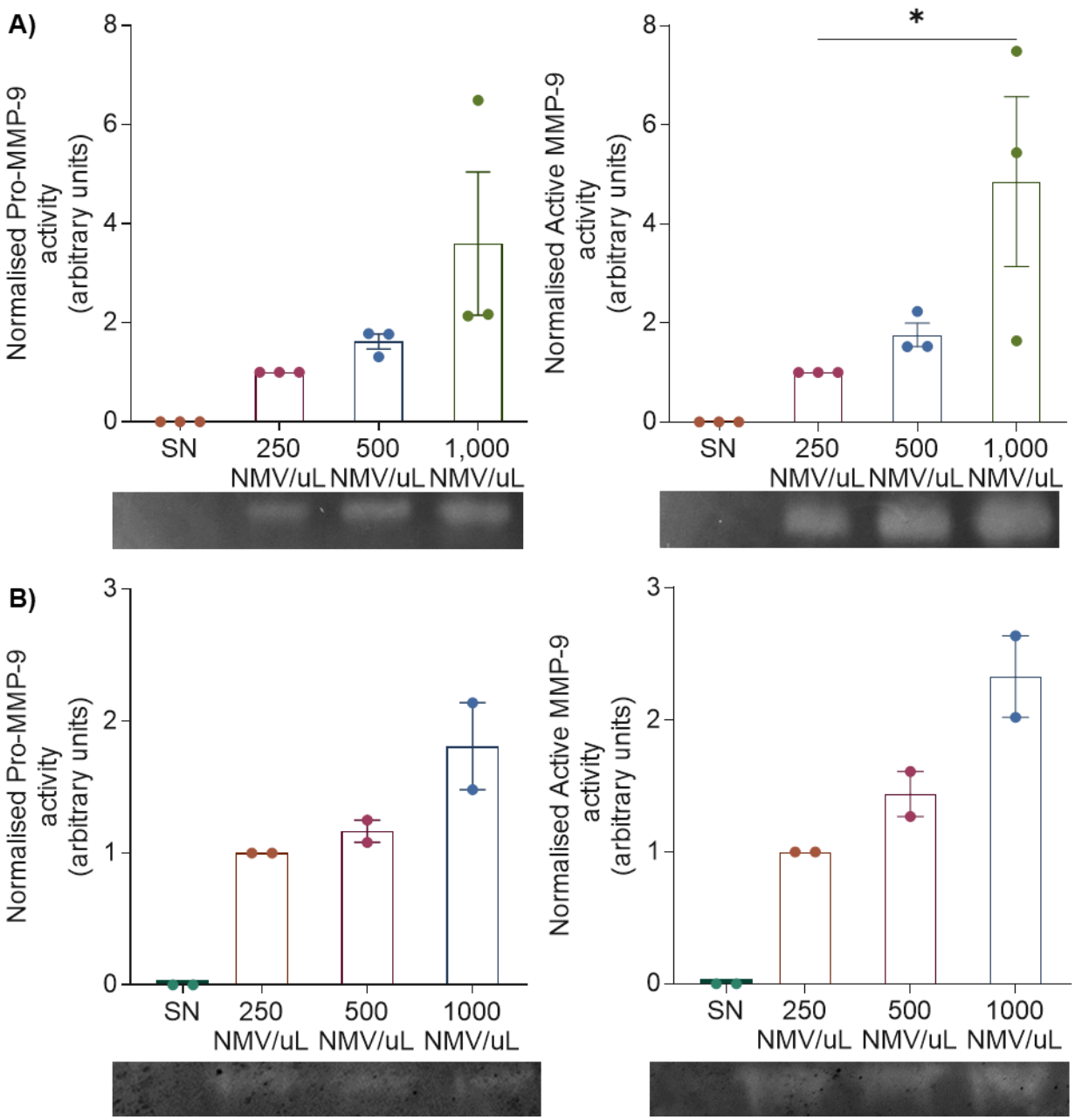


Figure 3.9. Pro- and active matrix metalloproteinase-9 activity in neutrophil microvesicles from native low-density lipoprotein-stimulated and unstimulated neutrophils*. Neutrophil microvesicle (NMV) lysate from* ***(A)*** *native low-density lipoprotein (nLDL)-stimulated and* ***(B)*** *unstimulated neutrophils was analysed by electrophoresis on an SDS-PAGE gel containing gelatin. Proteases are separated according to molecular weight, allowed to refold to regain enzymatic function and then incubated in the gelatin gel. Active proteases degrade the gelatin. Active proteases appear as clear bands where the gelatin has been degraded. The proteins responsible for these bands were identified by reference to a molecular weight marker and intensities quantified using ImageJ software. Representative images of the gelatin gels are included under the corresponding graphs for illustrative purposes. Data presented as band intensity normalised to the 250 NMV/µL concentration ±SEM, n=2-3.*

3.4.7 Results summary

- nLDL stimulates neutrophils to produce NMVs above resting levels.
- Different protease contents were observed between NMVs from resting neutrophils and those from nLDL-stimulated neutrophils.
- nLDL stimulation of neutrophils had no effect on quantity or activity of NE or MMP-9 contained within NMVs compared to NMVs from resting neutrophils.

3.5 Discussion

3.5.1 Native low-density lipoprotein stimulates neutrophil microvesicle biogenesis

Data presented in section 3.4.1 indicates neutrophils release NMVs above unstimulated levels following stimulation with nLDL. The number of NMVs produced in response to nLDL exceeded the number produced by unstimulated neutrophils indicating nLDL stimulates NMV biogenesis. Previous work has suggested LDL particles are often misidentified as MVs using flow cytometry (Sódar et al., 2016). Analysis by flow cytometry and nano particle tracking confirmed nLDL particles occupy a similar size range as NMVs, potentially leading to inaccurate quantification of nLDL induced NMVs. Particle tracking data showed nLDL particles had a single peak between 105-315nm with a modal diameter of 195nm, whilst NMVs demonstrated a more heterogeneous population with ranges from 50-1,000nm but with a similar modal diameter of 165nm. Therefore, I sought to control for this potential nLDL contamination. 50µg/mL nLDL was prepared in PBS, but without neutrophils. This control was prepared using the same procedure used to isolate NMVs. Quantification of nLDL particles in these samples revealed contamination was low, but not necessarily biologically insignificant. When accounting for nLDL contamination, although the number of NMVs produced in response to nLDL was reduced, it was still over three times higher than the number produced by unstimuated neutrophils. It is possible that some NMVs will have nLDL on their surface and may act as vehicles for the specific delivery of nLDL to the endothelium. Indeed, Sódar et al showed via transmission electron microscopy (TEM) that LDL particles associate with isolated MVs *in vitro* (Sódar et al., 2016).

To further investigate nLDL stimulation of neutrophils calpains 1 and 2 were inhibited prior to nLDL treatment. Inhibition of calpains 1 and 2 reduced NMV biogenesis 41.9% compared to neutrophils treated with nLDL but not with inhibitors. However, NMV biogenesis was not completely prevented and the decrease in NMV release did not reach significance. This suggests there may be calpain-independent mechanisms for NMV biogenesis. For example, MVs are known to be shed during

apoptosis (Coleman *et al.*, 2001; Schiller *et al.*, 2012) and nLDL may be acting through this mechanism. In addition, neutrophils are likely to be somewhat activated following the isolation procedure and therefore may produce NMVs prior to calpain inhibition. Another possibility is that contamination of NMV samples with residual nLDL particles may also mask the reduction in NMV release following calpain inhibition (figure 3.2).

3.5.2 Protease contents of neutrophil microvesicles from unstimulated and native low-density lipoprotein stimulated neutrophils

Having established that NMVs are released in response to nLDL treatment of neutrophils the second aim of this chapter was to determine if NMVs from nLDL-stimulated neutrophils differ in their protease content from NMVs generated by unstimulated neutrophils.

Neutrophils have been shown to contain a wide repertoire of matrix degrading proteases (Dorweiler *et al.*, 2008; Garratt *et al.*, 2015; Herman *et al.*, 2001) that have been linked with plaque rupture (de Nooijer *et al.*, 2006; Gough *et al.*, 2006; Herman *et al.*, 2001). I hypothesised these proteases are relevant to degradation of the ECM and erosion of the endothelium associated with stable plaques that have produced thrombi. Whilst previous studies suggest that changes to the neutrophil environment led to production of NMVs with differing proteomes (Dalli *et al.*, 2013) there is no available evidence on the effect nLDL stimulation has on NMV protease contents. Screening of 35 different proteases from samples of NMVs from unstimulated and nLDL-stimulated neutrophils pooled from multiple different healthy donors offered the most efficient method of establishing reliable differences in protease content between these two NMV populations.

In NMVs from nLDL-stimulated neutrophils, 7 proteases were detected compared to 9 proteases in NMVs from unstimulated neutrophils. MMP-8 and MMP-9 were the most abundant protease in both NMV populations. MMP-8 and MMP-9 have been previously detected in NMVs from adherent neutrophils and neutrophils in suspension (Dalli et al., 2013) and have been linked with plaque disruption (Peeters *et al.*, 2011; Sluijter *et al.*, 2006) however, most of the research has focused on models of plaque rupture, not erosion. Nevertheless, MMP-8 inactivation has been shown to limit lesion formation in $ApoE^{-/-}$ mice and interestingly lesions demonstrated greater collagen content (Laxton *et al.*, 2009). Degradation of collagen and other ECM proteins is a key part in the hypothesised mechanism of plaque erosion making MMP-8 and MMP-9 relevant factors in this process. There is evidence to suggest MMP-9 also acts upon endothelial cells directly inducing apoptosis and the blockage of MMP-9 production in mice reduces atherosclerotic lesion size, indicating a role in plaque progression (Florence *et al.*, 2017). Differences in the relative abundance

of MMP-8 and MMP-9 were identified in NMVs from unstimulated and nLDL-stimulated neutrophils. Whereas MMP-8 was the most abundant protease in NMVs from unstimulated neutrophils, MMP-9 was most abundant in NMVs from nLDL-stimulated neutrophils. These data could suggest nLDL stimulation induces the specific incorporation of MMP-9 into NMVs. Conversely, it may be that nLDL stimulation induces greater MMP-9 release from neutrophils and simply by virtue of more MMP-9 being present within neutrophils more is incorporated into NMVs. The analysis of MMP-9 mRNA and protein concentrations within nLDL-stimulated and unstimulated neutrophils could help to answer this question.

NMVs from unstimulated neutrophils contained 3 further proteases not detected in NMVs from nLDL-stimulated neutrophils, namely urokinase (uPA), cathepsin X/Z/P and ADAM8. uPA has been previously reported in isolated neutrophils (Reichel *et al.*, 2011) and is interesting due to its role in thrombolysis (Kadir and Bayraktutan, 2020)**.** uPA coverts plasminogen to the thrombolytic protease plasmin (Kadir and Bayraktutan, 2020), suggesting that uPA is protective against thrombosis. Additionally, specific polymorphisms of uPA inhibitor, plasminogen activator inhibitor-1 (PAI-1) gene are linked to greater serum PAI-1 concentrations and a greater risk of myocardial infarction (Abboud *et al.*, 2010; Liu *et al.*, 2018a). In addition to its thrombolytic properties, plasmin can also further degrade ECM components. The exact effect of uPA in the context of plaque erosion is unclear however, it is interesting to note the difference in uPA expression in NMVs from unstimulated neutrophils compared to NMVs from nLDL-stimulated neutrophils. The lack of uPA in NMVs from nLDL-stimulated neutrophils could indicate nLDL stimulation induces production of more pro-thrombotic NMVs.

Among the cathepsins detected 3 were cysteine proteases, cathepsin S, cathepsin A and cathepsin X/Z/P. Cathepsin S and A were detected in both NMV populations whereas cathepsin X/Z/P was detected only in NMVs from unstimulated neutrophils. Cathepsin S has been previously linked with atherosclerosis (Liu *et al.*, 2006) with cathepsin S knockout mice demonstrating reduced atherosclerosis as assessed by plaque size (Sukhova *et al.*, 2003). Cathepsin X/Z/P has been reported to interact with integrins of ECs via its RGD-motif to support EC adhesion to the ECM (Lechner *et al.*, 2006).

Cathepsin V was detected only within NMVs from nLDL-stimulated neutrophils. Cathepsin V is a potent cysteine protease targeting the elastin components of the ECM and has been detected in human atheroma (Yasuda *et al.*, 2004). Stenotic aortic valves have previously been shown to more highly express genes for cathepsin V compared with controls (Helske *et al.*, 2006). When isolated aortic valves were treated with cathepsins S, K and V elastin fibres were degraded (Helske *et al.*, 2006). Cathepsin S appears to also have a role in calcification of atherosclerotic plaques. ApoE$^{-/-}$ mice deficient in cathepsin S demonstrated less calcification compared to controls (Aikawa *et al.*, 2009). Other studies have shown that whilst cathepsin K, S and V degrade elastin fibres and promote calcification, this process actually leads to protection of fibres from further degradation (Andrault *et*

al., 2019). Therefore, it is difficult to determine the exact effect of cathepsin V on promoting plaque disruption.

Matrix metalloproteinases and cathepsins exist in pro-enzyme forms and require conversion to their active forms to exhibit their maximal enzymatic effects. The protease array used did not distinguish between active and pro-forms, apart from in the case of proteinase-3. This means that although NMVs contain these proteases they are not necessarily active.

3.5.3 Enzymatic activity of neutrophil elastase and matrix metalloproteinase-9 in microvesicles from unstimulated and native low-density lipoprotein stimulated neutrophils

NE has also been linked with EC dysfunction by inducing apoptosis via the PERK/CHOP pathway of the unfolded protein response (UPR) (Grechowa *et al.*, 2017). Co-localisation of NE with apoptotic ECs in rupture-prone human plaques further supported the role NE may play in atherosclerosis (Grechowa *et al.*, 2017). Further work has associated NE with atherosclerotic plaques; typically atheromatous plaques colocalised with macrophages, but NE has also been detected in fibrous plaques too (Dollery *et al.*, 2003; Franck *et al.*, 2017). NE mRNA expression is significantly elevated in ApoE$^{-/-}$ mice following high-fat diet compared to WT controls and knockout of NE was shown to reduce atherosclerosis (Wen *et al.*, 2018). The proposed mechanism for this is through the impairment of macrophage cholesterol transport resulting in foam cell formation, limiting the application to stable, lipid-poor plaques. Nevertheless, this is an interesting association between NE and atherosclerosis. NE has also been shown to promote IL-1β release from ECs (Alfaidi *et al.*, 2015). What is also important to plaque erosion is the effect of NE on the degradation of the ECM. It has been established for decades that NE degrades ECM proteins, collagens type I and type III (Gadek *et al.*, 1980; Kafienah *et al.*, 1998) and more recently it has been shown to activate proteases, such as MMP-9 whilst also degrading inhibitors, such as TIMP-1 (Jackson *et al.*, 2010) therefore acting to exacerbate their action. This has been noted in cystic fibrosis (CF) where a higher MMP-9/TIMP-1 ratio was associated with NE and bronchiectasis (Garratt *et al.*, 2015).

To determine if neutrophils package NE into NMVs, ELISA analysis of lysed NMVs was performed and NE was measured in NMVs from both unstimulated and nLDL-stimulated neutrophils. To determine if the measured NE was active, a second ELISA was performed whereby proteolytic cleavage of a synthetic substrate results in fluorescence which is measured and compared to a standard curve created using purified recombinant NE. Significantly greater NE activity was measured following nLDL stimulation compared to unstimulated when expressed per 1×10^7

neutrophils. There was considerable variation in the nLDL stimulated conditions whereas measured NE activity in the unstimulated condition was more consistent. This likely reflects individual donor neutrophil responses to nLDL stimulation.

MMP-9 was the most abundant protease detected in NMVs from nLDL-stimulated neutrophils, however the precise concentration was still unknown. Therefore, ELISA was used to quantify and compare MMP-9 concentration in NMVs from unstimulated and nLDL-stimulated neutrophils. No significant difference in MMP-9 concentration per NMV was measured between nLDL and unstimulated conditions. Measurements of MMP-9 concentration varied widely in each NMV population reflecting considerable donor variability. However, as nLDL induces greater generation of NMVs, when MMP-9 was expressed per 1×10^7 neutrophils there was greater NMV-associated MMP-9 in total in the nLDL condition. This may suggest that MMP-9 levels are higher in neutrophils treated with nLDL or rather nLDL causes more MMP-9 to be packaged into NMVs.

As with the protease array, the ELISA did not differentiate between active and pro-forms of MMP-9. Therefore, to determine the activity of MMP-9 gelatin zymography was used. The data indicated a dose-dependent increase in MMP activity as quantified by measuring the size of the bands caused by gelatin degradation as concentrations of NMVs from nLDL-stimulated neutrophils increased. This semi-quantitative method confirmed the presence of MMP-9 in both the pro- and active forms. Greater degradation of gelatin was caused by NMVs from nLDL-stimulated neutrophils compared to NMVs from unstimulated neutrophils, indicating an increase in MMP-9 activity following nLDL stimulation. The presence of MMP-9 is important as it has the potential to degrade collagens I and III found in stable plaques (Bigg *et al.*, 2007) and can negatively affect EC integrity (Butin-Israeli *et al.*, 2016) which in combination may promote EC detachment from stable plaques.

I concluded that NMVs from both unstimulated and nLDL-stimulated neutrophils contained active MMP-9 and active NE, both of which are potent proteases previously associated with degradation of ECM components observed in stable plaques and with atherosclerosis more broadly. The method of neutrophil stimulation did not affect MMP-9 or NE packaging into NMVs as there no significant difference per NMV however overall concentration and activity of these proteases was increased in the nLDL condition as a consequence of much greater NMV production. This places the importance on the quantity of NMVs generated in response to the stimuli over any effects on NMV packaging.

3.5.4 Conclusion

The data presented in this chapter demonstrates that nLDL induces NMV release. Whilst abundant matrix degrading proteases were measured in NMVs from both unstimulated and nLDL-stimulated neutrophils, there were differences in proteome content and relative abundance of these proteases. Further analysis of two highly expressed proteases MMP-9 and NE revealed no statistically significant effect of nLDL stimulation on the concentration or activity per NMV. However, when the increased production of NMVs is considered the total amount of both NMV-associated MMP-9 and NE increased following nLDL stimulation of neutrophils.

In summary, NMVs containing active proteases capable of ECM degradation and inducing EC dysfunction, are released in greater number in response to nLDL stimulation. Greater number of NMVs increase the total amount of NMV-associated MMP-9 and NE released by neutrophils potentially contributing to erosion of stable atherosclerotic plaques.

3.5.5 Limitations

The aim of this chapter was to determine if the method of neutrophil stimulation effected the production and contents of NMVs. NMV production was assessed using an LSRII flow cytometer and calibration beads to determine the correct gates to detect the NMVs. Whilst flow cytometry is widely regarded as one of the most sensitive instruments for detecting NMVs, this particular model was operating at its detection limit. For this reason, many smaller NMVs may not have been counted at all leading to distortions in the data if one particular stimulation method favoured production of smaller NMVs versus another stimulation method.

The MISEV2014 guidelines on extracellular vesicle studies detail a number of recommended practices to follow when researching EVs and their function (Théry *et al.*, 2018). Quantification of EVs is recommended by two different methods such as particle number and protein concentration. In my experiments only particle number was routinely used to quantify NMVs and this represents a limitation of the study. The concentration of particular proteins was measured in specific experiments and equal concentrations of NMVs were found to contain similar concentrations of these proteins, for instance in the case of NE. However, this only applies to specific proteins and was not routinely done for all NMV preparations. The lack of a second quantification method could have reduced the accuracy NMV quantification especially in light of the issues I faced with contaminating nLDL particles appearing in the size range of NMVs. In addition, the guidelines require high-resolution microscopy images of single EVs. These images were not acquired during my project and in

retrospect this was an oversight. The main reason for this was an assumption that NMVs produced in response to nLDL and that shared all the characteristics of NMVs produced in response to fMLP, high-resolution confocal images of which had already been acquired by the Ridger group, would be generated in the same manner from the neutrophil surface. During a time when time, equipment and funds were limited due to the Covid-19 pandemic other experiments were prioritised over obtaining images of single NMVs. In future studies, it would be necessary to confirm the formation of NMVs in response to nLDL is the same as it is in response to fMLP.

Another key part of this chapter was to characterise the contents of NMVs. For this I focused on the protease content of the NMVs due to the hypothesis that NMVs may be involved in ECM degradation and EC detachment. This limited our scope and meant other proteins contained within NMVs were ignored. Many of these proteins could have affected the function of ECs. Even when considering just proteases only a proportion of human proteins were screened for. Many other proteases were likely present with some potentially having a substantial effect on ECs or the ECM. Amongst the proteases that were screened, some were present within NMV from unstimulated neutrophils and not in NMVs from nLDL NMVs and vice versa. Proteases that display this specific incorporation into NMVs depending on the stimulation method are interesting and may uncover differences in the effects of NMVs from different population of neutrophils. A significant limitation of this chapter is that these proteases were not investigated further, and an additional general limitation of the study as a whole is the absence of any comparison in effect of NMVs from nLDL-stimulated neutrophils and NMVs from unstimulated neutrophils.

4 Effect of neutrophil microvesicles on human coronary artery endothelial cell function and matrix degradation using an *in vitro* model

4.1 Introduction

The endothelium is present at the interface between the arterial wall and flowing blood and in healthy vessels helps to reduce the risk of thrombosis by providing a physical barrier to the pro-thrombotic extracellular matrix (ECM) and through synthesis and release of anticoagulant factors, such as thrombomodulin and platelet inhibitors, such as nitric oxide (NO) (Yau *et al.*, 2015). In regions experiencing disturbed blood flow the endothelium shifts to a more inflammatory phenotype, promoting the recruitment of leukocytes, synthesis of pro-inflammatory factors and modulation of the ECM (Qu *et al.*, 2020; Russo *et al.*, 2020).

Healthy endothelial function depends on the close interaction with the ECM, the loss of which can induce a form of apoptosis known as anoikis (Aoudjit and Vuori, 2001). Neutrophils and NMVs contain a wide variety of proteases capable of promoting this excessive degradation of ECM components; for instance MMP-9 and neutrophil elastase degrade collagens (Bigg *et al.*, 2007) and elastin (Masood *et al.*, 2015), respectively. The detachment of ECs, either because of weakened interaction with degraded ECM or apoptosis, is hypothesised to be involved in the initiation of thrombosis at sites of plaque erosion. The areas of ECM exposed by eroded ECs can facilitate platelet recruitment and aggregation through interaction with collagen receptors expressed on the platelet membrane (Induruwa *et al.*, 2018; Maurice *et al.*, 2006; Nieswandt *et al.*, 2001).

Apoptosis is a key process associated with plaque erosion with studies linking it to erosion-prone lesions (Durand *et al.*, 2004; Franck *et al.*, 2017). Post-stenotic regions in rabbit arteries, resembling eroded plaques, demonstrated increased EC apoptosis and crucially EC detachment, the hallmark of plaque erosion (Sumi *et al.*, 2010). This is perhaps unsurprising given the evidence that disturbed blood flow promotes EC apoptosis (Chaudhury *et al.*, 2010; Tricot *et al.*, 2000). However, previous work investigating plaque erosion found neutrophils induce apoptosis and EC detachment following EC culture on the TLR2 agonist, hyaluronan (HA) (Quillard *et al.*, 2015). A correlation between neutrophil and NET abundance in smooth muscle cell-rich plaques and apoptotic ECs was also identified in human atheromata (Quillard *et al.*, 2015) identifying neutrophils as possible contributors to the mechanism of plaque erosion.

There are many factors that can induce EC dysfunction and thereby promote detachment, however the precise combination of factors that culminate in plaque erosion has not been fully described. It is likely that several competing processes determine whether ECs are lost from stable plaques. ECM degradation, EC detachment and apoptosis promote erosion whilst proliferation and migration of ECs compensate and protect the plaque from erosion. These processes are likely to be continual, both in diseased and healthy vessels, and are important in normal haemostasis. However, an imbalance in these processes that favours EC dysfunction will likely shift plaques toward a more erosion-prone phenotype, displaying greater EC loss and fewer protective mechanisms.

There are likely to be many factors involved in maintaining this balance, one of which is the influence of neutrophils on the endothelium. Neutrophils have been associated with atherosclerosis for many years (Cochain *et al.*, 2018; Ionita *et al.*, 2010) and are the most prevalent leukocyte subset in thrombus specimens (Riegger *et al.*, 2016). Neutrophils have also been implicated in the mechanisms of plaque erosion through promoting EC detachment and facilitating thrombus formation via the generation of NETs (Franck *et al.*, 2018; Quillard *et al.*, 2015). Previous work has also demonstrated that neutrophils influence the endothelium through other mechanisms. NMVs released by activated neutrophils were shown to deliver pro-inflammatory micro-RNA-155 (Mir-155) to endothelial cells, increasing NF-κB activation and monocyte recruitment (Gomez *et al.*, 2020).

Due to the association between neutrophils and atherosclerosis and the known interaction between NMVs and the endothelium, the ability of NMVs to degrade the ECM and modulate EC apoptosis, detachment, proliferation and wound healing response was investigated.

4.2 Hypothesis and Aims

It was hypothesised that NMVs degrade components of the ECM and promote EC dysfunction that in turn contributes to plaque erosion.

The work presented in this chapter uses an *in vitro* model to address three aims:

Aim 1 – To determine if NMVs can degrade analogues of the extracellular matrix.

Aim 2 – To determine if NMVs can promote endothelial cell apoptosis and detachment that contribute to endothelial cell erosion.

Aim 3 – To determine if NMVs can impair endothelial cell proliferation and wound healing response that could compensate for endothelial cell erosion.

4.3 Methods

The ability of neutrophil microvesicles (NMVs) isolated from unstimulated and nLDL stimulated neutrophils to lyse matrix proteins was assessed using a fluorescently conjugated gelatin substrate (method section 2.10). NMVs were lysed via freeze/thawing cycles to release proteases contained within and samples of increasing concentrations prepared in sterile filtered PBS. Lysed NMVs were incubated with fluorescein conjugated gelatin for 4 hours at 37^{o}C. Digestion of this substrate yields fluorescent peptides proportional to proteolytic activity, and this fluorescence was measured by a plate reader. TIMP-1 was co-incubated with NMVs to inhibit proteolytic activity of MMPs.

For the following assays of cell function primary human coronary artery endothelial cells (HCAECs) were cultured statically in cell culture plates or under atheroprone LOSS flow conditions in Ibidi µ-slides on matrices containing collagen type III and low molecular weight hyaluronan (LMW-HA). HCAECs were treated with NMVs. Please note when NMVs are mentioned without details of how they were generated this is referring to NMVs generated by nLDL-stimulated neutrophils. No comparison was made between the effects of NMVs from unstimulated neutrophils and nLDL-stimulated neutrophils on HCAEC function.

Detachment of HCAECs was assessed by treatment with optimised concentrations of trypsin-EDTA and imaging by phase contrast microscopy (methods section 2.15). Proliferation was assessed under static conditions by staining for Ki-67, a protein present during all active stages of the cell cycle, but crucially not detected in resting cells (methods section 2.17). Sterile cell gaps were produced in statically cultured confluent HCAEC monolayers using cell culture inserts (Ibidi, Germany) and wound healing response measured by time-lapse phase microscopy (methods section 2.18). Apoptosis was assessed in HCAECs cultured statically and under atheroprone LOSS by flow cytometric measurement of activated caspase-3 and 7 (figure 4.1) (methods section 2.16).

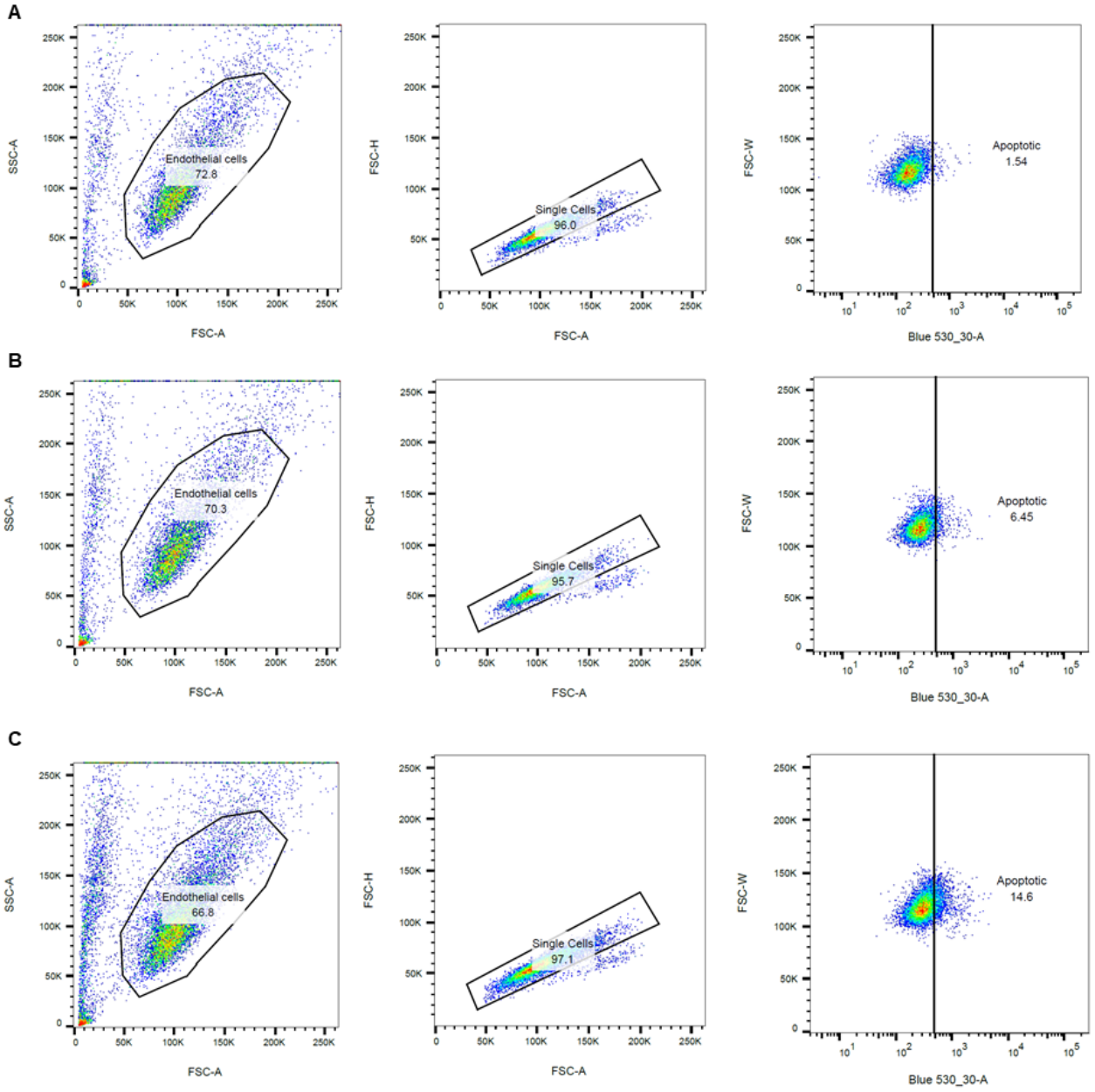


Figure 4. 1. Flow cytometry gating strategy for identifying apoptotic human coronary artery endothelial cells. A) *Unlabelled isolated human coronary artery endothelial cells (HCAECs) were identified based on side scatter (SSC) and forward scatter (FSC) plots and single cells identified. HCAECs were plotted as FSC vs blue 530-30 filter to quantify fluorescence from caspase-3-7 green detection reagent. Gates were set on unlabelled HCAECs. This gating strategy was applied to* ***B)*** *supernatant treated control HCAECs and* ***C)*** *NMV treated HCAECs.*

4.4 Results

4.4.1 Native low-density lipoprotein neutrophil microvesicles are internalised by human coronary artery endothelial cells

Previous work has uncovered NMVs produced in response to fMLP bind to and are internalised by HCAECs (Gomez *et al.*, 2020). To confirm NMVs produced in response to nLDL do the same, internalisation experiments were conducted. Confirming NMVs are internalised by HCAECs provided the foundation for further hypotheses that NMVs alter the function of these HCAECs.

HCAECs were incubated with PKH-26 labelled NMVs and imaged by confocal microscopy. NMVs were visualised within HCAECs after 2 hours. Analysis of images in two planes confirmed NMVs were localised within HCAECs rather than merely be attached to their surface (figure 4.2).

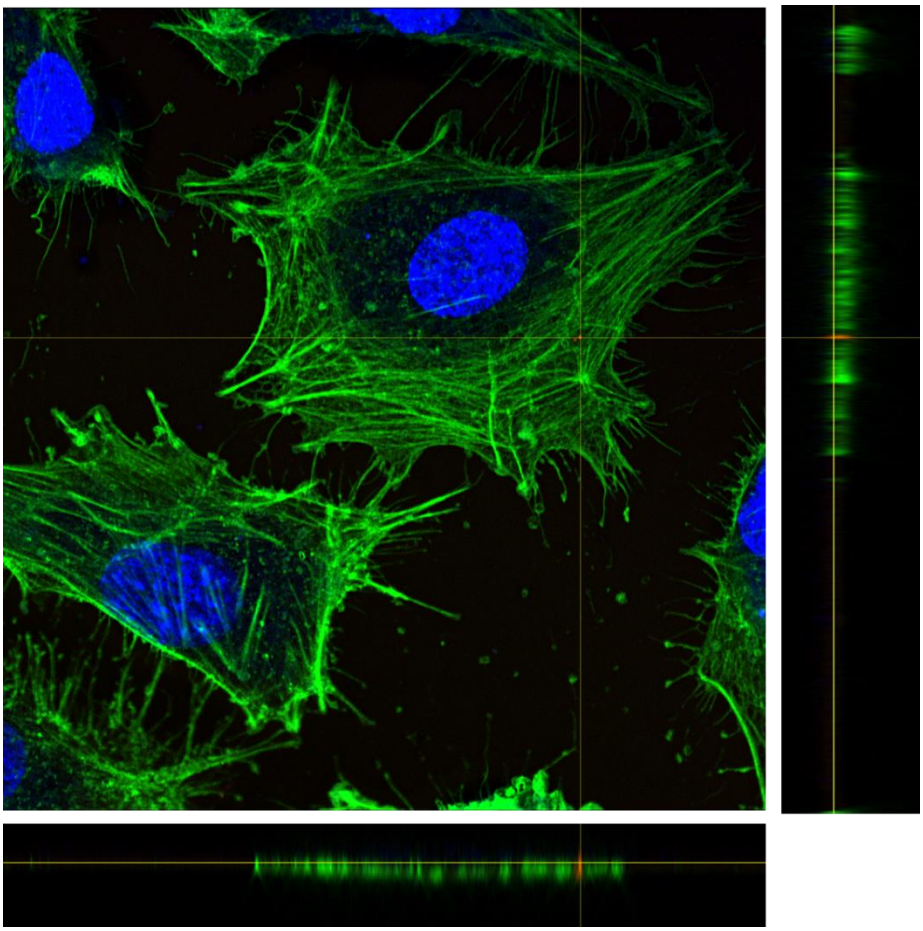


Figure 4. 2- Internalisation of neutrophil microvesicles by human coronary artery endothelial cells. *Human coronary artery endothelial cells (HCAECs) were cultured statically and treated with PKH-26 labelled native low-density lipoprotein (nLDL) neutrophil microvesicles (NMVs) for 2 h prior to fixation with 4% paraformaldehyde and staining with TO-PRO-3 (blue) and FITC-phalloidin (green). HCAECs were imaged by fluorescence confocal microscopy and analysed using ImageJ software. Orthogonal views demonstrated the presence of NMVs within HCAECs.*

4.4.2 Microvesicles from unstimulated and native low-density lipoprotein stimulated neutrophils degrade gelatin

I hypothesised that NMVs contain active proteases. This was tested by incubating NMVs with DQ-gelatin (*ThermoFisher, USA)* which fluoresces following degradation (section 2.16). DQ-gelatin was incubated with NMV supernatant control, 60,000 NMVs, 120,000 NMVs or 240,000 NMVs from unstimulated neutrophils or neutrophils stimulated with nLDL. These concentrations were chosen to provide a range of doses to assess if a dose-dependent effect was present. The 240,000 NMV condition was chosen as the highest dose consistently achievable from a typical neutrophil isolation. The number of available donors was severely limited as a result of COVID-19 restrictions forcing us to be more conservative with our NMV doses. Initial experiments demonstrated good degradation from the chosen doses, so these were repeated. Fluorescence was measured over time and the area under the curve quantified for statistical analysis (figure 4.3). NMVs from unstimulated and nLDL-stimulated neutrophils degraded gelatin in a dose-dependent manner. Supernatant controls from unstimulated neutrophils induced minimal degradation with degradation increasing as the concentration of NMVs from unstimulated neutrophils rose. Although 240,000 NMVs from unstimulated neutrophils induced greater degradation than the supernatant control this did not reach statistical significance. The supernatant control from nLDL-stimulated neutrophils induced minimal degradation which increased as the concentration of NMVs from nLDL-stimulated controls increased. 240,000 NMVs from nLDL-stimulated neutrophils induced significantly greater degradation compared with the supernatant control from nLDL-stimulated neutrophils. Addition of TIMP-1 to 240,000 NMVs from nLDL-stimulated neutrophils significantly reduced degradation (62.2% decrease, SEM±5.24, n=4, p=0.0068) to levels similar to supernatant control. Whilst a dose-dependent increase in degradation was observed in NMVs from both unstimulated and nLDL-stimulated neutrophils, there was no significant difference in the level of degradation induced by NMVs from unstimulated versus nLDL-stimulated neutrophils at each concentration. These data indicate that NMVs from both unstimulated and nLDL-stimulated neutrophils are capable of degrading analogues of the ECM in a largely MMP-dependent manner.

Whilst these data indicating NMVs can degrade the ECM are interesting, *in vivo* NMVs would not encounter ECM in isolation and therefore I attempted to model the effect of NMVs on DQ-gelatin degradation when a confluent HCAEC monolayer was present above the DQ-gelatin. To assess if incubation of HCAECs with NMVs from nLDL-stimulated neutrophils enhanced gelatin degradation, HCAECs were seeded on DQ-gelatin substrate and treated with NMVs from unstimulated (figure 4.4A) and nLDL-stimulated neutrophils (figure 4.4B). Treatment with NMVs led to percentage increases in gelatin degradation compared to supernatant control (mean 202.7% increase, SEM±31.1, n=2). Co-incubation of HCAECs with NMVs and TIMP-1 led to a small reduction in gelatin degradation compared with nLDL only, but was still elevated compared to supernatant control.

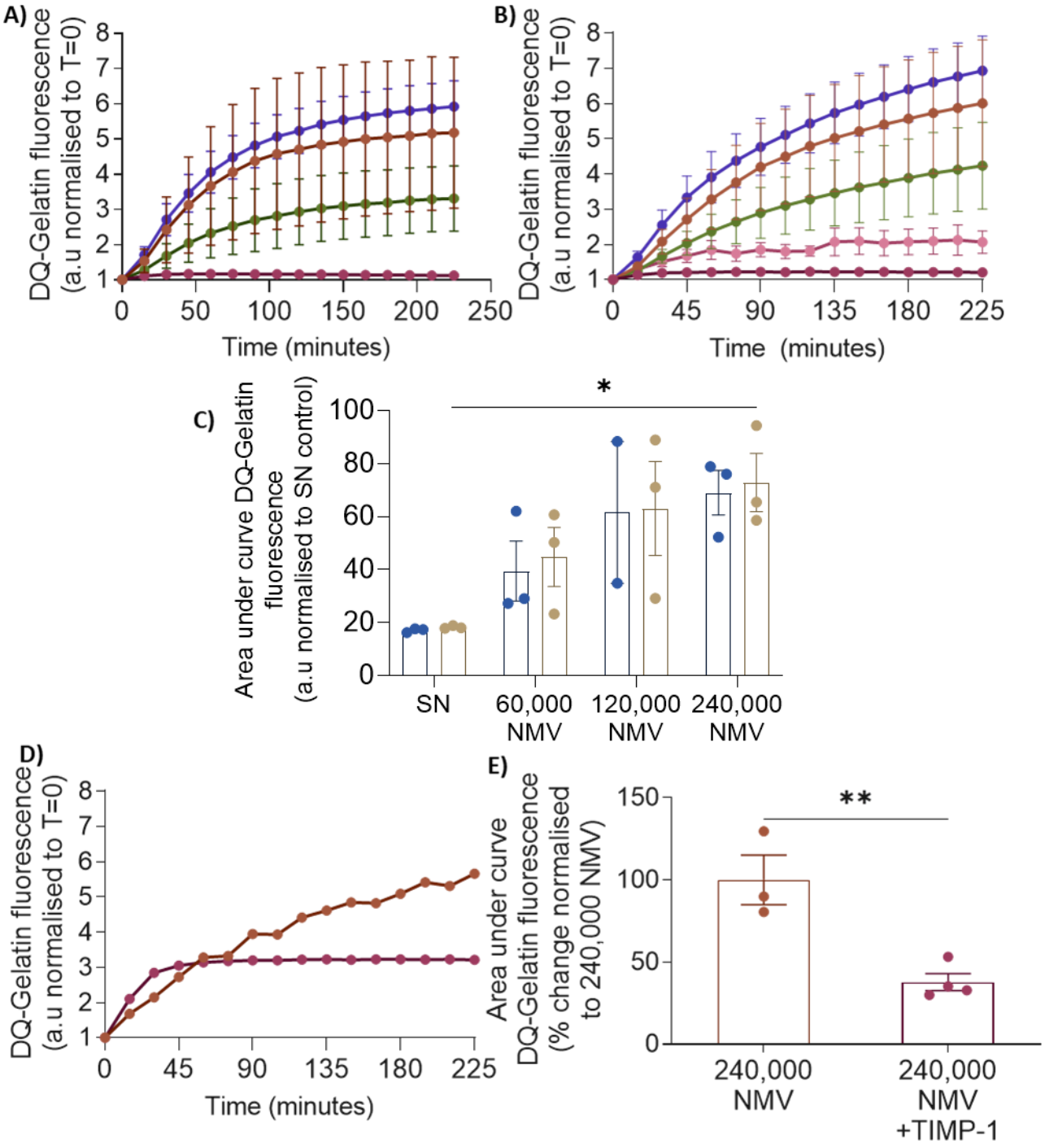


Figure 4.3. Degradation of gelatin by neutrophil microvesicles from unstimulated and native low-density lipoprotein-stimulated neutrophils. *Neutrophil microvesicle (NMV) lysate was incubated with DQ-gelatin. Fluorescence emitted following degradation was quantified using a plate reader. NMVs from* ***(A)*** *unstimulated and* ***(B)*** *native low-density lipoprotein (nLDL)-stimulated neutrophils at 60,000 (green), 120,000 (orange), 240,000 (purple) NMVs, 240,000 NMVs + TIMP-1 (pink) or supernatant control (red) were incubated with DQ-gelatin. Fluorescence was measured and normalised to time=0.* ***(C)*** *Area under the curve analysis was performed on NMVs from unstimulated (blue) and nLDL-stimulated (yellow) neutrophils. Significance assessed by ordinary one-way ANOVA with Tukey's multiple comparisons. Data expressed as mean ±SEM, n=3.* ***(D)*** *0.05U/mL collagenase A (orange) and 0.1U/mL matrix metalloproteinase-9 (MMP-9) (red) were analysed to validate the assay.* ***(E)*** *Incubation of NMVs from nLDL-stimulated neutrophils only (orange) and NMVs from nLDL-stimulated neutrophils with 800ng TIMP-1 (red) with DQ-gelatin. Data expressed as mean ±SEM, n=3. TIMP-1 significantly reduced DQ-gelatin degradation as assessed by unpaired t-test. * p=<0.05 ** p=<0.01.*

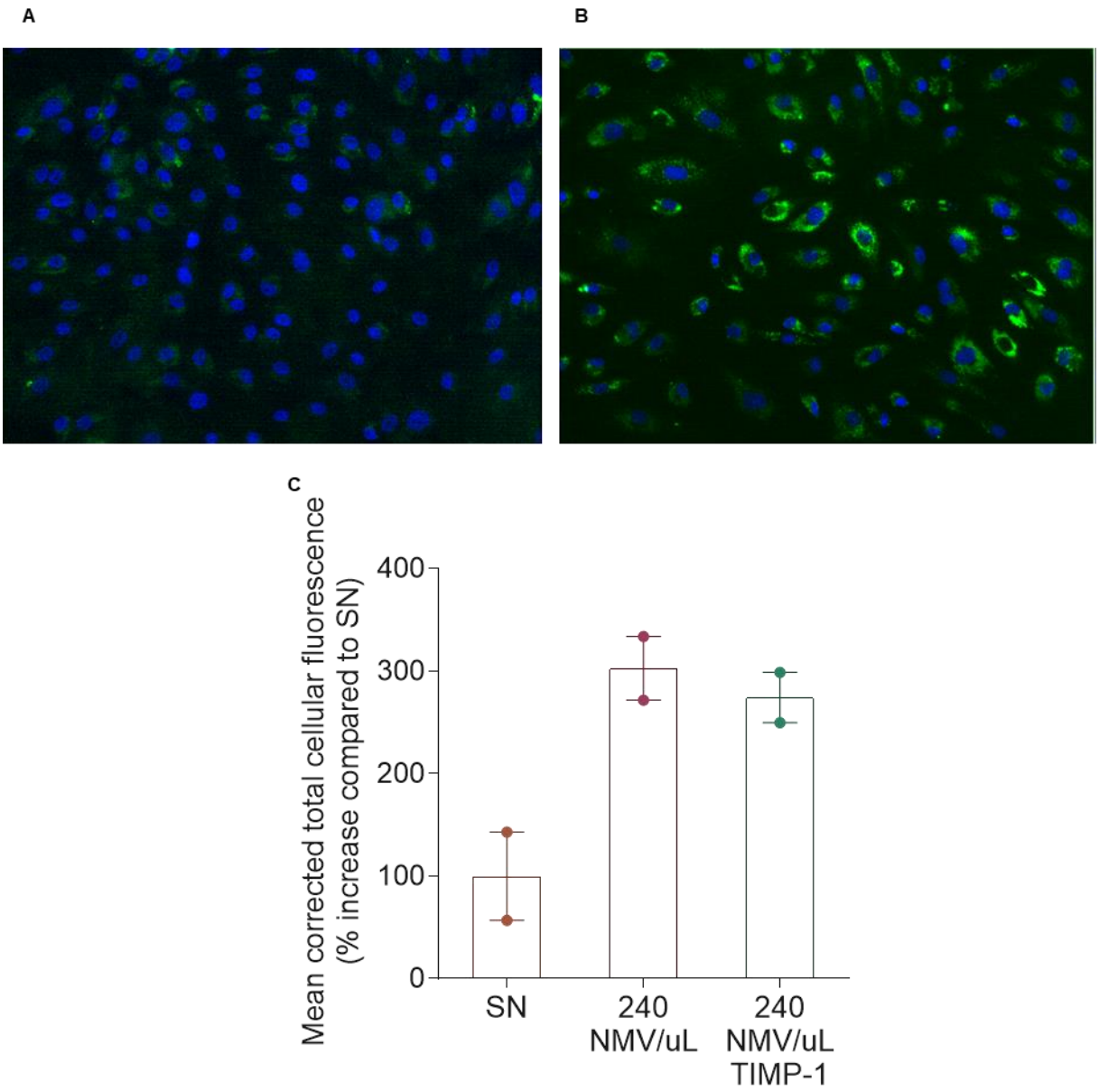


Figure 4. 4. DQ-gelatin degradation observed during human coronary artery endothelial culture with neutrophil microvesicles. *Human coronary artery endothelial cells (HCAECs) were seeded on 100 µg/mL DQ-gelatin and cultured statically for 24 hours with either* ***(A)*** *a supernatant control,* ***(B)*** *neutrophil microvesicles (NMVs) from native low-density lipoprotein (nLDL)-stimulated neutrophils or NMVs from nLDL-stimulated neutrophils + 1 µg TIMP-1. HCAECs fixed with 4% paraformaldehyde and labelled with DAPI. Widefield fluorescence microscopy was used to quantify resulting fluorescence from DQ-gelatin degradation. Data was analysed using ImageJ software and cellular fluorescence measured per cell. Data expressed as mean corrected cellular fluorescence ± SEM, n=2. Statistical significance assessed by one-way ANOVA with Tukey's multiple comparisons test.*

4.4.3 Optimisation of collagen III and low molecular weight hyaluronan concentrations for future culture conditions

Following on from the determination that NMVs were capable of degrading gelatin matrix, I explored culturing HCAECs on matrices more closely resembling the ECM of eroded plaques. Analysis of atherosclerotic lesions had identified collagen type III and low molecular weight hyaluronan (LMW-HA) as associated with eroded plaques (Kolodgie *et al.*, 2002; Kolodgie *et al.*, 2004; Pedicino *et al.*, 2018). Collagen type III of increasing concentrations was used to coat tissue culture plates for 1 hour prior to HCAEC seeding and static culture at 37°C for 24 hours. Fixed HCAECs were labelled with DAPI and imaged by fluorescence microscopy. Increasing concentrations of collagen type III had little effect on total number of adherent HCAECs. However, concentrations of 70 µg/mL and 100 µg/mL significantly increased the number of adherent HCAECs compared with the previously used substrate, 1% gelatin (figure 4.5A). 70 µg/mL was chosen as the optimal concentration as it was the lowest collagen type III concentration that resulted in significantly greater HCAEC adherence (mean =388, SEM±7.1, n=3, p=0.027) compared to 1% gelatin (mean =326, SEM±23, n=3).

Culturing ECs on a LMW-HA substrate has been shown to increase apoptosis (Quillard *et al.*, 2015) and, therefore, it was important to optimise this concentration to avoid excessive detachment. To optimise the concentration of LMW-HA, increasing concentrations of LMW-HA were incorporated into 70 µg/mL collagen type III and HCAECs cultured as above. The number of adherent HCAECs was lower and apoptosis increased in each LMW-HA condition compared with the collagen type III only condition (figure 4.5 B-C). 5 µg/mL LMW-HA was chosen as the optimal concentration as a decrease in the number of adherent HCAECs was observed (mean =288, SEM±26.6, n=3, p=0.0082) compared to collagen III only (mean =388, SEM±7.1, n=3), but apoptosis was only minimally increased at this concentration. These optimised conditions were used in future assays investigating HCAEC detachment and apoptosis.

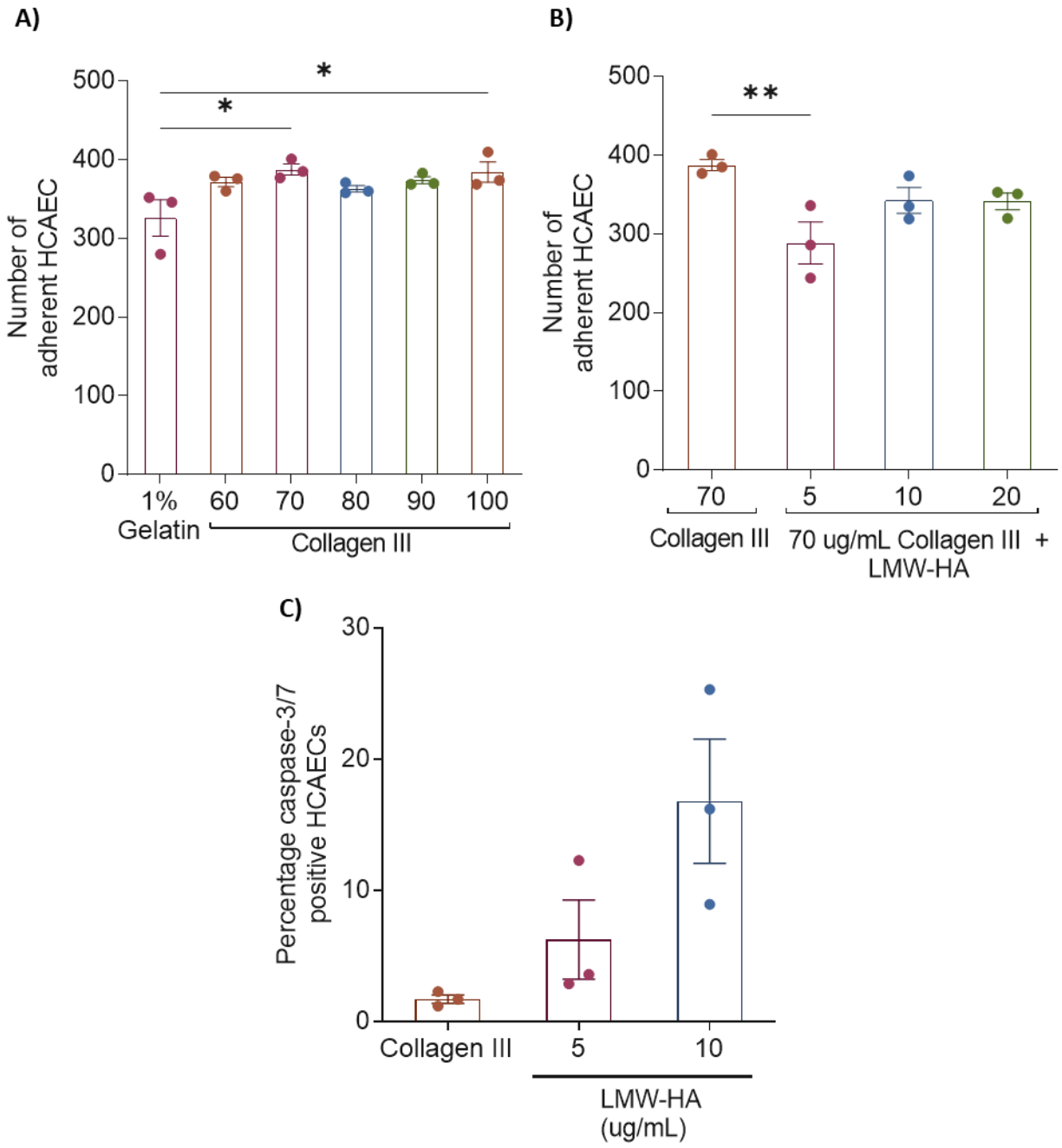


Figure 4. 5. Optimisation of collagen type III and low molecular weight hyaluronan for human coronary artery endothelial cell culture. (A) *Human coronary artery endothelial cells (HCAECs) were cultured statically on increasing concentrations of collagen type III or 1% gelatin prior to fixation with 4% paraformaldehyde, staining with DAPI and quantification. HCAECs were cultured on an optimal concentration of collagen type III spiked with increasing concentrations of low molecular weight hyaluronan (LMW-HA) and* ***(B)*** *total number of HCAEC and* ***(C)*** *the percentage of apoptotic cells were quantified, n=3. HCAECs were imaged by widefield fluorescence microscopy and analysed using ImageJ software. Data expressed as mean ±SEM and assessed by one-way ANOVA with Dunnett's multiple comparisons test. Control condition = SN CTRL. *=p<0.05, **=p<0.01.*

4.4.4 Optimisation of trypsin-induced human coronary artery endothelial cell detachment

To assess the ability of NMVs to induce HCAEC detachment, I replicated a previously published experimental design in which endothelial cell detachment was measured by light microscopy following incubation with diluted trypsin-EDTA (Quillard et al., 2015). The endothelial cells, matrix and culture media in these experiments differed from the previous authors and therefore the method required optimising to obtain reliable results.

Washed HCAECs cultured on collagen III matrix were incubated with increasing concentrations of trypsin-EDTA diluted in sterile PBS. Following a 3-minute incubation with trypsin-EDTA, HCAECs were placed in MV2 culture media and imaged by light microscopy. Unsurprisingly, a dose-dependent increase in the percentage of detaching HCAECs was observed as trypsin-EDTA concentrations increased. I hypothesised that due to NMVs containing active matrix degrading proteases they would contribute to increased HCAEC detachment. However, this would be unlikely to overcome HCAEC adherence to a collagen III matrix alone. Rather, *in vivo* they would act in concert with other factors to increase the propensity of HCAECs to detach. For this reason, the trypsin-EDTA concentration was optimised to induce moderate detachment in control HCAECs. Detachment above control levels would be attributed to the effects of NMV treatment. The experiments performed by Quillard et al used 25 µg/mL trypsin-EDTA. However, this concentration induced excessive levels of detachment of HCAECs in the present study. Detachment was lower at trypsin-EDTA concentrations of 12.5 µg/mL and 6.25 µg/mL (figure 4.6A). A concentration of 6.25 µg/mL was selected as it reliably produced moderate detachment of HCAECs whilst allowing the effects of NMV treatment to be observed. Detachment with 6.25 µg/mL also exhibited minimal variability.

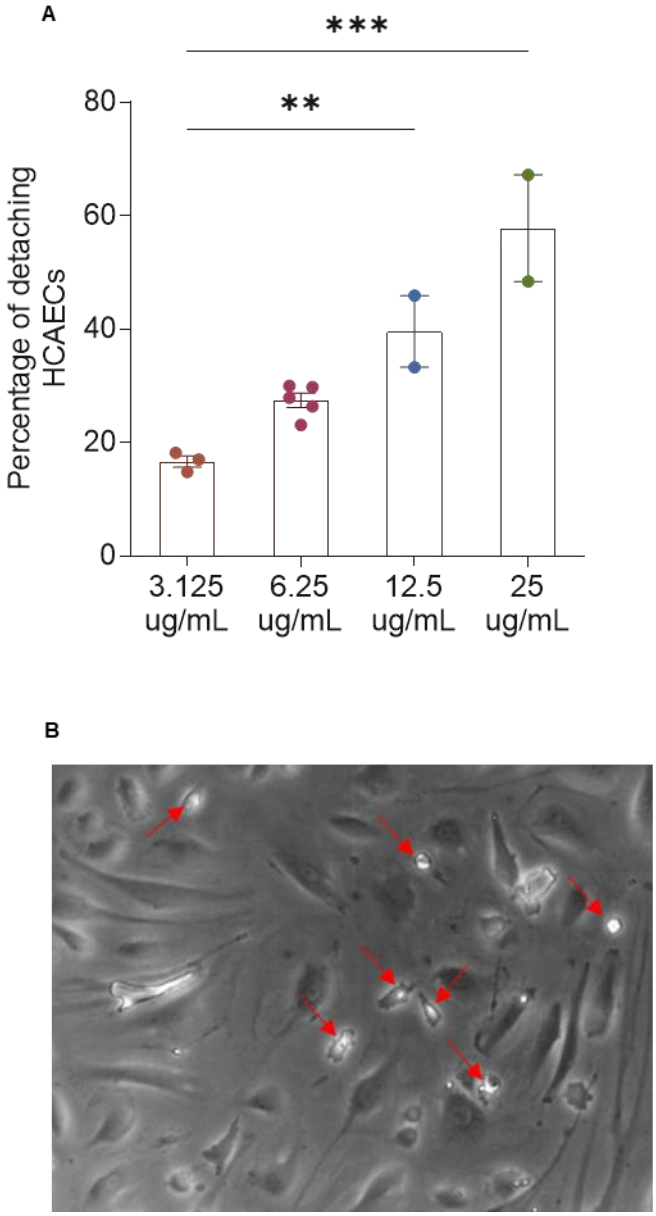


Figure 4. 6. Optimisation of trypsin-EDTA concentration for detachment assay. *Human coronary artery endothelial cells (HCAECs) were cultured statically on 70 µg/mL collagen type III for 24 hours prior to washing with PBS and incubation with trypsin-EDTA for 3 min to induce detachment. HCAECs were washed gently with fresh MV2 cell culture media to inhibit residual trypsin and imaged by phase contrast microscopy. Detaching cells quantified from 3 fields of view. No significant increase in detachment in 6.25µg/mL vs 3.125µg/mL (p=0.147).* ***(A)*** *Percentage of detaching cells presented for four different trypsin-EDTA concentrations, n=2-5.* ***(B)*** *Detaching cells (red arrows) identified as rounded up highly refractile cells. Data expressed as mean percentage of detaching HCAECs ±SEM. Statistical significance assessed by one-way ANOVA with Dunnett's multiple comparisons test. Control condition = 3.125 µg/mL condition. **=p<0.01, ***=p<0.001.*

4.4.5 Microvesicles from low-density stimulated neutrophils induce human coronary artery endothelial cell detachment

Following determination that NMVs contain active proteases, it was hypothesised that NMVs induce detachment of HCAECs from the ECM. HCAECs were statically cultured on collagen III before treatment with NMVs (section 2.17)**.** Negative control HCAECs treated with NMV supernatant exhibited a low percentage of detaching cells. NMVs increased the percentage of detaching HCAECs with 60 NMV/µL, 120 NMV/µL and 240 NMV/µL treatment conditions resulting in a dose-dependent increase in detaching HCAECs. Treatment with 240 NMV/µL significantly increased detaching HCAECs (mean =23.84%, SEM±3.02, n=7) compared with supernatant control (mean =9.11%, SEM±0.88, n=7, p=0.0003) and 60 NMV/µL (mean =13.88%, SEM±1.38, n=5, p=0.04) conditions (figure 4.7 A). It was still possible that whilst NMVs were increasing the detachment of HCAECs, they were not necessarily doing so through the action of their proteases. The possibility remained that NMVs induced some other HCAEC dysfunction predisposing them to detachment. To ascertain whether detachment was specifically the result of the action of proteases, TIMP-1, an inhibitor of MMPs, was included alongside 240 NMV/µL. When TIMP-1 was added to the 240 NMV/µL condition the percentage of detaching HCAECs significantly decreased (mean =10.3%, SEM±2.4, n=4, p=0.0052) compared with 240 NMV/µL only condition (mean =23.84%, SEM±3.02, n=7) returning the percentage of detaching HCAECs to levels comparable to the supernatant control (figure 4.7 A). To give further confidence that our assay was working correctly and to confirm MMPs found in NMVs could induce HCAEC detachment I included a recombinant human MMP-9 condition. 2.5 units/mL MMP-9 induced detachment comparable with the 120 NMV/µL condition. However, due to significant variability, significance was not reached compared to negative control.

I speculated that a potentially significant number of HCAECs may have become detached in response to MMP-9 prior to the addition of trypsin, therefore distorting our results. However, when total numbers of HCAECs were quantified, no significant difference was observed between any conditions (figure 4.7 B).

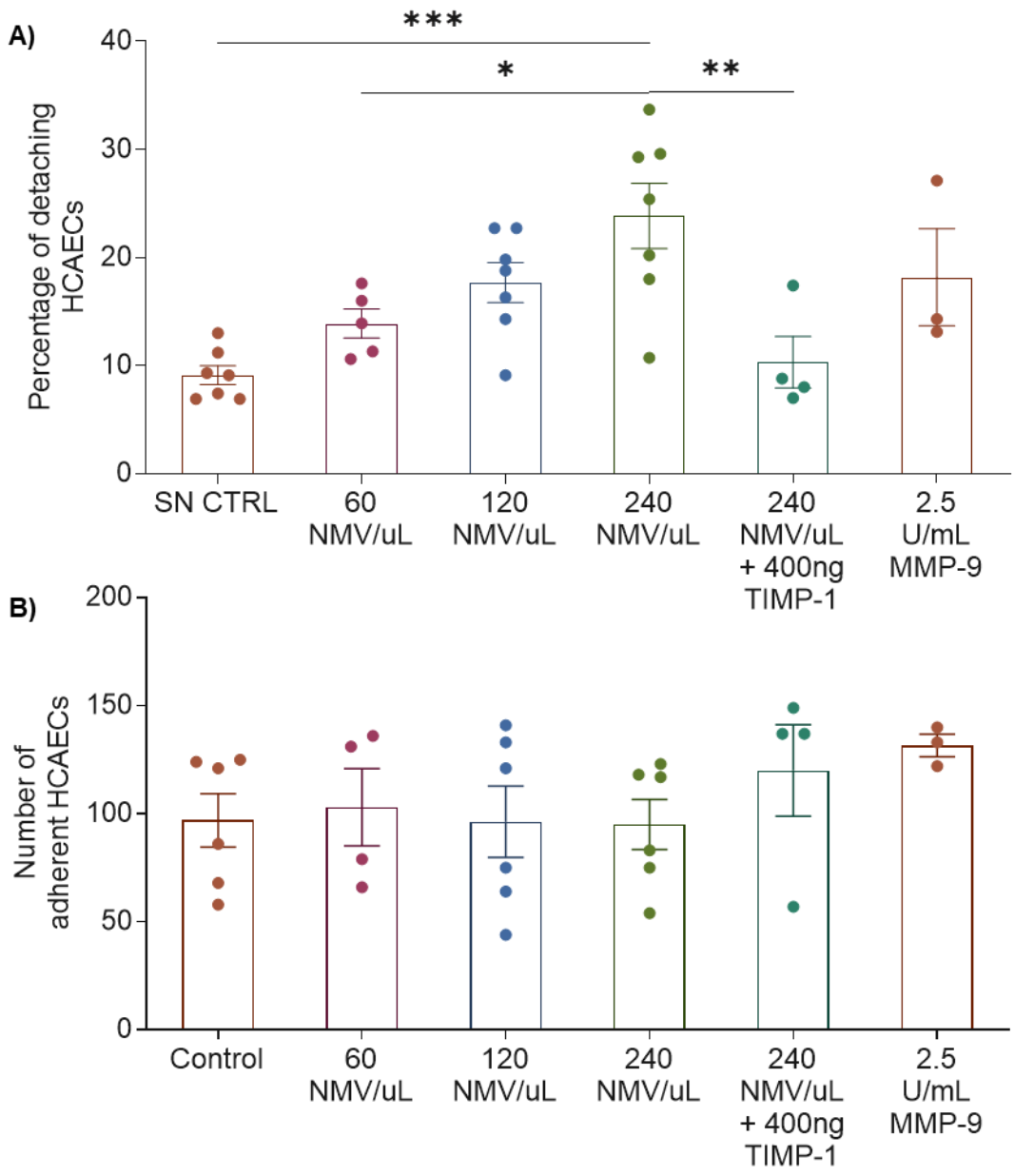


Figure 4. 7.Neutrophil microvesicles induce human coronary artery endothelial cells detachment. *Human coronary artery endothelial cells (HCAECs) were cultured on 70µg/mL collagen type III before treatment with increasing concentrations of neutrophil microvesicles (NMVs). HCAECs were treated with 6.25 µg/mL trypsin-EDTA to induce detachment.* ***(A)*** *Detaching HCAECs were quantified by widefield phase-contrast microscopy and* ***(B)*** *total number of HCAECs quantified in each condition, n=3-7. Data expressed as mean ±SEM. Statistical significance assessed by one-way ANOVA with Tukey's multiple comparisons test.*

4.4.6 Human coronary artery endothelial cell detachment under atheroprone conditions

Statically culturing HCAECs does not represent the *in vivo* conditions found around atherosclerotic plaques. The plaque apex experiences UHSS whilst the downstream plaque shoulder experiences LOSS. This LOSS promotes EC dysfunction (see section 4.1.). To model the region downstream of plaques, HCAECs were cultured under low oscillatory flow conditions prior to incubation with NMVs. Incorporation of low molecular weight hyaluronan (LMW-HA) into collagen type III matrix allowed for further investigation into the effect of ECM components found in eroded plaques.

Treatment with NMVs increased HCAEC detachment when cultured on both collagen type III and collagen type III + LMW-HA compared to supernatant treated controls. HCAECs cultured on collagen III under LOSS demonstrated greater detachment than observed in static experiments (see section 4.4.6) in both the supernatant treated control and NMV condition, indicating LOSS increases the propensity for HCAECs to detach.

HCAECs cultured on collagen type III + LMW-HA demonstrated a greater baseline level of detachment in the supernatant treated control indicating LMW-HA may promote HCAEC detachment. When cultured on LMW-HA HCAECs demonstrated significantly increased detachment (mean =34.7%, SEM±3.5, n=3, p=0.039) following NMV treatment (figure 4.8A), but no significant difference was observed when HCAECs were cultured on collagen III only (figure 4.8B). Similar percentage increases in detachment following NMV treatment were observed in both conditions implying the LMW-HA does not increase the ability of NMVs to exacerbate HCAECs detachment.

These data summarised in figure 4.8 demonstrate that LOSS, LMW-HA and NMVs increase the propensity for HCAECs to detach from a collagen III matrix.

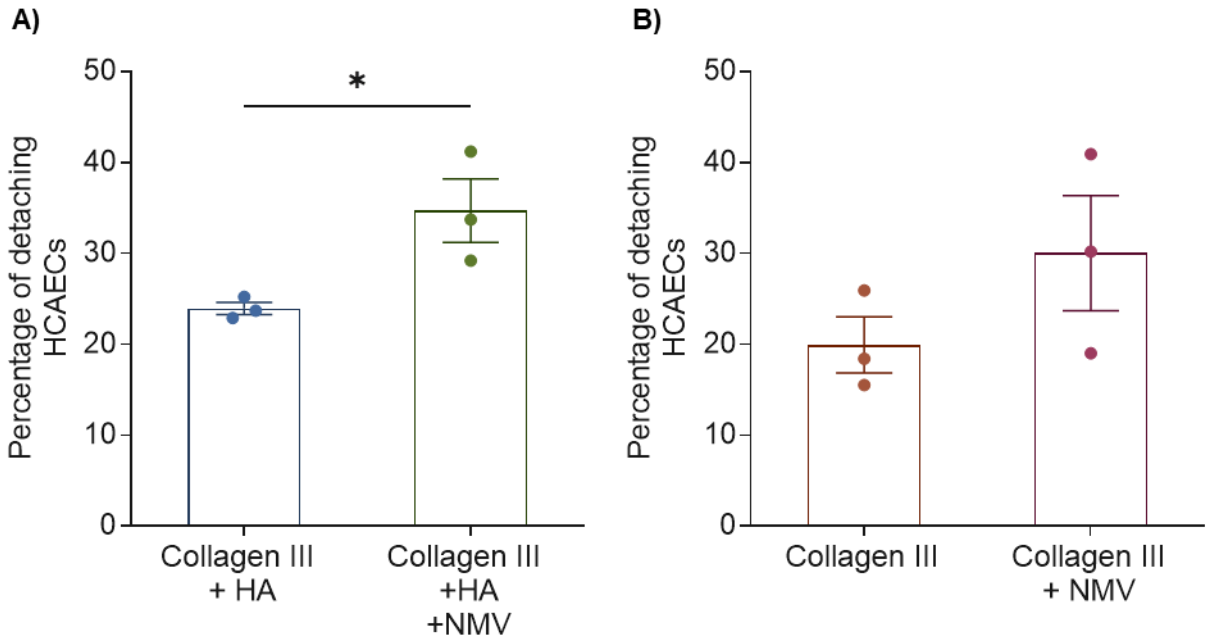


Figure 4. 8. Neutrophil microvesicles induce detachment of human coronary artery endothelial cells under low oscillatory shear stress. *Human coronary artery endothelial cells (HCAECs) were cultured on 70 µg/mL collagen type III or 70 µg/mL collagen type III + 5 µg/mL low molecular weight hyaluronan (LMW-HA) for 24 h prior to exposure to oscillatory shear stress at 4 dyn/cm^2 for 72 h. HCAECs were then treated with neutrophil microvesicles (NMVs) for 24 h. Incubation with 240 NMV/µL induced increased the percentage of HCAEC detachment in* ***(A)*** *the collagen type III (n=3) and* ***(B)*** *collagen type III + LMW-HA (n=3) conditions compared to supernatant controls. HCAECs were imaged by widefield phase contrast microscopy. Data expressed as mean ±SEM. Statistical significance assessed by unpaired t-test. *=p≤0.05.*

4.4.7 Neutrophil microvesicles increase human coronary artery endothelial cell apoptosis

HCAEC viability relies heavily on the interactions with the ECM. If these interactions are disrupted this can induce anoikis, a form of programmed cell death induced when adherent cells lose contact with the ECM. Prrevious data presented in this thesis suggests NMVs contain active proteases (section 3.4.5 – 3.4.6) and can induce HCAEC detachment (section 4.4.5) from a collagen substrate. Additionally, previous work indicated NMVs increase NF-κB activation in ECs via delivery of mir-155 (Gomez et al., 2020). Mir-155 reduces the expression of BCL6 which is a negative regulator of NF-κB leading to enhanced NF-κB activation. NF-κB as a transcription factor modulates the expression of many genes and whilst it is generally regarded as involved in anti-apoptotic responses there is evidence to suggest NF-κB activation is, in some instances, pro-apoptotic (Radhakrishnan and Kamalakaran, 2006; Ryan et al., 2000). It was therefore hypothesised that NMVs increase apoptosis in HCAECs. Statically cultured HCAECs were incubated with NMV supernatant, 2,000 NMV/µL or 1µM staurosporine (positive control) for 20 hours before analysis by flow cytometry for the presence of active caspase-3/7. Staurosporine is a positive control as it reliably induces apoptosis in ECs. NMV treatment significantly increased the percentage of caspase-3/7-positive HCAECs (mean =515.7% increase, SEM±88.45, n=4, p=0.0011) compared to supernatant controls (figure 4.9 A) as well as significantly increasing caspase-3/7 median fluorescence intensity (MFI) (mean =82.5% increase, SEM±18.84, n=4, p=0.0066) compared to supernatant treated controls (figure 4.9A).

Of course, in the vasculature ECs are not exposed to static conditions, rather they are exposed to complex shear stress patterns as a result of the fluid dynamics governing blood flow over a plaque and close to arterial bifurcations. Blood flow in atheroprone regions exerts low and oscillatory shear stress (LOSS) upon ECs, inducing expression of genes promoting atherogenesis and apoptosis (Demos et al., 2020; Jenkins et al., 2013; Rocha et al., 2018). HCAECs were cultured under LOSS (4dyn/cm^2) to better model the environment of ECs at the downstream plaque shoulder and determine if the pro-apoptotic effect of NMVs proved additive to the pro-apoptotic effect of LOSS or was masked by it.

The percentage of caspase-3/7-positive HCAECs increased following treatment with NMVs compared to supernatant treated controls, however this did not reach statistical significance (figure 4.9 B). Addition of both NMV and LMW-HA significantly increased the percentage of caspase-3/7-positive HCAECs (mean =79.8% increase, SEM±9.7, n=3, p=0.016) (figure 4.9B). Median fluorescence intensity (MFI) also increased following NMV treatment, however again this did not reach statistical significance.

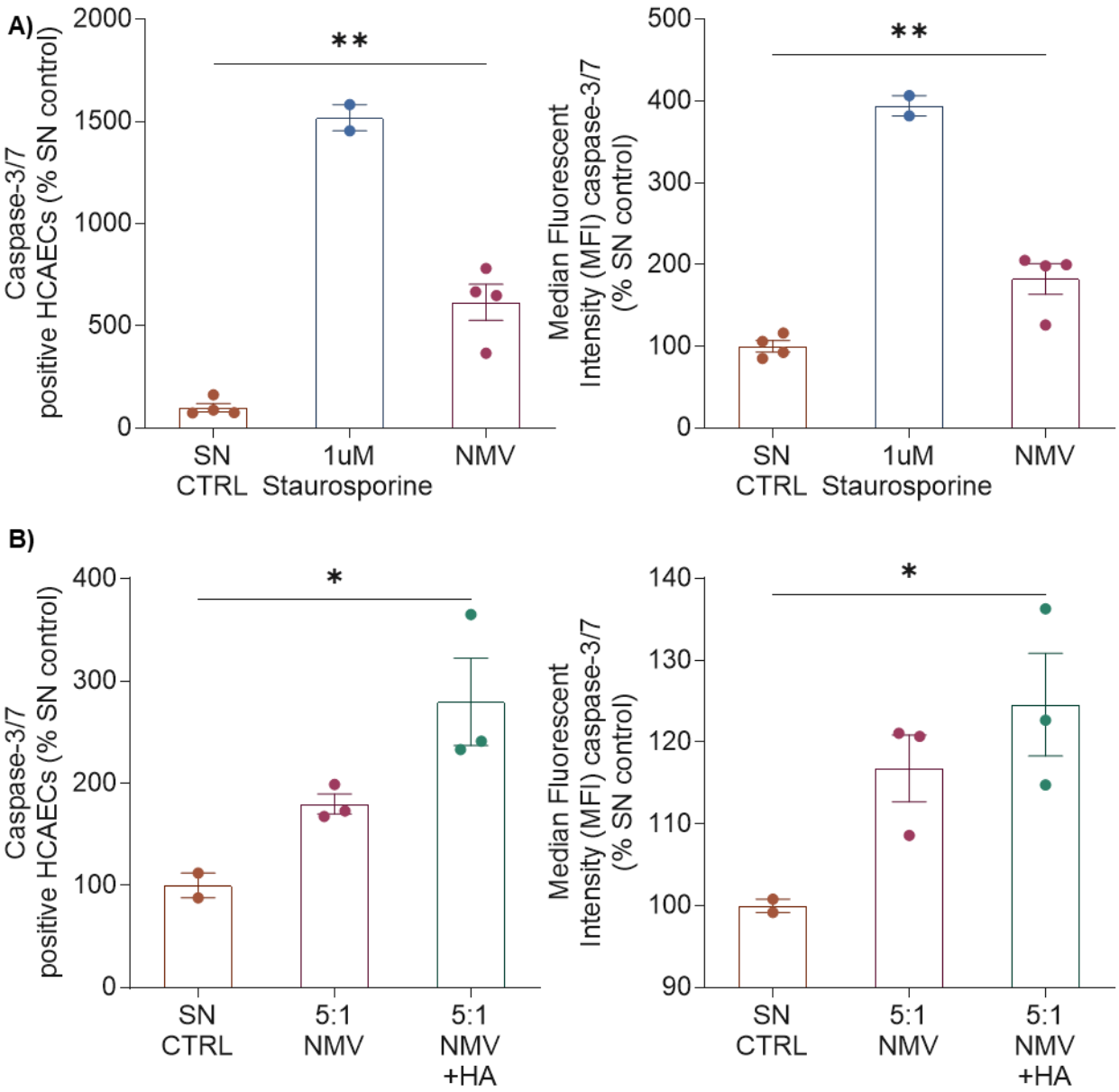


Figure 4.9. Neutrophil microvesicles induce apoptosis in human coronary artery endothelial cells. (A) *HCAECs cultured statically prior to treatment with supernatant control or neutrophil microvesicles (NMVs) for 20 h. (Left) Percentage of caspase-3/7 positive HCAECs and (right) median fluorescence intensity (MFI), n=2-4.* ***(B)*** *HCAECs cultured under low oscillatory shear stress for 72 h prior to addition of supernatant or NMVs for 20 h. Apoptosis assessed by measuring the (left) percentage of caspase-3/7 positive HCAECs and (right) MFI by flow cytometry, n=2-3. Data analysed using FlowJo software. Data expressed as mean ±SEM and assessed for significance using a one-way ANOVA with Dunnett's test. Control condition = SN CTRL * = P <0.05, ** = P<0.01.*

Incorporation of LMW-HA into the collagen type III matrix alongside NMV treatment significantly increased apoptosis compared to supernatant treated HCAECs on a collagen type III matrix only (mean =24.6% increase, SEM±6.28, n=3, p=0.0369).

4.4.8 NMVs reduce proliferation in statically cultured human coronary artery endothelial cells

It is likely that ECM degradation, EC detachment and apoptosis promote erosion whilst proliferation and migration of ECs compensate and protect the plaque from erosion. Having determined that NMVs increase HCAEC detachment and apoptosis, the effect of NMVs on HCAEC proliferation was investigated.

HCAECs were cultured under static conditions with supernatant control or varying concentrations of NMVs and measured proliferation by staining with a fluorophore conjugated Ki-67 antibody and imaging with widefield fluorescence microscopy. Proliferation was presented as the percentage of HCAECs positively labelled for Ki-67. HCAECs treated with supernatant control were the most proliferative. Proliferation decreased in a dose-dependent manner as the NMV concentrations increased with proliferation being significantly decreased in the 960,000 NMV/mL condition (mean =48.7% decrease, SEM±4.8, n=4, p=0.0037) compared with supernatant treated control (figure 4.10).

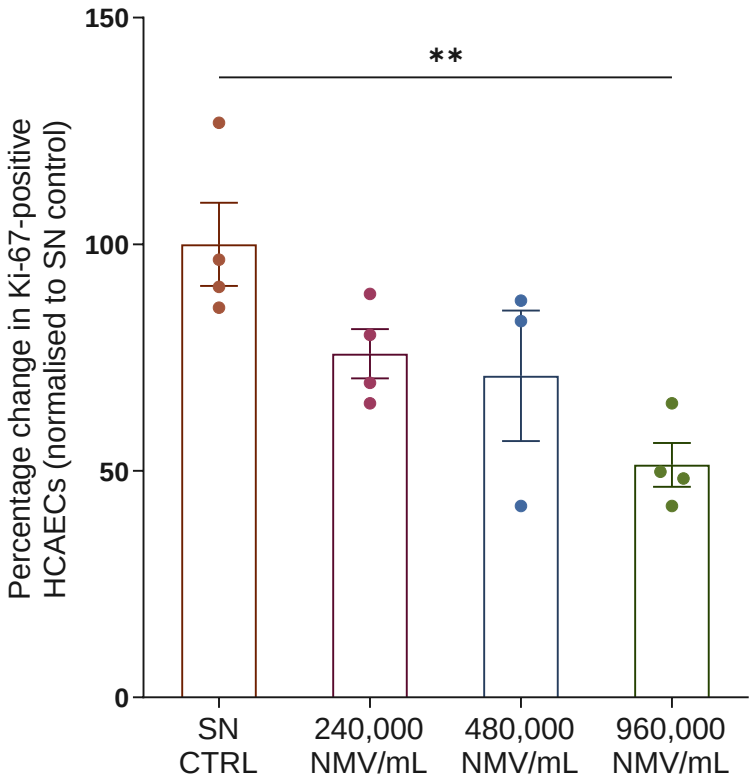


Figure 4. 10. Neutrophil microvesicles reduce proliferation in human coronary artery endothelial cells under static conditions. *Human coronary artery endothelial cells (HCAECs) were cultured statically on 70 µg/mL collagen III for 24 h prior to treatment with neutrophil microvesicles (NMVs) for a further 24 h. Proliferation was assessed by staining for Ki-67 and imaging via fluorescence microscopy. Data expressed as mean percentage proliferation normalised to the superntant control ±SEM, n=3. Statistical significance assessed by ordinary one-way ANOVA with Dunnett's test. Control condition = SN CTRL. ** = p<0.01.*

4.4.9 Human coronary artery endothelial cell proliferation under atheroprone conditions

Having determined that NMVs decrease proliferation in statically cultured HCAECs I next investigated if NMVs caused similar effects in HCAECs cultured under atheroprone flow conditions. Confluent HCAEC monolayers were cultured in 0.4 luer μ-slides and exposed to atheroprone flow for 72 hours prior to treatment with supernatant or NMVs.

No statistically significant difference was observed in the percentage of proliferating HCAECs in either condition with percentages of proliferating HCAECs very low in both conditions (figure 4.11). Fully confluent monolayers of HCAECs were necessary in the μ-slides to ensure adherence under flow and contact inhibition, the process whereby cell cycle arrest occurs in a confluent monolayer, greatly reduces endothelial cell proliferation making any further decrease induced by NMV treatment difficult to observe.

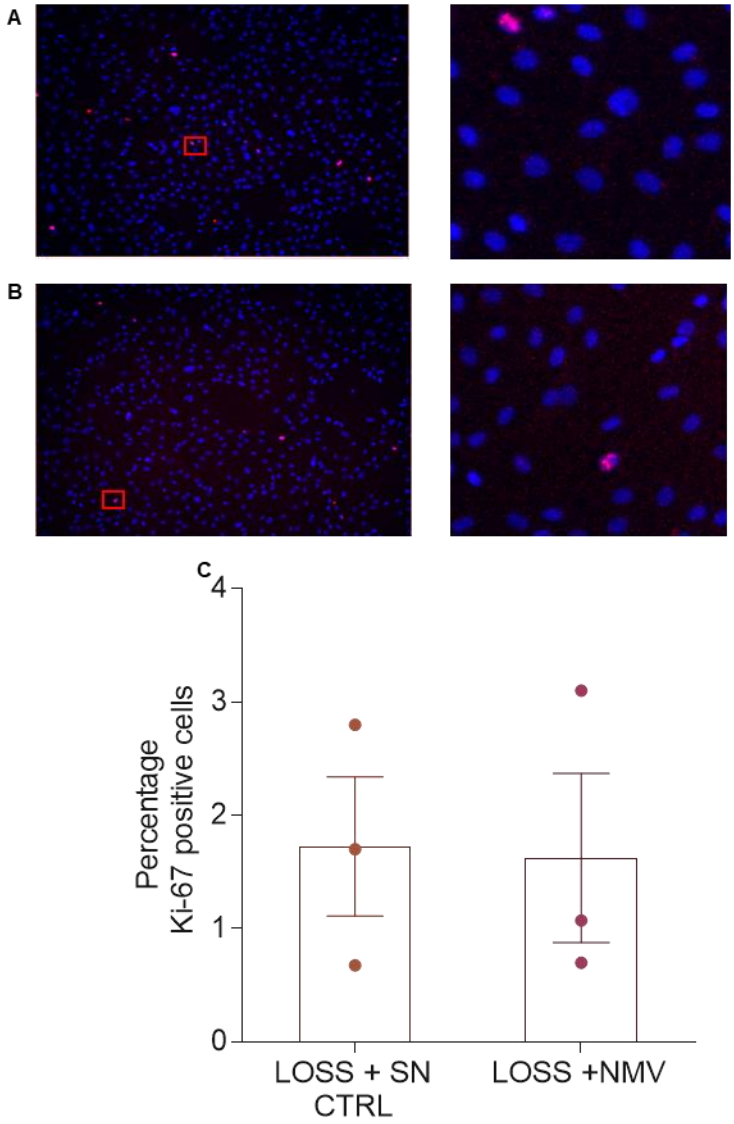


Figure 4. 11. .Human coronary artery endothelial cell proliferation under low oscillatory shear stress. *Human coronary artery endothelial cells (HCAECs) were seeded on 70ug/mL collagen type III and cultured statically for 24 h prior to exposure to low oscillatory shear stress (LOSS) for 72 h. Neutrophil microvesicles (NMVs) or supernatant control were added for a further 24 h under low oscillatory shear stress. HCAECs were fixed in 4% paraformaldehyde and labelled with DAPI and anti-Ki-67 antibody. Representative images of* ***(A)*** *supernatant treated and* ***(B)*** *NMV treated HCAECs. Right image shows enlarged image. HCAECs exposed to HCAECs were imaged by widefield fluorescence microscopy and analysed by ImageJ software.* ***(C)*** *Percentage of HCAECs displaying Ki-67 staining. Data expressed as mean ±SEM, n=3.*

4.4.10 Neutrophil microvesicles impair human coronary artery endothelial cell wound recovery response

Whilst processes such as extracellular matrix degradation and endothelial cell apoptosis which contribute to the removal of endothelial cells overlying a fibrous plaque are important in plaque erosion, it is likely that the impairment of processes protective against erosion may also contribute. Following erosion of endothelial cells from the plaque, the underlying pro-thrombotic extracellular matrix is exposed to blood flow, raising the risk of thrombosis. When endothelial cells encounter a 'wound' that exposes their underlying matrix, they migrate to the opposing cell front to close the gap (figure 4.12). If this ability to recover a wound is impaired, the matrix remains exposed for longer, increasing the risk that a thrombotic event will occur. I hypothesised that NMVs impair the wound recovery response of HCAECs cultured under static conditions.

Gaps were created in HCAEC monolayers by placing wound healing assay inserts (*Ibidi, Germany*) into a 6 well plate as described in section 2.20 of the methods chapter. HCAECs were then cultured with NMVs or supernatant for 16 hours at 37^{o}C and 5% CO_2 and imaged every 30 minutes by automated time-lapse phase-contrast microscopy. Wound recovery was assessed as the percentage of cell gap area closed over time.

Initially, the wound recovery response was comparable in both conditions, but from 5 hours onwards the rate of wound recovery observed in the two populations of HCAECs began to diverge. Supernatant-treated HCAECs maintained a linear trend whilst the rate of wound recovery in NMV-treated HCAECs began to decrease. After 16 hours NMV-treated HCAECs had closed a significantly smaller percentage of the cell gap (mean =59.34%, SEM±3.41, n=4, p=0.0058) compared to supernatant-treated HCAECs (mean =76.04%, SEM±2.08, n=4) as assessed by an unpaired t-test. 16 hours was chosen as the time point for measurement as it consistently fell within the linear phase of wound recovery in supernatant control HCAECs. These data summarised in (figure 4.12) suggest NMVs impair the ability of HCAECs to close a cell gap and repair a wound.

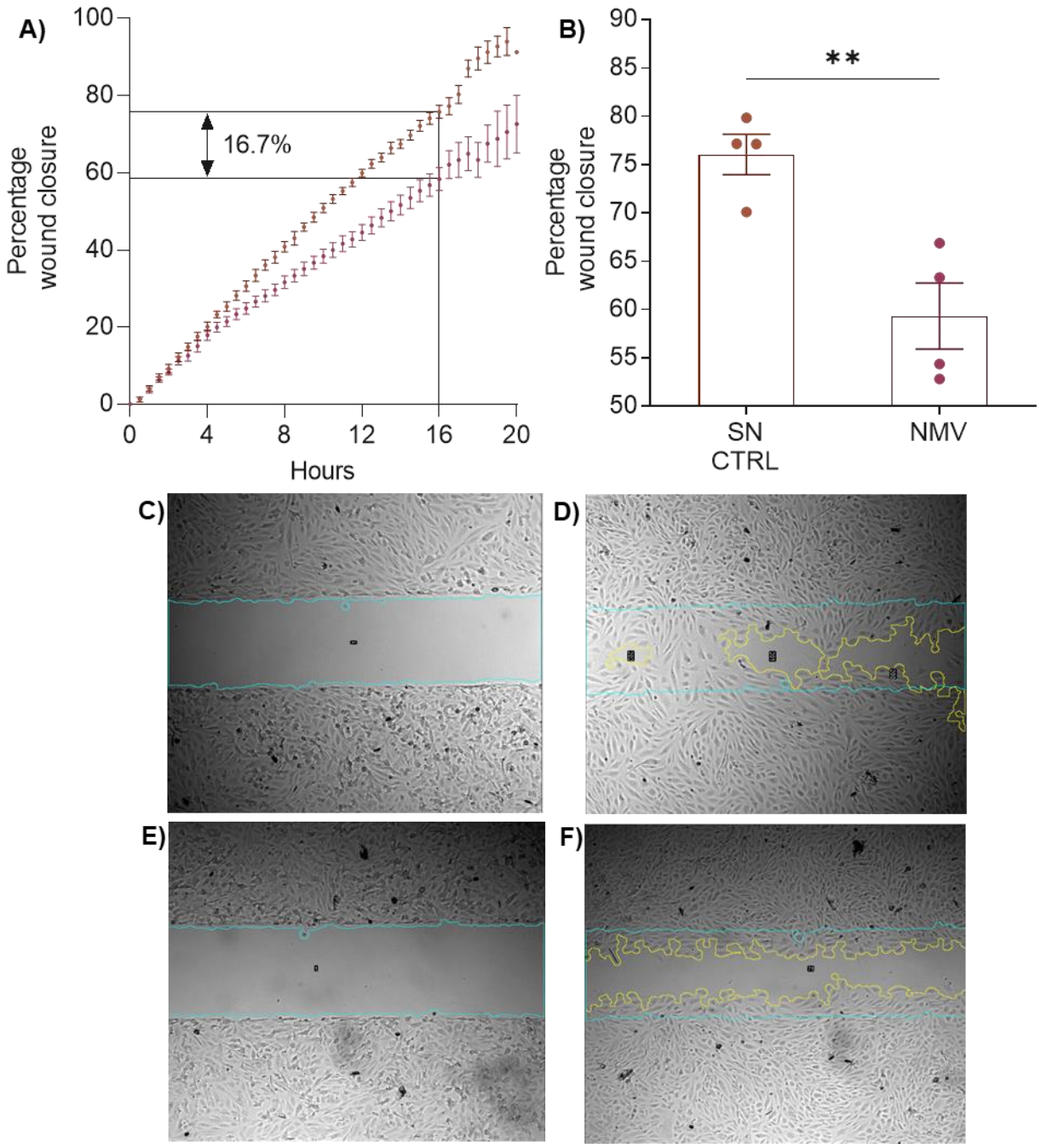


Figure 4. 12. Neutrophil microvesicles impair the wound healing response of human coronary artery endothelial cells. *Human coronary artery endothelial cells (HCAECs) were cultured statically in cell culture inserts either with neutrophil microvesicles (NMVs) or supernatant control. Cell inserts were removed to leave a cell gap and HCAECs were imaged over 20 h by time-lapse phase contrast microscopy.* ***(A)*** *Percentage of wound area closed by supernatant treated and NMV-treated HCAECs over 20 hours. Data expressed as mean ±SEM, n=4. A time-point consistently within the linear range was chosen (16 hours) and compared.* ***(B)*** *Percentage of wound area closed by supernatant-treated and NMV-treated HCAECs after 16 hours. Data expressed as mean ±SEM, n=4. Representative wound healing response by supernatant treated HCAECs at* ***(C)*** *0 h* ***(D)*** *and 16 h. Representative wound healing response by NMV-treated HCAECs at* ***(E)*** *0 h and* ***(F)*** *16 h. Statistical significance assessed by unpaired t-test ** P=≤0.01. Link to video files: https://drive.google.com/drive/folders/1FqpcyhkjI8Ed3YMn9v1Y-f0y9dXG_Kzs?usp=sharing*

4.4.11 Results summary

In conclusion, the data presented in this chapter demonstrates NMVs promote *in vitro* loss of HCAECs and reduce the ability of HCAECs to recover from this loss.

- NMVs degrade analogues of the ECM in an MMP-9 dependent manner
- NMVs increase the propensity for HCAECs to detach under static and atheroprone flow conditions
- NMVs increase HCAEC apoptosis under static and atheroprone flow conditions
- NMVs reduce HCAEC proliferation under static conditions
- NMVs reduce HCAEC wound healing response

4.5 Discussion

4.5.1 Neutrophil microvesicles increase extracellular matrix degradation and endothelial detachment

Having established NMVs from both unstimulated and nLDL-stimulated neutrophils both contain active proteases, I aimed to determine if there was an overall difference in the capacity of both populations to degrade analogues of the ECM. For this Iused fluorescently labelled gelatin. I identified a dose-dependent increase in degradation in NMVs from both unstimulated and nLDL stimulated neutrophils. Co-incubation of NMVs with tissue inhibitor of metalloproteinase 1 (TIMP-1), an irreversible inhibitor of a broad range of MMPs, significantly reduced gelatin degradation implicating MMPs as significant factors in ECM degradation. Previous results in the present study identified MMP-8 and MMP-9 in NMVs from nLDL-stimulated neutrophils (section 3.4.4), both are potent enzymes involved in matrix degradation and have been linked with plaque destabilisation (Peeters *et al.*, 2011; Sluijter *et al.*, 2006). Next, I determined that degradation of gelatin by HCAECs treated with NMVs was significantly higher than those treated with supernatant control. However, this increase was only marginally decreased by co-incubation with TIMP-1 suggesting a significant portion of the gelatin degrading proteases were of EC origin. It is possible that stimulation with NMVs increases expression of EC proteases and future experiments could be performed to investigate this.

Following on from the ECM degradation experiments, it was hypothesised that NMVs may facilitate the detachment of HCAECs, a key component of plaque erosion. There is evidence to support this hypothesis; MMP-9 has been previously shown to degrade junctional proteins between epithelial cells and decrease epithelial cell integrity (Butin-Israeli *et al.*, 2016) and neutrophils have been shown to promote EC detachment in an *in vitro* model of plaque erosion (Quillard *et al.*, 2015). HCAEC detachment was assessed using a previously published method (Quillard *et al.*, 2015). A higher proportion of statically cultured HCAECs demonstrated a propensity to detach following treatment with NMVs compared with HCAECs treated with supernatant controls (figure 4.7). This effect was dose-dependent and largely inhibited by incubation with TIMP-1. To better model HCAEC detachment within plaques and to better understand how ECM components affect HCAEC detachment, HCAECs were cultured on collagen type III supplemented with low molecular weight hyaluronan (LMW-HA) and exposed to LOSS. The ECM provides a substrate for endothelium to adhere, and the specific components of the ECM can modulate the function of ECs. ECs interact with the ECM via integrins and these interactions modulate EC responses to pathogenic stimuli, such as disturbed flow (Chen *et al.*, 2015). Kolodgie et al identified differences in the accumulation of particular ECM proteins in eroded plaques compared with stable and ruptured plaques. Eroded plaques demonstrated greater staining for HA, versican and collagen type III (Kolodgie *et al.*, 2002). CD44, the receptor for hyaluronan, was also identified at the interface between plaque and thrombus in eroded plaques (Kolodgie *et al.*, 2002). Further support for the role of HA in plaque erosion was provided by Pedicino et al who found increased expression of the enzyme hyaluronidase 2 (HYAL2), responsible for generating pro-inflammatory low molecular weight HA (LMW-HA), in peripheral blood mononuclear cells (PBMCs) of patients with plaque erosion compared with patients with rupture (Pedicino *et al.*, 2018). Patients with eroded plaques also showed greater expression of the splicing variant of CD44 known as CD44v6 (Pedicino *et al.*, 2018). Interestingly, when HCAECs were cultured on collagen type III under LOSS the percentage of detaching cells increased compared with statically cultured HCAECs. The percentage of detaching HCAECs increased further when cultured on collagen type III supplemented with LMW-HA (figure 4.8). Regardless of substrate, the proportion of detaching cells increased following treatment with NMVs. Together, these data indicate that NMVs promote HCAEC detachment, via the degradation of the collagen matrix by MMPs, a process exacerbated by the LMW-HA rich eroded plaques. LMW-HA is known to be pro-inflammatory and to induce EC dysfunction via TLR2 stimulation, likely creating an environment in which ECs are prone to detachment (Quillard *et al.*, 2015). Some investigations into local fluid dynamics of eroded plaques have identified regions of ultra-high shear stress (UHSS) at the plaque apex as the stimulus for thrombus formation (McElroy *et al.*, 2021) whilst alternative studies have correlated high oscillatory shear index (OSI) with plaque erosion (Thondapu *et al.*, 2021). Previous work by our group has shown NMVs preferentially adhere to ECs under low shear stress and not under shear stresses above 10 dyn/cm^2 (appendix fig 1). This rules out the prospect of NMVs influencing the plaque apex. However, whilst thrombus formation may initially propagate from these UHSS regions the

dysfunction of downstream endothelium may play a crucial role in the propagation of these thrombi. EC detachment and subsequent exposure of pro-thrombotic collagens downstream from the plaque may exacerbate initial thrombosis.

4.5.2 Endothelial cell apoptosis induced by neutrophil microvesicles

Whilst our data supported the hypothesis that degradation of the ECM by MMPs within NMVs promoted HCAEC detachment, it was important to investigate alternative mechanisms through which NMVs may be inducing HCAEC dysfunction and subsequent detachment. One potential mechanism was apoptosis. Durand et al identified apoptosis as a relevant process in thrombosis demonstrating that induction of EC apoptosis in rabbit femoral arteries promoted endothelial detachment and thrombosis compared to saline treated control arteries (Durand *et al.*, 2004) and this has since been replicated (Xu *et al.*, 2009). Further studies have linked apoptosis specifically with plaque erosion; for example Franck et al showed via immunohistochemistry of human plaques that the rate of apoptosis is greater in erosion-prone plaques compared to rupture-prone plaques and further to this presented data correlating neutrophil adherence to erosion-prone plaques with this increased rate of EC apoptosis (Franck *et al.*, 2017). Previous work by our group has shown NMVs can adhere to ECs via interactions between CD18 and ICAM-1, preferentially under LOSS conditions (Gomez *et al.*, 2020). I aimed to investigate if NMVs could induce HCAEC apoptosis under both static and LOSS conditions. Our data suggests NMVs significantly increase apoptosis in HCAECs under both flow conditions (4.4-8). In static HCAECs the percentage increase in apoptosis following NMV treatment rose over 500% compared to supernatant controls. Interestingly, under LOSS the percentage increase was 79% (figure 4.9). This may be explained by the greater absolute percentage of apoptosis in the supernatant control under LOSS compared to static supernatant controls. Under LOSS conditions that already promote apoptosis (Chaudhury *et al.*, 2010; Tricot *et al.*, 2000) the pro-apoptotic effects of NMVs may have been masked. Nevertheless, a significant increase in apoptosis was observed following treatment. Culturing HCAECs on a matrix containing LMW-HA further increased the rate of apoptosis above NMVs only. This supported our earlier data which showed increasing concentrations of LMW-HA induced HCAEC apoptosis under static conditions (figure 4.5) and previously published studies that showed LMW-HA and alternative TLR2 agonists, lipoteichoic acid and Pam3, induced EC apoptosis (Quillard *et al.*, 2015). The data presented here suggests LOSS and the presence of LMW-HA in eroded plaques likely predisposes HCAECs to apoptosis and that *in vitro*, this is exacerbated by NMVs. Previous work by our group demonstrated no effect of NMVs on EC apoptosis. However, there are some key differences that may explain this disparity. Firstly, the data presented in this thesis used NMVs generated in response to nLDL whereas the

data in Gomez *et al* used NMVs generated in response to fMLP. Secondly, HCAECs were incubated with NMVs from nLDL-stimulated neutrophils for 20 hours compared to just 2 and 4 hours and thirdly, apoptosis in this study was assessed via detection of active caspase-3/7 whereas Gomez *et al* used annexin V binding. In combination, these experimental differences may explain how different effects were observed.

4.5.3 Neutrophil microvesicles reduce wound healing response in human coronary artery endothelial cells

Cellular processes that act to close gaps in the endothelium created when ECs detach are likely to be protective against plaque erosion. These processes include proliferation and EC wound healing response. Significant increases in these processes could negate the effects of enhanced EC loss. Indeed, disturbed flow results in increased EC proliferation compared to laminar flow perhaps to counteract a higher EC turnover in these regions (Obikane *et al.*, 2010; Wang *et al.*, 2021). There is little evidence of any effect of neutrophils on EC proliferation. However, neutrophil extracellular traps (NETs) have been demonstrated to increase proliferation of pulmonary endothelial cells as well as EC motility (Aldabbous *et al.*, 2016). Whilst not a direct comparison, our data contradicts these findings, instead indicating NMVs result in reduced proliferation in statically cultured HCAECs (figure 4.10). Culturing HCAECs under static conditions was not ideal due to the effect shear stress has on EC proliferation (Sedlak and Clyne, 2020; Warboys *et al.*, 2014). An attempt was made to assess proliferation under LOSS however, due to the need to culture HCAECs to confluency to maintain adherence following the application of shear stress and the fact that confluency largely inhibits HCAEC proliferation it was not possible to detect proliferation.

Our data indicates NMVs inhibit HCAEC wound healing response after a short pre-incubation. This is supported by previous work that has found NMVs, produced in response to fMLP stimulation, inhibit wound healing by delivering myeloperoxidase (Slater *et al.*, 2017). Furthermore, Butin-Israeli et al recently demonstrated that NMVs produced in response to fMLP, IFN-γ and TNF-α were sufficient to promote double strand DNA breaks and impaired DNA synthesis in intestinal epithelial cells (IECs). MVs contained abundant miR-23a and miR-155 which they specifically delivered to IECs to downregulate LB1 and RAD51 (Butin-Israeli *et al.*, 2019). Subsequent inhibition of miR-23a and miR-155 significantly reduced mucosal wound healing response (Butin-Israeli *et al.*, 2019). The investigators in these studies used epithelial cells and isolated neutrophil MVs using higher centrifugal forces than necessary for MVs, likely leading to inclusion of exosomes as well of MVs. Nevertheless, these studies support our findings that NMVs impair wound healing responses, a

potentially relevant process to plaque erosion. It is important to note that proliferation of cells was not inhibited during the wound healing experiments, therefore some of the reduction observed in wound recovery could be accounted for by reduced proliferation. Counter to this, visual inspection of the wound healing videos revealed very few ECs at the wound edge divided in either the NMV-treated or control groups suggesting proliferation is likely a small contributor to the wound healing response observed in these experiments.

It is interesting to note that neutrophils have been reported to exert the opposite effect, instead acting to enhance the wound healing response in epithelial cells (Sumagin *et al.*, 2016). Whilst NMVs are released by activated neutrophils there is evidence to suggest their effects may differ and this disparity in function may be explained by which factors are packaged into NMVs by the parent neutrophil. For example, in response to activation, neutrophils have been shown to upregulate expression of miR-103; however miR-103 was not detected in released MVs (Butin-Israeli *et al.*, 2019).

4.5.4 Conclusion

The data presented in this chapter demonstrate NMVs are capable of increasing key functions of HCAECs that promote erosion. For instance, NMVs degrade analogues of the ECM and promote the loss of HCAECs by promoting their propensity to detach in an MMP-dependent manner as well as increasing apoptosis. Detachment and apoptosis were further enhanced following culturing of HCAECs under atheroprone LOSS conditions on a matrix incorporating the erosion specific protein LMW-HA, indicating characteristics of an eroded plaque may create an environment that already promotes HCAEC loss. NMVs also reduce the rate of HCAEC proliferation and speed of wound healing that could otherwise compensate for a greater HCAEC loss.

4.5.5 Limitations

As with all *in vitro* models there were significant limitations to this experimental approach. For instance, whilst peer-reviewed studies on the composition of the ECM at sites of plaque erosion were used to inform our decisions on what ECM components to use in our model, there was no information on specific quantities or even relative proportions of each component. We attempted to optimise the proportions of ECM components to ensure growth of a healthy endothelial cell monolayer however, it is quite possible that this was incorrect. In fact, it could be argued that an unhealthy endothelium is one of the key factors required for plaque erosion to occur and that this is not reflected in my model. In addition, other ECM components not mentioned in the literature or present in much smaller quantities could have been overlooked in my model. For instance, versican is present within the ECM beneath eroded plaques but because it is less abundant and is also present in the ECM

beneath ruptured plaques it was not included instead hyaluronan specifically found in eroded plaques was prioritised. It is possible interactions between versican and hyaluronan may have specific effects on ECs that this model would not elicit and therefore could not be measured. Related to this issue is the problem of recreating the morphology of a plaque in a 2-D environment. Peer-reviewed computational modelling experiments were used to inform the flow parameters chosen to most accurately recreate the conditions experienced by ECs downstream of a plaque however, these are unlikely to recreate the environment accurately. Additionally, the absence of other cell types, such as smooth muscle cells, mean the ECs were cultured in isolation which is far from the case *in vivo*.

In some cases, it was not possible to culture ECs under flow. For instance, in the wound healing assay the cell culture inserts were only compatible with cell culture plates and not the slides required for use with the Ibidi flow system. Measuring the response of ECs under static conditions may allow us to determine the effects of NMVs on wound response but it is not reflective of the *in vivo* environment and there is no guarantee that ECs behave the same way when exposed to flow.

With regard to the NMV preparations used in these experiments there are some limitations. As recommended in the MISEV2014 guidelines regarding EV experiments (Théry *et al.*, 2018) we compared the effect of our NMV preparation to an EV-depleted fluid (i.e the supernatant control) however we neglected to compare the effect of NMVs with MVs present in the final preparation originating from other cell types. Whilst I am confident the number of contaminating MVs was low due to low numbers of contaminating cells in my neutrophil preparations, the possibility of low numbers of MVs from another cell type having a disproportionately large effect on ECs can not be completely discounted. In future studies it would be beneficial to investigate the effects of MVs from other cell types to i) determine if the effect observed is due to NMVs and ii) whether this effect is specific to NMVs rather than a general trait of MVs.

5. Role of neutrophil microvesicles in regulating platelet activation and endothelial cell thrombogenicity

5.1. Introduction

Stable plaques in the vasculature can go undetected for many years and, in the majority of cases, only become symptomatic following thrombosis, when a significant portion of the vessel becomes obstructed. The process of thrombosis is, in part, governed by circulating platelets. Following activation platelets can adhere to the vessel wall and aggregate to form a thrombus. The soluble factors that initiate this process and the cellular pathways responsible for transducing these signals in platelets have been well characterised (Yun *et al.*, 2016). In the context of plaque erosion, it is not clear what initiates platelet recruitment.

MVs are produced continuously by cells of the immune system including neutrophils and therefore are present in the blood where they interact with a wide array of cell types. Of particular interest in the context pf plaque erosion is the interaction of NMVs with platelets due to the association between neutrophil-platelet interactions and thrombosis (Lisman, 2018). Platelets express P-selectin on their surface and neutrophils are known to express PSGL-1, a ligand for P-selectin. This interaction in combination with CD11b/CD18 facilitates adhesion between neutrophils and platelets (Maugeri *et al.*, 2009). These interactions activate neutrophils, leading to phagocytosis of activated platelets and generation of NETs (Clark *et al.*, 2007; Maugeri *et al.*, 2014). Previous work has established the presence of PSGL-1 on NMVs (Gomez *et al.*, 2020; Nolan *et al.*, 2008) suggesting the potential for NMVs to bind platelets. Numerous factors present in the circulation can initiate platelet activation including ADP, thrombin and tissue factor (TF) (Yun *et al.*, 2016). Neutrophils and NMVs have been reported to express TF (Darbousset *et al.*, 2012; Kambas *et al.*, 2014) indicating that NMVs also have the potential to regulate platelet activation.

The healthy endothelium plays a crucial role in preventing platelet recruitment by physically separating the prothrombotic subendothelial matrix from platelets in the bloodstream and expressing anti-thrombotic factors such as thrombomodulin and protein C (Van de Wouwer *et al.*, 2004; Wang *et al.*, 2018b). Thrombomodulin and protein C work in concert to reduce coagulation. Thrombomodulin binds to circulating thrombin, a potent pro-thrombotic factor, thus reducing circulating concentrations (Adams and Huntington, 2006). Protein C is activated when bound to thrombin and this activation is enhanced in the presence of thrombomodulin (Yang *et al.*, 2006). Protein C binds and cleaves pro-thrombotic factors Va and VIIIa (Liaw *et al.*, 2000). Reduced expression of these factors has been previously linked with atherosclerosis (Laszik *et al.*, 2001). However, activated ECs can also promote the recruitment of platelets through the expression of Von

Willebrand factor (VWF) (Lopes da Silva and Cutler, 2016) and TF (Solovey *et al.*, 2010). The anti-thrombotic effect of ticagrelor may be the result of reduced EC TF expression and activity (Reiner *et al.*, 2017). VWF has been known for decades as a potent activator of platelets through binding of GPIba (Kroll *et al.*, 1991).

Platelets express a variety of surface molecules involved in adhesion to activated ECs including platelet GPIb-IX-V or GPIIb/IIIa binding to EC VWF, and GPIbα binding EC intercellular adhesion molecule 1 (ICAM-1) (Bombeli *et al.*, 1998; Manon-Jensen *et al.*, 2016). P-selectin mediates platelet adhesion to leukocytes such as neutrophils and monocytes as well as having roles in stabilising platelet aggregates (Merten and Thiagarajan, 2000). P-selectin is stored within α-granules in resting platelets but is rapidly translocated to the surface following platelet activation by stimuli including ADP and thrombin. For this reason membrane expression of P-selectin is a commonly used marker of α-granule exocytosis (Linden, 2013). In addition to P-selectin, several other pro-thrombotic factors are stored and released by α-granules during platelet activation. These include VWF, fibrinogen and platelet factor 4 (PF4) (Maynard *et al.*, 2010).

The purpose of the work presented in this chapter was to explore the effects of NMVs on thrombosis by examining the impact they have on i) the direct binding to and activation of platelets and ii) the activation of ECs to promote platelet recruitment.

5.2. Hypothesis and Aims

It was hypothesised that NMVs promote thrombosis by binding to and activating platelets and by promoting pro-thrombotic EC gene expression changes (figure 5.1).

The work presented in this chapter addresses 5 aims:

Aim 1 – To determine if NMVs form complexes with resting platelets

Aim 2 – To determine if NMVs promote platelet P-selectin expression

Aim 3 – To determine if NMVs promote platelet aggregation

Aim 4 – To determine if NMVs promote pro-thrombotic gene expression in ECs

Aim 5 – To determine if NMV treatment enhances platelet adhesion to ECs

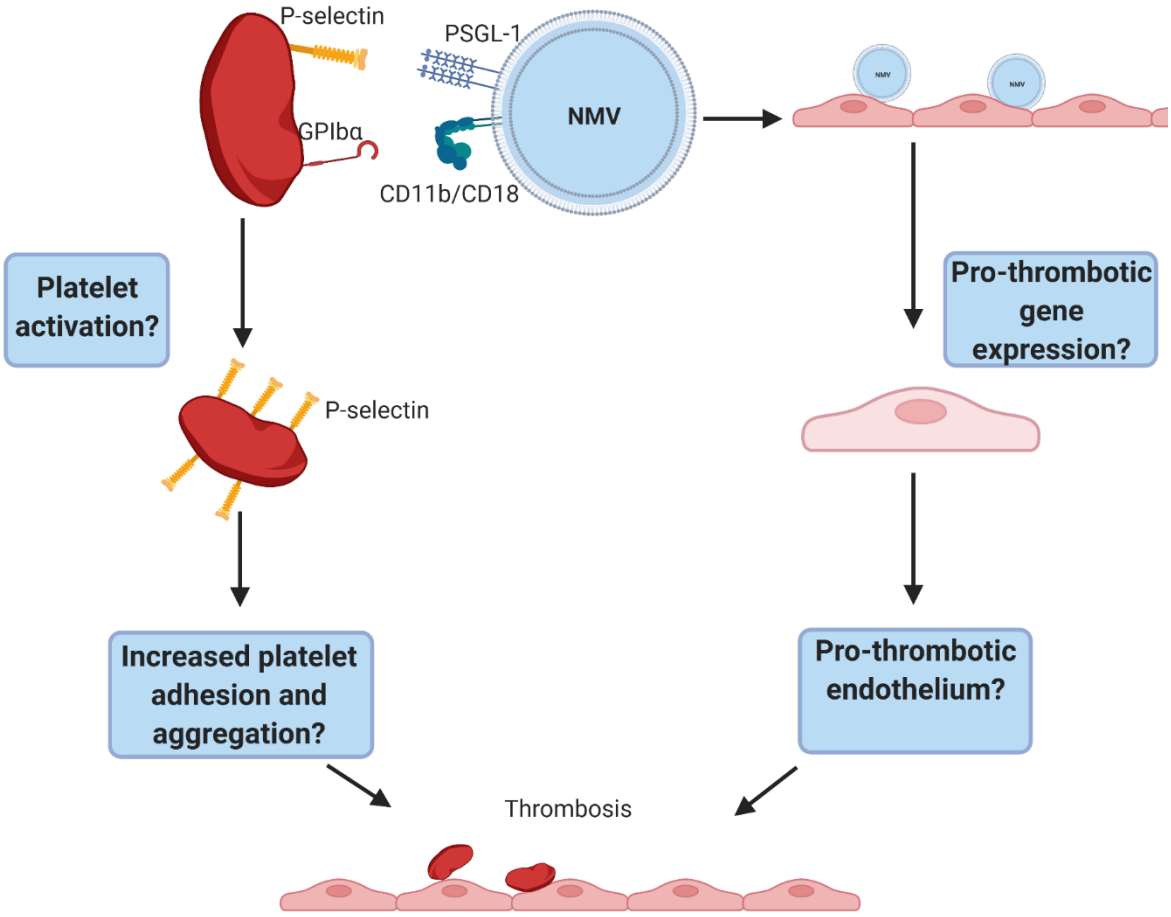


Figure 5.1. Hypothesis schematic. *Neutrophil microvesicles (NMVs) increase the risk of thrombosis by binding to resting platelets via PSGL-1 and CD11b/CD18 binding to P-selectin and GPIbα, respectively, leading to platelet activation as defined as P-selectin expression. In addition, NMVs bind to endothelial cells leading to increased pro-thrombotic gene expression. Created with Biorender.com.*

5.3. Methods

Platelet-rich plasma (PRP) was prepared from whole human blood from healthy volunteers via centrifugation and platelets isolated following a series of centrifugation and washing steps as outlined in methods section 2.21.

The interaction between NMVs and platelets was investigated by flow cytometry. Resting platelets were identified by CD42a labelling and subsequently probed for the expression of the neutrophil specific marker CD66b indicating the presence of NMVs. CD66b levels were compared to an isotype labelled control.

To measure P-selectin expression, resting platelets were incubated with NMVs in conjunction with ADP for 10 minutes prior to antibody labelling. To identify platelet-NMV complexes resting platelets were incubated with NMVs only for the same period prior to labelling of the neutrophil marker CD66b. In both cases platelets were analysed by flow cytometry (figure 5.2).

Platelet aggregation was assessed using a PAP-8E aggregometer (Bio/Data) and haematology analyser (Sysmex)**.** Platelets were treated with collagen and increasing concentrations of NMVs and aggregation measured over time.

The effect of NMVs on EC gene expression was measured using RT-qPCR and focused on the expression of 3 pro-thrombotic genes F3, VWF and SERPINE1, and 2 anti-thrombotic genes PROCR and THBD. HCAECs were cultured on 70 µg/mL collagen III and 5 µg/mL LMW-HA under 4dyn/cm^2 for 72 hours prior to treatment with 1:1 ratio of NMVs:HCAEC for a further 24 hours.

Platelet adhesion to HCAECs was assessed by fluorescence microscopy. Briefly, HCAECs were cultured statically or under LOSS conditions prior to treatment with NMVs for 24 hours. Resting platelets were labelled with the red fluorescent dye PKH-26 and added to HCAECs for 30 minutes. HCAECs were washed thoroughly with fresh media and HCAECs imaged to identify adherent platelets.

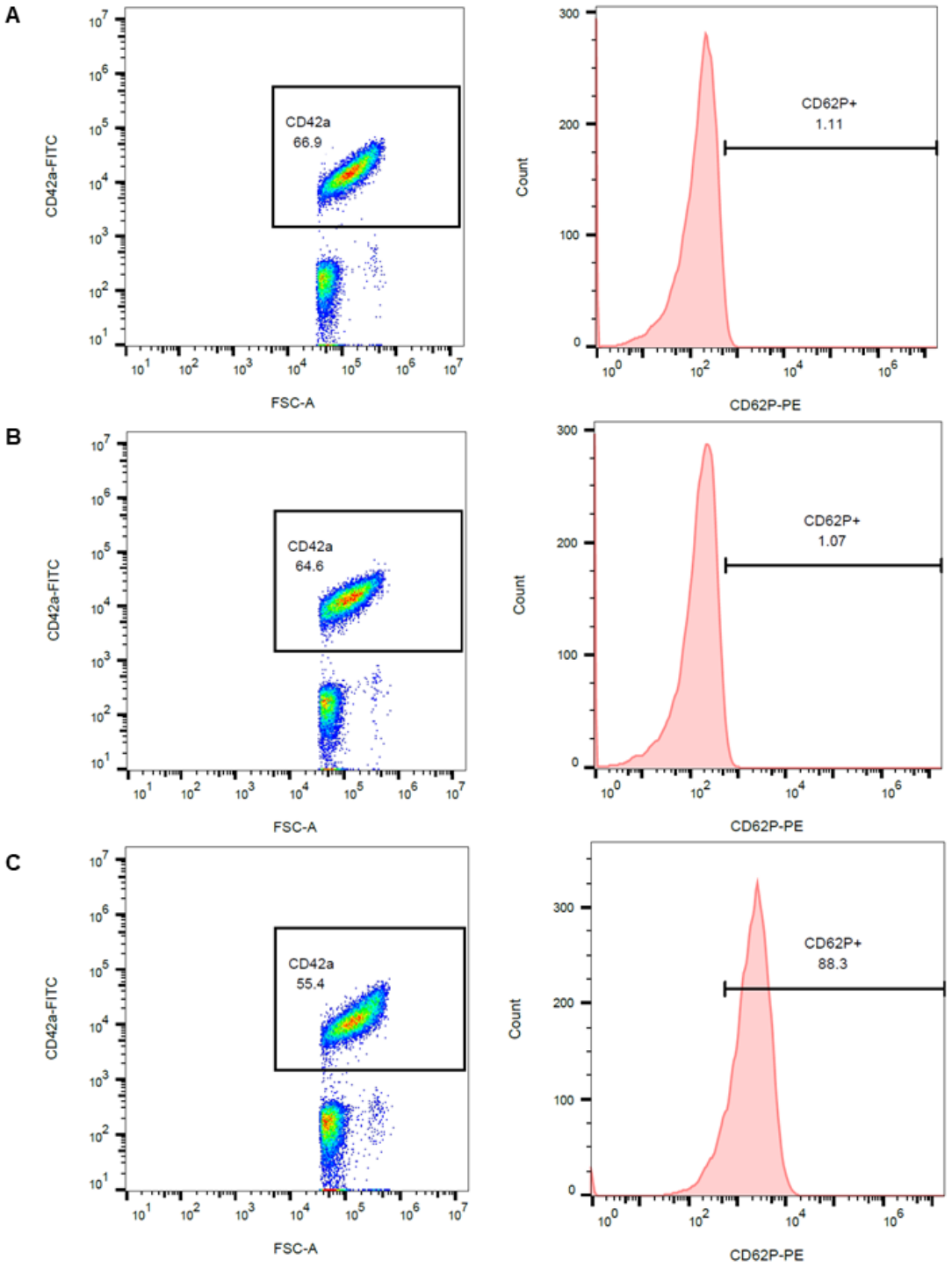


Figure 5. 2. Gating strategy for identification of human platelets and quantification of CD62P expression. *Human platelets were fixed and labelled with anti-CD42a antibody. CD42a-positive population was selected (left) and analysed for CD62P expression (right). Gates were set on* ***A)*** *isotype control labelled platelets and used to analyse* ***B)*** *unstimulated platelets and* ***C)*** *ADP-stimulated platelets using FlowJo software.*

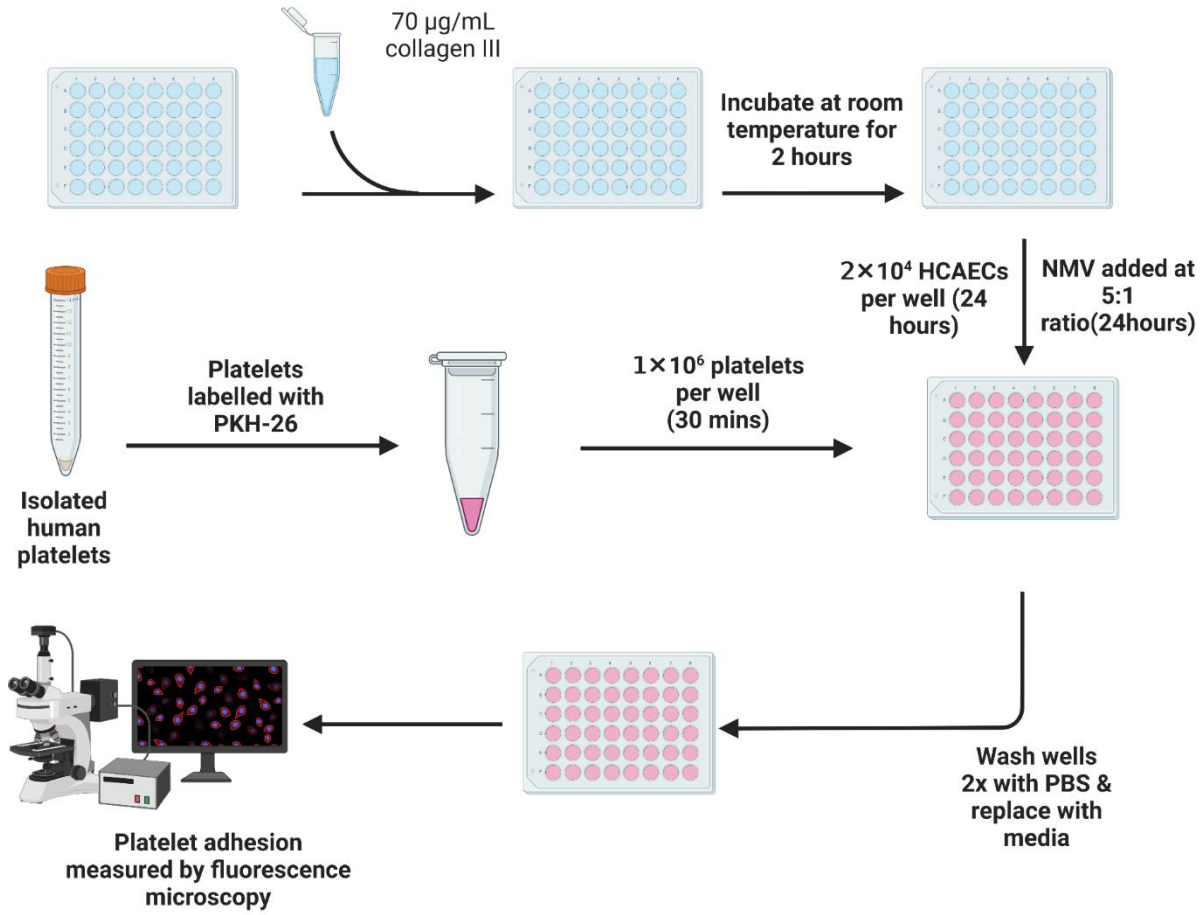


Figure 5. 3. Schematic of platelet adhesion experiment. *Wells of a 48-well plate were coated with collagen type III prior to addition of human coronary artery endothelial cells (HCAECs). HCAECs were cultured for 24 hours before neutrophil microvesicles (NMVs) were added at a 5:1 ratio of NMVs to HCAECs for a further 24 hours. HCAECs were washed twice with PBS and fresh MV2 media added. Platelet adhesion was assessed by fluorescence microscopy. (Created with Biorender.com).*

5.4. Results

5.4.1. Interaction between neutrophil microvesicles and resting platelets

NMVs present in the circulation will encounter and potentially interact with numerous other cell types potentially altering their functions. A potential interaction with circulating platelets could represent a significant mechanism through which NMVs could influence thrombosis. The interaction between platelets and neutrophils has uncovered mechanisms through which they bind, and platelets trigger neutrophil activation and NET release (Maugeri *et al.*, 2014).

To ascertain whether NMVs interact with platelets *in vivo*, freshly isolated resting platelets were dual labelled with CD42a and the neutrophil specific marker CD66b in the absence of isolated NMVs, and expression compared to isotype-labelled controls. Median fluorescence intensity (MFI) was measured for isotype control and CD66b labelled platelets and normalised to MFI for the isotype control.

Platelets labelled for CD66b demonstrated a greater normalised MFI relative to platelets labelled with isotype control (mean =30.7% increase, SEM±2.76, n=3, p=0.0072) (figure 5.3 A). Absolute percentages of CD42a-positive and CD66b-positive platelets were low in both the isotype control and CD66b-labelled conditions. CD66b labelled platelets exhibited a greater mean percentage of CD66b-positive platelets than isotype control labelled platelets when compared to unlabelled platelets (figure 5.3 B). This increase was assessed by a paired t-test but did not reach statistical significance.

These data suggest that NMVs adhere to platelets *in vivo* as platelets were not treated *in vitro* with any isolated NMVs; therefore, any CD66b detected on the platelets was the result of interactions between platelets and NMVs in the blood.

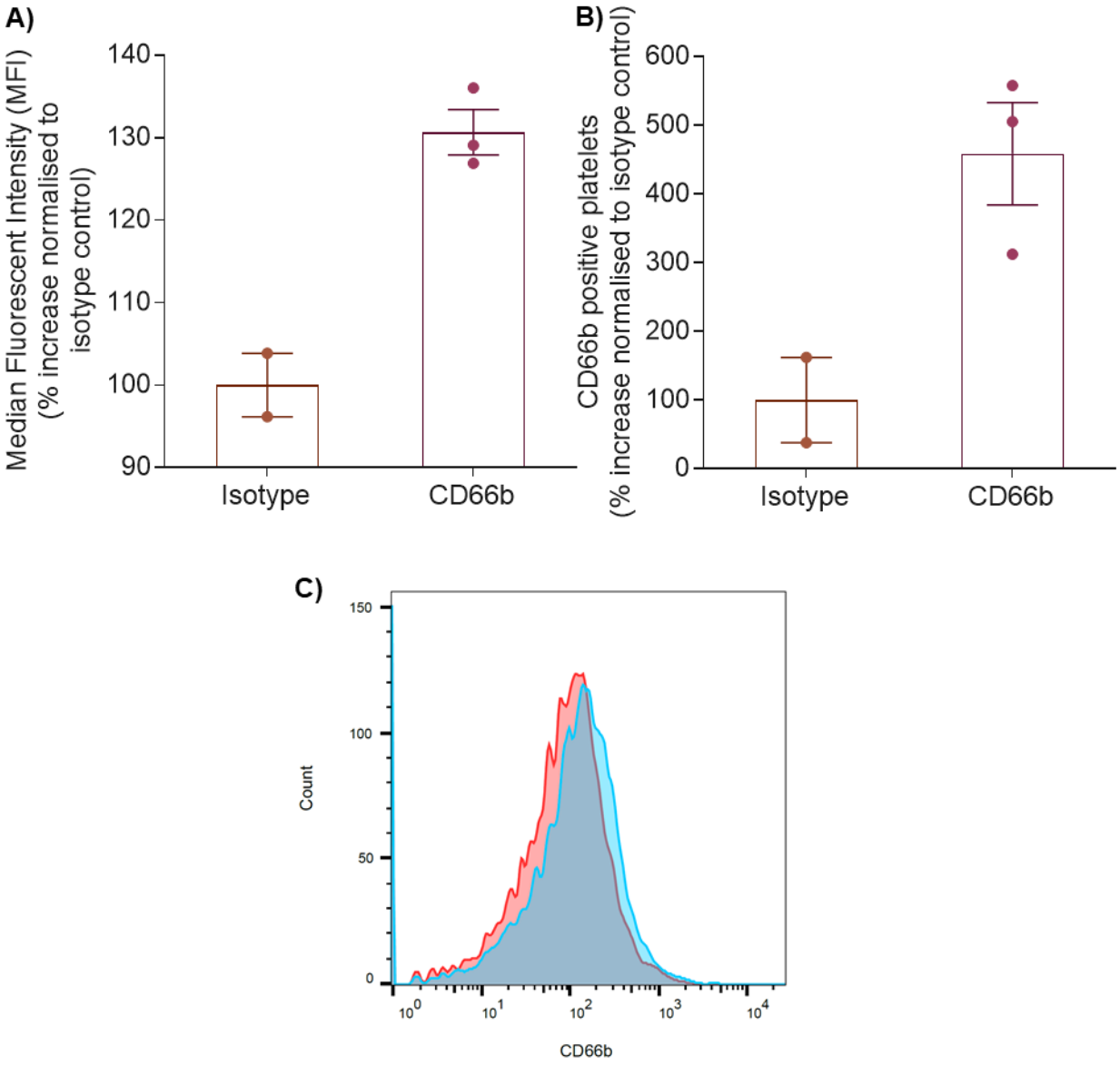


Figure 5.4. CD66b expression detected on resting platelets. *Resting platelets were isolated from platelet-rich plasma (PRP) and labelled with CD42a antibody and either CD66b antibody or an isotype control.* ***(A)*** *The normalised median fluorescence intensity (MFI) measured in isotype control labelled platelets and CD66b labelled platelets, n=2-3.* ***(B)*** *The normalised percentage of platelets positive for CD66b, n=2-3.* ***(C)*** *Representative histogram illustrating the shift in fluorescence between isotype control (red) and CD66b (blue) labelled platelets. Data was normalised to the isotype control and presented as mean percentage increase ±SEM. Statistical significance assessed by a paired t-test.*

5.4.2. Effect of neutrophil microvesicles on platelet P-selectin surface expression

Following activation, platelets rapidly translocate P-selectin to their surface. I investigated whether treatment with NMVs could enhance platelet P-selectin expression on the surface of platelets. To assess this, I incubated PRP with ADP and either NMVs or a supernatant control. ADP was included as an additional stimulus as NMVs alone were insufficient to induce increases in P-selectin expression. Platelets were labelled with antibodies to CD42a and P-selectin prior to analysis by flow cytometry.

Quantification of the median fluorescence intensity for P-selectin indicated a modest but significant increase in P-selectin expression following NMV treatment (mean =10% increase, SEM±1.16, n=3, p=0.025) compared to supernatant control (figure 5.4 A). Quantification of P-selectin positive platelets indicated no change in the number of P-selectin expressing platelets following NMV treatment compared with supernatant controls. A high percentage of platelets were P-selectin positive in both NMV treated and supernatant control platelets (figure 5.4 B).

These data suggest that NMVs increase the expression of P-selectin on the surface of platelets but do not increase the proportion of platelets expressing P-selectin.

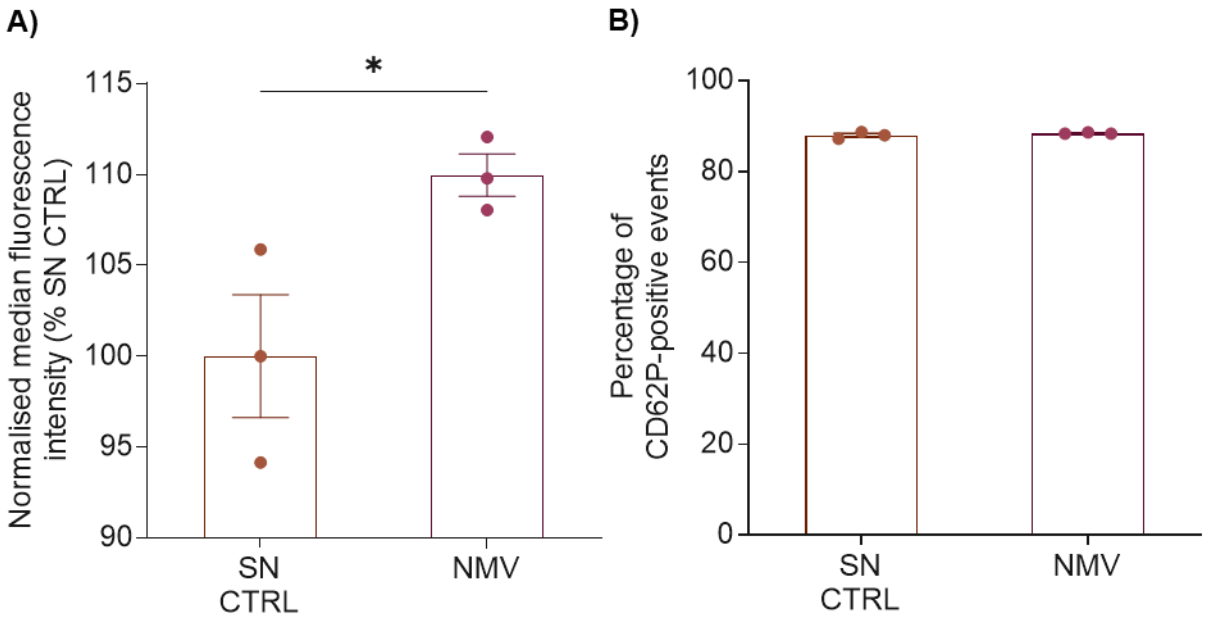


Figure 5. 5. Effect of neutrophil microvesicles on platelet P-selectin expression. *Freshly isolated platelets were incubated with neutrophil microvesicles (NMVs) or a supernatant control in combination with 5μM ADP prior to labelling with anti-CD42a and anti-CD62P antibodies. Fixed platelets were assessed for P-selectin expression by flow cytometry.* ***(A)*** *Percentage change in median fluorescence intensity (MFI) of P-selectin in supernatant (SN) control and NMV treated platelets (n=3).* ***(B)*** *Percentage of P-selectin positive platelets in SN control and NMV-treated platelets (n=3). Data expressed as mean normalised to SN control ±SEM. Statistical significance assessed by unpaired t-test. * p=≤0.05.*

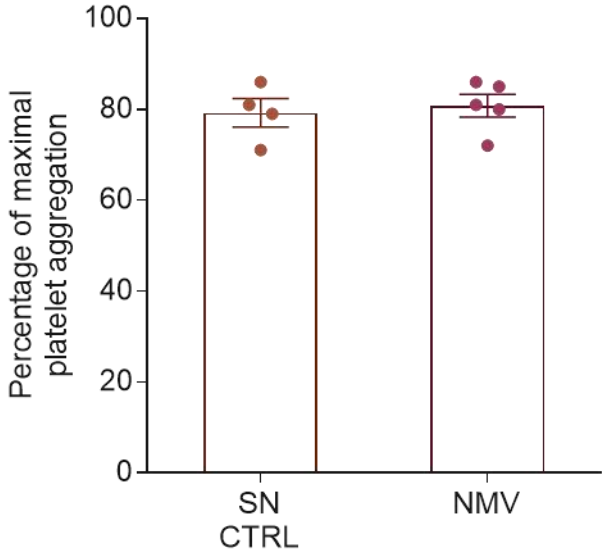
5.4.3. Effect of neutrophil microvesicles on platelet aggregation time

The initial binding of platelets to endothelium or subendothelial matrix is the initial step in thrombosis, however for a significant clot to form platelets must also aggregate. This process is largely driven by cross-linking of fibrinogen between platelets and stabilised by P-selectin.

Due to P-selectin being a marker of platelet activation it was hypothesised that NMVs may enhance platelet activity. To test this, it was determined if NMV incubation enhanced platelet aggregation in response to a collagen agonist.

The data summarised in figure 5.5 show minimal effects of NMVs on platelet aggregation. Supernatant treated platelets demonstrated very similar percentage aggregation compared to NMV treated platelets. These data indicate NMVs have no effect on the propensity for platelets to aggregate.

A)



B)

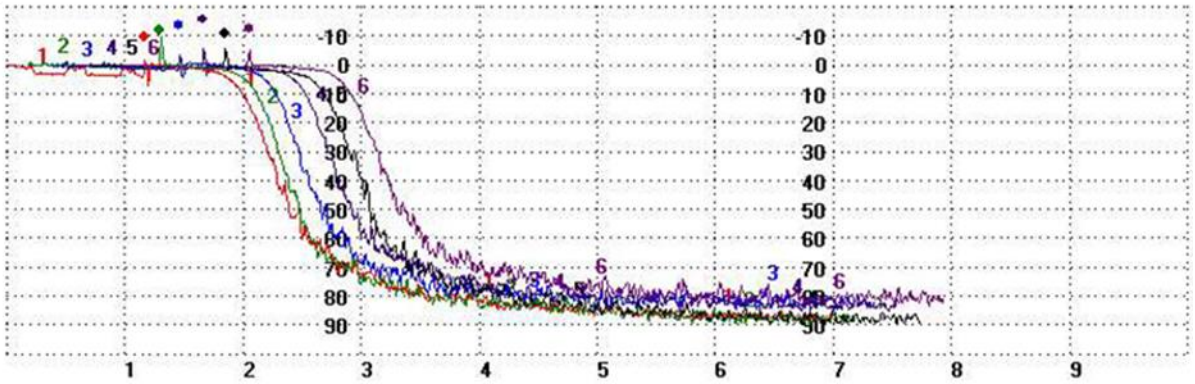


Figure 5. 6. Neutrophil microvesicles do not affect platelet aggregation. *Freshly prepared platelet-rich plasma (PRP) was incubated with neutrophil microvesicles (NMVs) or a supernatant (SN) control prior to addition of 0.25μM horm collagen. Platelet aggregation was assessed by light transmission aggregometry (LTA).* ***(A)*** *Percentage of maximal aggregation of platelets in NMV-treated compared to supernatant treated platelets (n=4-5).* ***(B)*** *Representative plot showing maximal percentage platelet aggregation over time. Supernatant controls (red, green and blue), NMV treated platelets (purple, black and pink). Data expressed as mean ±SEM. Statistical significance assessed by unpaired t-test.*

5.4.4. Effect of neutrophil microvesicles treatment on human coronary artery endothelial cell thrombosis related gene expression

In addition to evaluating the direct effects of NMVs on platelets I also investigated whether NMVs could promote thrombosis through their action on ECs. Previous work in our group has shown that NMVs bind to and are internalised by HCAECs under shear stress (appendix figure 1) and can alter HCAEC gene expression (Gomez *et al.*, 2020). To assess this, the relative expression of 3 pro-thrombotic genes (F3, VWF and SERPINE1) and two anti-thrombotic genes (THBD and PROCR) was assessed by RT-qPCR in NMV treated and supernatant control treated HCAECs cultured under LOSS.

The data summarised in figure 5.6 shows the mean relative expression of VWF (A) in the NMV treated group increased compared to the supernatant treated control, however this did not meet statistical significance. There was no observable change in relative expression levels of genes F3 (B) or SERPINE1 (C) in the NMV treated group compared to supernatant treated controls.

The mean relative expression of genes THBD (D) and PROCR (E) decreased in the NMV treated group compared to supernatant treated controls, however this did not meet statistical significance.

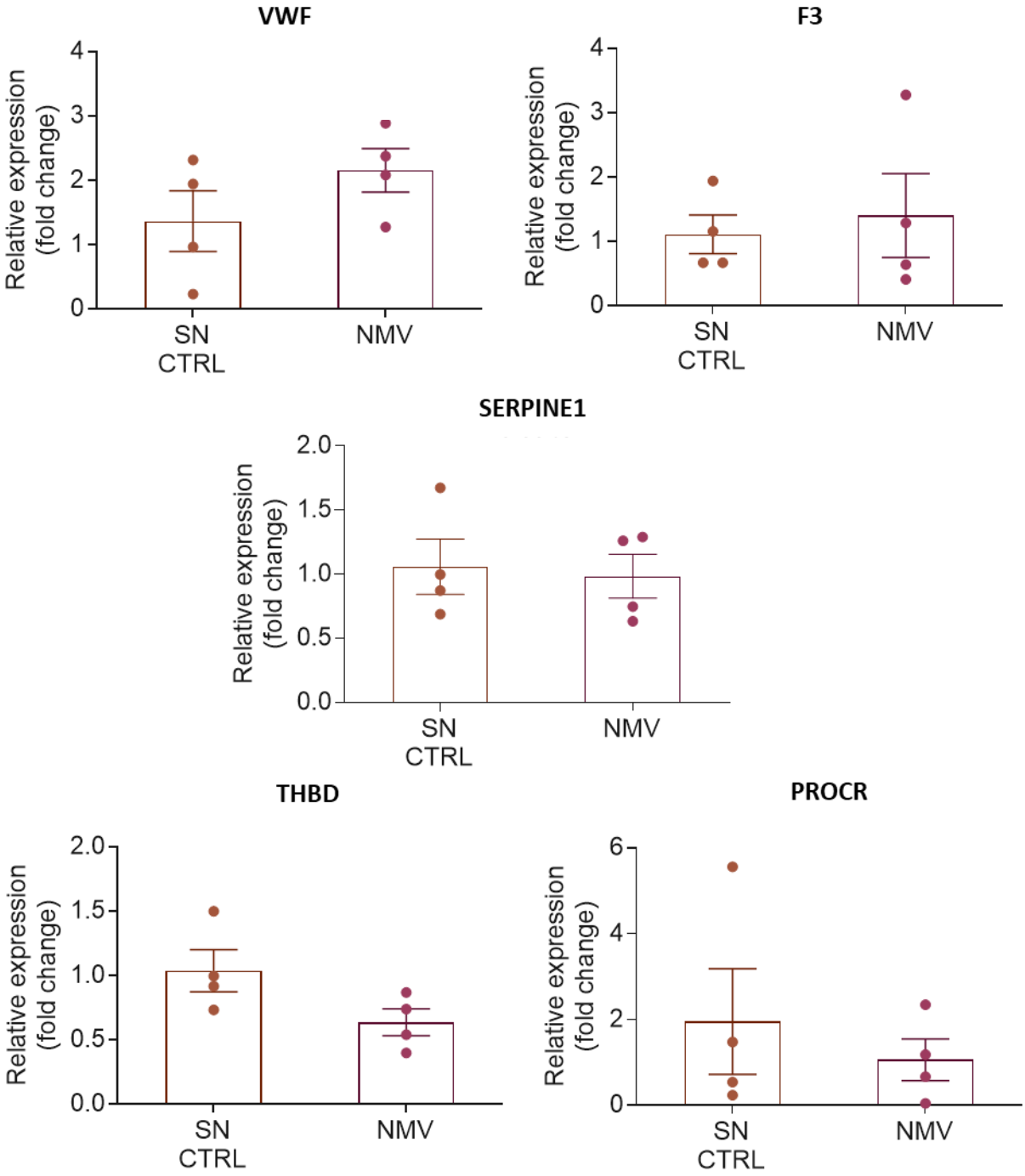


Figure 5. 7. Effect of neutrophil microvesicles on pro- and anti-thrombotic human coronary artery endothelial cell gene expression. *Human coronary artery endothelial cells (HCAECs) were cultured under low oscillatory shear stress (4 dyn/cm^2) for 72 h prior to treatment with neutrophil microvesicles (NMVs) or an NMV supernatant (SN) control for a further 24 h. Relative expression levels of VWF (von Willebrand factor), F3 (tissue factor), SERPINE1 (plasminogen activator inhibitor 1), THBD (thrombomodulin) and PROCR (protein C receptor) were analysed by RT-qPCR and normalised to expression of the reference gene, GAPDH. Data expressed as relative fold change relative to GAPDH ±SEM (n=4). Statistical significance assessed by a paired t-test.*

5.4.5. Effect of neutrophil microvesicles treatment on platelet-endothelial cell interactions.

Since NMVs appeared to form complexes with platelets and modestly increase the expression of platelet P-selectin, the effect of treatment of HCAECs with NMVs on platelet adhesion to HCAECs was investigated. HCAECs were cultured statically prior to treatment with 5:1 NMV:HCAEC ratio or a supernatant control for 24 hours. Freshly isolated platelets were fluorescently labelled and incubated at a 50:1 platelet:HCAEC ratio for 30 minutes. The number of adherent platelets, total percentage area they occupied and mean platelet aggregate size was quantified using ImageJ software.

The data summarised in figure 5.7 demonstrates statically cultured HCAECs treated with NMVs exhibited significantly greater numbers of adherent platelets compared with supernatant-treated controls (mean =52.5% increase, SEM±15.84, n=6, p=0.015). This was reflected in a significantly greater percentage area occupied by platelets following HCAEC NMV treatment (mean =1.2%, SEM±0.12, n=6) compared to supernatant-treated controls (mean =0.78%, SEM±0.09, n=6, p=0.021). However, no significant difference was observed in mean platelet aggregate size between NMV-treated and supernatant-treated HCAECs

These data indicate NMVs increase the propensity for platelets to adhere to HCAECs under static conditions but do not promote aggregation of platelets.

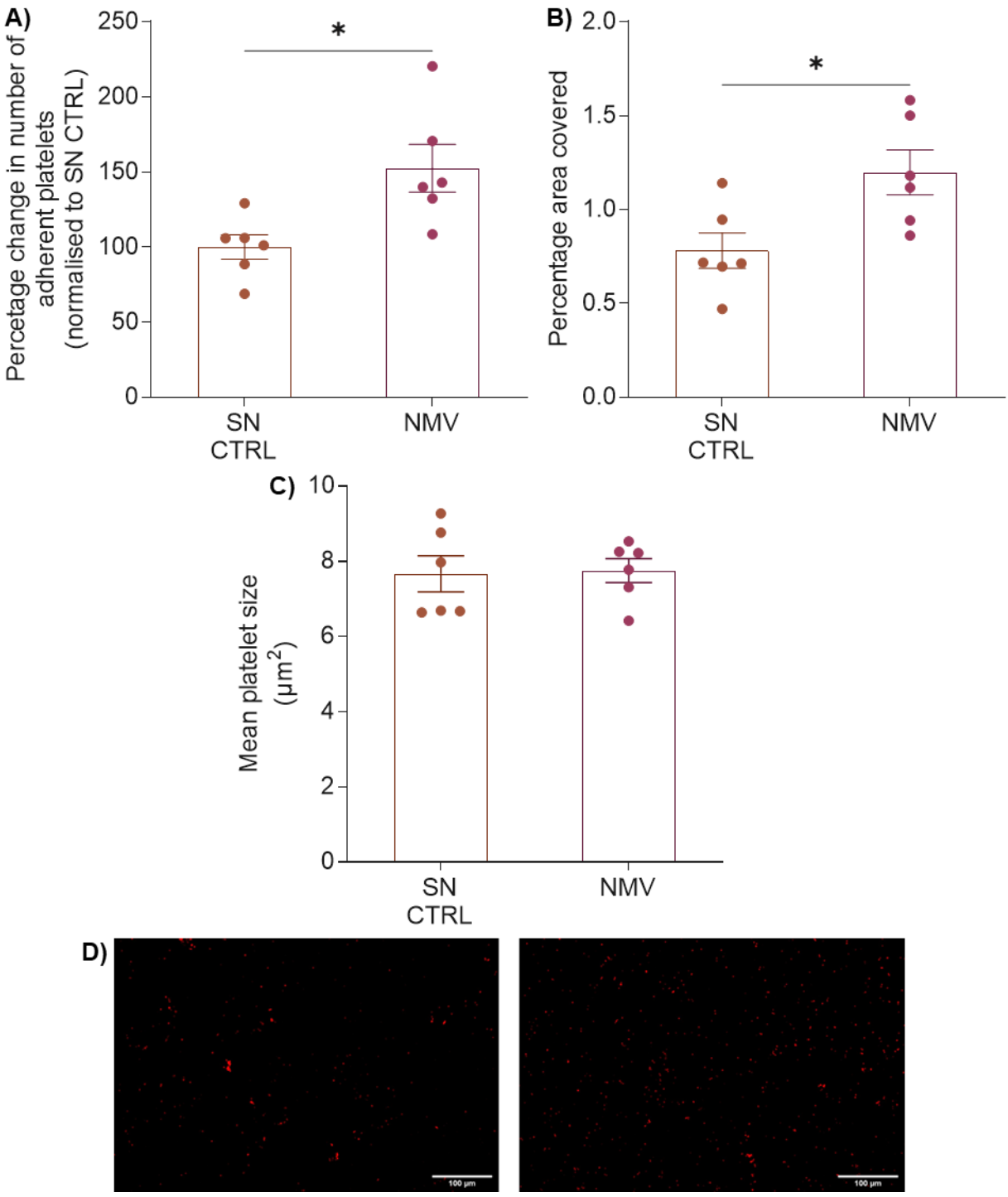


Figure 5. 8. Effect of neutrophil microvesicles on platelet adhesion to static human coronary artery endothelial cells. *Human coronary artery endothelial cells (HCAECs) were cultured statically for 24 h prior to treated with a 5:1 neutrophil microvesicle (NMV):HCAEC ratio or NMV supernatant (SN) control for a further 24 h. HCAECs were washed and incubated with PKH-26 labelled platelets at a 50:1 platelet:HCAEC ratio for 30 min. HCAECs were thoroughly washed to remove non-adherent platelets and then imaged by widefield fluorescence microscopy. Images were analysed by ImageJ software.* ***(A)*** *Percentage change in adherent platelets (n=6).* ***(B)*** *Percentage change in area covered by adherent platelets (n=6).* ***(C)*** *Mean platelet aggregate size (n=6).* ***(D)*** *Representative images of labelled platelets (red) adhered to HCAECs treated with supernatant (left) and NMVs (right). Data expressed as mean ±SEM. Statistical significance assessed by unpaired t-test. * p=≤0.05*

5.4.6. Effect of neutrophil microvesicles on platelet-endothelial cell interactions under low-oscillatory shear stress

Static conditions do not accurately reflect the *in vivo* environment of either HCAECs or platelets and both are known to modulate their functions in response to shear stress. In order to model these shear stress effects, HCAECs were cultured under LOSS for 72 hours prior to treatment with a 5:1 NMV:HCAEC ratio or supernatant controls for 24 hours. HCAECs were then incubated with a 50:1 platelet:HCAEC ratio for 30 minutes under the same LOSS conditions. Adherent platelets were analysed by widefield fluorescence microscopy and quantified using ImageJ software.

The data summarised in figure 5.8 shows that treatment of HCAECs with NMVs did not significantly change the number of adherent platelets recruited, the percentage area covered by platelets, or the mean platelet aggregate size compared to supernatant controls.

These data suggest that, under LOSS, NMVs do not increase the recruitment of platelets to the endothelium.

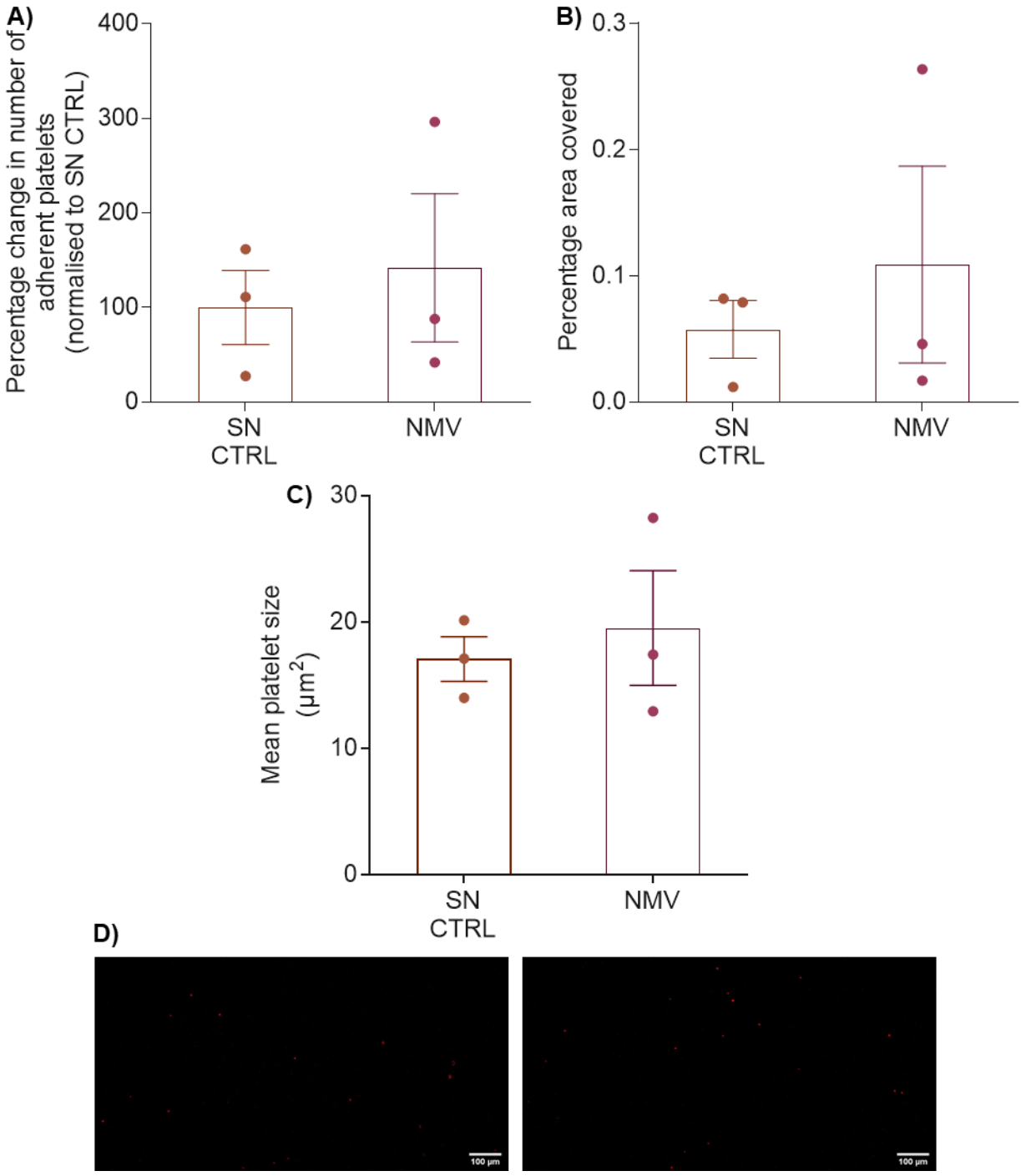


Figure 5. 9. Effect of neutrophil microvesicles on platelet adhesion to endothelial cells under low-oscillatory shear stress. *HCAECs cultured under low oscillatory shear stress (4 dyn/cm^2) for 72 h prior to treatment with a 5:1 neutrophil microvesicle (NMV):HCAEC ratio or NMV supernatant (SN) control for a further 24 h. HCAECs were washed and incubated with PKH-26 labelled platelets at a 50:1 platelet:HCAEC ratio for 30 min. HCAECs were thoroughly washed to remove non-adherent platelets and then imaged by widefield fluorescence microscopy. Images were analysed by ImageJ software.* ***(A)*** *Percentage change in adherent platelets (n=3).* ***(B)*** *Percentage change in area covered by adherent platelets (n=3).* ***(C)*** *Mean platelet aggregate size (n=3).* ***(D)*** *Representative images of labelled platelets (red) adhered to HCAECs treated with supernatant (left) and NMVs (right). Data expressed as mean ±SEM. Statistical significance assessed by unpaired t-test.*

5.4.7 Results summary

- NMVs bind resting platelets
- NMVs increase platelet P-selectin expression
- NMVs have no impact on platelet aggregation time
- NMVs induce pro-thrombotic gene expression changes in endothelial cells
- NMV treatment of endothelial cells increases platelet adhesion under static conditions
- No significant difference in platelet adhesion under low-oscillatory shear stress conditions

5.5. Discussion

5.5.1. Neutrophil microvesicles form complexes with platelets

Platelets and neutrophils can adhere to each other through platelet P-selectin and the counter receptor P-selectin glycoprotein ligand 1 (PSGL-1) expressed by neutrophils (Gardiner *et al.*, 2001). Due to the mechanism through which NMVs are generated, neutrophil PSGL-1 is also incorporated onto the surface of NMVs (Gomez *et al.*, 2020). I therefore hypothesised that NMVs, like their parent neutrophils, can also interact with platelets and potentially alter their function. MVs of monocyte/macrophage origin have been shown to interact with activated platelets through PSGL-1 and phosphatidylserine (PS) (Del Conde *et al.*, 2005). In this study, TF was transferred from MVs to activated platelets increasing the TF-VIIa activity of platelets (Del Conde *et al.*, 2005). Additionally, cancer MVs expressing TF reduced the occlusion time for venules and arterioles. This pro-thrombotic state was abolished in the presence of a P-selectin blocking antibody (Thomas *et al.*, 2009). More recently, salivary MVs responsible for TF activity were shown to accumulate on the platelet surface and increase fibrin deposition in a whole blood perfusion model (Yu *et al.*, 2018b).

Platelets were identified and gated using CD42a as a marker. From this population of platelets, the expression of the neutrophil specific marker CD66b was measured. Our data showed resting platelets isolated from whole blood displayed significant CD66b expression compared to isotype controls, indicating the presence of NMVs on the platelet surface. Whilst there is the possibility that the CD66b detected on the platelets could have originated from previous interactions with neutrophils

this is unlikely. There are no reports of neutrophils transferring CD66b onto platelets and it is also unlikely that I detected platelet-neutrophil complexes instead of platelet-NMV complexes as binding to neutrophils that are approximately 6 times the size of platelets would have moved the platelets out of our previously established platelet gate. As the isolated platelets were not treated with isolated NMVs *in vitro* these data indicate that the adherence of any NMVs to platelets occurred either *in vivo* prior to blood being drawn or during the processing of the blood samples. Previous studies indicated MVs bound activated platelets due to the increased expression of P-selectin by activated platelets. The binding of NMVs to platelets in our experiments most likely occurred as a result of limited platelet activation occurring from the preparation of the blood samples. To assess if NMVs adhered more to activated platelets, further experiments incubating NMVs with increasingly activated platelets would be necessary.

Given the significant increase in CD66b expression on platelets, NMVs likely do adhere to platelets and this interaction may alter platelet function.

5.5.2. Neutrophil microvesicles increase platelet P-selectin expression

Having established NMVs adhere to platelets, it was hypothesised that this interaction drove increased platelet activation, thus increasing the risk of thrombosis. To address this, platelet P-selectin expression was quantified. P-selectin is a widely used marker of platelet activation due to its presence on the platelet surface only following activation-induced degranulation (Linden, 2013).

Preliminary experiments demonstrated that NMVs were not capable of activating resting platelets alone and therefore, it was not surprising to learn that NMVs did not increase the percentage of P-selectin positive platelets in combination with ADP. ADP is a potent stimulator of platelet P-selectin expression (Anderson *et al.*, 2020). NMVs do not appear to induce P-selectin expression in platelets but were able to increase the expression of P-selectin in ADP stimulated platelets. The counter receptor for P-selectin is PSGL-1 (Del Conde *et al.*, 2005), which is highly expressed on neutrophils (Xu *et al.*, 2007) and NMVs (Gomez *et al.*, 2020). Greater expression of P-selectin on the platelet surface likely results in greater NMV adherence to these platelets as indicated by previous work investigating monocyte/macrophage MVs (Del Conde *et al.*, 2005). In a population of both resting and activated platelets the activated platelets, expressing more P-selectin, likely adhere to greater numbers of NMVs and this higher degree of NMV binding may be sufficient to drive an increase in P-selectin expression that the lower degree of NMV binding to resting platelets is incapable of inducing. However, the potential mechanism for this process would require investigation. Additionally, if NMVs bind to P-selectin this may prevent the ability of labelled P-selectin antibody to bind. In this case the P-selectin signal is likely to be an underestimate in the NMV treated condition.

Having quantified platelet activation via P-selectin expression I next investigated if NMVs decrease platelet aggregation time. Platelet coagulation time is governed by multiple platelet receptors including P-selectin and GPIIb/IIIa (Yakushkin *et al.*, 2011). GPIIb/IIIa binds to VWF to facilitate recruitment of platelets to damaged endothelium but also binds fibrinogen to cross-link platelets allowing their aggregation (Yakushkin *et al.*, 2011).

Coagulation time was assessed by light transmission aggregometry (LTA). PRP was pre-incubated with NMVs prior to platelet activation with 0.25 µg/mL horm collagen (native equine tendon collagen I). No difference was detected in the percentage platelet aggregation following NMV treatment compared to supernatant control (figure 5.5). When considered together with the P-selectin data this finding is perhaps surprising given that I found an increase in P-selectin MFI following NMV treatment (figure 5.4 A). However, platelet aggregation is not solely governed by P-selectin expression (Sangkuhl *et al.*, 2011).

From these data I am unable to conclude NMVs have a measurable effect on platelet function despite evidence suggesting they can adhere and may increase P-selectin expression in already activated platelets. The main obstacle to interpreting these data is the low number of experimental repeats. These were an unfortunate consequence of a limited number of healthy volunteers available to donate blood. Our efforts to find suitable donors was severely hampered by COVID-19 restrictions specifically by an inability to recruit donors not employed within the Royal Hallamshire Hospital and many eligible donors working from home.

5.5.3. Effect of neutrophil microvesicles on platelet-endothelial cell interaction.

The endothelium also plays a crucial role in thrombosis and generally protects against thrombosis, with platelet adhesion and aggregation occurring only when the endothelium is damaged, and the pro-thrombotic sub-endothelial matrix becomes exposed. However, platelets can also adhere to endothelium activated by disturbed blood flow (Qu *et al.*, 2020), for instance through the interaction between platelet GPIb-IX-V and endothelial VWF (Massberg *et al.*, 2002). NMVs are internalised by ECs and can alter gene expression (Gomez *et al.*, 2020). The effect of NMVs on expression of selected pro and anti-thrombotic EC genes to promote platelet recruitment was therefore investigated. Endothelial genes involved in the coagulation cascade were investigated. Expression of genes encoding for pro-thrombotic factors VWF, TF and PAI-1 was measured alongside genes for anti-thrombotic factors THBD and PROCR. VWF is expressed by ECs and released from Weibel-Palade bodies (WPB) (Valentijn *et al.*, 2010) and cleaved from the EC surface by ADAMTS13 (Turner *et al.*, 2009; Xiang *et al.*, 2011). VWF binds subendothelial matrix collagens and platelet

glycoproteins facilitating the recruitment of platelets to damaged endothelium (Romijn *et al.*, 2001). Enhanced VWF levels could increase recruitment of platelets to areas of plaque erosion. A higher relative expression of VWF was measured in ECs cultured under LOSS when incubated with NMVs compared to that seen with the supernatant control. VWF release as mature multimeric glycoprotein is regulated by three secretory pathways, constitutive release, basal release from WPB bodies and regulated release from WPB bodies (Lopes da Silva and Cutler, 2016). The size of VWF multimers determines their thrombogenicity with ultra large VWF (UL-VWF) multimers being the most thrombogenic. ECs constitutively release less thrombogenic low molecular weight VWF (LMW-VWF) to the subendothelial matrix, but also release UL-VWF multimers both towards the apical side of ECs and into the subendothelial matrix via basal release from WPB. However, the majority of VWF, especially the UL-VWF form, is released from WPB following stimulation with a secretagogue (Lopes da Silva and Cutler, 2016). Release of mature VWF protein was not investigated in this project, largely due to time restraints, and in future work this would be investigated. Therefore, it is not possible to conclude NMVs increased VWF release by ECs however, due to constitutive and basal WPB release it is possible to speculate that increased VWF gene expression may lead to greater release of VWF via these pathways. Additionally, NMVs may induce the regulated release of VWF from WPB thus leading to a large increase in VWF secretion. However, this would require further investigation.

No changes were observed in the expression of genes encoding TF or SERPINE1 following NMV treatment, however reduced expression of the anti-thrombotic genes THBD and PROCR were observed following NMV treatment (figure 5.6). THBD encodes thrombomodulin (TM) a cell surface glycoprotein that binds and neutralises α-thrombin and activates protein C, a potent anti-coagulant (Esmon *et al.*, 1982)**.** TM has been shown to significantly reduce atherosclerosis in mice models through binding of thrombin. Alterations to the TM thrombin-binding domain reduced thrombin clotting time and abolished the inhibition of thrombin-induced expression of EC adhesion molecules observed in WT mice (Wei *et al.*, 2011). PROCR encodes endothelial protein C receptor (EPCR) which binds protein C and in combination with TM bound to thrombin further activates protein C (Dahlbäck and Villoutreix, 2005). Protein C exerts anti-thrombotic effects by binding and inhibiting coagulation factors FVIIIa and FVa (Dahlbäck and Villoutreix, 2005; Pellequer *et al.*, 2000). Reduction in protein C has also been linked with atherosclerosis in type II DM (Matsumoto *et al.*, 2007).

Although the lack of statistical significance means care must be taken in interpreting these data, the trends observed in relative gene expression suggest NMVs may promote a more pro-thrombotic EC phenotype. Due the variation observed it is likely these experiments were underpowered. Variation arose, at least in part, as a consequence of using NMVs isolated from whole blood of different volunteers and using primary HCAECs from multiple donors. One way to reduce this variation would be to use primary HCAECs from a single donor however, this was deliberately avoided in order to

ensure any effects I observed were reliable and not the result of an anomaly in one specific HCAEC donor.

Further analysis of the protein expression of TM and EPCR would be necessary to conclude the observed gene expression changes manifested in changes to protein expression. In combination these RT-qPCR data indicated that NMVs promote a pro-thrombotic response in ECs cultured under LOSS. However, these experiments would need to be repeated further in order to increase reliability in the results and allow for better interpretation of the data.

Next, the combined effects of NMVs on the recruitment of platelets to ECs were investigated. Based on the RT-qPCR data it was hypothesised that NMVs would enhance recruitment of platelets to ECs. This was investigated by treating HCAECs cultured statically or under LOSS conditions with NMVs and then fluorescently labelled platelets. Greater recruitment of platelets to statically cultured HCAECs following treatment with NMVs was observed when compared to supernatant control (figure 5.7). A 50% increase in the number of adherent platelets and a similar increase in the percentage area that was covered by platelets indicated NMVs were significantly increasing platelet recruitment. Due to the propensity for activated platelets to aggregate, the mean size of platelet aggregates was also quantified. Analysis revealed that whilst platelet aggregates occurred in both NMV and supernatant control conditions, there were no significant differences in mean aggregate size. This suggested no increased propensity for platelets to aggregate on NMV treated HCAECs. Platelets are influenced by shear stress conditions and static conditions do not effectively replicate the environment of an atherosclerotic plaque. Therefore, platelet adhesion was investigated under LOSS conditions representative of the downstream plaque region. LOSS has been shown to induce pro-inflammatory and pro-thrombotic EC activation (Demos *et al.*, 2020; Heo *et al.*, 2011; Jenkins *et al.*, 2013). Platelets were added under the same flow conditions and allowed to adhere. Based on clear results of the static experiments I replicated the methods using the same NMV:HCAEC and platelet:HCAEC ratios. Interestingly, I observed no difference in adherent platelet number, percentage area covered or mean size of the platelet aggregates following NMV treatment (figure 5.8). Whilst the ratio of NMVs and platelets was the same as previous experiments on static HCAECs the concentration of both was markedly reduced in the experiments under shear stress. As a result, I observed fewer adherent platelets under flow compared to static conditions. If a greater concentration of platelets were used in future experiments a difference may become apparent. I know NMVs adhere under the flow conditions chosen (Gomez *et al.*, 2020), however the reduced NMV concentration may have been insufficient to generate significant gene expression changes in enough of the HCAECs to increase platelet recruitment.

5.5.4 Conclusion

The data summarised in this chapter indicate that NMVs may contribute towards platelet activation and platelet adhesion to the endothelium. Our data suggests NMVs are capable of binding to resting platelets and increasing platelet P-selectin expression. Whilst this had no effect on platelet aggregation time there was an increase in platelet adhesion to the endothelium. This may in part be the result of additional pro-thrombotic effects of NMVs on the endothelium, namely the increased expression of genes encoding VWF and decreased expression of genes encoding THBD. In combination, the increase in platelet P-selectin and a shift towards a more pro-thrombotic endothelium may result in an increased risk of thrombosis. The interpretation of these results is limited due to low number of individual experimental repeats and the lack of a clear trend when platelet adhesion experiments were replicated under LOSS conditions.

5.5.5 Limitations

The aim of this chapter was to establish the effect of NMVs on platelets and their interaction with ECs. Initially, I established that NMVs did adhere to platelets in circulation via detecting neutrophil specific CD66b on the platelet surface. However, no investigation was done to determine if NMVs would bind more to activated platelets compared with resting platelets. This is a limitation as activated platelets are more relevant in the context of thrombosis than their resting counterparts.

Additionally, a limitation of this chapter is the relatively low n numbers present in the RT-qPCR data compared to the variation in the data. Whilst the data indicates some trends there are too few repeats to establish if this is significant or not. For this reason, little can be inferred from the data.

When the effect of NMVs on platelet adhesion to ECs was investigated I found NMV treatment of ECs increased platelet adhesion under static conditions. However, this was not replicated in the experiments conducted in the presence of flow. This is a limitation of the study as flow is integral to the process of platelet recruitment to the endothelium and means we must use caution when interpreting the results of the experiment under static conditions. In addition, whilst the effect of EC treatment with NMVs was tested, the effect of platelet treatment with NMVs was not investigated. This is a limitation of the study as we showed NMVs do increase platelet P-selectin expression and incorporating NMV treatment of platelets would more accurately reflect the environment *in vivo* and could have led to more significant results in the experiment under flow.

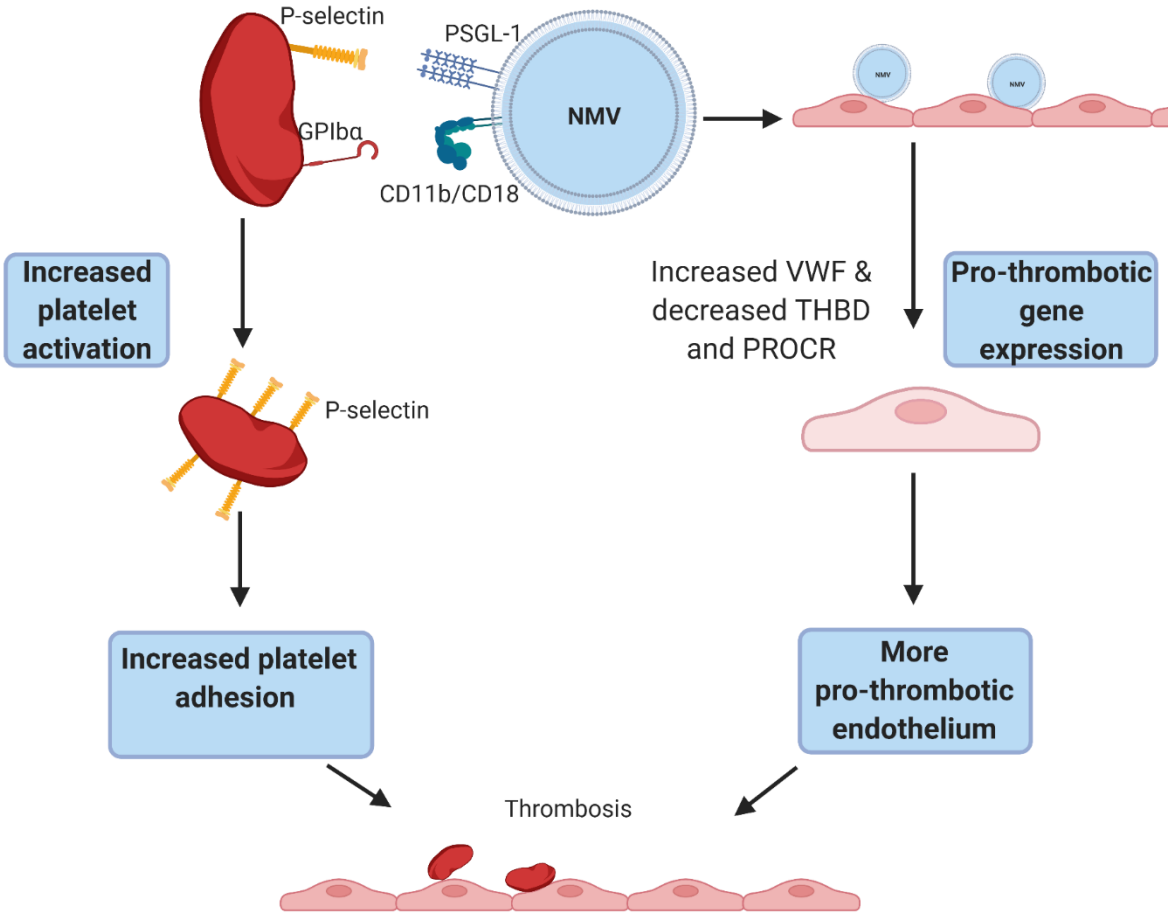


Figure 5. 10. Summary results. *Neutrophil microvesicles (NMVs) increase the risk of thrombosis by binding to resting platelets via PSGL-1 and CD11b/CD18 binding to P-selectin and GPIbα, respectively, leading to platelet activation as defined as P-selectin expression. In addition, NMVs bind to endothelial cells leading to increased pro-thrombotic gene expression. Our data supports our hypothesis that NMVs induce platelet P-selectin expression and drive a pro-thrombotic gene expression changes in HCAECs. Created with Biorender.com*

6 General discussion

6.1. Study summary

The role of neutrophils in atherosclerosis in general has likely been underappreciated. Limited observations of neutrophils associated with plaques contributed to this. However, correlations between circulating neutrophil numbers and atherosclerotic lesion size (Drechsler *et al.*, 2010) and the effect of neutrophil depletion on limiting plaque development (Zernecke *et al.*, 2008) has changed this perception. Whilst the lack of neutrophils observed in plaques may be accounted for by technical limitations in detecting neutrophils or due to their short life span, recent work has described how neutrophils may impart their effect on the endothelium via the release of MVs (Gomez *et al.*, 2020) promoting inflammation, monocyte recruitment and plaque development. Recently, work has uncovered a role for neutrophils and NETs in the less well understood mechanism of plaque erosion and thrombosis (Franck *et al.*, 2017; Quillard *et al.*, 2015). The aim of the studies presented in this thesis were to investigate the role of NMVs in the processes that contribute to plaque erosion. I hypothesised that NMVs would promote responses in endothelial cells likely to contribute towards an increased risk of erosion. The main findings from these investigations are summarised below and in figure 6.1.

1. NMVs are produced *in vitro* in response to native low-density lipoprotein at levels significantly above unstimulated neutrophils.

2. NMVs contain a library of proteases and are capable of degrading components of the extracellular matrix in an MMP-dependent manner, promoting endothelial cell detachment.

3. NMVs influence a variety of cellular processes in endothelial cells such as increasing apoptosis and decreasing both wound healing and proliferation.

4. NMVs interact with resting platelets and increase P-selectin expression of activated platelets.

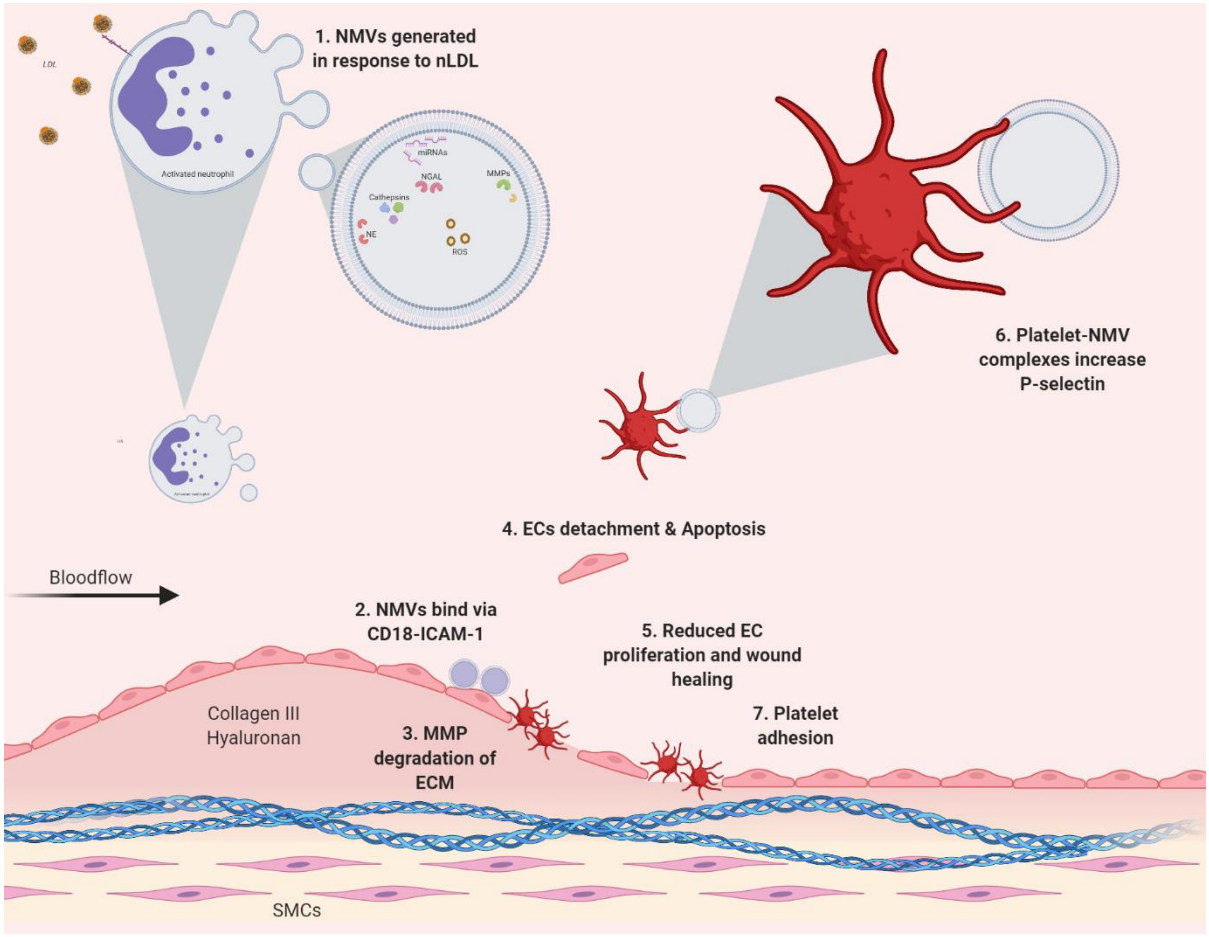


Figure 6. 1. Thesis summary diagram. 1) *Neutrophil microvesicles (NMVs) are generated in response to stimulation by native low-density lipoprotein (nLDL) present in the circulation.* ***2)*** *NMVs adhere to human coronary artery endothelial cells (HCAECs) and* ***3)*** *degrade the extracellular matrix (ECM) through matrix metalloproteinase (MMP) activity resulting in* ***4)*** *increased HCAEC detachment and apoptosis.* ***5)*** *NMVs also reduce HCAEC proliferation and the wound healing response.* ***6)*** *NMVs form complexes with platelets in the circulation that may increase platelet activation and P-selectin expression resulting in* ***7)*** *platelet adhesion. SMC (smooth muscle cells), NE (neutrophil elastase), ROS (reactive oxygen species), miRNA (micro-RNA). NGAL (neutrophil gelatinase-associated lipocalin). Created with Biorender.com*

6.2. Wider implications and future work

6.2.1. Effect of stimuli on neutrophil microvesicle contents

The data presented in this thesis indicate NMVs are generated in response to nLDL and are generated in much greater number compared to unstimulated neutrophils. These data may explain why high-fat overfeeding results in greater NMV production (Gomez *et al.*, 2020) and could identify a link between LDL levels, a known risk factor in CVD, and levels of NMVs. Whilst LDL is closely associated with plaque rupture and the reduction of LDL levels is related to a reduced risk of plaque rupture (Kurihara *et al.*, 2020) this association has not been reported for cases of plaque erosion. However, a meta-analysis of patients with eroded plaques found similar levels of LDL to patients with plaque rupture and significantly higher levels than those found in patients with calcified plaques (Yamamoto *et al.*, 2019b). Our data suggests that LDL is an effective stimulus of NMV generation and therefore efforts to reduce circulating LDL levels, primarily through preventative strategies such as alterations to diet, could help to reduce the potentially harmful effects of NMVs. Neutrophils express receptors for LDL in addition to scavenger receptors for oxidised LDL which is known to promote neutrophil activation (Palvinskaya *et al.*, 2013). The ability of neutrophils to generate reactive oxygen species (ROS) allows for the possibility that neutrophils oxidise native LDL which subsequently acts upon neutrophils to promote NMV generation. In future studies it would be interesting to block these receptors prior to LDL treatment to determine if NMV formation is specifically stimulated by nLDL binding to neutrophils or the result of generated oxLDL. The most efficient way to investigate this would likely be by blocking the nLDL receptor (LDLR) on the neutrophil surface with a blocking antibody prior to stimulation with nLDL and comparing NMV numbers to a LDLR blocked control condition.

Levels of circulating MVs in the plasma are likely important in the pathogenesis of many diseases and have been implicated in the pathogenesis of atherosclerosis (Chironi *et al.*, 2006). Our data indicates that NMVs are elevated following nLDL stimulation and have dose-dependent effects on ECM degradation and HCAEC function. This suggests higher levels of NMVs in response to high circulating nLDL levels may increase the risk of plaque erosion. Therefore, analysis of circulating NMV concentrations may represent a good biomarker for the risk of plaque erosion in patients. Through a simple blood test individuals at higher risk of erosion could be identified and treated earlier. Of course, this relies on a firm link between NMVs and plaque erosion being established through further work. MVs have also been identified specifically in atherosclerotic plaques (Canault *et al.*, 2007; Leroyer *et al.*, 2007) however, there is less research involving NMVs directly in atherosclerotic plaques. This may in part be because of the neglected role of neutrophils in atherosclerosis. Much of the research has focused on infiltrating macrophages however, more

recently neutrophils have gained more interest and have been associated specifically with eroded plaques (Franck *et al.*, 2017). Previous work by our group has shown NMVs are preferentially recruited to the endothelium under atheroprone flow conditions (Gomez *et al.*, 2020) however, direct detection of NMVs specifically interacting with eroded plaques has not be reported. This could well be due to the lack of a good *in vivo* model of plaque erosion or the ability to effectively model eroded plaques *in vitro*. However, recent studies have studied plaque erosion in $ApoE^{-/-}$ mice. Electric injury to the LCCA produced fibrous plaques resembling the features of eroded plaques. Following recovery of the endothelium 4 weeks later, disturbed flow was induced over these lesions through the placement of a constrictive cuff on the artery (Franck *et al.*, 2017). Recently, the development of the 'Catchup' mouse model has allowed the specific visualisation of murine neutrophils. This has been achieved through specific expression of tdTomato within the neutrophil-specific Ly6G locus (Hasenberg *et al.*, 2015) and has the advantage of allowing fluorescent labelling and *in vivo* visualisation of neutrophils without *ex vivo* manipulation. This model has not yet been used for studying NMVs, and it is unclear how efficiently any NMVs generated by tdTomato labelled neutrophils would express tdTomato. However, this model may in future allow for murine NMVs generated *in vivo* to be tracked throughout different tissues. This could include NMVs within fibrous plaques. Catchup mice subjected to left common carotid artery (LCCA) injury and fibrous plaque formation, as described by Franck *et al*, would offer a useful model for investigating NMVs in the context of plaque erosion.

Whilst the numbers of NMVs generated significantly increased following native LDL treatment, the concentration of MMP-9 and NE per NMV did not significantly differ, indicating that nLDL treatment did not lead to an enrichment of proteins within NMVs. However, due to the significant increase in the number of NMVs generated following nLDL stimulation the total amount of both MMP-9 and NE packaged in and released through NMVs increased. This suggests it is the quantity of NMVs generated that is most important rather than the effect of the stimuli on NMV packaging and that the focus should be on reducing NMV generation to limit any negative effects attributed to NMVs. Considering our findings show nLDL is a good stimulator of NMV generation and given previous findings correlating NMV numbers with high-fat diet improving diets may represent an effective target in reducing the burden of disease.

Due the focus of this thesis on ECM degradation and the abundance of MMP-9 observed in NMVs, MMP-9 was chosen for further investigation. However, there were more proteases identified that may have important roles in the pathology of plaque erosion. Interestingly, whilst I identified no significant difference in the concentration of MMP-9 and NE between NMVs from unstimulated and nLDL-stimulated neutrophils I did observe some differences in the overall protease repertoire of both populations of NMVs. Most proteases detected were present in NMVs from both unstimulated and nLDL-stimulated neutrophils, however there were examples of proteases present in just one population. For example, cathepsin V was detected only in NMVs from nLDL-stimulated neutrophils.

Further investigations would be necessary to confirm that these differences in protease content were reliable and the result of differences in stimulation method rather than individual donor differences. However, these data were produced by pooling multiple donors in order to reduce the effect of individual donor differences distorting the results. These data indicate that NMV contents reflect the stimulus and environment of the parent neutrophil at the time of NMV generation. This could result in a particular NMV population being produced in response to infection and another to high cholesterol levels for example. Data from previous studies supports this, for example Dalli *et al* found that NMV contents are influenced by the environment of the parent neutrophil (Dalli *et al.*, 2013). If the protease content of NMVs is altered by stimulation method, then it is possible other effector molecules are also altered and this could result in different populations of NMVs being produced with very different effects depending on the neutrophil stimulus. An important area for future research to investigate would be how these changes in NMV cargo effect NMV function and what effects other neutrophil stimuli have on NMV cargo. Our data concerns only the effects of NMVs from nLDL-stimulated neutrophils on HCAECs, but in future it would be interesting to investigate if NMVs produced in response to other stimuli, for instance fMLP and PMA exert different effects. As already noted, previous studies found no effect of NMVs from fMLP-stimulated neutrophils on HCAEC apoptosis (Gomez *et al.*, 2020) whilst apoptosis was increased following treatment with NMVs from nLDL-stimulated neutrophils. This disparity in results could indicate neutrophils are highly responsive to their environment and generate NMVs to reflect this. Further to this, an investigation of the different effects of different NMV populations and a comparison of their proteomes could specific proteins that underpin these effects.

6.2.2. Neutrophil microvesicle effects on extracellular matrix degradation

Neutrophils are rich in MMPs (Ong *et al.*, 2017) and other proteases (Jackson *et al.*, 2010; Lee *et al.*, 2015; Pham, 2008) capable of degrading components of the ECM and the data presented in this thesis suggests neutrophils package MMP-8 and MMP-9, along with other proteases, into NMVs. A high protease content indicates NMVs could have important roles in regulating the ECM, with changes in the ECM linked to atherosclerosis (Chistiakov *et al.*, 2013). NMVs may provide a mechanism for targeted delivery of concentrated proteases directly to atheroprone regions of the vasculature as NMVs are known to preferentially adhere to these regions (Gomez *et al.*, 2020). In support of this, I demonstrated NMVs induce significant degradation of gelatin, a process significantly reduced by TIMP-1. NMVs were only assessed for their protease content and not their protease inhibitor contents. However, their ability to degrade gelatin suggests their contents are richer in proteases than inhibitors. Previous studies have shown neutrophils release TIMP-free MMP-9 with

strong proteolytic activity (Ardi *et al.*, 2007). TIMPs, specifically TIMP-1, inhibit MMP-9 but previous studies have shown neutrophils do not express TIMP-1, even following stimulation with IL-8 and PMA (Jönsson *et al.*, 2011). In contrast, unstimulated neutrophils express TIMP-2 and this increased following stimulation with IL-8 and PMA (Jönsson *et al.*, 2011). TIMP-2 inhibits MMP-2 most strongly however, does still inhibit MMP-9 to a lesser degree. Interestingly, previous investigations have identified TIMP-1 as a biomarker of cardiac mortality and myocardial infarction (Cavusoglu *et al.*, 2006; Hansson *et al.*, 2011). This somewhat paradoxical finding may result from an upregulation of TIMP-1 in response to high levels of MMPs.

I also found NMV treatment increases areas of gelatin degradation around the perimeter of HCAECs. However, it was not determined if this was the result of NMV-derived proteases or NMV-induced upregulation of EC-derived proteases. Analysing EC MMP expression by RT-qPCR and ELISA would be useful in shedding light on this. ECs are known to express MMP-2, MMP-8 and MMP-9 (Herman *et al.*, 2001; Liu *et al.*, 2018b; Magid *et al.*, 2003; Taraboletti *et al.*, 2002) which could contribute to degradation of the ECM scaffold they rely on for adhesion. To investigate this, HCAECs could be incubated with NMVs, and ELISA and gelatin zymography used to measure the presence of active proteases compared to an equal concentration of NMVs only. In addition, the expression of MMP coding genes could be analysed by RT-qPCR following NMV treatment and compared to untreated control HCAECs. From these experiments it could be possible to determine if NMVs increase HCAEC MMP release and whether this is due to enhancing the rate at which HCAECs secrete MMPs extracellularly or increasing MMP gene expression, or both.

6.2.3. Detachment and apoptosis

Previous studies have demonstrated the pro-inflammatory effects of NMVs on endothelial cells (Gomez *et al.*, 2020; Hong *et al.*, 2012). In this study I attempted to assess their effect on endothelial cells in an environment mirroring that of plaque erosion. Endothelial cells were cultured on ECM components present within eroded plaques and where practical under atheroprone flow conditions experienced at the downstream region of the plaque.

I found NMVs facilitated EC detachment in an MMP-dependent manner. These findings indicate a potentially important role for NMVs in inducing erosion of ECs. EC detachment is a central component of plaque erosion and neutrophils have been implicated in this process previously (Quillard *et al.*, 2015). It is not completely clear if EC erosion causes thrombosis, or if ECs become eroded as a result of thrombosis. However, due to the exposure of pro-thrombotic ECM following EC erosion the consensus is that this process initiates thrombosis. I also observed an increase in EC

apoptosis following NMV treatment. Together, these observations indicate NMVs may well contribute towards loss of the endothelium, a key hallmark of erosion. Whilst interesting, future work would endeavour to uncover the cellular mechanisms through which these effects manifest in ECs. For example, in the case of apoptosis NMVs have already been reported to alter endothelial gene expression via mir-155 in a pathway involving NF-κB (Gomez *et al.*, 2020). Alterations to NF-κB signalling could explain the changes in apoptosis observed however, disruption of EC interactions with the underlying ECM can also induce a form of apoptosis, known as anoikis (Taddei *et al.*, 2012). By establishing the mechanism through which NMVs induce apoptosis it could be possible to block this cellular pathway, potentially reducing the harmful pro-apoptotic effects of NMVs on the endothelium.

6.2.4. Wound healing and proliferation

Having determined NMVs increase HCAEC detachment and apoptosis it was important to assess cellular processes that may compensate for these increases. I chose to investigate proliferation and wound healing response as two mechanisms through which the endothelium could recover from erosion. I showed NMVs induced a decrease in both the rate of proliferation and wound healing response in HCAECs. I interpreted these effects as exacerbating the erosion of ECs induces by increased apoptosis and detachment. If the endothelium is less well able to recover from the damage it follows that the damage caused will accumulate, potentially culminating in an eroded plaque prone to thrombosis. Previous work supports our findings. For example, Slater *et al* found NMVs inhibited epithelial cell wound healing through the delivery of MPO. Reduced wound recovery and proliferation resulted from cell cycle arrest, actin fibre dysfunction and impaired cell spreading (Slater *et al.*, 2017). Additionally, there is evidence to suggest the ECM has wide ranging effects on ECs including the ability to modulate EC wound healing responses and proliferation (Raines, 2000). The effects of ECM components on wound healing and proliferation were not investigated in this thesis. However, I did show LMW-HA increased apoptosis in HCAECs and therefore it would be reasonable to hypothesise that LMW-HA may also have additional effects on proliferation and wound healing that may exacerbate the effects of NMVs. This would be an important question to consider in future investigations. Additionally, a broader range of ECM components could be investigated focusing primarily on ECM components found in greater concentrations in eroded plaques compared with ruptured plaques. This work could potentially uncover how differences in the fundamental structure of eroded plaques influence the mechanisms that drive erosion.

In relation to both proliferation and wound healing responses, future experiments would also seek to identify the mechanism through which NMVs may be exerting their effects. Whilst there are many mechanisms through which NMVs could be acting previous work by our group indicates a promising area may be the delivery of micro-RNAs to the endothelium (Gomez *et al.*, 2020). Mir-155 was shown to be delivered and modulate gene expression via NF-κB. Future micro-RNA sequencing of NMVs could potentially identify candidates with known effects in ECs dysfunction and subsequent silencing of these micro-RNA targets could confirm their activity.

Further experiments in future would address some unanswered questions raised in chapter 4. Initially, this would include aspects that were planned for chapter 4 but were not feasible due to technical issues and time restraints. The effect of LOSS on the proliferation of NMV treated HCAECs would make these data more translational to the *in vivo* environment of eroded plaques. As discussed previously our method of Ki-67 staining as a marker of proliferation was not sufficient to detect proliferation in confluent HCAECs. Flow cytometry may provide a more sensitive method to detect small differences in staining intensity compared to fluorescence microscopy.

6.2.5. Pro-thrombotic gene expression

Changes in EC pro-inflammatory gene expression induced by NMVs have been reported previously (Hong *et al.*, 2012). In this thesis, I investigated the effect of NMVs on expression of thrombosis-related genes and observed increased expression of pro-thrombotic VWF and decreased expression of anti-thrombotic THBD. This indicates NMVs may skew ECs towards a more pro-thrombotic phenotype, thus increasing the risk of thrombosis. VWF is known to be linked to cardiac events (Kucharska-Newton *et al.*, 2009; Spiel *et al.*, 2008) and stroke (Wieberdink *et al.*, 2010) and has been implicated in atherosclerotic lesion size, neutrophil, macrophage and monocyte content, and MMP content (Doddapattar *et al.*, 2018). Increased expression of HCAEC VWF increases platelet recruitment to these cells (Doddapattar *et al.*, 2018) and our data on platelet adhesion under static conditions show more platelet adhesion following NMV treatment compared to controls, supporting the hypothesis of a more pro-thrombotic endothelium. Decreased THBD expression observed in HCAECs treated with NMVs also suggests a more pro-thrombotic environment. THBD encodes the surface protein thrombomodulin which acts to decrease the pro-thrombotic effects of another protein, thrombin, thus reducing atherosclerotic lesion formation (Wei *et al.*, 2011).

Due to time restraints the RT-qPCR data were not followed up with protein expression analysis, however this would be the necessary next step in investigating whether NMVs induce pro-thrombotic changes in ECs. This is perhaps most important in the case of VWF due to the manner in which VWF is stored. VWF protein is stored within Weibel-palade bodies (WPBs) in ECs and released

following stimulation. In further studies it would be interesting to determine if the increase in VWF gene expression results in an increase in VWF protein secretion by ECs. In addition, it would be interesting to determine whether NMV could induce the secretion of stored VWF from WPBs.

6.2.6. Neutrophil microvesicle effect on platelet activation and adhesion

Platelets are central to the process of thrombosis. In plaque erosion, where the cause of thrombosis is less apparent than in plaque rupture, subtle changes in platelet activation and propensity to adhere could provide useful insights into how thrombosis initiates and propagates. Platelets are known to adhere to neutrophils via interactions between platelet P-selectin and neutrophils PSGL-1 (Kappelmayer and Nagy, 2017), as well as GPIbα to CD11/CD18 (Wang *et al.*, 2005). Whilst there is considerable research concerning the interactions between neutrophils and platelets for instance, the role of NETs in thrombosis (Lisman, 2018; Martinod *et al.*, 2013) there is little regarding the role of NMVs in this process. However, Rossaint *et al* demonstrated a link between the interaction of neutrophils with platelets in the production of thromboxane A_2 (T_xA_2), a process mediated by NMVs. T_xA_2 is a pro-thrombotic mediator that promotes platelet activation and aggregation. Co-incubation of neutrophils with fMLP and platelets significantly enhanced NMV production, which was reduced following blocking of both GPIbα or P-selectin. These NMVs also exhibited increased arachidonic acid content. Internalisation of NMVs by platelets delivered this arachidonic acid to cyclooxygenase 1 (Cox1)-rich compartments of the platelets. Cox1 synthesises T_xA_2 from arachidonic acid and through a feedback loop increases neutrophil adhesion to the endothelium by enhancing EC ICAM-1 protein expression (Rossaint *et al.*, 2016). Given the crosstalk between platelets, neutrophils and NMVs I investigated whether NMVs generated in response to the endogenous stimuli, nLDL rather than bacterial fMLP, promote platelets to adhere to the endothelium and aggregate. Our data indicates that NMVs adhere to platelets, however as shown by Rossaint *et al* NMVs are likely internalised meaning our measurements likely underestimate of the proportion of platelets having interacted with NMVs. CD11/CD18-dependent uptake of NMVs by platelets was reported by Rossaint *et al* however, the role of P-selectin was not addressed. Specifically blocking P-selectin and PSGL-1 and repeating our binding experiment would shed light on the binding mechanism. If this is, as I expect, the mechanism of interaction then activation of platelets would likely enhance these interactions due to the increased expression of P-selectin. This could be tested by activating platelets to varying degrees and quantifying the adherence of NMVs. Alongside this, experiments to quantify internalisation of NMVs by platelets would inform this investigation.

Our data also demonstrate NMVs induced an increase in platelet P-selectin expression and promoted platelet adhesion to ECs. The interaction between NMVs and platelets could represent a potential therapeutic target for reducing the risk of thrombosis. Disrupting the interaction between NMVs and platelets via blocking PSGL-1 and CD18 could help to reduce this effect on platelet activation. However much more research is necessary to determine what effect NMV-platelet interactions have on the risk of thrombosis *in vivo* and whether targeting this interaction could provide significant benefits. Follow up experiments blocking NMV-platelet interactions in a mouse model of atherosclerosis may provide useful insight on this question.

In the current work, when assessing platelet adhesion to ECs, ECs were pre-treated with NMVs and then freshly isolated platelets. Therefore, any increase in platelet adhesion to ECs observed would likely be due to the effect of NMVs on EC gene expression. This neglects the potential effect NMV may have on platelets directly and is a limitation of the study. I have presented data showing NMVs enhance platelet P-selectin expression and therefore it is likely this could influence adhesion to the endothelium. Even if our data suggests this enhanced P-selectin expression did not increase platelet aggregation. Additional experimental conditions are required in future investigations whereby untreated ECs are exposed to NMV-treated platelets and NMV-treated ECs are exposed to NMV-treated platelets. This experiment, including both NMV-treated ECs and NMV-treated platelets would more accurately reflect the *in vivo* environment and would allow us to determine through which cell type NMVs exert the greatest pro-thrombotic effects. In other words, is platelet adhesion governed more by the effect of NMVs on platelet activation (e.g. P-selectin expression or T_xA_2 production) or the effect on ECs (e.g. increased vWF expression). Furthermore, a limitation of this study is that under atheroprone flow conditions I did not observe a significant increase in platelet adhesion to HCAECs following NMV treatment. This could be due to the pro-thrombotic effects LOSS conditions have on ECs, masking the more subtle effect of NMV treatment. Additionally, it could be a dosage issue. Under LOSS conditions the NMVs have much less contact time with the HCAECs compared to when used under static conditions. Increasing the concentration of NMVs used under LOSS conditions may allow the results of the static experiments to be replicated.

Whilst additional experiments detailed above to further investigate the effects of NMVs on platelet function would be informative in investigating the mechanisms responsible, the priority should be to increase the n numbers of already conducted experiments to gain more reliable results that would allow for better interpretation. As noted previously, COVID-19 restrictions severely restricted our ability to recruit healthy volunteers to donate blood. Due to our reliance on freshly isolated neutrophils and platelets this limit held back progress. Due to limited availability of NMVs it was not feasible to run dose-response experiments for every assay I used. Instead, I used concentrations of NMVs established from earlier experiments, for instance the apoptosis and proliferation assays and applied them to new experiments where possible. Future experiments would determine optimal NMV

concentrations for each cellular process being investigated and each assay type. This may uncover dose-dependent effects of NMVs not identified in the current study.

6.3. Conclusions

The data presented in this thesis demonstrate NMVs are generated in response to native LDL and induce responses within endothelial cells that could promote atherosclerotic plaque erosion. Given the importance of endothelial cell removal in the context of plaque erosion our observations that NMV promote extracellular matrix degradation, endothelial cell detachment and endothelial apoptosis suggest NMVs may contribute to the loss of the endothelium. Our data also suggests NMVs reduce proliferation and the wound healing response in endothelial cells potentially reducing the ability of the endothelium to recover and further promoting plaque erosion. Further to this I presented data suggesting NMVs may interact with platelets, increasing their activation and adhesion to the endothelium. In summary, these data suggest a potential role for NMVs in the processes that contribute to atherosclerotic plaque erosion.

Based on the findings of this thesis a number of new hypotheses are appropriate. These include:

- NMVs contain effector molecules other than proteases that alter endothelial cell apoptosis, proliferation, and wound healing responses.
- Inhibiting the protease activity of NMVs may reduce the impact of NMVs on ECM degradation and EC detachment.

6.4. COVID-19 impact statement

The disruption caused by the COVID-19 pandemic substantially impeded my ability to complete experiments reducing the amount of experimental data presented in this thesis. In addition, experiments suffered from being underpowered in some cases, limiting the ability to draw meaningful conclusions from the results. There were several key components of this project which rendered it particularly vulnerable to disruption.

More than half of my PhD was completed during the pandemic including the first 4 months from March 2020 where all lab work was suspended. This sudden stoppage resulted in many experiments being abandoned, in some instances meaning valuable reagents and samples were discarded.

Once lab work was able to resume, a lack of healthy volunteers to donate blood slowed my progress. All my experiments relied upon access to freshly isolated neutrophils and/or platelets from whole blood. My ability to recruit healthy donors was substantially restricted due to several factors outside of my control. For instance, many of the regular donors were staff members who were required to work from home or were isolating. Recruitment was restricted further by our inability to recruit donors from outside the hospital due to restrictions on members of the public entering the department and wider hospital property. Furthermore, due to neutrophils being sensitive cells of the immune system and to avoid influencing my experiments it was important to exclude donors who had recently received a positive COVID-19 test result or who had experienced symptoms in the last 2 weeks. These factors in combination severely restricted the pool of available donors and since volunteers may donate blood only every two weeks, I quickly exhausted the limited supply and struggled to collect enough whole blood for my experiments.

Efforts were made to mitigate the impact of a reduced pool of available donors. I coordinated with other groups using whole blood in their experiments to maximise the number of neutrophil and platelet isolations I could perform. Additionally, whenever volunteers were available, every effort was made to isolate the neutrophils and generate NMVs which were subsequently frozen and stored for future use when fewer volunteers were available. This allowed me to complete more experiments than would otherwise have been possible. However, this was not feasible with platelets which had to be freshly isolated for reliable results.

Access to the labs was also restricted even once return to campus authorisation was granted. Due to room occupancy limits in the tissue culture lab, it was often difficult to schedule experiments due to high demand. This was especially true for experiments that required access to the lab on several consecutive days. The impact of this was reduced somewhat by good organisation and planning experiments in advance however, unforeseen circumstances such as last-minute donor cancellations made this challenging. Fewer colleagues being authorised to the use the labs meant there were fewer people available ask for support during experiments. Previously what would have been a simple conversation with a technician or fellow PhD student to resolve a problem would now take considerably longer due to all communication occurring via email.

I also experienced problems with reagent availability due to global shipping delays and shortages of certain materials. For instance, our order of primary endothelial cells was significantly delayed meaning all cell-based experiments were postponed for many weeks. This issue was compounded by the need to spend grant funds by the specified deadlines. As the lockdown had delayed resumption of experimental work it was now necessary to spend all of the remaining grant money

much earlier in my project than planned. With over 1.5 years of my PhD remaining, it was necessary to plan most of the future experiments I aimed to complete during my project and purchase the necessary materials. This was incredibly challenging because future experiments normally build upon the results generated by previous experiments. As these experiments were often delayed, I had to design my future experiments with little experimental data to inform my choices.

Without these delays and disruptions, I would have completed more repeats of my experiments to increase the n numbers. This would have allowed for better interpretation of the results and stronger conclusions to have been drawn. Additionally, with fewer disruptions it may have been possible to investigate some of the areas of interest that arose during my project. For instance, determining the mechanism of action behind the increased apoptosis and reduced proliferation that was observed.

Despite all the challenges faced during this project, I believe this thesis represents an interesting contribution to the field of NMVs and their role in plaque erosion and certainly encourages future research on this topic.

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