The University Of Sheffield.

Modulation of Endothelial Interleukin-1β Inflammation.

By:

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Dedication.

This work is lovingly dedicated to my family and friends. A very special gratitude for my mother Mrs Najia Ali, her support, encouragement, and constant love have always been with me and sustained me throughout my life, without you I would never be here. My sister Ms. Hajer Abdeljalel, thanks for your patience with your cancer despite the tough course of chemotherapy and I hope once you read this to be always happy and healthy. My younger sister Ms. Sara Abdeljalel for your kindness and devotion, and your endless support for Hajer during her tough journey of a treatment, you are very special to me. Finally, I dedicate this work to all of my friends and the rest of my family who have always been supportive throughout the process.

Preface.

The amelioration of atherosclerosis is one of the most demanding aspects in the management of coronary artery disease. In cardiovascular medicine, despite the new discoveries in research, patients with coronary artery disease still suffer from complications of atherosclerosis. The aim of this thesis is to shed new light on the pathogenesis of atherosclerosis and to propose a new mechanism to modulate endothelial inflammation in order to mitigate atherosclerosis, both *in vitro* and *in vivo* using different approaches.

This thesis describes two projects, *in vitro* work on endothelial interleukin-1beta releasing mechanism(s) and *in vivo* studies on defining the cardio-protective roles of omega-3 fatty acids. These seemingly disparate projects are connected by a central role for the proinflammatory cytokine interleukin-1beta. The studies are described in separate chapters but the work is drawn together in chapter (8) and concluded in chapter (9).

Acknowledgments.

Studying a PhD at the University of Sheffield was one of my life turning points. I feel both fortunate and intimidated to be writing this dissertation after a four-year experience as a scientific researcher. Working previously as a clinical cardiologist, I feel fortunate because I now have one of the best lab-based research ever produced with a challenge of translating this study into clinical cardiology. I was able to publish a part of this study and I have done that because of help of many great people.

Thanks must go first and foremost to my supervisors: Professor Sheila Francis and Dr. Janet Chamberlain for their time, encouragement and their generous expertise throughout this project. In particular, I am very grateful to Professor Sheila Francis for providing countless hours of support, patience and her continuum friendships during my PhD. Her exquisite attention to details and demands for excellence were very helpful in successfully finishing this project.

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Summary.

Atherosclerosis, complex chronic inflammatory disease, has a heterogonous aetiology. Endothelium is critically involved in the pathogenesis of atherosclerosis by producing proinflammatory cytokines, including interleukin-1 beta (IL-1β). However, the mechanism by which IL-1β is released is unknown. Neutrophil elastase (NE; a potent serine protease) has been shown to cleave proIL-1β *in vitro*. Therefore, I hypothesised that NE induces IL-1β secretion from endothelial cells (ECs).

I found that NE cleaves proIL-1β in ECs and causes significant secretion of mature IL-1β into supernatant. The release is via extracellular vesicles (EVs), associated with a transient increase in intracellular Ca^{2+}. The released IL-1β is significantly attenuated by inhibition of NE, but not caspase-1. Intracellularly, IL-1β is detected within LAMP-1 positive organelles only after NE treatment. Two distinct populations of vesicles, containing IL-1β are found: at early time points, intracellular vesicles (100-200μm), associated with detection of MV shedding enriched of IL-1β; however, at later time points, IL-1β was detected inside ECs in (>200μm) multivesicular bodies (MVBs) containing exosomes.

In a second study, in experimental atherosclerosis, I attempted to manipulate inflammation using omega-3 fatty acids (n3FAs). I hypothesised that docosahexaenoic acid (DHA), the main n3FAs in fish oil, would inhibit inflammation by an IL-1β driven mechanism. I found that DHA significantly decreased high blood pressure and left ventricular mass induced by high fat diet in $ApoE^{-/-}$ mice. Interestingly, this is associated with a reduction in distal vessel atheroma and plasma proinflammatory markers. Locally, DHA also significantly attenuates eNOS and endothelial IL-1β expressions.

This study reveals a hitherto unexplained mechanistic link between NE expression in atherosclerotic plaques and concomitant bioactive IL-1β secretion from ECs, highlighting the possibility of targeting NE to control IL-1β-induced atherosclerosis. It also sheds a light, for the first time, on how DHA can act as an anti-atherogenic agent through its effects upon IL-1 system.

Detailed Contents.

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Symbols and Abbreviations.

Abbrev.	*Full name.*
<	Less than.
>	More than.
%	Percentage.
/	Per.
+ve	Positive.
approx.	Approximately.
α-SMA	Alpha-smooth muscle actin.
ALA	α-Linolenic acid.
$ApoE^{-/-}$	Apolipoprotein E knockout mice.
ATP	Adenosine 5′-triphosphate.
BAF-1	Bafilomycin A1.
BCA	Brachiocephalic artery.
BP	Blood pressure.
CAD	Coronary artery disease.
Ca^{2+}	Calcium.
$[Ca^{2+}]_i$	Intracellular or cytosolic calcium.
CG	Cathepsin G.
cm	Centimetre.
DHA	Docosahexaenoic acid.
DW	Distilled water.
DBP	Diastolic blood pressure.
DAB	3,3 Diaminobenzine.
ECs	Endothelial cells.
EM	Electron microscopy.
ELISA	Enzyme-linked immunoassay.
eNOS	Endothelial nitric-oxide synthase.
ECs	Endothelial cells.
EDTA	Ethylenediaminetetraacetic acid.
EGTA	Ethylene glycol tetraacetic acid.
EVs	Extracellular vesicles.
ECM	Extracellular matrix.
ECS	Extracellular space.
FS	Fractional shortening.
FO	Fish oil.
HUVEC	Human umbilical vein endothelial cells.
HCAEC	Human coronary artery endothelial cells.

HDL-C	High-density lipoprotein cholesterol.
h	Hour.
HSS	High shear stress.
IHD	Ischemic heart disease.
IL-1	Interleukin-1.
IL-1β	Interleukin-1 beta.
IL-1α	Interleukin-1 alpha.
IL-6	Interleukin-6.
IL-8	Interleukin-8.
IL-1RI	Interleukin-1 receptor type I.
IL-1RII	Interleukin-1 receptor type II.
IL-1ra	Interleukin-1 receptor antagonist.
LDH	Lactate dehydrogenase.
LAMP-1	Lysosomal associated membrane protein-1.
LSS	Low shear stress.
LDL-C	Low-density lipoprotein cholesterol.
$LDLr^{-/-}$	Low-density lipoprotein receptors knock out mice.
LV	Left ventricles.
LVEF	Left ventricular ejection fraction.
LVFS	Left ventricular fractioning shortening.
LVH	Left ventricular hypertrophy.
LVM	Left ventricular mass.
LPS	Lipopolysaccharide.
MVBs	Multivesicular bodies.
min.	Minute.
MVs	Microvesicles.
MCP-1	Monocytes chemoattractant protein 1.
µg	Microgram.
µl	Microliter.
mL	Millilitre.
MI	Myocardial infarction.
MBP	Mean blood pressure.
NE	Neutrophil elastase.
NF-κB	Nuclear factor κ-light-chain-enhancer of activated. B cells.
NEIII	Neutrophil elastase type III inhibitor.
n3FAs	Omega-3 fatty acids.
NO	Nitric oxide.
NOX	NADPH oxidase.
Ox-LDL	Oxidised LDL.

OSI	Oscillatory shear index.
ORS	Oil red O stain.
PS	phosphatidyl serine.
PBS	Phosphate-buffered saline.
PFA	Paraformaldehyde.
SEM	Standard error of the mean.
SFM	Serum free media.
SBP	Systolic blood pressure.
SMC	Smooth muscle cell.
TLR	Toll like receptor.
TLR4	Toll like receptor type 4.
TNF-α	Tumor necrosis factor-α.
TG	Triglycerides.
TC	Total cholesterols.
TE	Transthoracic echocardiogram.
YVAD-CHO	Caspase-1 inhibitor.
v/v	Volume/volume.
VSMC	Vascular smooth muscle cells.
w/v	Weight/volume.
WSS	Wall shear stress.

Note: abbreviations that are mentioned in this thesis more than three times are fully defined here.

Chapter (1) General Introduction.

OUTLINE:

1.1. Overview of General Introduction:

Cardiovascular disease (CVD) is the leading cause of death worldwide (Kovacic and Fuster, 2011), particularly for those under the age of 60 years (Nowbar et al., 2014). The vast majority of these deaths are attributed to coronary artery disease (CAD). CAD, is one of the clinically diagnosed CVD, is worrisome as it is both prevalent and has a poor prognosis (Kovacic and Fuster, 2011). The main underlying process that contributes to CAD is atherosclerosis, a narrowing or hardening of large and medium-sized arteries, which leads to ischemia or infarction either in the heart or the brain (Fauci, 2008).

Despite advances in disease management, therapies are still insufficient to mitigate atherosclerosis and, consequently, CAD risk and mortality (Van Tassell et al., 2013). The growing epidemic in metabolic risk factors (e.g. diabetes, hyperlipidaemia and obesity), smoking and hypertension have led to increase incidence of acute and recurrent ischemic heart disease (IHD) (Libby, 2015). IHD was classically attributed to occur as a result of metabolic, rather than an inflammatory, disturbance, particularly in glucose and lipid metabolism and that was linked to progression of the disease and its complications. However, reports of inflammatory responses have begun to emerge since the 1970s (Kushner et al., 1978, Ross, 1999). Subsequently, several observational studies have documented an increase in plasma pro-inflammatory mediators, including C-reactive protein (CRP), a surrogate marker for interleukin-1 (IL-1), a major inflammatory culprit in atherosclerosis, in patients with acute coronary events (Libby, 2002).

In atherosclerosis, endothelial dysfunction and low-density lipoprotein cholesterol (LDL-C) sub-endothelial precipitation are the earliest changes in the vasculature (Palinski et al., 1989, Glass and Witztum, 2001). As a result, a cascade of inflammatory changes, starting with inflammatory cell recruitment and release of cytokines and chemokines occurs (Libby, 2003).

Interleukin-1 beta (IL-1β) is among the cytokines that have been studied in both humans and animals in order to ameliorate atherosclerosis (Chamberlain et al., 2006, Galea et al., 1996). This cytokine plays a pivotal role in the process of atherosclerosis from the enhancement of monocyte recruitment (Rollins et al., 1990,

Kirii et al., 2003) to smooth muscle cell proliferation (Nathe et al., 2002) and plaque instability (Monaco et al., 2009). As a consequence of this, IL-1β has been targeted in many studies (Dinarello, 2005, Kirii et al., 2003) in an attempt to discover a novel therapy that might be helpful in the management of atherosclerosis and CAD.

In inflammation, IL-1β is considered as a 'dangerous' cytokine and in order to keep its levels negligible, in a disease free state, production of the pro-inflammatory cytokine IL-1β is controlled by an auto-regulatory mechanism. A natural competitive inhibitor, IL-1 receptor antagonist (IL-1ra) competes with IL-1β binding at the receptor level without downstream signalling effects (Dinarello, 1996). Additionally, a decoy receptor, IL-1 Receptor II (IL-1RII), binds to IL-1β without any signalling impact (Dinarello, 2005, Colotta et al., 1994). The role of IL-1ra as an anti-atherogenic drug has been studied in myocardial infarction (MI) (Abbate et al., 2008) and hypertension (Mauno et al., 2008), although its use has not yet entered in wider cardiologic practice. Unresolved questions remain regarding the complex physiology of IL-1β and its cellular of origin in atheromatous plaques.

Epidemiological, population-based and clinical studies highlight the effectiveness of prevention (primary or secondary) of IHD risk factors in controlling of the disease. One of the effective preventive strategies is the introduction of omega-3 fatty acids (n3FAs). These fatty acids show a considerable cardio- protective role, particularly in patients with a high risk of IHD and atherosclerosis (Mozaffarian and Wu, 2011). Omega-3 FAs are a part of the polyunsaturated fatty acid (PUFA) family obtained from the diet. Although free fatty acids like these have never been used as a therapy for an already established atherosclerotic lesion, they are used as supplements for high-risk patients (Yates et al., 2014). However, the mechanism of action of these free fatty acids has not been fully elucidated.

The next sections of this chapter will discuss different aspects raised in the literature concerning the potential roles of IL-1β in atherosclerosis and hypertension. Additionally, it will examine the possible relationship between n3FAs, inflammation due to IL-1β, and atherosclerosis. Finally, it will draw relevant conclusions leading to a hypothesis and objectives for this thesis.

1.2. Atherogenesis and Inflammation:

Atherosclerosis develops in approximately (approx.) 1% of the worldwide population and presents as an abnormal occlusion of arteries, that leads to life threatening complications, including myocardial (MI) and cerebral infarction (Libby, 2003). The disease develops due to endothelial dysfunction, and an excessive accumulation of macrophages and lipid laden cells, as a result of a maladaptive body response to high plasma cholesterol and its derivatives, in the subendothelial space (Hansson, 2005).

Although atherosclerosis is a multifactorial disease, it is currently appreciated that hyperlipidaemia and hypertension are the main causative factors (Hansson and Hermansson, 2011). However, substantial evidence has suggested that, in atherosclerotic plaques, mixed patterns of inflammatory cells have been identified (Weber and Noels, 2011). Furthermore, bacterial infection can induce atherosclerosis in susceptible mouse strains (Chi et al., 2004). Moreover, even with lipid lowering therapy and changing life-style patterns, the risk of death from atherosclerosis and CAD remains relatively high (Grundy et al., 2004, Nowbar et al., 2014). This strongly suggests that inflammation can be considered as an additional targetable process in atherogenesis.

In response to atherogenic stimuli, inflammatory cells adhere and migrate to the subendothelial space, mainly at the sites of bifurcation with disturbed blood flow (Libby et al., 2014). Strong evidence demonstrates that oxidised LDL-C (ox-LDL-C) is a major contributory factor for atherogenesis (Nievelstein et al., 1991, Tabas et al., 2007) and many studies have shown that ox-LDL-C is taken up by macrophages and vascular smooth muscle cells (VSMCs) in the arterial wall via scavenger receptors, such as CD36, and scavenger receptors type A & B (SRA & SRB), leading to accumulation of cholesterol esters and subsequent formation of foam cells, the hallmark of the fatty streak stage (Ward et al., 2009b, Libby, 2002, Glass and Witztum, 2001, Moore et al., 2013).

Previous studies have demonstrated that the endothelium of diseased vessels also expresses adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1)

and vascular cell adhesion molecule 1 (VCAM-1), that play a fundamental role in the rolling and thus migration of circulating cells into the vessel walls (McEver, 2015). Moreover, monocytic chemoattractant protein; MCP-1, the major chemoattractant protein, produced by various inflammatory cells in plaques, including endothelium, VSMCs, and macrophages, in response to IL-1 (Kirii et al., 2003).

The process of atherogenesis is shown diagrammatically in figure (1.1).

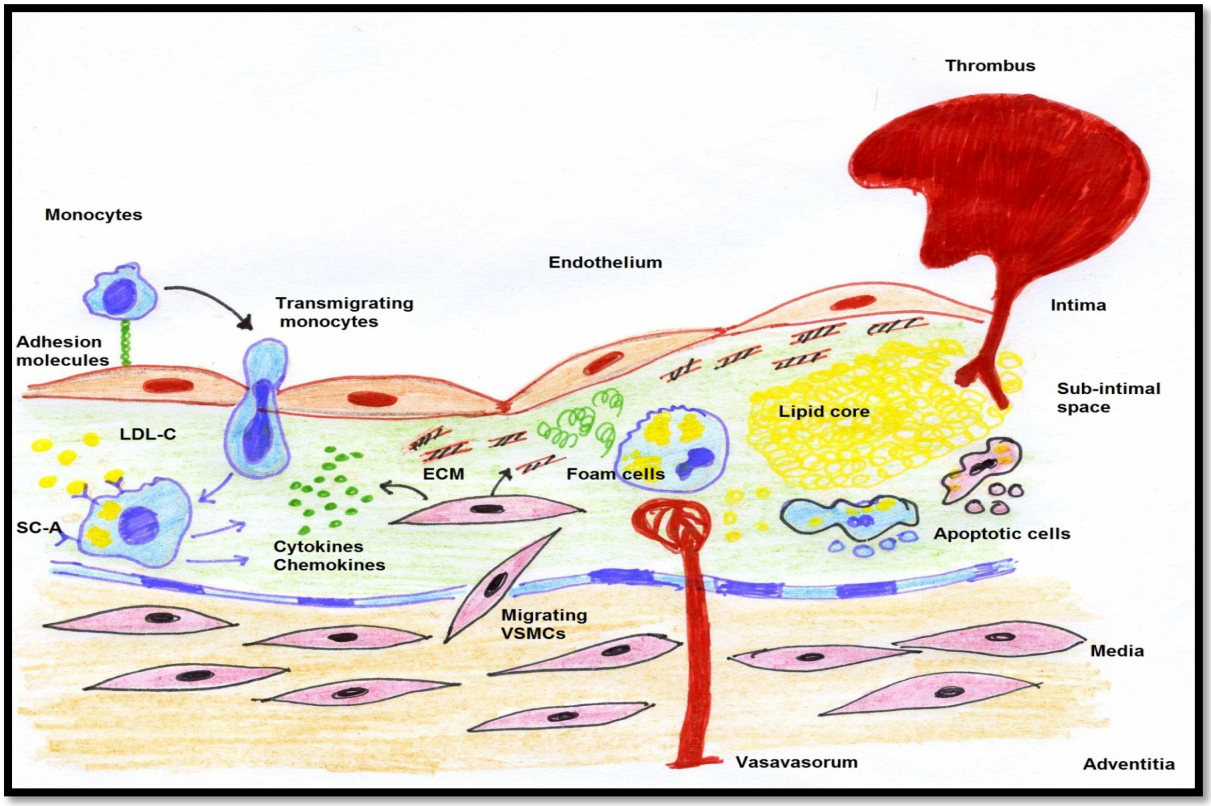

Figure 1.1 Atherosclerosis, lesion formation and plaque rupture. Oxidised LDL-C enhances the expression of adhesion molecules on the surface of endothelial cells. The circulatory monocytes adhere and roll on the endothelium before their migration into the sub-endothelial layer. The process of migration is fostered by chemo-attractants released by inflammatory and endothelial cells; macrophage chemo-attractant protein-1 (MCP-1). In the sub-intimal space, the monocytes differentiate into macrophages, which express their scavenger receptors. Scavenger receptors, particularly scavenger receptor type A (SR-A) and CD36 mediate the engulfment of oxidised LDL-C into macrophages, resulting in foam cells. Cytokines released by foam cells and VSMCs stimulate macrophages to release matrix metalloproteases, which dissolve the cap. Furthermore, an increase in the size of necrotic core, which consists of apoptotic foam and VSMCs, leads to rupture of the plaque. Platelets adhere to the erosion sites at the shoulders and thrombin is precipitated at the site.

1.2.1. Fate of Atheroma:

After migration of smooth muscle cells (SMCs) from the media into the sub-intimal space in response to cytokine activation, they proliferate and release extracellular

matrix (ECM) proteins (Libby, 2002). The resulting lesion is usually complex and bulky with some backward extension into the lumen. This arterial remodelling is due to the elasticity of the external elastic lamina (Tabas et al., 2015), which appears as a “bulge” in coronary angiograms (Glass and Witztum, 2001). However, at this stage, the disease is difficult to diagnose, as the majority of patients are asymptomatic (Tabas et al., 2015).

The growth and progression of the fibrous cap lesion takes decades before the symptoms of ischemia appear as a result of obstruction (Libby et al., 2014). Alternatively, the plaque might rupture with subsequent platelet activation and thrombus formation (Hansson, 2005). It is generally accepted that plaque stability is a determinant for the clinical complications and MI manifestations (Tabas et al., 2015).

Plaque instability is usually characterised by a large lipid core (>20-30% of plaque area) (Davies, 1996). Two additional factors that may predispose plaque rupture are a thin fibrous cap (Virmani et al., 2006) and released proteases that are secreted by inflammatory cells and these dissolve parts of the cap, especially the shoulder region (Glass and Witztum, 2001, Galis et al., 1994). Cytokines play a significant role at this stage by stimulating macrophages to release matrix metalloproteases (MMPs) and neutrophil elastase (NE) (Galis et al., 1994, Dollery et al., 2003).

A third factor that enhances plaque instability is an increasing number of apoptotic cells (mainly derived from foam cells and VSMCs) (Tabas et al., 2015), which is also thought to be mediated by cytokines and IL-1 (Geng et al., 1996).

The resulting thrombosis or calcification at the sites of ruptured plaque (Insull, 2009) leads to narrowing or complete obstruction of the vascular lumen (Libby and Pasterkamp., 2015). If this obstruction/narrowing occurs at the coronary arteries, symptoms of myocardial infarction or ischemia appear (Seropian et al., 2014).

1.3. The central role of the endothelium in atherogenesis vascular disease:

1.3.1. Endothelial cell dysfunction and atherogenesis:

Mechanical or functional disruption of the endothelial layer is an additional observation in unstable plaques (Virmani et al., 2000). In addition, dysfunctional endothelium has been considered as the one of the earliest (Casino et al., 1993) and persistent vascular changes in atherosclerosis (Suwaidi et al., 2000).

Although macrophages, SMCs and platelets are important in atherosclerotic plaque formation, endothelial cells (ECs) also play a central role. ECs in healthy vessels play a protective role by maintaining the vascular tone and secretion of anti-inflammatory mediators such as nitric oxide (NO) (Aird, 2008). However, in a disease prone state, the endothelium enhances atheroma formation by several mechanisms (Bonetti et al., 2003). Endothelial dysfunction allows precipitation of LDL-C into the subintimal space (Libby, 2003). The endothelium also produces and expresses adhesion molecules to promote inflammatory cell migration into the subintima (Bombeli et al., 1999). Experimental studies have shown that inflamed ECs, when stimulated, produce considerably large amounts of proinflammatory cytokines, including interleukin (IL)-6 (Jirik et al., 1989) and IL-8 (Bonetti et al., 2003).

1.3.2. NO, NOS, and NADPH oxidase and their roles in atherogenesis:

NO is an important vasodilator that is secreted by the healthy endothelium and maintains vascular tone (Forgione et al., 2000). Recent evidence shows that deficiency of NO impairs vasodilatation and thus increases vascular resistance and risk of hypertension (Fauci, 2008).

NO is synthesised by converting L-arginine to L-citrulline in the presence of endothelial nitric oxide synthase (eNOS) and a cofactor tetrahydrobiopterin (BH4) (Tayeh and Marletta, 1989, Rabelink and Luscher, 2006). Among the three isolated isoforms of nitric oxide synthase (NOS), eNOS and neutral NOS (nNOS) are the primary forms in a healthy endothelium (Kawashima and Yokoyama, 2004) whereas iNOS (inducible NOS) is the predominant form in hypertension and atherosclerotic lesions (Behr-Roussel et al., 2000, Kawashima and Yokoyama, 2004, Werner et al.,

2003). The mechanism by which iNOS is increased in hypertension and atherosclerosis is unknown. One explanation is that, in response to inflammation, eNOS induces expression of iNOS and this, in turn, inhibits eNOS activity and impairs the endothelial function (Connelly et al., 2005).

A diminishment in the amount of eNOS substrates (either L-arginine or BH4) results in the formation of oxygen free radicals. The process of producing superoxide radicals (SOR) by eNOS is commonly known as uncoupling (Werner et al., 2003; Clapp et al., 2004; Li et al., 2006). In other words, the uncoupling of eNOS is an important source of SOR and endothelial dysfunction in hypertension. This effect can be reversed by the addition of BH4, hence restoring the endothelium dependent vasodilatation (Setoguchi et al., 2001). Alternatively, SOR can be produced by NADPH oxidase (NOX). There are at least 5 isoforms of NOX. Among them, NOX1, NOX2 (Cave, 2009) and NOX4 (Ellmark et al., 2005) are the most commonly isolated isoforms in cardiovascular diseases and atherosclerosis.

1.4. The interleukin-1 signalling pathway and its roles in atherosclerosis:

In addition to NO/eNOS system, studies on atherosclerotic plaques identified a myriad of proinflammatory cytokines that orchestrate the process of inflammation and atherogenesis. The IL-1 family has received a great deal of attention due to identification of IL-1 system in different stages of atheromatous human lesions (Ray, 2014, Satterthwaite et al., 2005), and studies on mouse models of atherosclerosis suggest a particular role for IL-1 signalling pathway in the disease (Chamberlain et al., 2006, Kirii et al., 2003).

The IL-1 family consists of 11 members whose production is closely linked to inflammation, and pioneering work by Dinarello and colleagues identified IL-1 as a biomarker of chronic inflammation (Dinarello, 2009). In inflammatory cells, closely related genes code two functionally different proteins, including IL-1β and L-1α. However, both cytokines bind to the same receptors, including interleukin- 1 receptor type I (IL-1RI) and IL-1RII (Dinarello, 2009).

IL-1α is produced in an active form that binds to the plasma membrane, acts locally and is released upon cell death (Dinarello, 1996). Therefor, IL-1α has been more linked with local inflammation and it participates less in systemic inflammation. On the other hand, IL-1β is the main circulating isoform of IL-1 (Dinarello, 1991), produced as an inactive form (proIL-1β) that requires enzymatic activation and cleavage, for example, by caspase-1 (Brough and Rothwell, 2007) or serine proteases (Black et al., 1991) to generate the active mature form (IL-1β).

The main focus of this thesis is on IL-1β due to its broadly circulating effects. However, the roles of IL-1α as endogenous priming for inflammation are discussed briefly throughout the work.
The synthesis, processing and release of IL-1β are tightly controlled by several mechanisms. The plausible roles of IL-1β as an atherogenic and hypertensive mediator, and its signalling and secretion mechanisms are discussed in detail in the sections that follow.

1.4.1. IL-1β is a key player in atherosclerosis:

1.4.1.1. Role of IL-1β in early lesions:

IL-1β appears to be crucial in the formation of fatty streaks and atherosclerotic plaques. Recent research using atherogenic mouse strains highlights the importance of IL-1β in early atherosclerosis. For instance, the fatty streak lesions in Apo-lipoprotein E knockout mice ($ApoE^{-/-}$) show significant regression after interleukin-1 receptor antagonist (IL-1ra) injection (Elhage et al., 1998). This contrasts with another study involving $ApoE^{-/-}$/$IL\text{-}1ra^{-/-}$ mice, which demonstrated no significant changes in the size of the foam cell lesion of the proximal aorta (Devlin et al., 2002).

However, the role of IL-1β in fatty streaks cannot be excluded. Evidence to support this is that the lack of IL-1β in $ApoE^{-/-}$/$IL\text{-}1\beta^{-/-}$ mice results in a considerable reduction in the lesion size, and this is associated with a lowering of adhesion molecules such as ICAM-1 and VCAM-1 expressions and MCP-1 levels (Kirii et al., 2003). Thus, IL-1β plays a key role in the expression of these endothelial adhesion molecules (Tamaru et al., 1998). IL-1β also enhances VSMC migration and proliferation (Nathe et al., 2002). Collectively, these effects are fundamental in triggering and development of atherosclerosis.

1.4.1.2 IL-1β and plaque instability:

The role of IL-1β in advanced atherosclerosis has been conflicting in many laboratory studies. High levels of IL-1β have been linked with an increase in the number of infiltrating macrophages (Isoda et al., 2004, Merhi-Soussi et al., 2005). As previously mentioned in section (1.2.1), this increase is associated with plaque instability and rupture, as these cells are more likely to undergo apoptosis (Libby and Pasterkamp., 2015).

In addition, IL-1β promotes the release of MMP-1, which has been shown to play a role in dissolving the cap (Monaco et al., 2009). In contrast, and controversially, Alexander and colleagues have postulated that advanced atherosclerosis in mice lacking IL-1RI are more prone to rupture. They report that this effect is associated with reduced MMP3 expression (Alexander et al., 2012). However, Chamberlain *et al.* have found that IL-1 plays a significant role in the formation of advanced lesions in response to high fat feeding (Chamberlain et al., 2009). Thus, although IL-1β is a key parameter in advanced atherosclerosis, the suppressive or the promoting role of IL-1β needs to be further elucidated.

1.4.2. The hypertensive effects of IL-1β:

Despite extensive clinical and experimental studies, the role of IL-1β in high blood pressure and hypertension is not completely understood. A prospective population-based study in Finland has found that IL-1β levels are elevated in hypertensive, and reduced in normotensive, individuals (Mauno et al., 2008). Conversely, another prospective study conducted on postmenopausal women in the USA has shown no variations in the levels of IL-1β in both hypertensive and normotensive participants (Wang et al., 2011b).

However, it could be argued that, in the first study, the elevated levels of IL-1β are probably due to the presence of an underlying pathology like atherosclerosis in those high risk individuals whereas the absence of such finding in the second study may be a result of measurements of IL-1β only once during the period of study. This is supported by a study by (Peeters et al., 2001) that tested blood samples of 23 essential hypertensive patients and showed that the levels of IL-1β significantly increased only after *in vitro* stimulation of circulating monocytes with lipopolysaccharide (LPS).

Some laboratory-based animal studies have been carried out to discover the molecular basis by which IL-1β induces its hypertensive effects. An important finding is that ApoE$^{-/-}$ / IL-RI$^{-/-}$ mice on a high fat diet had a lower blood pressure than ApoE$^{-/-}$ strains. The attenuated effect of blood pressure is associated with an increase in NO bioavailability and a decrease in SOR levels and NOX4 expression (Chamberlain et al., 2009). Surprisingly, in another study, NOX4 expression is suppressed by IL-1β in C57BL6/J mice and caused a transient reduction in the levels of SOR (Ellmark et al., 2005).

Similar to the human studies, the suppressive effect of IL-1β in C57BL6/J mice on NOX4 and SOR could be due to the absence of atherogenesis in these mouse strains. Also, it could be argued that the suppression of IL-1β is enhanced in the presence of the high fat in the diets, which fits with the clinical implications of the risk development of hypertension in patients consuming high fat in their diet (Fauci, 2008, Krauss et al., 2000).

1.4.3. The IL-1 system:

The IL-1 system is complex and in-line with its pleiotropic effects, three main levels of control have been shown to reduce the potent proinflammatory effects of IL-1β: (i) Control of IL-1 receptor activation (Dinarello, 2005); (ii) Downstream activation, controlling signal transduction effects; and (iii) Control of IL-1β synthesis and release (Keller et al., 2008, Kahlenberg and Dubyak, 2004, Wewers, 2004). Figure (1.2) shows the full details of control.

(i) Control of IL-1 receptor activation:

IL-1β signals via ubiquitously expressed cell surface receptors known as interleukin-1 receptor type I (IL-1RI) (Figure 1.2, step 1) (O'Neill and Dinarello, 2000, Cohen, 2014). The cytosolic segment of these sets of the receptors contains a Toll-like domain that functions in the innate body responses (Dunn et al., 2001).

The main expressed types of Toll-like receptors (TLRs) in atherosclerosis are TLR2 and TLR4 (Edfeldt et al., 2002, Schoneveld et al., 2008). These receptors have been shown to be activated by a variety of ligands such as LPS (Andersson et al., 1992, Kaspar and Gehrke, 1994), and ox-LDL (Xu et al., 2001).

As a results of IL-1/IL-1RI complex, IL-1 receptor accessory protein (IL-1RAcP), an intracellular co-receptor protein, is activated and induces downstream signal transduction (Weber et al., 2010).

A natural competitive inhibitor, IL-1ra, binds to IL-1RI (same affinity as that of IL-1β) but it does not induce any downstream signalling effects (Dinarello, 2009, Dunn et al., 2001, Schreuder et al., 1997). Animal based studies have suggested that IL-1ra, secreted predominately by the endothelium (Dewberry et al., 2000) and monocytes (Andersson et al., 1992), binds tightly to, and blocks, IL-1RI from signalling (Dinarello, 2009).
As a result, the synthetic form of IL-1ra, Anakinra, has been introduced in clinical trials as an anti-cytokine drug in septic shock (Eichacker et al., 2002). Subsequently, attention has been paid on using of IL-1ra in multiple chronic inflammatory diseases, including rheumatoid arthritis (RA) (Dinarello, 2005), type 2 diabetes mellitus (DM) (Larsen et al., 2007) and more recently in post MI patients (Ridker PM., 2011).

IL-1β suppression can also be achieved by IL-1 binding to interleukin-1 receptor type II (IL-1RII). This is a decoy receptor and, when bound to IL-1β, detach into the circulation without any inflammatory effects (O'Neill and Dinarello, 2000). Consequently, IL-1ra and IL-1RII (and soluble IL-1RI, IL-1RAcP) can provide negative feedback regulators to IL-1 signalling whose abundance (controlled at transcriptional levels) may prevent the unfavourable responses of IL-1 (Dinarello et al., 2012).

(ii) Downstream activation, controlling signal transduction effects:

After binding of the IL-1 ligand to its receptors, a complex of IL-1RI and IL-1RAcP, results in a downstream activation and assembly of two intracellular proteins, including myeloid differentiation primary response gene 88 (MYD88) (Figure 1.2, step 1) and interleukin-1 receptor–activated protein kinases (IRAKs) (Brikos et al., 2007). Mice lacking in MYD88 or IRAKs, especially IRAK4 showed defective IL-1 signalling (Suzuki et al., 2002). As a result of (auto)phosphorylation of IRAKs, a series of intracellular phosphorylation and activation takes place as illustrated in figure (1.2, step 2) (Kawagoe et al., 2008, Cao et al., 1996). Subsequently, nuclear factor-kappa B (NF-κB), a cytoplasmic transcriptional factor, is activated (Figure 1.2,

step 3) (Walsh et al., 2008) and is responsible for the synthesis of the IL-1β precursor (proIL-1β). NF-κB activation requires phosphorylation of the inhibitor of nuclear factor B (IκB) kinase (IKK) (Li et al., 1999), which subsequently phosphorylates and thus promotes IκB destruction (Zandi et al., 1998).

As a result of IκB degradation, the two subunits of NF-κB are released and translocate to the nucleus (Figure 1.2, step 4), which is a central step in NF-κB activation (Weber et al., 2010).

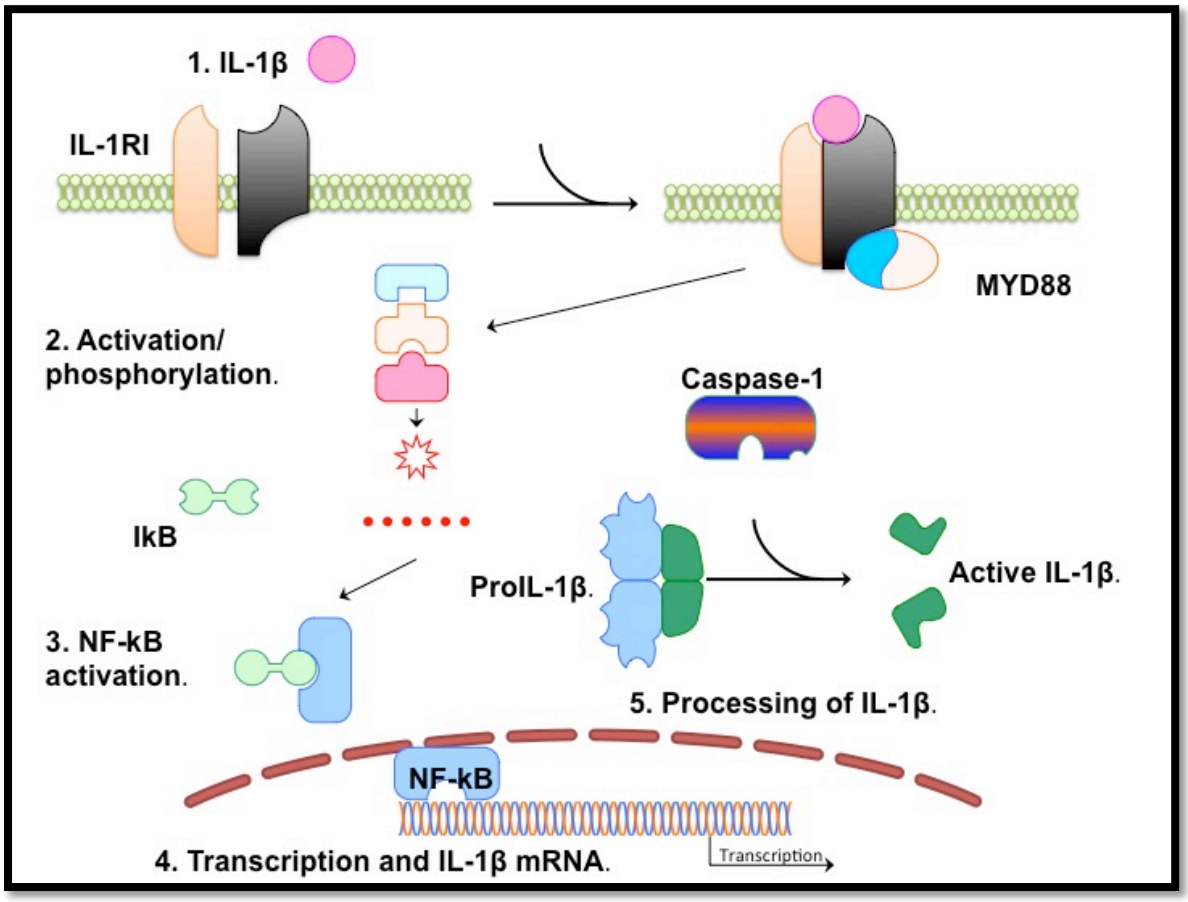

Figure 1.2. The molecular mechanism of IL-1β, steps of IL-1β processing and release. Step (1): IL-1β binds to IL-1RI. **Step (2):** This results in a cascade of activation and phosphorylation. **Step (3):** NF-kB is activated by degradation of IkB. **Step (4)**: NF-kB translocation into the nucleus and subsequent transcription and mRNA IL-1β formation and proIL-1β generation. **Step (5):** After IL-1β precursor transcription and translation, the inflammasome is assembled and procaspase-1 is activated. Caspase-1 cleaves proIL-1β and subsequently activeIL-1β is released.

(iii) Control of IL-1 activation and release:

As a result of NF-kB activation, proIL-1β accumulates in the cytoplasm in response to various ligand binding, by activation of TLRs/IL-1RI (Takeuchi and Akira, 2010). In immune cells, the biologically inactive proIL-1β (31kDa) is converted by a proteolytic cleavage via caspase-1 into active/mature IL-1β (17kDa) (Thornberry et al., 1992), which itself is regulated by an assembly of a multi-protein complex; an inflammasome (Figure 1.2, step 5) (Dinarello, 2007, Brough and Rothwell, 2007).

In vitro studies suggested that activation of the inflammasome complex requires a second stimulus (Schroder and Tschopp, 2010) and, in monocytic-derived cells, adenosine 5′-triphosphate (ATP) has been demonstrated to increase IL-1β secretion by activating the plasma membrane receptors, P2x7 (Netea et al., 2009). However, in ECs, P2x7 receptors are expressed in substantially low levels and subsequently the release of IL-1β in response to ATP activation is relatively inefficient (Wilson et al., 2007), suggesting that ECs may secrete IL-1β by an alternative yet unknown mechanism(s).

Moreover, *in vivo* models of inflammation demonstrated that IL-1β is detected in plasma and other body fluids of caspase-$1^{-/-}$ animals (Stehlik, 2009, Joosten LA, 2009, Couillin et al., 2009), raising a controversy to the *in vitro* findings and suggesting an alternative IL-1β secretory mechanism without the need to caspase-1/inflammasome activation.

In addition to caspase-1, other cysteine proteases such as caspase-4 and caspase-5 have been implicated in IL-1 secretion. For instance, transgenic expression of human caspase-4 in mice supported caspase-1 activation and enhanced IL-1 and IL-18 release in response to LPS (Kajiwara et al., 2014). However, and recently, Vigano *et al* have identified caspase 4 and caspase-5 as being crucial downstream targets of LPS activation in human monocytes, without the need to activate caspase-1 (Vigano et al., 2015).

Additionally, Murine caspase-11 (a member of caspase-1 subfamily and is most homologus to human caspase-4) has been shown to be induced by LPS activation (Wang et al., 1998). Caspase-11 does not directly process proIL-1 but overexpression of caspase-11 stimulates proIL-1 processing by caspase-1 (Kang et

al., 2002). On the other hand, Fas receptor signalling activates caspase-8 in macrophages and dendritic cells, leading to maturation of IL-1 by a caspase-1/caspase-11 independent mechanism (Bossaller et al., 2012). Further studies are needed to dissect the exclusive and overlapping roles between these proteases as well as their involvement in the canonical caspase-1/inflammasome pathway. Moreover, in ECs the role of the caspases is still unclear and it would be interesting to investigate the possible contribution of the inflammatory caspases in IL-1 secretion.

1.4.4. The mystery of IL-1β secretion:

Despite the exquisite biological control mechanisms, the mechanism of IL-1β trafficking outside the cells into the extracellular environment remains poorly understood. It is generally agreed that the leaderless IL-1β is secreted by a non-classical pathway of protein secretion. This means that IL-1β lacks a signal peptide, which is responsible for directing secretory proteins through an endoplasmic reticulum (ER)-Golgi pathway of protein secretion (i.e. classical pathway of protein secretion) (Rubartelli et al., 1990).

Most secretory proteins, such as tumour necrosis factor-alpha (TNF-α) and IL-6, have a leader or a signal sequence of 13-30 amino acids in close proximity to their N-terminus that directs the proteins a cross-translation to the ER membrane (Milstein et al., 1974). The secretory proteins then translocate to the Golgi apparatus to undergo a further glycosylation and are subsequently packaged into secretory vesicles that fuse to the plasma membrane and release their contents outside the cells (Kaiser and Schekman, 1990).
IL-1β, however, is one among handful proteins that lacks the typical signalling sequence (Auron et al., 1984) and data shows that inhibiting the process of the transfer to the ER using chemicals, for example, does not ameliorate IL-1β secretion (Andrei et al., 1999, Nickel and Rabouille, 2009, Rubartelli et al., 1990). This raises the question of how IL-1β is released into the extracellular environment.

Moreover, in patient plasma IL-1β is known to have a short half-life of approximately 6-8 minutes (Ray, 2014), despite the sustained IL-1 dependent inflammation in chronic inflammatory diseases (Dinarello, 2007). The sustained effect may suggest

that IL-1β is transported and protected from degradation by packaging, possibly in vesicles to exert its distal endocrine effects.
As a result, in the literature, different mechanisms for IL-1β secretion have been proposed that do not suggest a unified mechanism for IL-1β release but rather suggest that the secretion is different in different cell types and is stimulus specific (Lopez-Castejon and Brough, 2011).

The proposed mechanisms by which IL-1β is released, such as lysosome-regulated secretory routes (Rajamaki et al., 2010), microvesicle (MV) shedding (MacKenzie A, 2001) and exosomal mediated release (Rabouille et al., 2012) are summarised in the following sections. Understanding the mechanism by which the bioactive IL-1β is secreted may help in identifying new anti-IL-1β therapies and help in understanding how the inflammatory process develops during vascular disease.

1.4.4.1. Release of IL-1 by lysosomal exocytosis:

In vitro, several mechanisms by which IL-1β may make its cellular exit have been proposed. The first and the oldest mode of IL-1β secretion was thought to be following cell death (Wewers, 2004). However, IL-1β secretion seems to be more complicated than a result of simple cell lysis. An interesting proposal by Andrei *et al* (1999) is that IL-1β is released by lysosomal exocytosis. Lysosomes are small intracellular organelles that are usually surrounded by a single layer of plasma membrane and contain hydrolases that are maintained in their active state to digest engulfed pathogens or autophaged molecules (Rauova and Cines, 2013).

However, lysosmal exocytosis and release of their contents have been described in multiple secretory cells, including activated platelets and macrophages (Mullins and Bonifacino, 2001). Thus, in essence, lysosomes can act as secretory rather than terminal digestive compartments, within secretory cells.
The secretory lysosomes have features of both digestive lysosomes and secretory organelles (Nickel and Rabouille, 2009). However, secretory lysosomes are distinguished from the conventional lysosomes in that they undergo a regulated secretion (Blott and Griffiths, 2002).

In monocytes, secretory lysosomes are well appreciated in the secretory pathway of IL-1β (Andrei et al., 2004). Following caspase-1 activation, secretory lysosomes fuse to the plasma membrane and release their IL-1β contents by a process of exocytosis (Keller et al., 2008). The most commonly studied stimuli in immune cells that release IL-1β by this pathway are ATP (Qu et al., 2007) or cholesterol crystals (Rajamaki et al., 2010).

This mechanism is partially understood, yet it seems to be linked to a signal recognition peptide that directs the lysosomes to the plasma membrane to undergo exocytosis (Ghossoub et al., 2014, Andrews, 2000).

1.4.4.2. IL-1β secretion by microvesicle shedding:

Microvesicle (MV) shedding from the plasma membrane as a tool of non-conventional protein secretion was firstly proposed by Chargaff and West in 1946 (Gyoergy et al., 2011). The release of MVs as they bud off from the plasma membrane is induced by an increase in cytosolic calcium $[Ca^{2+}]_i$ levels. MV release is enhanced by calcium ionophore in platelets and monocytes, and decreased by the Ca^{2+} chelating ethylene glycol tetraacetic acid (EGTA) (Gyoergy et al., 2011).

MV shedding, containing active IL-1β, was firstly observed by (MacKenzie A, 2001) in THP-1 monocytes treated with ATP. The shedding was preceded by flipping of the inner layer of the plasma membrane, phosphatidyl serine (PS), into the outer layer following P2x7 activation (MacKenzie A, 2001). MV shedding as a route of IL-1β secretion was also proposed in other cell types such as dendritic cells (Pizzirani et al., 2007) and macrophages (Asgari et al., 2013). However, how IL-1β is released from the MVs in order to bind to its receptors is yet to be described.
Data suggests that ATP stimulated MVs are able to secrete their contents, including IL-1β, once they are in contact with their target sites (Pizzirani et al., 2007), and this may provide evidence on how the protected IL-1β may be released at its sites of action. However, whether this is applicable to ECs is yet to be elucidated.

1.4.4.3. Exosomes are an additional secretory route for IL-1:

Inside cells, proIL-1β has been detected in the cytosol. However, the mature form was isolated from vesicles that are endolysosomal in nature (Andrei et al., 2004,

Lopez-Castejon and Brough, 2011). As a result, a fundamental question on how cytosolic IL-1β gets into the endolysosomes is raised, along with how these endosomes could be involved in IL-1β secretion. An intracellular compartment, within endolysosomes, that has been linked to IL-1 secretion, is multivesicular bodies (MVBs) (Qu et al., 2007).

MVB genesis and their functions are relatively unclear. Studies show that growth factor stimulation can increase their membrane inward invagination and formation of intraluminal vesicles (ILVs) (White et al., 2006). In addition, MVBs are detected in certain cell types where they may bind to lysosomes and thus act as degradation compartments (Stoorvogel et al., 2002). Alternatively, in secretory cells, they may act as temporary storage compartments and release ILVs outside of the cells as a type of exosome (Piper and Katzmann, 2007).

Proteins such as endothelial growth factor (EGF) (Felder et al., 1990) and tetraspanins (Heijnen et al., 1998) are released by exocytosis of MVBs and exosome release (Denzer et al., 2000). The detection of mature IL-1 within these compartments in monocytes (Andrei et al., 1999), may suggest that, inside the cells, IL-1 is sorted by intermediate vesicles prior to the formation of MVBs and exosomal release.

Endothelial IL-1 production and release is partially understood and little has been done to evaluate which of the above-mentioned pathways are involved. ECs can produce IL-1 in large amounts (Wilson et al., 2007) and whether lysosomal or endolysosomal pathways are predominant in ECs is relatively unknown. Moreover, the trigger for the secretory process of IL-1 from ECs remains unclear.

1.5. Neutrophil elastase as a possible inflammatory trigger to endothelial IL-1 secretion:

In atherosclerosis, remodeling of the ECM has long been known to occur (Virmani et al., 2000), but the multitude of the cell types and the molecular mechanisms involved has only recently emerged (Kashiyama et al., 2011). The proinflammatory mediators that are released by infiltrating cells create environmental changes and ECM degradation, within the lesion and thus promote plaque instability (Libby, 2009,

Dollery and Libby, 2006). One study has implicated a plausible role for the potent serine protease, NE in that process and lesion instability (Dollery et al., 2003).

NE despite its name, has been detected in different cell types, including SMCs (Kim et al., 2011), monocytes (Campbell et al., 1989) and macrophages (Dollery et al., 2003). In addition, NE can act as a signalling mediator that drives inflammation in ways other than its known proteolytic functions (Korkmaz et al., 2010). NE is a proteolytic enzyme that cleaves different components of the ECM (Pham, 2008). It also has been shown to play a crucial role in the innate body defense in response to pathogens. For example, mice lacking in NE have defective microbicidal activities with susceptibility to infection, especially to gram-ve bacteria (Belaaouaj et al., 1998).

Tissues are protected from the excessive destructive/inflammatory nature of NE by expressing natural inhibitors. NE inhibitors are either produced locally at the site of inflammation, such as elafin, or systemically by the liver, such as α-1-antitrypsin (Williams et al., 2006). α-1-antitrypsin, detected with high blood levels, inhibitory activity are decreased in chronic inflammation where it can be cleaved and deactivated by matrix metalloproteases (Shapiro, 2002) and oxidative stress (Taggart et al., 2000), leaving NE activity unchecked in chronic inflammatory diseases.

The next section will focus on the possible roles that NE may play in atherosclerosis and provides evidence on how NE can be a direct mediator for IL-1 dependent inflammation.

1.5.1. NE and its cellular of origin:

NE is a proteinase stored in the azurophil granules of neutrophils (Pham, 2008, Chua and Laurent, 2006) and unlike other proteases, has broad biological functions. Although proteases were identified in the early 20th century, NE was not discovered until 1968 by Janoff and Scherer (Takahashi et al., 1988a). The name was given due to its ability to release soluble substances from the insoluble elastin, yet recent studies show that its function is extended to dissolve other components of ECM such as collagen, fibrin, and cadherin (Lee, 2001).

The NE gene is located on the short arm of chromosome 19 and is not expressed in mature neutrophils, but only in the myelomonocytic cell lineage (Takahashi et al.,

1988b), which may explain why NE has also been identified in macrophages (Dollery et al., 2003).

Serine proteases, including NE, CG, and proteinase 3 (PR3) were detected at different levels and functions in the granules of monocytes and neutrophils (Henriksen and Sallenave, 2008, Pham, 2008). Despite this extensive knowledge, however, it is still difficult to define the biological activities of NE.

1.5.2. Emerging roles for NE in inflammation:

An unresolved issue in understanding the mechanism of action of NE is its wide range of activities. Besides its ECM degrading roles, NE has been reported to enhance the production of the chemokine IL-8 in HEK293, via TLR4 (Devaney et al., 2003), suggesting an alternative mechanism of action. Similarly, in human umbilical cord endothelial cells (HUVECs), the levels of IL-8 inside the cells were significantly increased following NE treatment (Henriksen et al., 2004).

Although *in vitro* studies have revealed the involvement of TLR4 in NE signalling, animal studies have suggested that elastase causes cytokine release in a TLR-independent manner. It is postulated that NE activates the ASC-inflammasome complex and thus induces production of pro-inflammatory mediators (Couillin et al., 2009). Emerging evidence suggests that NE is also involved in the synthesis and release of the growth factor, TGF-β (transforming growth factor-beta), in human bronchial smooth muscle cells, associated with NF-κB activation (Lee et al., 2006). Furthermore, it has been widely accepted that NE can enhance production of IL-6 (Lee, 2001), CCL15, CXCR4 and CXCL2 (Pham, 2008) and thus can promote inflammation by maintaining chemotaxis and migration of inflammatory cells. By contrast to its secretagogic effects, the intracellular signals for this enzyme are unclear.

1.5.3. NE effects may be mediated by IL-1β:

Like other inflammatory mediators, NE may enhance IL-1 production in many inflammatory conditions; however, this has not yet been directly studied in atherosclerosis.

Although the underlying mechanism remains incompletely understood, caspase-1 deficient mice showed no impairment in IL-1 levels in synovial fluids (Guma et al., 2009), emphasising the presence of an alternative mechanism by which IL-1 is produced.

Likewise, caspase-1 deficient mice have no significant reduction in IL-1β mediated arthritis, but pro-IL-1β is attenuated after the cells from these mice are incubated with NE and this was associated with an increase in levels of IL-1β which, in turn, was improved after NE inhibitors were added (Joosten LA, 2009).
Recently, Schreiber *et al*., have shown a significant reduction in IL-1β production in the kidneys of $NE^{-/-}$ mice (2012). A more recent study by Warnatsch and collagues has suggested a significant reduction in atheroma formation in $NE^{-/-}/PRO3^{-/-}$ mice with a reduction in plasma and plaque IL-1β expression (2015).
Collectively, this suggests that NE may play a role in enhancing IL-1β mediated inflammatory effects in animal models, yet precise details in IL-1β mediated endothelial and vascular injury have not been fully elucidated.

1.5.4. NE as an atherogenic mediator and its roles in IHD:

Different families of proteases, including NE, MMPs and cathepsins have been suggested to modulate ECM in atherogenesis (Henriksen and Sallenave, 2008). Indeed, there is substantial overlap in the substrates among these three families. However, NE is distinct in having broad functions and being released in active form from cells in large amounts either by cell degranulation (Pham, 2008) or within neutrophil extracellular traps (NETs) (Warnatsch et al., 2015) at the site of inflammation. On the other hand, MMPs and cathepsins require activation by a cascade of proteolysis that is regulated by gene expression (Klein and Bischoff, 2011). It has been shown that NE plays a key role in this process, thus modulating the function of the other proteases that are well characterised in plaque instability such as MMPs (Dollery and Libby, 2006).

Growing evidence suggests that neutrophils from patients with IHD show signs of activation and degranulation (Goldmann et al., 2009), with abundant NE detected in ruptured coronary lesions of patients who died from acute MI (Naruko et al., 2002). In addition, serum NE antigen levels were relatively high in patients with unstable

angina (UA) and these levels were predictive of future MI (Smith et al., 2000). It is also interesting to note that levels of NE increase with the major coronary risk factors in experimental animals (Garcia-Touchard et al., 2005), suggesting a direct role for NE as an important trigger in atherosclerosis. However, future work to confirm this is required.

1.6. CAD prevention and control:

Inflammation and atherosclerosis is an on-going disease process manifested in elderly patients with dreadful complications. As part of interventional strategies, preventive medicine is a growing alternative to control the disease. The second part of this thesis and, therefore, this introduction, focuses on the roles and the mechanism of actions of various types of PUFAs in atherosclerosis and their protective roles.

1.6.1. Dietary control:

It is well appreciated that hyperlipidaemia, namely hypercholesterolemia, is one of the main causative factors of CAD. In addition to dietary cholesterol, the risk of hypercholesterolemia and thus CAD is increased with high consumption of dietary saturated fatty acids (Kinsell et al., 1952).

Therefore, the current approach to control CAD is to reduce the high fat diet intake, and an efficient strategy for that is to replace dietary saturated fat with PUFAs. Fish oil (FO) and the bioactive fatty acids found within, mainly docosahexaenoic acid (DHA; 22:6 n-3), are suggested to benefit CAD risk in clinical trials (Dyerberg and Bang, 1979).

1.6.2. Omega-3 FAs: Nomenclature:

Fatty acids (FAs), carboxylic acids, have variable numbers of carbon (C) atoms: from two to more than thirty. They are either saturated or unsaturated depending upon the presence of double bond (=) between the carbon atoms. Saturated FAs have no double bonds whereas monosaturated FAs have a single double bond. PUFAs, more than one (=), are either omega-3 or omega-6.

PUFAs are also commonly referred using systematic and common names. For example, DHA is described as 22:6 n-3 where 22 is the total number of C in the chain, 6 is the number of the (=), and n-3 (the first double bond is located at the third carbon atom from the methyl terminal). This nomenclature helps in differentiating between different types of PUFAs within the family. For instance, in omega-3 FAs, the first double bond (=) is located at C3 (referred as n-3) whereas omega-6, the first (=) is at C6 (n-6) in the molecule (Yates et al., 2014).

1.6.3. Omega-3 FAs: Types and dietary sources:

N3FAs, naturally occurring PUFAs, cannot be produced within the human body and, thus, must be provided in diet. These essential FAs are present in different quantities, in different types of fish (Calder, 2004). Table (1) shows the different types of n3FA and their oily fish sources.

Depending on the dietary habits and the availability of the fish derived food, it is estimated that daily intake of n3FAs in the UK is <250mg/day (Buttriss, 1992, Calder, 2002). In the absence of fish derived food, α-Linolenic acid (ALA) is considered as a source of n3FAs in the human diet.

ALA can be enzymatically converted in human tissues into EPA and DHA (Calder, 2004). However, recent studies revealed that this endogenous conversion is no longer efficient due to enzymatic deficiency, especially in males (Burdge et al., 2002), and thus ALA cannot replace the other types of n3FAs in the diet. Therefore, it is crucial to consume other types of n3FAs to increase their amount in human tissues and achieve the benefits they provide.

Table 1, Omega-3 Fatty Acids: Types and dietary origin:

Types	Dietary source	Amount: g/100g	Nomenclature
ALA	Flaxseed oil.	53.3	18:3*
DHA	Salmon, farmed Sardines, Atlantic Tuna, White Tuna, light.	1,104 509 629 237	22:6
EPA	Herring, Atlantic Sardines, Atlantic Tuna, White Tuna, light.	909 473 233 91	20:5
DPA	Salmon, farmed Sardines, Atlantic Tuna, White Tuna, light.	393 0 18 17	22:5

Abbreviations: ALA; α-Linolenic acid, DHA; docosahexaenoic acid, EPA; eicosapeantoic acid, DPA; docosapentaenoic acid. *18:3; 18 carbon atoms with three double bonds. Adapted and permitted from (Mozaffarian and Wu, 2011).

1.6.4. N3FAs and Cardiovascular disease; risk control and research evidence:

The interest in n3FAs has substantially grown since 1970s, when a study on the population of Inuit, Greenland, showed a marked reduction in CAD risk with an average of 10% reduction of the predicted risk despite their high fat intake (Dyerberg and Bang, 1979). Furthermore, the Japanese are known to consume high quantities of n3FAs (DHA and EPA) in their sea-derived food and thus exhibit the lowest risk to develop CAD (Yano et al., 1988). Subsequently, clinical trials have been conducted, but have shown mixed findings.

1.6.4.1 Clinical trials:

Accumulating evidence from secondary preventive trials has shown that n3FA consumption, either in fish or as supplements, may have a substantial impact on cardiovascular mortality due to coronary events (Mozaffarian and Wu, 2011). Although not all studies have confirmed this effect, recent studies have suggested that high n3FA supplementation in recent MI patients markedly reduces the all cause mortality.

For instance, the DART study (Diet And Reinfarction Trial) conducted on 2033 surviving MI male patients showed a 29% reduction in all cause mortality among patients advised to increase their daily intake of FO (Mozaffarian and Wu, 2011). Moreover, the Japanese EPA (eicosapeantoic acid) lipid intervention study (JELIS) showed a 19% reduction in the incidence of CAD, including MI and UA in patients that received EPA (1.8g/day) over a duration of 5 years (Yokoyama et al., 2007).

Conversely, the Alpha Omega trial, studying 4837 patients with a previous history of MI given n3FA supplementation did not show any significant benefit in the group received n3FAs (Kromhout et al., 2010). It could, however, be argued that, in this study, the patients received relatively low amounts of n3FAs (400mg EPA/DHA per day) and that the benefit cannot be achieved in patients with established coronary lesions.

Collectively, several studies document clinically that n3FA supplementation may have a significant benefit on CAD. All these studies tested the effects of n3FAs on major CV risks. However, relatively little is known on the individual (purified) types of n3FAs such as DHA and ALA on the coronary events and atherosclerosis. Furthermore, these studies were carried out without a clear understanding of the molecular mechanism(s) by which n3FAs exert their cardio-protective roles.

1.6.4.2. Animal studies:

Although recent clinical studies revealed a wide gap in understanding the molecular mechanisms of different types of n3FAs and their cardio-protective roles, animal studies using genetically susceptible species have shown their global anti-inflammatory effects, detailed below:

1.6.4.2.1. Omega-3 fatty acids and dyslipidemia:

Dyslipidemia (high LDL-C and triglycerides and low high density lipoprotein cholesterol (HDL-C)) is one of the best known precipitating factors for atherosclerosis and hypertension (Libby, 2002). The lipid lowering effects of n3FAs have been controversial in many studies, mainly those on mice.

Currently, mice are the most studied species for atherosclerosis as they can be genetically manipulated to induce atherosclerosis. Two genotypes of mice, low

density lipoprotein receptor knock out mice ($LDLr^{-/-}$) and apolipoprotein E knock out mice ($ApoE^{-/-}$) are commonly used to study atherosclerosis by inducing hyperlipidaemia (Zhang et al., 1992).

N3-FAs are known to play a fundamental role in the lowering of plasma triglyceride (TG) levels and this has been well tested in animals (Chang et al., 2010, Frenoux et al., 2001). The mechanism by which n3FAs could lower plasma TG has been attributed to the suppressive effect of n3-FAs on the hepatic triglycerides and very low-density lipoprotein synthesis (Mozaffarian and Wu, 2011). However, the LDL-C lowering effects of n3FA have been less clear.

1.6.4.2.2 Omega-3 fatty acids and changes in plaque composition:

It is debated whether n3-FAs may potentially affect atherosclerotic plaque composition and stability. Growing evidence indicates that coronary vascular lesions are associated with low plasma EPA and DPA (docosapentaenoic acid) levels and more lipid accumulation (Amano et al., 2011). Moreover, n3-FAs may reduce the arterial uptake of LDL-C (Chang et al., 2010) and oxidation (Frenoux et al., 2001). The inhibitory effect of n3-FAs on LDL-C uptake has been attributed to their NF-κB suppressive activity (De Pascale et al., 2009).

Although Sacks and his colleagues reported in 1995 that fish oil had no impact on advanced atherosclerosis (Sacks et al., 1995), recent studies suggest that these fatty acids do have a potential role. This is due to their ability to be incorporated into the plaque (Cawood et al., 2010, Chang et al., 2010, Thies et al., 2003) and thus change its structure. When n3-FAs are incorporated into the plaque, they replace the arachidonic acid (A.A), a precursor to inflammatory mediators known as eicosanoids, with its enzymes. Eicosanoids, such as leukotriene B4 (LTB4) and thromboxane A2 (TXA2), are potent vasoconstrictor and thrombogenic agents (Calder, 2002).

In addition, these free fatty acids reduce the number of macrophages (Chang et al., 2010, Thies et al., 2003) and increase the number of lymphocytes (Cawood et al., 2010) at the sites of lesions. The reduction plaque macrophages is attributed to their ability to suppress chemoattractant production (Endres et al., 1995). Furthermore, they attenuate the levels of adhesion molecules and thus decrease inflammatory cell

migration (Verschuren et al., 2011, Cawood et al., 2010, Chen et al., 2003) and MMPs (Cawood et al., 2010). They also inhibit VSMC proliferation *in vitro* (Terano et al., 1999). As a consequence of all these effects, it could be concluded that n3-FAs might maintain the stability of plaque and reduce the chance of rupture. However, the molecular basis of these changes, particularly in atherosclerosis needs further elucidation.

1.6.5. Omega-3 fatty acids and IL-1β:

Omega-3 fatty acids may have an IL-1β suppressive effect. A randomised trial has shown that high intracellular ALA and EPA is associated with a reduction in serum IL-1β levels (Caughey et al., 1996). Another randomised study has indicated that high plasma DHA levels are correlated with IL-1β inhibition, and that this suppression is at its maximum after LPS stimulation (Vedin et al., 2008). A recent study tested mice fed a n3-FA rich diet with LPS and reported that IL-1β levels decreased significantly and that this decline was mediated by TLR4 stimulation (Vijay-Kumar et al., 2011). A further mechanism by which n3-FAs have an IL-1β suppressive effect may be at a nuclear level, by inhibition of NF-κB (Boudreau et al., 2001).

1.6.6. Omega-3 fatty acids have anti-hypertensive effects:

In contrast to their anti-atherogenic roles, the anti-hypertensive roles of n3-FAs are thought to be multifactorial. A randomised controlled study carried out on middle-aged men showed that, despite the lowering effect of fish oil on blood pressure, this effect was insignificant (Vandongen et al., 1993). However, the most recent randomised controlled study conducted on women aged more than 39 years has proposed that the risk of hypertension significantly decreased with the intake of n3-FAs (Wang et al., 2010).

Despite this, the molecular basis of the anti-hypertensive effect of n3-FAs is relatively unknown. One possible explanation for their effect on blood pressure is cyclooxygenase-2 (COX-2) suppression (Massaro et al., 2006, Matsumoto et al., 2009). Cyclooxygenase has two isoforms; one is cyclooxygenase-1 which is expressed normally by the endothelium and has no pathological role. However, COX-2 is an enzyme that catalyses A.A into prostaglandin-E2 (a potent vasoconstrictor parameter) and is expressed only in inflammatory cells (Calder,

2002). Alternatively, the ability of n3-FAs to restore NO production and endothelial function and decrease SOR production (Richard et al., 2009) could account for the anti-hypertensive effect.

Table (2) shows the most recent clinical/animal based research conducted on the n3-FAs and cardiovascular disease.

Table 2. N3-FAs and CAD: a summary of the relevant studies on CAD.

Reference	Type of study	Population	Results	Interpretation
(Amano et al., 2011)	Observational.	368 patients ACS, males& females, 8 months.	ACS associated with low levels of, DPA& EPA but not DHA. High % LV& low FV%.	N3 -FAs have a cardio-protective effect. Also can modify AS in the foam cell stage.
(Verschuren et al., 2011)	Animal.	Two groups, one transgenic mice (n=7) and the other; ApoE-Leiden mice. Fish oil and placebo (16 months).	CRP levels dropped after IL-1β stimulation. ICAM-1& E-Selectin levels were lower in fish oil group.	N3-FAs might have an anti-IL-1β effect shown by lowering IL-1β mediators (CRP, ICAM-1& E-Selectin).
(Vijay-Kumar et al., 2011)	Animal.	Two groups (n=10). One fed fish oil, the other group fed sat-fats for 60 days, tested LPS stimulation.	In fish oil group, levels of IL-1β declined after LPS challenge. N3-FAs mediate IL-1β inhibition through TLR4.	An *in vivo* study showing an IL-1β suppressive effect of n3-FAs. However, receptor expression needs to be tested to validate.
(Cawood et al., 2010)	Observational, RCT.	61 patients with carotid AS. Omacor (DHA& EPA) for 102 days.	The plaque morphology changed with 100%, 13% incorporation of EPA, DHA, respectively. There was significant reduction in the number of foam cells and an	It is clear that n3-FAs maintain the stability of already developed lesions by lowering the number of inflammatory cells and thus decreasing the

			increase in the number of lymphocytes. ICAM-1& MMP-1 levels were decreased.	chance to rupture.
(Chang et al., 2010)	Animal.	Diabetic mice strains L1 (n=12) fed chow, SAT, omega-3 rich diets for 12 weeks.	Reduction in TG, LDL-C levels in n3-FA group. Reduction in the arterial uptake of LDL-C. Suppression of macrophage infiltration.	N3-FAs play an important role in the earliest stages of AS by limiting the LDL-C uptake and macrophage infiltration. However, the mechanism by which doing so is not illustrated in this study.
(Kromhout et al., 2010)	The Alpha Omega Trial: Double-blind, placebo-controlled trial.	4837 patients with MI for 3.5 years. Males& females (60-80 years). April 2002-December 2006.	DHA& EPA in combination showed no significant impact on the coronary vascular event, neither the lipid profiles of those patients.	The patients were not followed regularly. Also, they were on anti-hyperlipidemic medications, so the lipid profile cannot be interpreted accurately.
(Matsumoto et al., 2009)	Animal.	5 weeks rats exhibited type-2 DM with endothelial dysfunction fed EPA (100/ 300mg/kg/d) for 4 weeks.	Isolated mesenteric artery showed no significant changes with 100mg EPA. However, with 300mg dose these rats exhibited significant reduction in their blood pressure and improvement in HDL-C. Enhancement in Ach-induced NO relaxation, decreased COX-2 and NF-	N3-FAs might improve the blood pressure by lowering COX2 levels and its metabolites. Furthermore, the improvement in the level of NO and the vasodilator effect might be considered as a potential impact of n3-FAs on blood pressure.

			κB expressions.	
(Vedin et al., 2008)	The OmegAD study. Double-blind, placebo-controlled study.	204 patients with CAD ingested fish oil for 6 months.	Significant drop in IL-1β only after LPS stimulation. IL-1β reduction correlated with plasma levels of DHA, not EPA.	It seems that n3-FAs lower IL-1β by a TLR-based mechanism. However, the preferential effect needs further studies.
(Yokoyama et al., 2007)	JELIS study, Prospective, randomised Cohort study.	18645 Hypercholestermic patients on statins. Men & women (75 years). 4.6 years. EPA-1800mg a day.	EPA reduced the non-fatal coronary events with 19%.	The study has been carried out in Japan. N3-FA levels are already high in Japanese patients.
(Massaro et al., 2006)	*In vitro* study.	Human saphenous vein endothelial cells were treated by DHA and IL-1α.	A decrease in the followings, after DHA pre-treatment 24 hours to IL-1α stimulated cells: COX2, NADPH oxidase & NF-κB.	The suppressive effect of DHA was enhanced in the presence of IL-1.

Abbreviations: ACS; acute coronary syndrome, CAD; coronary artery disease, % LV& FV; percentage of lipid volume and fibrous volume respectively, CRP; C-reactive protein, Sat-fats; saturated fats, RCT; randomised control trial, AS; atherosclerosis, TG; triglycerides, MI; myocardial infarction, LPS; lipopolysaccharides, TLR4; toll-like receptor type 4, HDL-C; high density lipoprotein cholesterol, LCL-C; low density lipoprotein cholesterol, DM; diabetes mellitus, DHA; docosahexaenoic acid, EPA; eicosapeantoic acid, DPA; docosapentaenoic acid, n3FAs; omega-3 fatty acids, ICAM-1; intercellular Adhesion Molecule 1, MMP-1; matrix metalloproteinase-1, Ach; acetylcholine, NO; nitric oxide, COX2; cyclooxygenase-2.

1.7. Hypothesis:

IL-1 has gathered substantial interest over recent years because experimental atherosclerosis responds specifically to IL-1 blockade using either soluble IL-1ra or monoclonal blocking antibodies. Although agents able to impair IL-1β effects have been considered to have a highly therapeutic impact, the mechanism of IL-1β secretion is still obscure.

In the backgrounds (introduction chapter 1), I began by discussing the possible mechanisms that underlie IL-1β modulation in atherosclerosis. I then addressed the emerging role of NE as a possible unconventional IL-1β secretion. Finally, I

highlighted possible roles of n3FAs as anti-atherogenic agents. This led me to two hypotheses.

Hypothesis (1):

NE enhances IL-1β secretion from vascular endothelium *in vitro* by a vesicular related mechanism.

Hypothesis (2):

DHA and ALA reduce atherogenesis in ApoE$^{-/-}$ mice fed a high fat diet via an IL-1 related mechanism.

1.8. Aims and Objectives:

The aims for this thesis were to:

1. Determine whether IL-1β release from endothelial cells could be induced by neutrophil elastase *in vitro*.
2. Investigate whether ALA and DHA might modulate IL-1β effects on experimental atherosclerosis in mice.

The objectives were:

a) To use an *in vitro* model, HCAECs (human coronary artery endothelial cells), to test the release of IL-1β following incubation with NE using ELISA.
b) Using this model to detect released forms of IL-1β using Western blots.
c) Ascertain if the released IL-1β was biologically active.
d) Begin to suggest mechanisms that might underpin the release of IL-1.
e) Test NE inhibitors in the *in vitro* system.
f) Measure the effects of DHA and ALA supplementation on experimental atherosclerosis and IL-1 expression in mice.

Chapter (2) General Materials and Methods.

OUTLINE:

2.1. Overview of the general materials and methods:

The experimental work conducted in this thesis is divided into two main parts. The *in vitro*, cell culture, work was performed to test whether NE induces IL-1β secretion from ECs and explore the underlying mechanism(s). The *in vivo* experiments were carried out to investigate the molecular mechanism of action of the two main types of n3FAs, particularly DHA and ALA in atherogenesis.

2.2. Materials and reagents:

Full details of reagents and antibodies used with their stock concentrations, working concentrations/doses, and their sources are shown in appendices (I & II).

2.3. Detailed Methods:

2.3.1. Cell culture:

All cell experiments were conducted in a laminar flow hood and cells incubated at 37^{o}C with 5% (v/v) CO_2 unless otherwise stated. To reduce the chance of infection, which may interfere with the interpretation of the results, strict sterile procedures were used at all times.

2.3.1.1. Human umbilical vein endothelial cells (HUVECs):

One of the aims of this project was to induce maximal IL-1β production within ECs using different combinations of cytokines, before testing for release. HUVECs are a commonly obtained type of ECs used frequently in research, as they are easily available with low costs. Cells were obtained from freshly collected cords of newly delivered mothers, with the collaboration of the maternity unit of Sheffield Teaching Hospital (STH), Sheffield, UK under an ethical approval (STH15599, REC ref 10/H1308/25, for details please see page 256). Additional cells were purchased from PromoCell (UK), pooled from several donors with their special media. These cells were supplied in a cryo-preservative vial and stored in liquid nitrogen at -154^{o}C until cultured.

2.3.1.1(A) Isolation of HUVECs from umbilical vein:

Freshly obtained cords were stored at +4^{o}C for at least 24h in minimum essential media (MEM) (see appendix III, section 1 for the preparation and the chemical

combinations of MEM). Cords that were at least 10 centimetre (cm) long, with no clamp marks, were used for the isolations. All other cords were discarded.

Following an established protocol (Jaffe *et al*., 1973), the umbilical vein was cannulated with a 14G plastic cannula and perfused with pre-warmed M199 (basic media, Gibco) media to remove all traces of blood. The other end of the cord was then ligated and the vein inflated with 0.1% (w/v) type VI collagenase (Sigma-Aldrich) in M199. Cells detached by the collagenase action were gently collected into a sterile universal tube and the cord was then flushed with 20ml pre-warmed M199 to collect the remaining ECs. The tube containing cells was centrifuged at 300g for 5 minutes at 24^{o}C, yielding a small pellet that was then re-suspended in 1ml complete growth media (CGM details in appendix III).
The cells were transferred into a T25 flask (25cm^{2}) pre-coated with 1% (w/v) gelatin/ distilled water (DW) (Sigma-Aldrich) and left overnight in the incubator. The media was then changed, after washing the cells with sterile phosphate buffered saline (PBS, pH 7.4) to remove unattached cells.

After the isolation process, the HUVECs initially showed small epithelioid clusters, however, after 2-3 days, these spread to the typical cobblestone monolayer appearance. The cells used in this study were collected from at least 20 different cords.

2.3.1.2. Human Coronary Artery Endothelial cells (HCAECs):

HCAECs from single donors were purchased from PromoCell and cultured in their supplemented media (details in appendix III), according to the supplier's instructions. The cells were stored in liquid nitrogen until used.
Following thawing, the cells were cultured in four T25 flasks and sub-cultured as described below. HCAECs were supplied at passage 2, therefore, the cells were cultured and sub-cultured until passage 3, and then they were either used experimentally or frozen and kept in liquid nitrogen until needed.

2.3.1.3. Sub-culturing protocol:

When the cells were at 80-90% confluence, they were passaged 1:3 to maintain a continuous growth. Over-confluent cells easily detach from the flask and die due to

exhausting the media and the nutrients. Furthermore, if the cells become over-confluent, there is a high possibility of them stopping IL-1β production and thus giving false results in this study.

The cells were washed once with PBS (pH 7.4) after the removal of the media. Pre-warmed trypsin solution (0.2% v/v in PBS and Ethylenediaminetetraacetic acid; EDTA) was then added to the cells (1ml for T25, 2ml for T75) and the cells were detached at room temperature. The trypsin solution was used to dissolve the junctions between the cells, but lengthy exposure can dissolve the lipid bilayer of the cells. Therefore, immediately after detaching the cells, the trypsin was diluted with 10 ml of PBS (pH 7.4) and the content was transferred to a universal tube and centrifuged at 300g at 24^{o}C for 5 minutes. The supernatants were then discarded and the pellet was re-suspended in 1ml CGM.
The final required amount of the media was calculated depending on the number of flasks needed; for instance, for three T75, 45ml of media was added to the cell solution and then 15ml of the solution containing cells was added to each flask. The flasks containing cells were placed in the incubator and the media changed every two days until the cells became confluent.

At a high passage number, ECs are known to lose their surface markers and have slower growth rates (King et al., 2003) and a lesser response to stimulation. Therefore, ECs at passage 2-4 were used for all experiments.

2.3.1.4. Dual-Step activation protocol:

CGM for each specific cell type was used in the stimulation experiments, except for NE stimulations, where serum free medium (SFM) was used.
The cells were seeded in 6-well plates at a density of $2x10^4$ cells/well 48 hours (h) before starting stimulation. A single T75 flask containing confluent cells was trypsinised and centrifuged to produce a pellet which was re-suspended in 1ml media and the cells were counted using a haemocytometer. The calculated density of cells was added to 1% (w/v) gelatin/DW pre-coated 6-well plates and supplemented further with 2ml of media/well. The cells were allowed to grow and reach 60-70% confluence (48h after plating). After changing the media and washing

the cells once with PBS (pH 7.4), the stimulating solution (2ml/well; detailed below) was added.

2.3.1.4A) Step (1) activation:

To up-regulate endogenous proIL-1β production by the cells, different combinations of pro-inflammatory cytokines were used for incubation times, as follows:

- TNF-α (10ng/ml) /interferon-gamma (INF-ɣ) (100ng/ml) for 48h then LPS (1µg/ml) was added for 24h.
- LPS (1µg/ml) /IL-1β (10ng/ml) for 48h.
- TNF-α/IL-1β (10ng/ml each) or
- TNF-α/IL-1α (10ng/ml each) for 48h.

Further details of the pro-inflammatory cytokines used to stimulate IL-1β synthesis and release are given in appendix (I).

After the given incubation periods, the media was discarded and the cells were washed three times with sterile PBS (pH 7.4), to remove the remaining IL-1β and/or serum in the media. The last wash was kept to be analysed by ELISA to check the competency of the washing.

2.3.1.4B) Step (2) activation:

To test IL-1β release, a freshly made mixture solution of NE (concentrations: 0.5, 1, 2µg/ml) in SFM was added for 30 minutes, 2h and 6h. Serum deprivation is known to cause caspase activation and apoptosis in ECs (Hogg *et al*., 1999), thus, stimulation for longer than 6h was not considered, to prevent apoptosis masking any release by other mechanisms.

In parallel experiments, cells were pre-incubated with neutrophil elastase type III inhibitor (NEIII; 500µM) (Karmaker M., 2012), caspase-1 inhibitor I (YVAD-CHO; 50µM) (Ward et al., 2010, Schumann et al., 1998), or bafilomycin A1 (BAF1; 50nM) (Gupta et al., 2012) for 30 minutes before the addition of NE.

At the end of the incubations, supernatants were harvested and centrifuged at 300g for 5 minutes to remove the cellular debris, then placed in eppendorf tubes, 1tube/well. Instant freezing with dry ice was used with the samples before being stored at -80^{o}C until analysis.

After washing (once) with ice cold PBS (pH 7.4), the cells were lysed with a lysis buffer (1% (v/v) triton-x100/PBS) by re-suspension and the adherent cells were scraped off the plates. The triton-x 100 was used as a detergent to detach and gently lyse the cells. The cells were collected in eppendorf tubes one tube/well and frozen in dry ice to prevent IL-1 degradation and stored at -80^{o}C until analysis was conducted.

2.3.1.5. IL-1β/ProIL-1β quantification using ELISA:

To directly measure the levels of IL-1, a quantitative sandwich enzyme-linked immunoassay (ELISA) was used. IL-1β and proIL-1β in the total supernatants or the cell lysates were detected using an IL-1β ELISA kit (DLB50) and proIL-1β ELISA kit (DLBP00), respectively (R&D Systems, UK), following the manufacturer's protocol.

Briefly, IL-1β standards were prepared at concentrations of 3.9, 7.8, 15.6, 31.2, 62.5, 125, and 250pg/ml to generate a standard curve. ProIL-1β standards were generated at concentrations: 10000, 1500, 750, 375, 187.5, 93.8, 46.9, 23.4pg/ml. 200µl of standard, controls or samples were added to a 96 well plate and incubated for 2h at room temperature. The plate was washed three times with washing buffer before addition of IL-1β or proIL-1β conjugated antibody (200µl/well) for an hour. After further washing, substrate solution was added to the wells and protected from light. The samples were left for 20 minutes before stop solution was added and the absorbance read at 450nm. The average concentrations of the samples in pg/ml were then calculated from the standard curves.

In proIL-1β ELISA, the capture antibody coated to the microplate is a specific for the first 116 residues in the precursor of IL-1β whereas the detection antibody is a specific for the mature form. Therefore, the assay did not detect the mature form alone but instead it is specific for the intact proform.

2.3.1.6. Measurement of NE activity:

To assess the proteolytic activity of NE and test whether NE remains active over the duration of 6 hours, a chromogenic substrate that is specifically cleaved by NE and generates photometric products was used (Figure 2.1).

NE activity was assessed spectrophotometrically using a highly specific synthetic substrate (324696 Elastase Substrate I, MeOSuc-Ala-Pro-Val-pNA; 100µM) as previously described (Lee et al., 2006).

In Brief, samples (supernatant and lysate) were added to the assay buffer (0.45 Tris-Base and 2M NaCl; pH. 8.0) containing Elastase Substrate I for 6h. The rate of the substrate cleavage was measured using a plate reader (ThermoScientific) at 410nm. The bioactivity of NE in the samples was then compared to a standard curve of substrate hydrolysed by a commercially provided NE (0, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25µg/ml).

The rate of the substrate cleavage was expressed as µg/µM where the stated µg of NE leads to 1µM of substrate cleavage. Figure 2.1 illustrates the mechanism by which NE cleaves the substrate as proposed by Schechter and Berger in 1967 (Korkmaz et al., 2010).

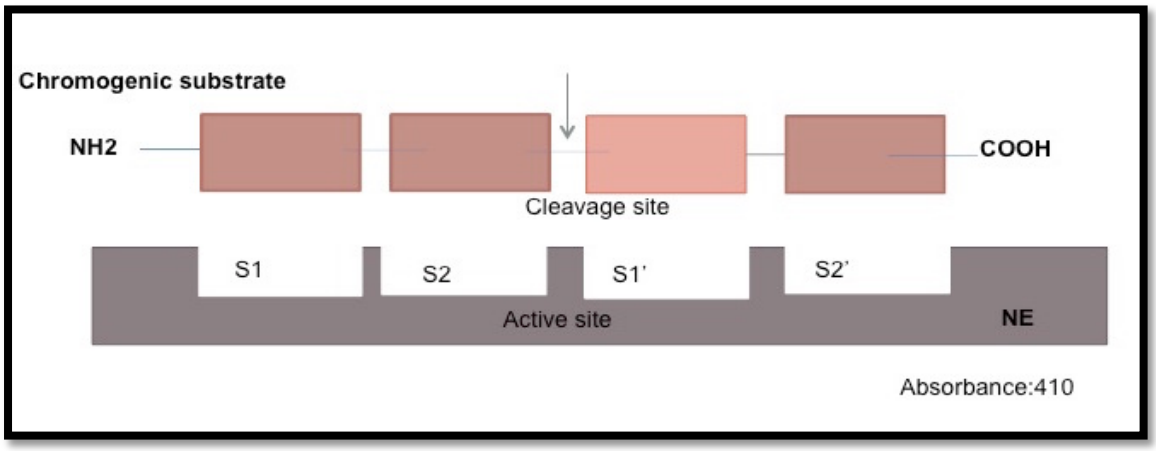

Figure 2.1. Schematic diagram illustrates the mechanism by which NE specifically cleaves the chromogenic substrate to measure its activity. The linear interaction between NE and the substrate demonstrates that each of the subset (S residual) on the elastase is binding to the upstream residual of the chromogenic substrate. Subsequent products elaborate colour changes that can be measured at 410nm absorbance (Permitted and adapted by (Korkmaz et al., 2010)) .

2.3.1.7. Cell Viability assays:

2.3.1.7A) Trypan Blue dye Exclusion:

ECs are highly susceptible to apoptosis and detach easily (Winn and Harlan, 2005). To rule out the possibility that NE may affect cell viability and cause EC death, trypan

blue dye exclusion was performed. This is a dye for dead cell identification and a quick way to differentiate between viable and dead cells.

The basic principle is that dead cells easily take up the dye, thus they appear dark blue whereas viable cells resist and hence appear colourless and refractive, under light microscopy. 50µl of 0.4% (v/v) trypan blue was mixed with 50µl of cell suspension. After mixing the solution, 20µl of trypan blue/cell suspension was pipetted into one of the two chambers of a haemocytometer. Accordingly, % of cell viability = number of unstained cells (viable) x 100 /total number of cells.

2.3.1.7B) Cytotoxicity assay:

Lactate dehydrogenase (LDH) is an intracellular enzyme found in all cell types and mediates the enzymatic oxidation of lactate to pyruvate. However, once the cells are damaged by stress, injury or chemicals, the intracellular enzyme is rapidly released outside the cells. Therefore, LDH quantification is a method that has frequently been used as an indication for cell death (Abe and Matsuki, 2000).To test whether LDH is released and the process of the cell death occurred as a result of NE actions, cell cytotoxicity was assessed using a LDH cytotoxicity assay (Promega, USA) according to manufacturer's instructions.

The percentage (%) of LDH released into the conditioned media was measured in freshly isolated supernatants after different time points as described (Wilson *et al*., 2007).

Briefly, in a 96 well-flat-bottom plate, 50µl of samples (supernatants) were mixed with 50µl of substrate. The mixture was protected from the light and left at room temperature for 30 minutes. Stop solution (50µl) was then added to stop the reaction and the absorbance was recorded at 490nm within 1h.

2.3.1.8. Cell apoptotic assay:

Many different approaches are used to study programmed cell death or apoptosis. Apoptosis is distinctive from cell necrosis or death in that the cells tend to change their morphology and shrinkage, with fragmentations in their nuclei and subsequent release of apoptotic bodies (Majno and Joris, 1995). As with cell viability, no definitive approach is used to define the cellular apoptosis and therefore many

techniques are used. In this thesis, I used caspase-3/7 activity assay as an indicator of apoptosis.

Caspase-3 is a single effector in the apoptotic pathway and it mediates activation of all other caspases in the pathway (Sebbagh et al., 2001). Apoptosis, detected via caspase-3/7 activity, was analysed by Caspase-Glo® 3/7 assay (Promega, USA), according to supplier's instructions.

In brief, HCAECs in 96 well plates were seeded at a seeding density of $6x10^3$ and grown until they reached 70% confluence. They were then stimulated with the above-mentioned conditions (see dual-step activation). Camptothecin (CPT) 1μM (Sigma-Aldrich, UK) (Jansen et al., 2012) was used to induce apoptosis in ECs as a positive control. At the end of the stimulations, the 96 well plates containing cells were removed from the 5% (v/v) CO_2 incubator to allow the plates to adjust to room temperature.
Caspase-Glo 3/7 Reagent (100μl) was then added to 100 μl of media containing cells, protected from the light, and left for 2h at room temperature. 50μl of the mixture from each well was then transferred to a corresponding well of a white 96 well plate. The luminescence of each sample and control was measured in a plate reading illuminometer according to manufacture's instructions (ThermoScientific).

2.3.1.9. Western blot analysis for IL-1β processing and release.

2.3.1.9A) Protein assay:

Quantification of the amount of protein in each sample is an important step before conducting immunoblotting, to ensure equal loading. I used a colorimetric detection, Bicinchoninic Acid (BCA)-based, protein assay (ThermoScientific), following the manufacturer's instructions.
Briefly, equal volumes of samples (supernatants and lysates) were added to a working solution provided by the kit, in a 96-well plate. After 30-minute incubation at 37°C, the plate was allowed to cool at room temperature and the resultant purple colour measured at 562nm. The absorbance of known protein (bovine serum albumin, BSA, standard curve), with known concentrations at a minimal detection level of 5μg/ml was used to calculate the concentrations of unknown proteins.

2.3.1.9B) Immunoblotting:

Western blotting, otherwise known as immunoblotting, is a basic technique used to separate proteins based on the differences in their molecular weights. The term "blotting" refers to transfer of samples from a gel to a membrane with a subsequent detection on the membrane surface, whereas "Western" or immunoblotting refers to detection of a specific antigen, using an antibody binding.

Samples (supernatants, concentrated using 10k Amicon filter devices (ThermoScientific), and lysates) at equal amounts of protein (15-20µg per lane) were separated on Novex 4-12% Tris-glycine gels (Invitrogen, Paisley, UK) at 200 voltage (V), prior to transfer onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) non-fat milk in 0.1% (v/v) PBS/Tween-20 (VWR International Ltd, Lutterworth, UK) for 1h to prevent any nonspecific binding of the antibodies to the surface of the membranes. The membranes were then incubated with primary antibodies, overnight at 4^{o}C, against IL-1β (mouse monoclonal, MAB201, 1µg/ml, R&D Systems), caspase-1 (rabbit polyclonal, ab17820, 1µg/ml, abcam, UK), α-tubulin (mouse monoclonal, 0.3 µg/ml, Sigma), NLRP3 (mouse monoclonal, 1µg/ml, ENZO, UK) or Lysosomal associated membrane protein-1 (LAMP-1, 0.1µg/ml, cell signalling, UK).

After washing 4 times for 5 minutes with washing buffer (PBS containing 0.1% (v/v) Tween-20) to remove unbound primary antibodies and reduce background staining, the membranes were incubated with secondary antibodies for 1h at room temperature (fluorescently labelled, 1:15000 dilution; Invitrogen, UK). The membranes were then again washed 4 times with the washing buffer to remove any unbound secondary antibodies and subsequently scanned with Odyssey according to the manufacturer's recommendation (LI-COR; Cambridge, UK).

All western blotting reagents were obtained from Life Technology, unless otherwise stated. Densitometry using LI-COR software was performed on at least 3 separate blots from three different experiments.

2.3.1.10. Direct (cell-free) effects of NE on recombinant IL-1β/proIL-1β:

NE is known to undergo spontaneous autolysis (Liu *et al*., 1999) and has a proteolytic activity against many cytokines such as TNF-α (Henriksen and Sallenave, 2008). For this reason, I tested the effect of NE (at the concentration used in stimulation experiments) on both mature IL-1β and proIL-1β standards. IL-1β standard (R&D Systems, UK) at a concentration of 125pg/ml and proIL-1β standard (R&D Systems, UK) at a concentration of 10000pg/ml were mixed with NE (1µg/ml) incubated for 30 minutes, 2h and 6h, at 37^{o}C. The samples were then stored at -80^{o}C prior to testing for NE cleavage, by ELISA and Western blot.

2.3.1.11. Luciferase assay and determination of IL-1β biological activity:

The bioactivity of the secreted IL-1β by HCAECs following NE stimulation was assessed using an IL-8 luciferase reporter assay as previously described (Kiss-Toth et al., 2000). Hela cells (Sigma-Aldrich, UK) were cultured in their supplemented media (DMEM, Appendix III) and were transfected using PolyFect transfection regent (Qiagen) according to the supplier's recommendations.

Briefly, Hela cells were plated in 96-well plates at 5x10^{3}; around 60% confluence. Transfection efficiency was calculated using EGFP (Green fluorescent protein) and measuring the activity of renilla in the assay. Cells with approx. >70% of estimated efficiencies were used in the bioactivity assay.

Hela cells were transfected with a total of 100ng DNA/well; including 60ng pIL-8-luciferase (luc) as a reporter and 40ng renilla (pRL-TK) as an internal control in the assay. After 24h, cells were stimulated with 0.1nM of rIL-1β as a control, or freshly harvested supernatants from HCAECs stimulated with NE for 6h. IL-1 beta neutralising antibody (1µg/ml, MAB201, R&D Systems) was used in some wells, to prove specificity.

After 6h incubation, cells were lysed with passive lysis buffer and kept at -20^{o}C overnight to ensure the complete lysis of the cells. On the second day, lysed cells were transferred to a white-bottomed plate (15µL) to be assayed using LARII and Stop and Glo reagents and luminescence intensity was measured using a plate reader (ThermoScientific).

Luciferase activity was calculated by normalising to the renilla luminescence measured in each well. Ratio was taken for each individual experiment and the activity of rIL-1β stimulated cells were used as a 100% and activities in NE supernatant were expressed as a percentage of this.

2.3.1.12. Annexin V binding to the cells and detection of MV shedding:

HCAECs ($3x10^4$) were plated in LabTek (Fisher) 8-well chamber slides and subjected to the above-mentioned stimulation conditions (Dual-step activation). AnnexinV-Alexa Fluor® 488 (Invitrogen) was then added to the cells at 5µl/well. MV shedding was visualised using image acquisition software (Inverted widefield fluorescence microscope Leica AF6000 Time Lapse) after the addition of NE in a 5%CO_2/ 37^{o}C (v/v) heated chamber. The images were captured after 10 minutes, 30 minutes, 2h and 6h and analysed using Image J software. EVs (0.1-1µm) were quantified per random field of cells.

2.3.1.13. Electron Microscopy (EM) for MV shedding and exosome release:

Electron microscopy is a type of microscopy that uses electrons to generate an image of a specimen. It has a higher resolution and magnification than the light microscopy and thus allows visualisation of smaller objects in a fine detail. Thus to observe MV morphology and shedding and exosome release from EC plasma membranes, EM was used.

HCAECs were treated, or left untreated, with NE for 30 minutes, 1h, and 2h. The cell pellet was prepared as described above (Section 2.3.1.4B) and fixed in 2% (w/v) paraformaldehyde (PFA) and 1.25% (w/v) glutaraldehyde in sodium cacodylate (w/v) overnight. Following washing in 0.1% (w/v) sodium cacodylate, the samples were dehydrated in a series of graded ethanol (70%, 80%, 90%, 100%, dry 100%) v/v and infiltrated in 50:50 London resin (LR) White/100% Ethanol LR (v/v) White over night. LR white was changed twice over a period of 8h and tissue polymerised in fresh LR White at 50^{o}C overnight before cutting into ultrathin sections.

Blocks were sectioned using a Reichart Ultramicrotome, generating 85nm thick sections, mounted on nickel grids. Sections were stained with saturated uranyl acetate w/v (30 minutes), washed in DW, stained with Reynold's Lead Citrate (5

minutes) and washed again in DW. Grids were viewed using an FEI tecnai TEM at 80Kv operating voltage. Images were recorded using a Gatan multiscan digital camera and analysed using Gatan digital micrograph software.

All reagents were kindly provided by Dr. Christopher J Hill (Department of Biomedical Science, University of Sheffield, Sheffield, UK).

2.3.1.14. Microvesicle (MV) isolation:

MV isolation was conducted as described (Jansen et al., 2012, Wang et al., 2011a). Briefly, to pellet MVs, supernatants were centrifuged at 20,000g for 15 minutes at 4°C. MV pellets were washed twice with PBS and stored at -80°C until analysis was conducted. The MV concentrations were quantified by total protein measurement using BCA protein assay (Pierce; Leicestershire, UK) according to manufacturer's instructions and as described in section 2.3.1.9.

2.3.1.15. Flow cytometry gating strategy for MV population:

A pellet of MVs (an average of 20µg from $2x10^4$ cells), resuspended in annexin V-binding buffer, was stained with annexin V PE-Cy7 fluorescence according to the manufacturer's recommendations (eBioscience, UK). LSR II flow cytometer (BD Biosciences) was used to quantify MVs. The upper size limit of MVs was defined in a logarithmic forward scatter/side scatter dot plot histogram using 0.9-2µm calibrated latex beads (FC Size Calibration Beads; Sigma-Aldrich).

The MV gate was defined by drawing around the 2µm bead population and excluding the first channel of forward and side scatter. Endothelial MVs were defined as events of size <2µm expressing Annexin V. Background fluorescence within the MVs gate was established by incubating labelled control antibodies with MVs. Each sample was run until 10,000 calibration beads were counted in the 2.5mm gate.

Data were acquired and analysed by Flow Jo software (TreeStar, Inc, Ashland, USA). Positive events in the gated area of interest were multiplied by a concentration factor to determine the number of the MVs per $2x10^4$ cells.

2.3.1.16. Measurement of intracellular free calcium mobilisation during NE stimulation:

Intracellular calcium in ECs $[Ca^{2+}]_i$ was measured as previously described (Storey et

al., 2000), using Fluo-4 Direct™ Calcium Assay Kit (Invitrogen), according to manufacturer's recommendations.

A HCAEC monolayer, in 96 well plates at $5x10^3$ cells/well, was stimulated with cytokines for 48h, or left untreated, then the media was disposed and cells were washed with PBS (pH 7.4, calcium free, Gibco). The cells were then coated with Fluo-4 in the dark for 30 minutes in 1% (w/v) Ca^{2+} free media (Gibco).

The cells were then washed and the media was replaced with serum free / calcium deprived media containing stimulants as described in section 2.3.1.4B. Calcium responses to NE were read spectroflurometrically using a plate reader at excitation 494nm and emission 516 nm. Ethyleneglycoltetraacetic acid (EGTA; 6mM, Sigma) and Ionophore A3784 (10μM, Sigma) were used as negative and positive controls, respectively.

2.3.1.17. Immunofluorescence staining:

Confluent layers of ECs were grown on fibronectin (Sigma) coated Lab II-Tek chamber slides (ThermoScientific) and incubated with NE for (30 minutes to 2h), or left untreated. The cells were then fixed in 4% (w/v) PFA at room temperature for 10 minutes. Excess PFA was removed by washing the cells with PBS (3x, pH 7.4) to and permeabilised using 0.3% (v/v) triton-X100/PBS for 10 minutes.

Antibody nonspecific binding was prevented by incubating the cells with a blocking buffer (PBS containing 1% (w/v) BSA) for 30 minutes before the addition of primary antibodies (anti-IL-1β, antiLAMP-1, or anti-NE; see appendix II for antibody concentrations and their sources), in a diluent of 0.3% (v/v) donkey serum/PBS for 1h. After washing with PBS and subsequent incubation with the appropriate fluorescently labelled secondary antibody (donkey anti-rabbit 448, donkey anti-mouse 555, and donkey anti-goat 640, Invitrogen), the cells were mounted in a 4',6-diamidino-2-phenylindole (DAPI) containing mounting media and analysed using confocal microscopy (Zeiss). Colocalisation analysis was performed using image J software.

2.3.1.18. Immunolabelling of NE:

Immunolabelling of NE was conducted as previously described (Houghton et al., 2010) using a Microscale Protein Labelling kit (Molecular Probes) according to

manufacturer's instructions. 50µg NE was used for the reaction. Final concentration of Alexa 647-NE was 0.1 mg/ml in a volume of 100 µl. NE inside ECs was observed using confocal microscopy and analysed using image j software.

2.3.1.19. Immunogold staining:

Sections were mounted on inert grids such as nickel or gold described in section 2.3.1.13. The grids (nickel) were washed in DW for 10 minutes. After washing, sections were incubated in TBS (tris buffered saline), pH 7.4 containing 10% (v/v) normal serum (from the same species as the secondary antibodies) for 1h. Samples were then stained with the appropriate specific primary antibody diluted 1:50 with TBS, pH 7.4, including 0.2% (w/v) BSA, for 2 hours.

The grids were washed in two changes of TBS (pH 7.6) for 5 minutes each, and then two changes of TBS (pH 8.2) for 5 minutes each. The immunogold conjugated secondary antibody was then added (diluted 1:200 with TBS, pH 8.2, including 0.8% (v/v) BSA) for 2 hours. After subsequent washing of the grids in TBS, pH 8.2, twice, for 5 minutes, the grids were stained with saturated uranyl acetate 1% (w/v) for 30 minutes, washed in DW, then stained with Reynold's Lead Citrate (5 minutes) and washed in DW.

Imaging was conducted using using a FEI tecnai TEM at 80Kv operating voltage. Images were recorded using a Gatan multiscan digital camera and analysed using Gatan digital micrograph software.

Reagents were kindly supplied by Dr. Christopher J Hill (Department of Biomedical Science, University of Sheffield, Sheffield, UK).

2.3.2. Animal experimental design:

Male ApoE$^{-/-}$ mice were bred in-house at the University of Sheffield. Food and water were given *ad libitum* under a controlled environment (Temperature; 22-25^{o}C, humidity; 55 ± 5 and 12h light cycle). At 8 weeks of age, the mice were housed individually and randomly separated into one of three groups:

- 12 mice fed high fat diet (HFD) with jelly alone.
- 12 mice fed HFD and ALA mixed with jelly.
- 12 mice fed HFD and DHA mixed with jelly.

2.3.2.1 Animal feeding:

2.3.2.1A) High fat diet:

The mice were fed a Western-type diet, high fat diet (HFD), containing 21% (w/w) fat, 0.15% (w/w) cholesterol and 0.296% (w/w) sodium (Special Diet Services, Witham, UK) over a 12-week duration. This HFD was used specifically to study the diet effects on atherosclerosis and hypertension as described (Chamberlain et al., 2009).

2.3.2.1B) Jelly feeding and drug delivery:

In order to dissolve the fatty acids efficiently and provide a constant daily intake, 99% purified fatty acids (Sigma-Aldrich, UK) were mixed with jelly. The jelly (blackcurrant) was purchased from a local supermarket in a concentrated form and before starting the experiment; the mice were trained on the jelly at least 1 week before adding the FAs. One cube of jelly was dissolved in 25ml boiling water and poured into 4 sterile plastic moulds. 180µl of FAs (equivalent to 300mg) were then mixed gently with the jelly and left at -20^{o}C for 1h. The set jelly was cut into equal pieces and kept at -20^{o}C for individual mouse feeding. The omega-3 FAs (DHA or ALA) were given at a final concentration of 300mg/kg/day as previously reported (Matsumoto et al., 2009).

2.3.2.2 Ethical issues:

All animal care and procedures were closely conducted under ASPA 1986, UK. The work was performed under personal licence PIL40/10381 and project licence PPL70/7992 (previously PPL40/3307).

2.3.2.3. Food intake and body weight monitoring:

The mice were monitored daily for their intake of jelly containing fatty acids. On a weekly basis, body weights were measured among the three studied groups. Measurements were taken to the nearest 0.1g and recorded from week 0, before starting the study, to week 12. Average body weights for mice per group were plotted per individual week for 12 weeks.

2.3.2.4. Blood pressure monitoring:

Systolic and diastolic blood pressure was measured in the mice using a tail-cuff (Visitech Systems, NJ, USA) as previously described (Chamberlain et al., 2009).

Briefly, the mice were subjected to a one-week of training before starting measurements in order to minimise stress levels. Blood pressure was measured in 4 mice per group, and 10 measurements per mouse per day were recorded on four different days per week for 12 weeks. The data were rejected if the systolic blood pressure was more than 200mmHg, less than 40mmHg, outside 2 standard deviation (SD) of the mean or had fewer than 4 valid readings. Per week measurements were also rejected if they had fewer than 3 valid readings.

2.3.2.5. Echocardiogram:

Echocardiogram is an ultrasonographic technique that has become a dominant cardiac imaging technique in day-to-day clinical practice. Quantification of cardiac chamber size and ventricular function assessment are the most frequently tasks performed by echocardiogram (Lang et al., 2006). Therefore, to assess the cardiac function of the mice during the duration of the study, transthoracic echocardiogram (TE) was conducted as previously described (Hameed et al., 2013). In brief, the mice were anesthetised using isoflurane delivered in oxygen in an isolator before placing the mice in a supine position. To minimise excessive heat loss, which may affect the measurements, the mice were placed on a heated platform and continuous rectal temperature was monitored.

During recording, the mice were maintained on approximately 0.5-1.5% (v/v) isoflurane in oxygen, delivered via a nasal cone. The level of anaesthesia was adjusted to achieve a heart rate of 500 ± 125 beats per minute (bpm).
Left ventricular (LV) function was recorded in 2D- short axis view at the level of left ventricular outflow tract (LVOT), whereas M-mode measurements were made for LV wall and cavity dimension (LVIDd). LV fractional shortening (LVFS) and ejection fraction (LVEF) and corrected LV mass (LVM) were assessed using automated analysis. Pulse wave Doppler was used to determine the cardiac output (COP), and aortic blood velocity.

The measurements were taken twice, at the beginning of the study (baseline) and just prior to the termination of the study, using an ultrasound imaging system (Vevo 770®, Visual Sonics, Toronto, Canada) and a RMV707B scan head was used.

2.3.3. End of study:

The mice were euthanased with an overdose of phenobarbital (200mg/kg, intraperitoneal (ip) injection). Blood was collected by cardiac puncture following the loss of the carpopedal reflex and before the complete cessation of breathing.
For plasma isolation, blood was collected using a 1mL heparinised syringe (1:100 in blood). At room temperature, blood was centrifuged at 3000g for 5 minutes using a microfuge R (Beckman Coulter, UK).

After the cessation of breathing, the chest wall was opened and the heart, the brachiocephalic artery and the whole aortae were harvested for histological analysis following 1mL perfusion of the right ventricle with PBS (pH 7.4) then 10% (v/v) formalin buffered saline (Appendix III, section 2).

2.3.4. Preparation of samples for erythrocyte fatty acid analysis:

Freshly collected blood, by cardiac puncture, was stored in 4ml EDTA collecting tubes (4 pooled samples of red blood cells (RBCs) from 4 different mice per group with a minimum volume of 1mL). The samples of blood were centrifuged at 3000g for 5 minutes and the plasma and buffy coat were aspirated and discarded.
The tubes containing red cell fractions were then labelled and stored at -80^{o}C until sent to BioLab, London, UK for analysis.

2.3.5. Plasma lipid & glucose measurements:

Plasma analysis for TG, total cholesterol (TC), HDL-C, LDL-C and glucose was performed at the Department of Clinical Chemistry (Royal Hallamshire Hospital, Sheffield, UK).

2.3.6. Plasma cytokine measurement:

The levels of plasma proinflammatory cytokines (TNF-α, IL-6, IL-8, IL-1β, IL-1α, MCP-1, RANTES; pg/ml) were measured using a BD™ Cytometric Bead Array (CBA, BD Biosciences; UK), according to manufacturer's recommendations. The analysis was performed by Ms Kay Hopkinson in the medical care facility, Medical School, Sheffield, UK.

2.3.7. Assessment of atherosclerotic lesions:

2.3.7A) Whole-mount atherosclerotic lesion analysis (Aorta):

The extent of atherosclerosis was assessed in the whole aortae by an *en face* method (Freigang et al., 2011). In brief, the aortae were perfused firstly with PBS and then 10% (v/v) formalin. After exposure of the whole aortae and removal of the adherent fats and intercostal vessels, the aortae were dissected from its origin in the heart to the aortic orifice of the diaphragm, under a dissecting microscope.

Aortae were fixed in 10% (v/v) formalin overnight at 4^{o}C then stored in PBS at 4^{o}C until pinning was conducted.

The aortae were opened longitudinally and stained with oil red O stain (ORS): the aortae were first rinsed in 60% (v/v) isopropanol and then stained with 0.3% (w/v) ORS (Sigma) in PBS. After 30 minutes of staining, the aortae were destained for 20 minutes in 60% (v/v) isopropanol and then further washing in DW was performed. The stained aortae were pinned, the lumen upwards, on a wax filled petri dish (15cm) using micro-needles (Fine Science Tools, Heidelberg, Germany). Using a digital camera connected to light microscope at 15x magnification, images were acquired. Lesion areas were analysed using NIS-elements analysis software (Nikon, UK). Atherosclerotic lesion in the whole aortae, arch and descending parts were quantified as % of the total surface area.

2.3.7B) Atherosclerotic lesion analysis in aortic root and brachiocephalic artery:

A second assessment of atherosclerosis was conducted in cross-sectional aortic sinuses and brachiocephalic sections as described (Daugherty and Whitman, 2003). Briefly, the hearts and brachiocephalic arteries (BCA) (the first branch of the aortic arch) were collected and stored in 10% (v/v) formalin overnight at 4^{o}C and then in PBS (4^{o}C) until they were embedded in paraffin wax.

The paraffin-embedded hearts (at the level of aortic valves) and brachiocephalic arteries were serially sectioned using a Leica RM2135 microtome (Leica Microsystems, Wetzlar, Germany). Sections (5µm thickness) were collected and stained with Alcian Blue & Elastic Van Gieson (AB/EVG) as described in detail below.

Mean lesion size was calculated from measurements of five serial sinus sections starting from the three cusp area. Analysis was conducted blinded per individual animal and the average of all sections (5) per mouse, used as n=1. The individual mouse analysis was discounted if there were fewer than 3 sections of brachiocephalic artery with atherosclerotic lesions or fewer than 4 sections with three leaflets for aortic valves. The % of atherosclerotic lesion to the total surface area was evaluated using NIS-elements software.

2.3.7B (i) Alcian Blue, elastic Van Gieson (AB/EVG) Staining:

The lesion area and plaque compositions (aortic and brachiocephalic sections) were assessed morphologically in histologically stained sections.

Briefly, the sections were dewaxed in xylene and rehydrated through graded alcohols: 100%, 90%, 70% and 50% (v/v) ethanol, and then rinsed in tape water. Sections were then oxidised with potassium permanganate (0.25% w/v) for 30 minutes. The sections were rinsed in tape water and bleached in 1% (w/v) oxalic acid. Following 3min, the nuclei were counterstained with Carazzi's haematoxylin for 2 minutes and differentiated for a few seconds in acid alcohol: 1% (v/v) hydrochloric acid (HCl) in 70% (v/v) ethanol. The sections were then exposed to hot running tape water for 5 minutes.

The slides were stained with 1% (w/v) Alcian Blue in 3% (v/v) aqueous acetic acid, pH 2.5, for 5 minutes and the stain was washed with tape running water. The slides were initially rinsed in 95% (v/v) ethanol before subsequent staining with Miller's Elastin for 30 minutes. After that, the slides were rinsed and differentiated in 95% (v/v) ethanol and distilled water before incubation in a Curtis Modified Van Gieson stain for 6 minutes.

Before mounting using DPX resin, the tissues were rehydrated in 100% (v/v) ethanol, then two changes of 100% (v/v) xylene.

2.3.7C) Analysis of Collagen content:

Collagen content in aortic and brachiocephalic sections were measured in martius scarlet blue (MSB) positive stained areas as described (West et al., 2014) and expressed as a % to the total surface area.

Briefly, the tissues were dewaxed and rehydrated in xylene and graded alcohols as described above. The tissues were then stained with 1% (w/v) Celestine blue for 5 minutes, drained, and counterstained with Harris' haematoxylin for 5 minutes.
After rinsing in tape water and differentiating in acid alcohol for few seconds, for 5min, the tissues were exposed to hot running tape water and then shortly rinsed in 95% (v/v) ethanol. Subsequent staining with 0.5% (w/v) martius yellow and 2% (w/v) phosphotungstic acid in 90% (v/v) ethanol was carried out for 2 min.

Ponceau de xylene solution (1% (w/v) ponceau de xylene / 2% (v/v) glacial acetic acid) was applied to stain the sections for duration of 10 minutes. The stain was differentiated using 1% (w/v) phosphotungstic acid for 5 minutes. After draining of the stained sections, the tissues were stained with methyl blue (5% (w/v) methyl blue in 10% (v/v) glacial acetic acid) for 10 minutes. The stain was washed off by rinsing of the slides in 1% (v/v) acetic acid for 10 minutes. The tissues were rehydrated through graded alcohol and two changes of xylene and then mounted using DPX mounting media. Analysis was performed using NIS-Elements software.

Alcohols and xylene were purchased from ThermoScientific whereas all stains and DPX resin were provided from VWR International Ltd (Lutterworth, UK) unless otherwise stated.

2.3.8. Immunohistochemistry:

Sections were used for immunohistochemistry as previously described (Chamberlain et al., 1999). Aortic sinuses were stained to assess IL-1β, IL-1α, IL-1ra, the anti-macrophage surface glycoprotein Mac-3, TLR-4, SMCs (α-smooth muscle actin; SMA), eNOS, von willebrand factor (vWF) and NE.
Briefly, formalin-fixed, paraffin-embedded samples were deparaffinised and rehydrated through decreasing concentrations of ethanol (as described above). Endogenous peroxidases were blocked with 3% (v/v) hydrogen peroxide/PBS. Sections were either treated or left untreated with a heat mediated antigen retrieval stage.

Heat-induced antigen retrieval was conducted in sodium citrate solution (10 mM, pH

6.0, Alfa Aesar, Ward Hill, MA) for 20 minutes in a water bath (95^{o}C). Slides were washed in PBS and incubated with 5% (w/v) BSA or 1% (w/v) non-fat milk in PBS for 30 minutes to block nonspecific immunological binding. Primary antibodies were diluted to the required concentrations (see appendix II for the individual antibody concentrations) and then either applied for 1h at room temperature (for SMA and vWF) or overnight incubation at 4^{o}C (for IL-1α, IL-1ra, IL-1β, NE, Mac-3, eNOS and TLR4).

Secondary antibody incubation was conducted for 30 minutes at room temperature using either fluorochrome-labelled antibodies for fluorescence microscopy or biotinylated antibodies (appendix II), followed by a 30 minute incubation with an avidin-biotin complex (Vectastatin Elite ABC kit (Vector Laboratories Inc)) and DAB to visualise the antigen, for light microscopy.
Nuclei were counterstained with Carazzi's haematoxylin. Negative (PBS instead of the primary antibodies) and positive controls (Lungs, kidneys and splenic tissues from male ApoE$^{-/-}$ mice fed HFD for 12 weeks) were used for individual staining conditions.

Analysis of all immunostaining was conduced using NIS-elements software (Nikon Instruments, Kingston upon Thames, UK).

2.3.9. Statistical analysis:

Data are expressed as mean ± standard error of the mean (SEM) and analysed using prism software (Version 6, GraphPad, San Diego, CA). For multiple comparison tests, one way analysis of variance (ANOVA) followed by Tukey's test was performed. For a comparison of two experimental groups, data were analysed by unpaired student's t test for normally distributed data. Blood pressure data were analysed by 2-way ANOVA followed by Tukey's post-test. Statistical significance was achieved when $p<0.05$.

Chapter (3) The Secretion of the Leaderless Interleukin-1β from Coronary Endothelium is Neutrophil Elastase Mediated.

OUTLINE:

3.1. Overview: Secretion of the leadeless IL-1 is NE mediated:

IL-1β has been implicated in several aspects of vascular inflammation (Dinarello, 2011b) and neointima formation (Chamberlain et al., 2006). It generally was assumed that IL-1β is produced predominantly by immune derived cells (Dinarello, 2009). However, work by Galea and colleagues shows that in IHD patients, atherosclerotic coronary arteries express IL-1β, predominantly within the endothelium (Galea et al., 1996). Therefore, IL-1 release from EC may play an important role. Moreover, experimental studies have indicated that cultured ECs synthesise considerably large amounts of IL-1β in response to different cytokine stimulations (Wilson et al., 2007, Libby et al., 1986a). However, IL-1β release from these cells is relatively inefficient.

IL-1β production is a two-step controlled process, requiring an initial stimulus for NF-κB activation and transcription of proIL-1β (31kDa) which, in turn, is cleaved by an inflammasome-activated caspase-1 (Schumann et al., 1998, Hogquist et al., 1991) into a biologically active isoform (17kDa) in response to a second hit stimulus before secretion (Dinarello, 2007). The cleavage of proIL-1β is a crucial step in the secretion process (Ward et al., 2010) and studies on monocytes show that caspase-1 (a cysteine protease) is a cardinal enzyme in this process (Keller et al., 2008).

There are other potential enzymes that cleave proIL-1β into the mature form, including serine proteases (neutrophil elastase, cathepsin G and proteinase 3) (Stehlik, 2009). It has previously been indicated that serine proteases cleave purified proIL-1β into a biologically active IL-1β *in vitro* at distinct sites to that of caspase-1, with production of 18kDa and 20kDa isoforms of IL-1β (Black et al., 1988, Hazuda et al., 1990, Stehlik, 2009). However, whether, and to what extent, these proteases could contribute to IL-1β release in live cells, including ECs is relatively unknown.

In this chapter, I sought to determine whether NE activates IL-1β secretion from vascular endothelium and study the underlying mechanism(s).

3.2. Brief Methods:

The methods used in this chapter are described in detail in chapter (2), briefly:

ECs were primed with different combinations of cytokines to induce IL-1 production followed by NE (1µg/ml) at different time points (30min, 2h and 6h), levels of released IL-1β were measured using ELISA. IL-1β processing and secretion were further confirmed using immunoblotting. Cell death during NE incubation was ruled out using LDH cytotoxicity and trypan blue assays. NE activity was measured spectrophotometrically using a highly specific synthetic substrate. Statistical analysis was performed on at least n=3 repeat experiments and data are expressed as mean ± SEM.

3.3. Interleukin-1 generation from vascular endothelium:

Circulating levels of IL-1β have been detected in patients with CAD and correlated with the severity of atherosclerosis (Van Tassell et al., 2013). Previous cell culture studies show that IL-1β is produced in ECs following incubation with pro-inflammatory cytokines (Wilson et al., 2007). I, therefore, sought to determine whether IL-1β is produced in un-stimulated HUVECs and whether the levels are different with different inflammatory stimuli.

Using ELISA, I showed that un-stimulated HUVECs did not produce any significant levels of IL-1β inside cells. However, intracellular levels of IL-1β were significantly increased following TNF-α/IL-1α stimulation (500 ± 121pg/ml) and IL-1β/LPS (430 ± 170pg/ml) compared to un-stimulated cells (0pg/ml) (Figure 3.1).
Treatment of ECs with a combination of TNF-α/INF-ɣ/LPS did not significantly stimulate any IL-1β production.

Subsequent experiments, therefore, used a combination of TNF-α/IL-1α as it gave the highest IL-1β generation within ECs.

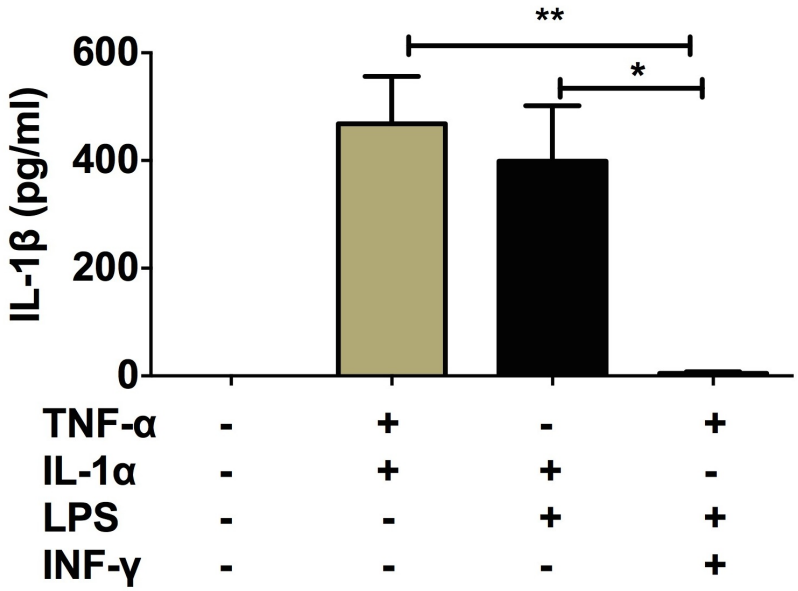

Figure 3.1. Generation of IL-1β within endothelial cells. Human umbilical vein endothelial cells (HUVECs) in 6-well plates were primed with different combinations of cytokines to up-regulate endogenous levels of proIL-1β. Levels of IL-1β were assessed by ELISA. Data are expressed as mean ± SEM, n=3. Data are analysed by One-way ANOVA followed by Tukey's test, *p<0.05, **p<0.01.

3.4. IL-1 stimulated secretion from HCAECs, by NE, is dose dependent:

To assess the contribution of NE in IL-1β secretion, cytokine-primed ECs (TNFα/IL-1α; 10ng/ml each; to up-regulate endogenous pro-IL-1β) were treated with varying concentrations of NE in serum free media for several incubation lengths (detailed in Chapter 2).

NE at 1µg/ml caused a significant (10 fold) increase in the release of IL-1β from cytokine-primed cells (198 ± 24.85pg/ml, p<0.0001, n=3) compared to primed cells without NE (12.1 ± 4.81pg/ml), after 2h of stimulation (Figure 3.2). In contrast, the release was not as great with higher concentrations of NE (2µg/ml) due to cell death (data not shown).

Subsequent experiments, therefore, used NE at 1µg/ml to give the highest amount of IL-1β release without a significant increase in cell death.

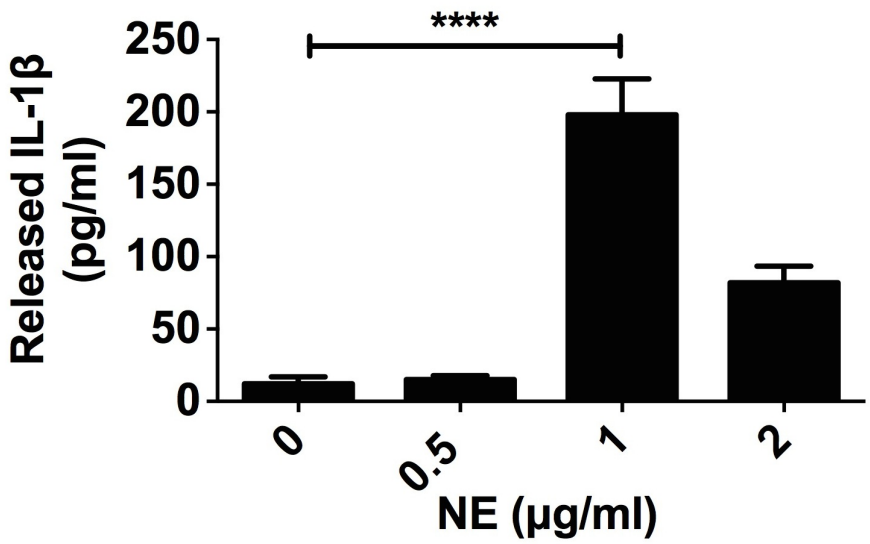

Figure 3.2. NE enhances IL-1 secretion in a dose dependent fashion. IL-1β released by HCAECs after 48h stimulation with cytokines (TNF-α/IL-1α; 10ng/ml) followed by NE activation for 2h, was assessed by ELISA. Cytokine-stimulated cells alone (0µg/ml) were used as controls. NE concentrations ranged from 0.5µg/ml to 2µg/ml. Data are mean ± SEM, analysed by One way ANOVA and Tukey's post-test, ****p<0.0001, n=3.

3.5. NE stimulated IL-1 secretion from HCAECs is time dependent:

To investigate the time effect on IL-1β release by NE, levels of IL-1β released into the supernatants were measured over 6h incubations with NE. The levels were measured using ELISA. IL-1β was detected in the media at all time-points tested. However, the released IL-1β was significantly increased at 6h, compared to 2h, incubation with 1µg/ml NE (272.8 ± 47.06 vs.103.8 ± 12.13pg/ml, p<0.001, n=3) (Figure 3.3A).

These levels equate to approximately 37% of the total intracellular IL-1β being released at 6 hours after NE stimulation compared to cytokine-primed cells alone (37 ± 2.018% vs. 3.3 ± 0.321%, respectively, p<0.0001, n=3) (Figure 3.3B) (section 3.6, figure 3.4B for intracellular levels).

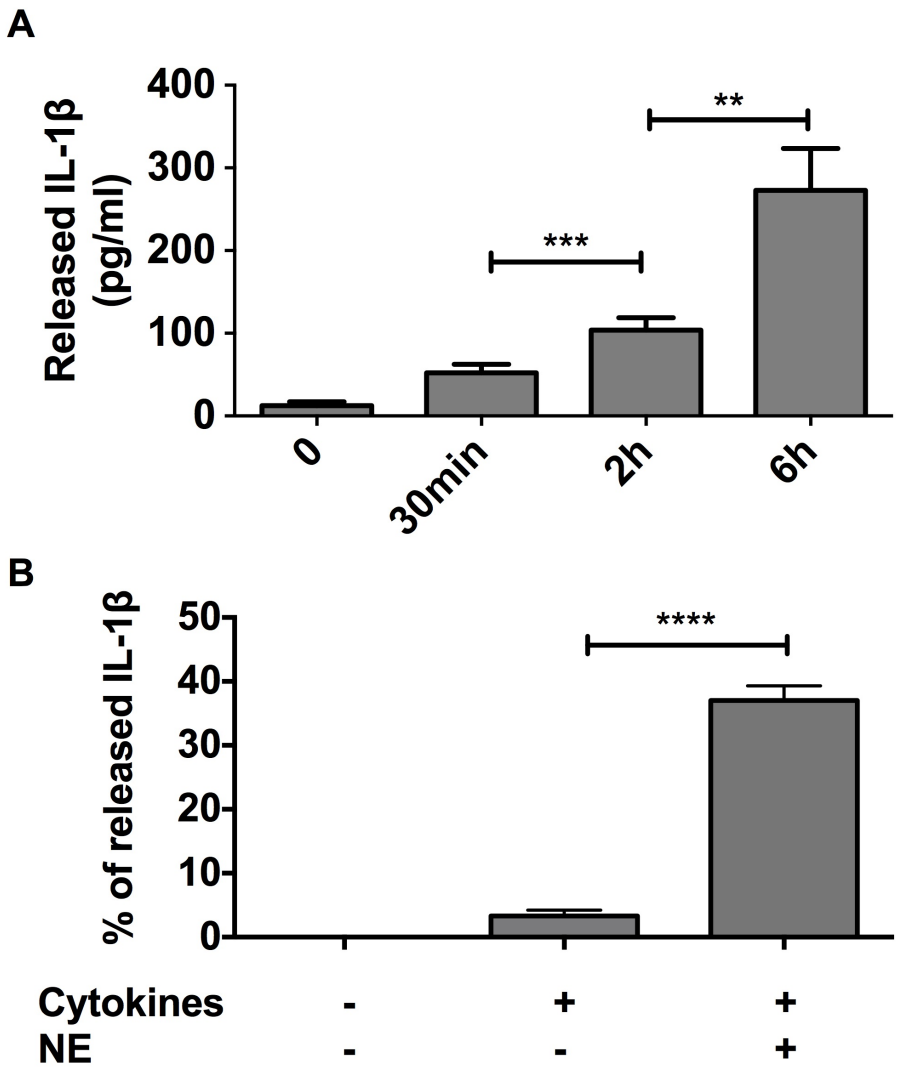

Figure 3.3. IL-1β stimulated secretion by NE is time dependent. A) Kinetics of IL-1β secretion by activated ECs plus NE treatment for increasing incubation times. Data represents mean ± SEM, n=3. Data are analysed by One way ANOVA and Tukey's post-test, **p<0.01, ***p<0.001. **B)** Percentage of the released IL-1β at 6h of NE stimulation (level of IL-1β in the supernatants divided by the total concentration of IL-1β (in the supernatants and lysates together) X 100). Data are from three independent experiments, n=3, mean ± SEM and analysed by One way ANOVA followed by Tukey's test, ****p<0.0001.

3.6. Interleukin-1 secretion by the endothelium is NE mediated and caspase-1 independent:

Although in unstimulated ECs no IL-1β was measured in the media, a small amount of IL-1β was released into the serum free media of cytokine-primed cells (Figure 3.4A). However, a 6h incubation with NE caused a significant increase in IL-1β secretion compared to cytokine stimulation alone (272.8 ± 50pg/ml vs. 55.5 ± 17.3pg/ml, p<0.001, n=5) (Figure 3.4A).

To confirm that this release was due to direct NE action, the cells were pre-treated with the potent, specific, irreversible, neutrophil elastase inhibitor (NEIII, Ki=10μM), resulting in a significant attenuation of IL-1β secretion compared to NE treated cells without the inhibitor (64.64 ± 47.73pg/ml vs. 272.8 ± 50pg/ml, $p<0.01$, $n=5$) (Figure 3.4A).

Caspase-1 is the cardinal enzyme for IL-1β secretion in immune derived cells (Keller et al., 2008). Therefore, to determine the involvement of caspase-1 in this system, cytokine-primed ECs were pre-treated with the caspase-1 inhibitor, YVAD-CHO and then with NE. No significant changes in the secreted levels of IL-1β were seen compared to NE alone (Figure 3.4A). Thus, NE-mediated IL-1β secretion in ECs appears to be independent of caspase-1 in this system and at the time-points studied.

In unstimulated EC lysates, and as expected, I could not detect IL-1β using ELISA. However, there was a significant IL-1β production following treatment with the proinflammatory cytokines (Figure 3.4B). The IL-1β levels in the lysates were not significantly altered following incubation with NE, or inhibitors, with an approximate average of 304 ± 37.6pg/ml (Figure 3.4B). Thus, NE treatment alone did not provoke IL-1β production in the cells, suggesting no direct effects of NE on IL-1β generation. There was also no effect seen on IL-1β production in cell lysates by NEIII or caspase-1 inhibitors (Figure 3.4B).

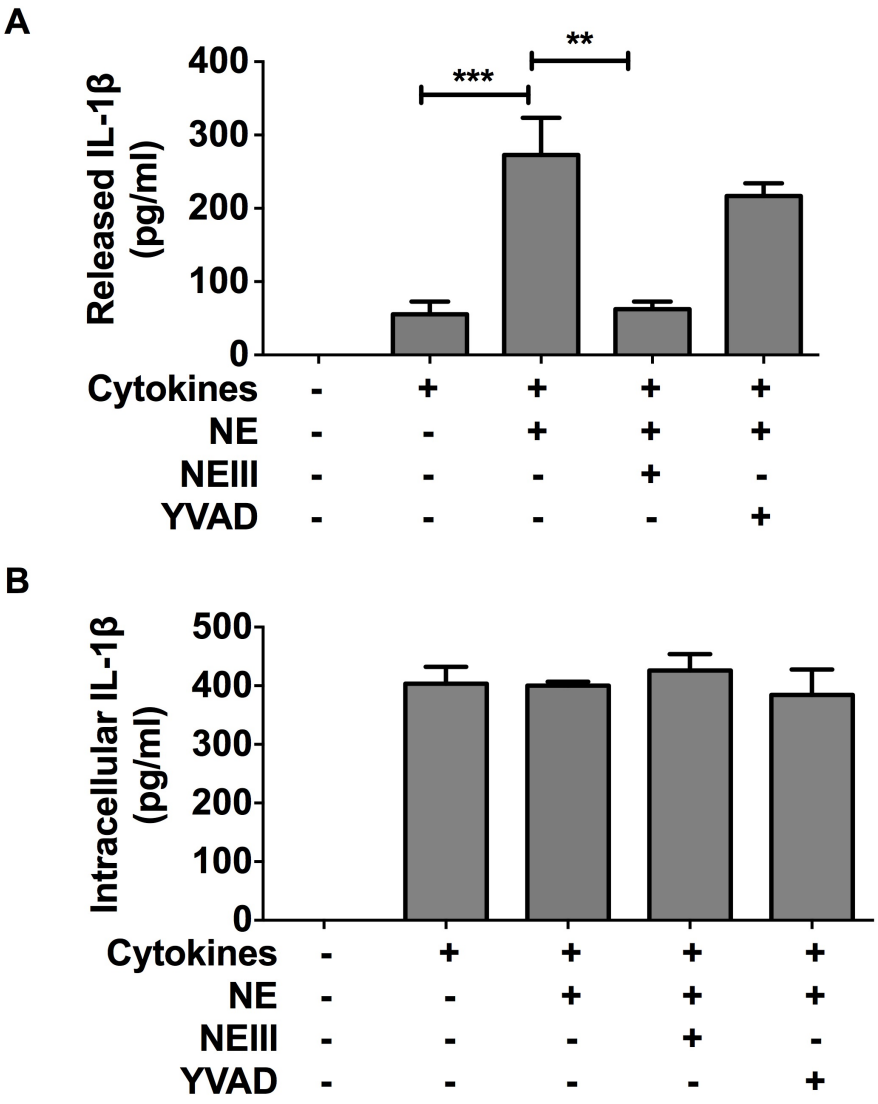

Figure 3.4. NE enhances IL-1 secretion by ECs by a caspase-1 independent mechanism. A) IL-1β release in HCAECs (primed for 48h with or without cytokines and incubated for 6h in serum free media alone or with NE (1µg/ml), measured in the presence or absence of inhibitors (NEIII; 500µM or YVAD; 50µM). **B)** Levels of IL-1β in the cell lysates. **(A-B)** measured by ELISA. Data are mean ± SEM, and analysed by One way ANOVA and Tukey's post-test, **p<0.01, ***p<0.001, n=5.

3.7. NE effects on proIL-1 in ECs:

To investigate whether NE has an impact on the levels of the IL-1β precursor either in the supernatants or cell lysates, a proIL-1β ELISA was used. Consistent with the previous findings, in the lysates, unstimulated ECs did not produce any proIL-1β (Figure 3.5A). However, following cytokine-treatment, a large amount of proIL-1β is produced, with an average of 4946 ± 340.2pg/ml (Figure 3.5A). Despite a slight decrease in levels of proIL-1β at a 6h stimulation with NE, this is not significantly

different to that of cytokine-primed cells (4058 ± 886.6 vs. 4946 ± 340.2pg/ml, n=3, figure 3.5A). Incubation of ECs treated with NE with NEIII or YVAD had no significant effect on proIL-1β levels (Figure 3.5A).

Without cytokine stimulation, no proIL-1β was detected in the supernatants (Figure 3.5B). However, cytokine-primed cells released a small amount of proIL-1β (139.2 ± 39.19pg/ml, figure 3.5B), which may suggest that the released IL-1β detected following cytokine stimulation (figure 3.4) was the pro-form. Following NE activation, very little proIL-1β was detected (Figure 3.5B), suggesting that either NE releases the mature or other isoforms of IL-1β but not the full-length proIL-1β.

In the supernatants from EC-primed with cytokines where cells were pretreated with specific NEIII before NE application, levels of proIL-1β increased compared to NE treated cells (234.1 ± 195.8 vs. 0pg/ml, n=3, figure 3.5B), although this was not significant. YVAD-CHO pretreatment showed a similar result to that of NE treated cells (with negligible levels of proIL-1β, figure 3.5B).

The levels of pro-IL-1β measured after shorter incubations with NE did not show any difference to those seen at 6h.

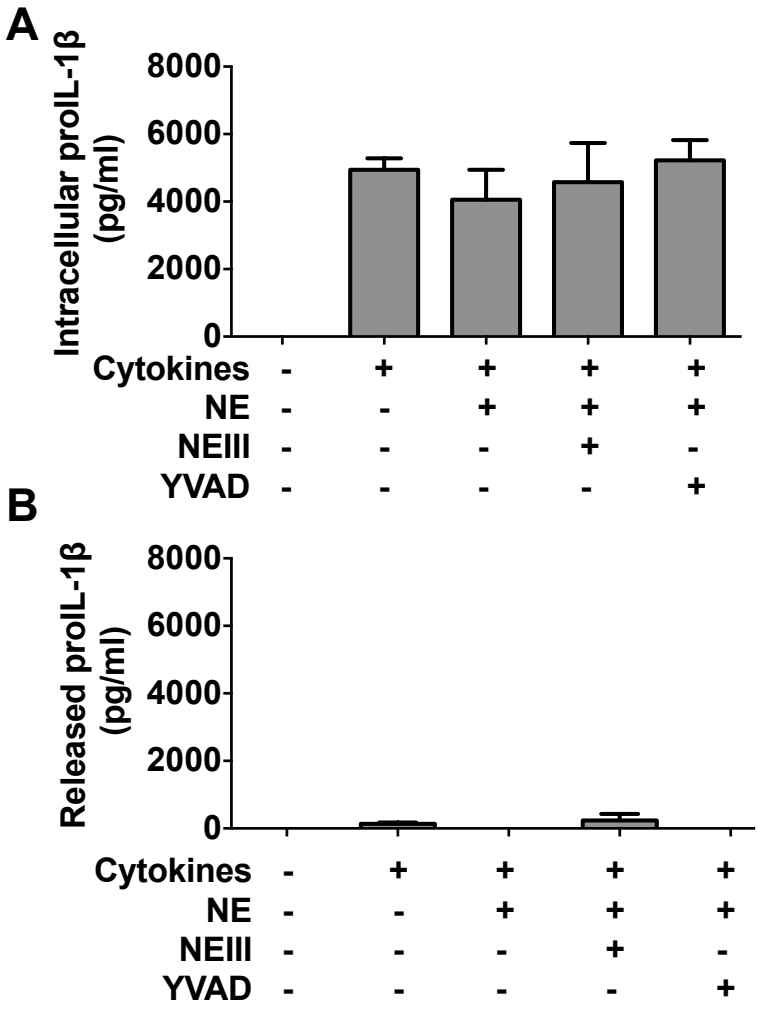

Figure 3.5. NE effects on proIL-1β in ECs. Levels of proIL-1β in EC lysates **(A)** and supernatants **(B)** after cytokines (IL-1α/TNF-α; 10ng/ml) and NE (1μg/ml, for 6h) treatment and/or NEIII (500μM) or YVAD-CHO (50μM), measured by ELISA. Data are mean ± SEM, n=3.

3.8. NE remains bioactive for the duration of the experimental procedures:

NE may undergo autolysis with prolonged incubation times (Korkmaz et al., 2010). Therefore, to confirm that NE remains bioactive during IL-1 secretion from ECs, the enzymatic activity of NE in the EC lysates and supernatants was measured using the rate of cleavage of a specific NE substrate (MeOSucc-Ala-Pro-Val-AFC) as an indicator of NE bioactivity.

NE activity was detected in both the supernatants and lysates at 6h of NE incubation. NE in the conditioned media of NE-treated ECs had a significantly higher bioactivity compared to cells without NE: cytokine-primed and unstimulated ECs

(5.416 ± 0.37552 vs. 0.0375 ± 0.0123µg of NE per µM of substrate, p<0.0001, n=3). NE activity was 7-10 fold less in the presence of NEIII, confirming the sensitivity of the assay (Figure 3.6A).

Interestingly, in the harvested lysates from ECs treated with NE, there was a significant increase in NE activity compared to controls (5.632 ± 0.713 vs. 0.180 ± 0.062 µg/µM, p<0.0001, n=3) (Figure 3.6B). This activity was decreased in the presence of NEIII (Figure 3.6B).

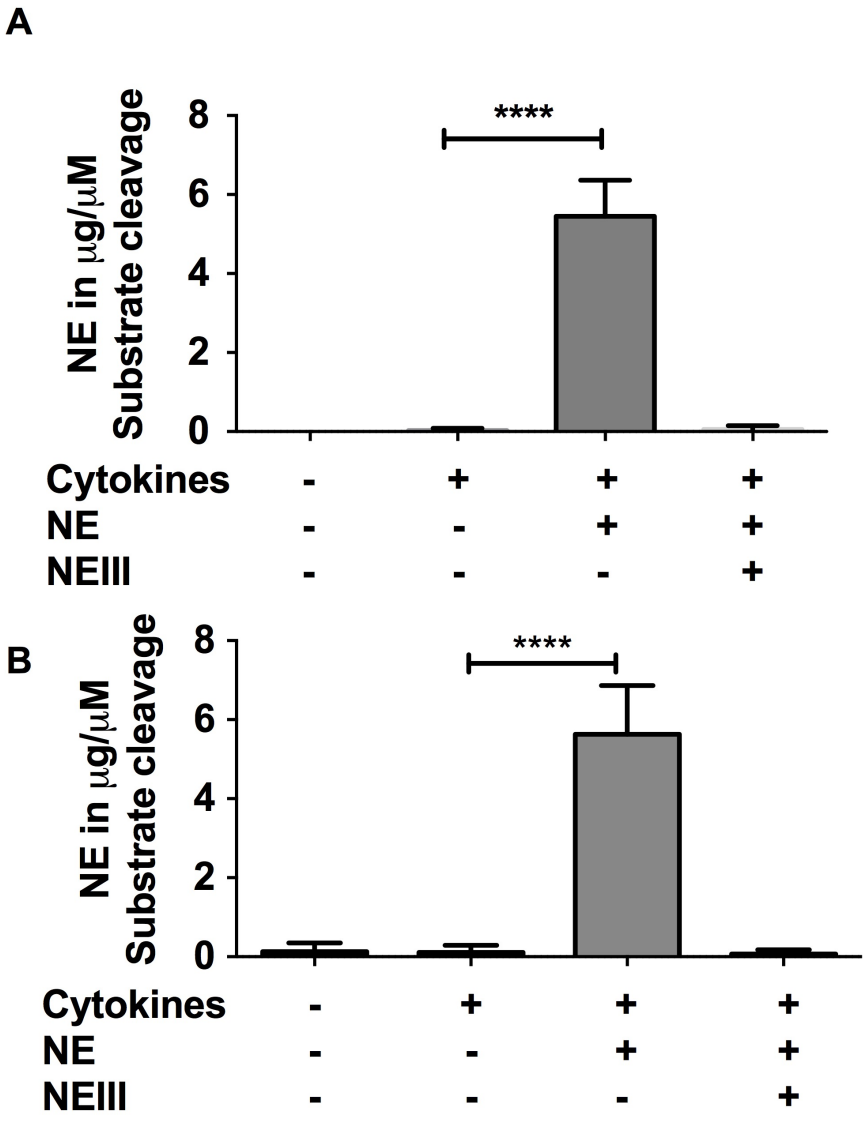

Figure 3.6. NE is bioactive for the duration of the study. Graphs showing increased NE activity in EC supernatants **(A)** and lysates **(B)** treated with NE for 6h compared to unstimulated cells. Data are analysed by One way ANOVA and Tukey's post-test, ****p<0.0001, n=3.

3.9. NE-mediated IL-1β secretion is not via cell death:

Cell death is another mechanism by which IL-1β is released from cells (Auron et al., 1987). Therefore, to rule out cell lysis as a possible mechanism for IL-1β release from ECs by NE effects, I first performed trypan blue dye exclusion to measure cell viability.

There was no significant difference in the percentage of viable cells following NE incubation at 6h compared to cytokine-primed cells or unstimulated ECs (83.3 ± 3.24 vs. 73.60 ± 2.30% in cytokine primed cells or 93.5 ± 4.44% in unstimulated ECs, respectively, n=3) (Figure 3.7A).

The cytosolic enzyme LDH leaks out of cells at an early stage of death (Abe and Matsuki, 2000). Therefore, I also sought to test whether there is an early release to LDH following NE incubation. Using a cytotoxicity assay to measure the cytosolic enzyme in the conditioned media, no significant increase in LDH levels were seen after NE treatment for 6h (Figure 3.7B).

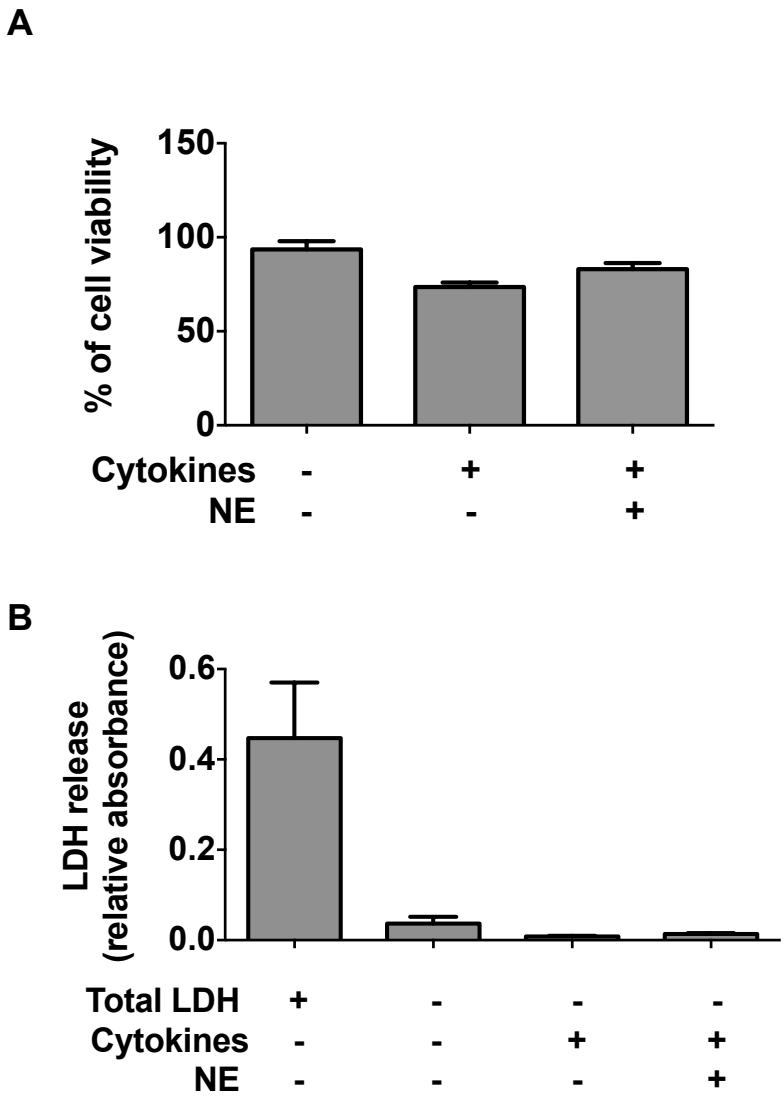

Figure 3.7. NE-mediated IL-1β secretion is not via cell death. A) Cell viability (%) was measured using trypan blue dye exclusion. Percentages of viable ECs were calculated as the number of unstained cells/total number of cells (stained + unstained) X 100. **B)** Lactate dehydrogenase (LDH) levels were measured in conditioned media in the presence or absence of NE for 6h. The detected LDH in cell lysates was used as an indicator to the total LDH inside the cells (positive control). Data are expressed as mean ± SEM, n=3, analysed by One-Way ANOVA followed by Tukey's test.

3.10. Apoptosis is not an alternative mechanism for IL-1 secretion:

Another alternative pathway for IL-1β secretion is by induction of apoptosis (Lopez-Castejon and Brough, 2011). To further confirm that IL-1β is released by a direct action of NE, and to also ask whether NE may have some role in the apoptotic signalling pathway, caspase-3/7 activity was tested in ECs.

Apoptosis was induced in ECs by treating with campthothecin (CPT) (1μM, for 6h, positive control). There was a significant increase in caspase-3/7 activation following CPT stimulation (106712 ± 15707 RLU), confirming the sensitivity of the assay (Figure 3.8).

Using this assay, it was clear that caspase-3/7 activation in NE treated cells was not significantly different from that in unstimulated ECs (46749 ± 7398 vs. 19368 ± 8489 RLU, respectively, n=3) (Figure 3.8).

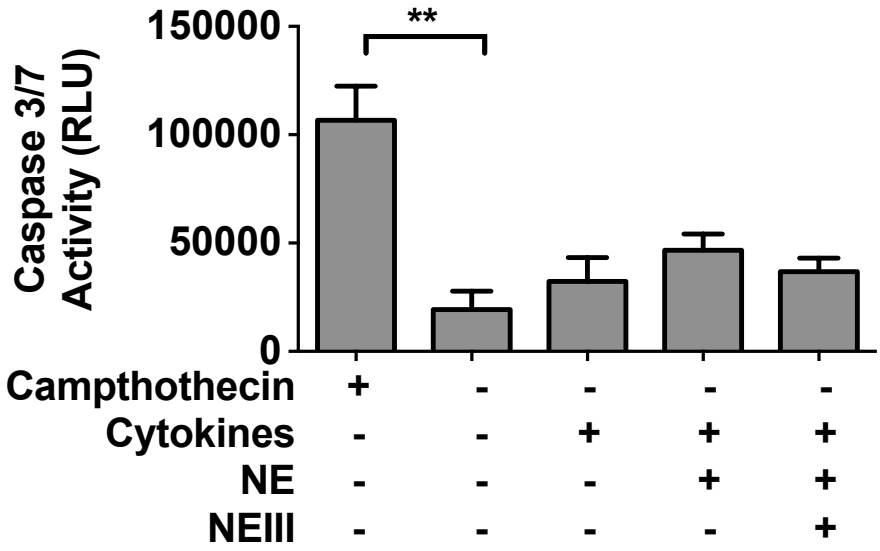

Figure 3.8. Analysis of caspase-3/7 activity in HCAECs. HCAECs in 96 well plates ($2x10^4$) were treated or left untreated with cytokines (TNF-α/IL-1α; 10ng/ml each) for 48h then subjected to NE (1μg/ml) in serum free media for 6h. Campthothecin (1μM, over 6h) was used to induce apoptosis as a positive control. Data are expressed as mean ± SEM and analysed by One-way ANOVA with Tukey's multiple comparison test, **p<0.01, n=3.

3.11. Neutrophil elastase cleaves proIL-1β in ECs in a time dependent manner, independent of caspase-1:

In vitro, some proteases, including NE, have been shown to process proIL-1β into different cleaved products of IL-1β (Black et al., 1988, Hazuda et al., 1990). The above shown ELISA results suggest that NE causes release IL-1β, but did not distinguish between the cleaved products of the active IL-1β. Thus, I investigated

which IL-1β cleaved products were present in cell lysates and supernatants by immunoblotting.

Unstimulated HCAEC lysates did not contain any detectable IL-1β, although full-length proIL-1β (31kDa) was seen in cytokine-primed cell lysates (Figure 3.9A). NE cleaved proIL-1β (31kDa), at all time points tested, with increased cleavage at 6h, evidenced by the appearance of bands for IL-1β at 20kDa and faint bands at 18 and 15kDa (Figure 3.9A). No IL-1β at 17kDa was detected in the lysates, suggesting either that the 17kDa is released immediately after cleavage or NE cleaves proIL-1β at a site distinct than that of caspase-1 and thus different cleaved products for IL-1β are generated.

Figure 3.9B shows a representative immunoblot for EC lysates incubated for 6h with or without NE in the present or absence of NEIII or YVAD-CHO. As expected, proIL-1β cleavage by NE is inhibited by NE inhibition, and caspase-1 inhibition had no effect on NE action. Densitometric analysis confirmed these findings and demonstrated a significant reduction in proIL-1β (31kDa) levels, normalised to α-tubulin, within the EC lysates treated with NE alone or NE+YVAD compared to the levels in cytokine primed ECs (1.605 ± 0.6050 vs. 4.479 ± 0.4606, $p<0.05$, $n=3$) (Figure 3.9C).

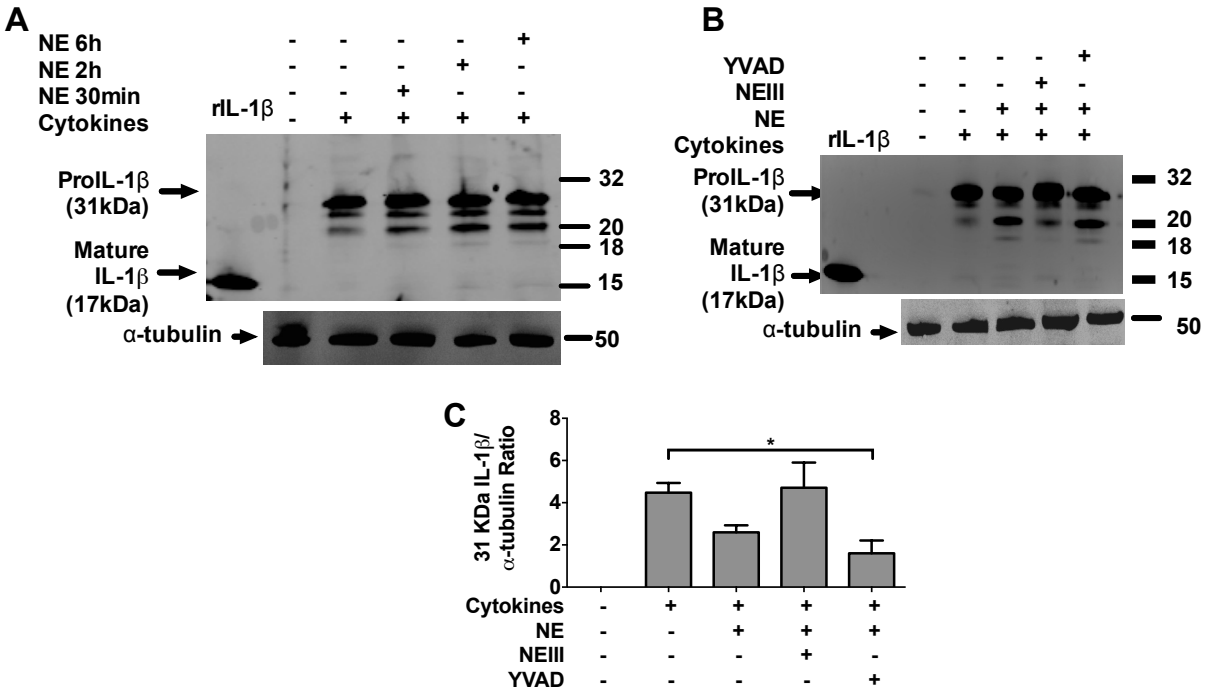

Figure 3.9 Neutrophil elastase cleaves proIL-1β in ECs in a time dependent manner, and is independent of caspase-1. A) HCAEC lysates from primed EC +/- NE for 30 minutes, 2h or 6h, assessed for IL-1β. **B)** IL-1β expression in EC lysates pre-treated with cytokines (TNF-α/IL-1α; 10ng/ml each) followed by NE (1μg/ml) for 6h in the presence or absence of inhibitors (NEIII; 500μM/ YVAD-CHO; 50μM). The blots are representative of three independent experiments with α-tubulin was used as a loading control. Recombinant IL-1β (rIL-1β; 20μg, 17kDa) was loaded as a positive control and represents the commonly detected isoform of the mature form, whereas proIL-1β (31kDa) indicates the inactive pro-form. **C)** Densitometry from three independent experiments. Data are mean ± SEM, analysed by One-way ANOVA and Tukey's post-test, *p<0.05.

In addition, NE induced maturation of IL-1β was not associated with procaspase-1 cleavage/activation or any changes in NLRP-3 levels in lysates as detected by Western blot (Figure 3.10 A&B). Taken together, these data clearly suggest that NE co-activation increases cytokine-induced IL-1β secretion in ECs by an inflammasome-caspase-1 independent pathway.

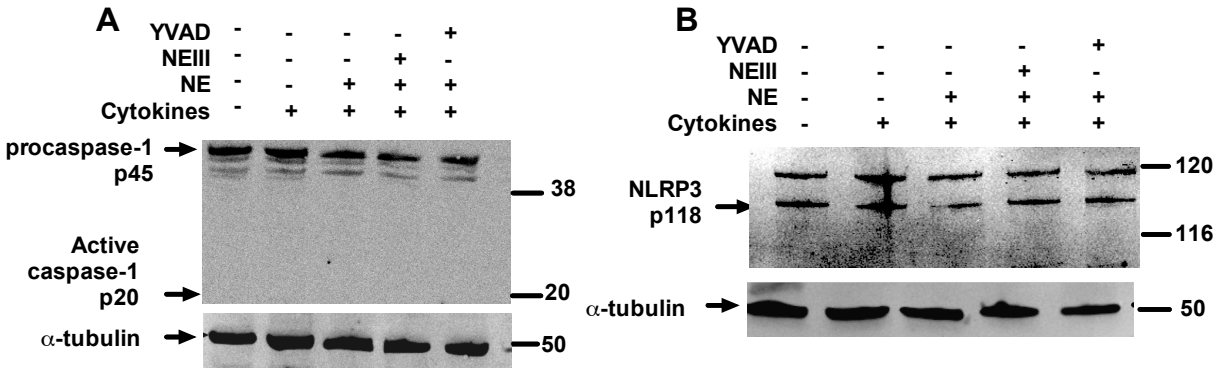

Figure 3.10. NE effects on caspase-1 and NLRP3 in ECs. Western blot analysis of cell lysates from primed EC +/- NE, assessed for Caspase-1 **(A)** & NLRP-3 **(B).** Equal protein loading was confirmed using α-Tubulin (a loading control) and loading 20μg of protein per lane. The representative blots are from three independent experiments.

In cell supernatants, Western blots confirmed the presence of proIL-1β following cytokine treatment, consistent with the proIL-1β ELISA data (see figure 3.5), however, after the addition of NE, three cleaved products of IL-1β: 20, 18 and 15kDa, were clearly detected at 6h (Figure 3.11). These isoforms disappeared when NEIII was added to NE treated cells (Figure 3.11), but were still present after YVAD treatment. The IL-1β release into surrounding media was associated with a significant decrease in the 31kDa proIL-1β in cell lysates (see figure 3.9), suggesting processing of IL-1 by NE.

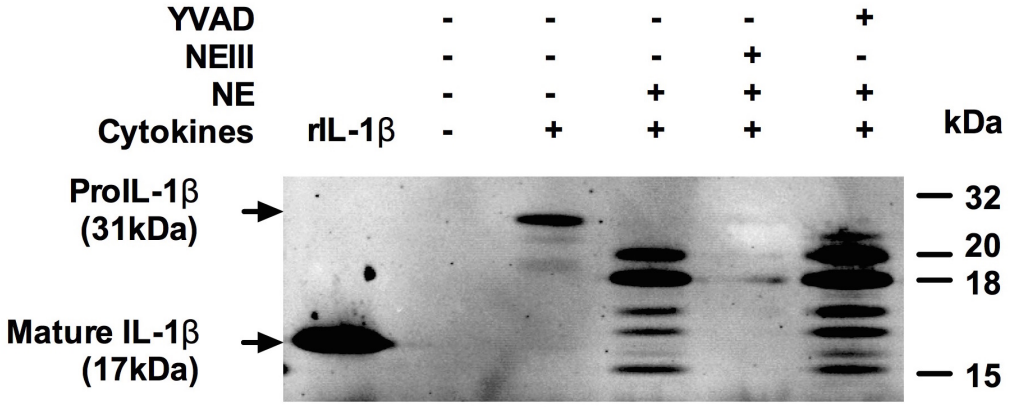

Figure 3.11. Neutrophil elastase causes the release of different isoforms of IL-1β. Supernatants from cells +/- NE for 6 hours were analysed for IL-1β isoforms using immunoblotting. Equal protein concentrations were loaded per lane (15μg/ml), and the representative blot is from three independent experiments.

3.12. *Neutrophil elastase selectively cleaves rproIL-1β but not rIL-1β in vitro:*

To confirm the direct effects of NE on the IL-1β precursor (31kDa) and rule out that NE cleaves the mature IL-1β (17kDa), recombinant (r) IL-1β (rIL-1β, 17kDa) and rproIL-1β (31kDa) were mixed with NE *in vitro* for 30 minutes, 2h and 6h at 37°C. IL-1β and proIL-1β were then assessed using ELISA and immunoblotting.

There was no significant difference in the levels of rIL-1β measured by ELISA in the presence or absence of NE in the studied time-points (Figure 3.12A). Immunoblotting analysis confirmed the absence of cleavage of rIL-1β by NE (Figure 3.12B).
However, rproIL-1β (31kDa) was cleaved by NE and the cleavage increased with longer time incubations (Figure 3.12C). These findings suggest that NE cleaves proIL-1β but has no effect on the mature form of IL-1β.

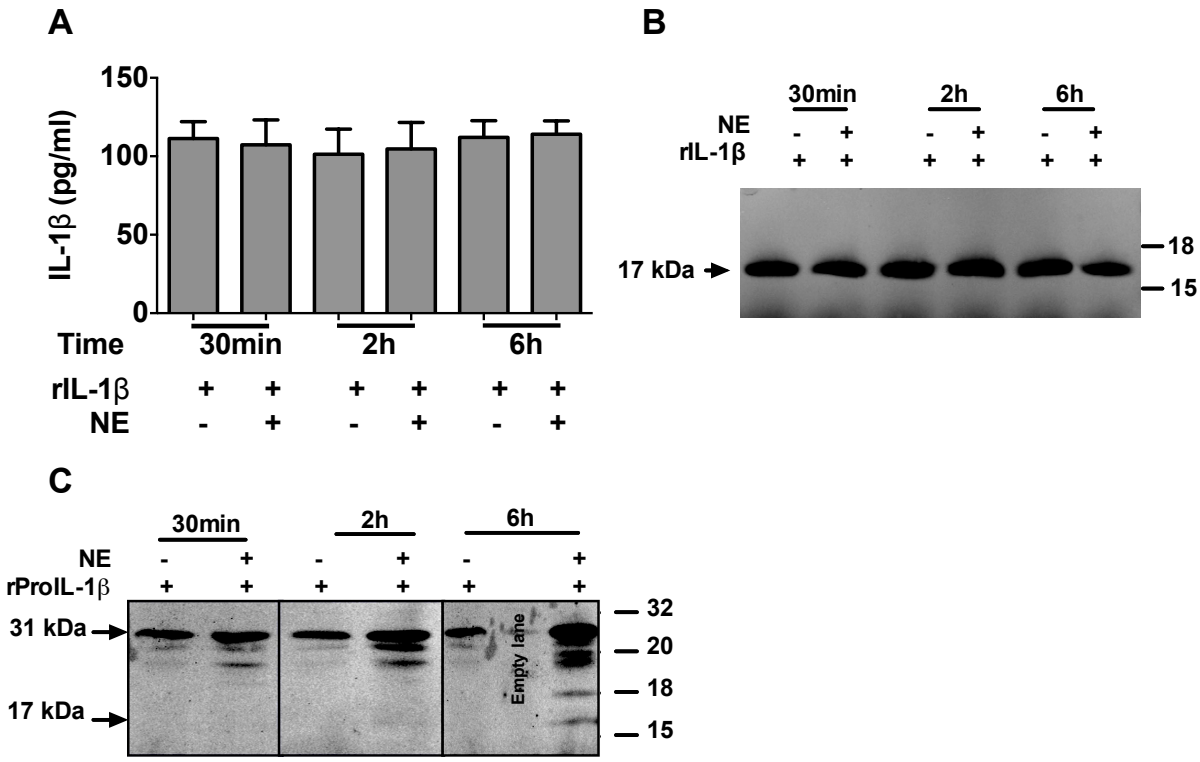

Figure 3.12. Pro-IL-1β is sensitive to neutrophil elastase *in vitro* but mature recombinant IL-1β is resistant to NE effects. A) ELISA and **(B)** Western blot for rIL-1β (125pg/ml) in the presence or absence of NE. Data are mean ± SEM, n=3. **C)** Recombinant proIL-1β (rProIL-1β; 10000pg/ml) was incubated at 37°C (in 5% CO_2; v/v) alone or in the presence of NE (1µg/ml) for 30 minutes, 2h, and 6h prior to detection of cleavage by immunoblotting. Representative western blots are from three independent experiments.

3.13. IL-1 released by NE stimulation is bioactive:

Having shown that NE causes the release of different isoforms of IL-1β, I then asked whether this processed IL-1β released into cell supernatants in response to NE activation was bioactive. I collected supernatants from NE-treated or untreated cells (6h treatment), applied them to HeLa cells expressing an IL-1RI responsive IL-8 reporter, and measured subsequent IL-8 release.

I compared the reporter assay output (IL-8) from media obtained from unstimulated or cytokine-primed EC +/- NE with a positive control (0.1nM recombinant IL-1β) and with interleukin-1 beta neutralising antibody (NAB). Supernatants isolated from NE activated ECs contain significantly increased IL-8 levels compared to unstimulated and cytokine-primed cells without NE, and this was completely abrogated by IL-1 NAB (Figure 3.13).

In order to confirm that the bioactivity was due to released IL-1β and not a result of direct NE effects on HeLa cells, NE (1µg/ml) was added to HeLa cells (as a spike) and this showed no significant IL-8 generation. These data indicate that the released IL-1β is indeed bioactive.

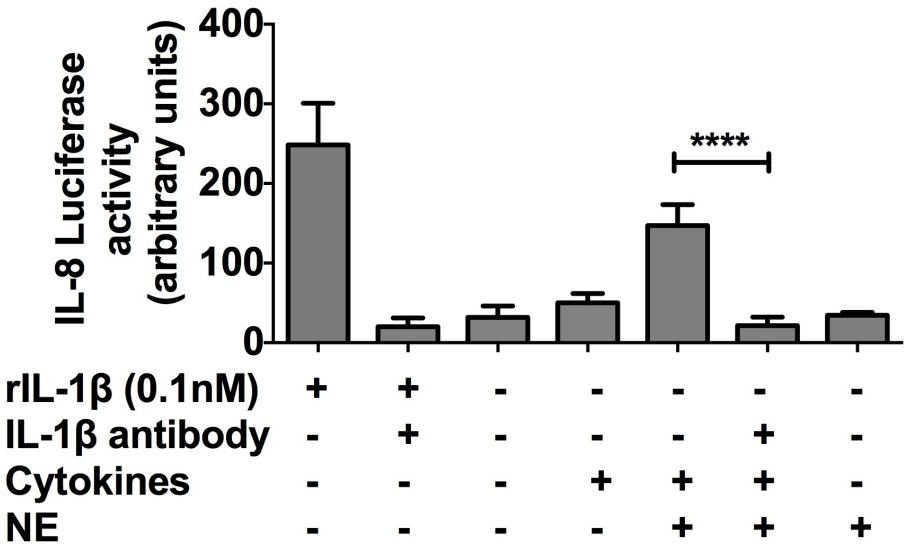

Figure 3.13. IL- 1β bioactivity measured by a luciferase reporter assay. HeLa cells with an IL-1 sensitive IL-8 reporter were exposed to harvested media from cytokines primed cells (TNF- α/IL-1α; 10ng/ml each) +/- NE (1µg/ml) for 6h or rIL-1β (0.1nM) in the presence or absence of IL-1β neutralising antibody (1µg/ml). Specificity for IL-1β is shown by reduction of IL-8 luciferase following incubation with IL-1 β neutralising antibody. Data are expressed as mean ± SEM and are analysed by One way ANOVA followed by Tukey's test, ****p<0.0001, n=3.

3.14. Summary:

In this chapter, I describe, for the first time, how coronary artery ECs release IL-1β, which has been a 'holy grail' of endothelial biology for many years. I report that a considerable amount of IL-1β is released from ECs in response to NE via a caspase-1 independent mechanism. Several lines of evidence support this:

1. IL-1β is only released from HCAECs after the addition of NE.
2. The release is dose and time dependent, with maximum release of IL-1β detected with 1μg/ml of NE at 6h.
3. The release is completely attenuated when a specific NE inhibitor (NEIII) is added to the cells, thus confirming that the secretion is by direct NE activity.
4. The specific caspase-1 inhibitor, YVAD-CHO (50μM), has no affect on IL-1 secreted into the supernatants after NE treatment.
5. Cell death as a possible cause of IL-1 release is ruled out in this system.
6. NE induces IL-1 secretion by cell activation is without the involvement of the apoptotic pathway as indicated by the findings of caspase-3/7assay.
7. Western blot for caspase-1 expression in the lysates revealed the absence of caspase-1 activation.
8. The absence of inflammasome activation is also confirmed using immunoblotting.
9. NE selectively cleaved proIL-1β in the cell lysates and *in vitro* whereas mature IL-1β is resistant to NE proteolytic activity.
10. There is an enhanced IL-1β biological activity in supernatants from cells incubated with NE, as confirmed by an IL-8 luciferase reporter assay.

3.15. Discussion:

The endothelium is fundamental in atherosclerotic plaque development, not only during early lesion development but also later by controlling plaque instability (Aird, 2008). In atherosclerosis, crosstalk between circulating cells, including monocytes, neutrophils and the endothelium can cause ECs to liberate soluble agents which, together with oxidised lipids (Ray, 2014) perpetuate the cycle of inflammation. Several lines of evidence suggest that IL-1β is an apical cytokine in this process (Morton et al., 2005, Dewberry et al., 2008, Rogus et al., 2008), yet its mechanism of release from ECs is largely unknown. Furthermore, the biological pattern of the crosstalk is not completely defined.

NE is secreted by inflammatory cells in atheroma (Dollery and Libby, 2006); however, its precise role in the development of atherosclerosis has yet to be proven. It was originally believed that NE has a limited function by just degrading ECM components, including elastin (Takahashi et al., 1988a). Current thinking, however, links NE to atherosclerosis and plaque instability (Leclercq et al., 2007, Dollery and Libby, 2006, Warnatsch et al., 2015), yet the way in which this occurs and the mechanism of action remain to be elucidated.

My hypothesis was that NE, released by circulating inflammatory cells, modulates IL-1β release by the endothelium and that this is important in the development of atherosclerosis. The presented data show that IL-1β can be released in significant quantities from the endothelium following stimulatory treatment with NE. This is the first study describing the involvement of NE in IL-1β release from the vascular cell wall.

Atherosclerosis is a chronic inflammatory condition; therefore, it was important to differentiate between IL-1 production in healthy (un-stimulated) endothelium and atherogenic (subjected to cytokines) endothelium. My data demonstrate that IL-1β in un-stimulated HUVECs is negligible, suggesting crucial roles of the prototypic cytokine in inflammation contrasting with a disease free state where the endothelium does not produce IL-1. This finding is also consistent with all previous studies (Marceau et al., 1992).

The main focus of this study was to maximise IL-1β synthesis by using a range of proinflammatory mediators in an EC culture model before looking for IL-1β release. The chosen cytokines were the commonly isolated mediators in atherosclerosis and its complications, namely TNF-α, INF-ɣ, LPS and IL-1α in differing combinations (Wilson et al., 2007). Interestingly, the greatest induction of IL-1β synthesis was in the combinations in which IL-1 was used to stimulate its own production. The ability of IL-1 to induce its own precursor is well known in previous publications (Schindler et al., 1990). This finding is also in agreement with Wilson and colleagues where they showed maximum IL-1 induction with a TNF-α /IL-1 combinations (Wilson et al., 2007).

The cytokines TNF-α plus IL-1α had the greatest impact on IL-1 production; therefore, these were used for the release study. As it is important to distinguish between the IL-1 added to the cells and the released cytokine, the cells were washed three times as per the assay and serial ELISA assays were conducted on the washings, showing no detectable levels of IL-1 in the wash, confirming that any IL-1 detected in the supernatants is in deed the released IL-1β.

Lee and colleagues in 2006 showed that NE induces secretion of TGF-β from bronchial SMCs by a dose-effect response (Lee et al., 2006). Therefore, I investigated IL-1β release in response to different concentrations of NE (0.5, 1, 2μg/ml). Interestingly, at 1μg/ml NE caused a significant increase in IL-1β secretion but higher doses of NE induced less secretion of IL-1β. The reduction in IL-1β release was associated with more cell detachment and enhanced cell death. This was confirmed by trypan blue dye exclusion. This is in agreement with (Lee et al., 2006, Smedly et al., 1986).
In contrast to my work, Gresnigt et al., 2012 showed that NE at 10μg/ml (5x the dose I used) decreased IL-1β levels in peripheral blood mononuclear cell (PBMC) supernatants. It could be argued that NE at lower doses has signalling triggering roles, and at higher concentrations it acts predominantly as a proteolytic enzyme. NE signalling effects at lower doses have been supported by (Schreiber et al., 2012, Devaney et al., 2003, Karmaker M., 2012). However, the nature of the dual functions of this enzyme and how significant this could be *in vivo* and in atherogenesis is yet to be examined.

I also showed in this chapter that NE enhances IL-1β secretion in a time dependent fashion, reaching a maximum at 6h incubation. Interestingly at 6h stimulation of NE, approximately 37% of the produced IL-1β is secreted. However, in immune derived cells it has been postulated that IL-1 is released as early as 30min following ATP activation and the longer the incubation the less release, with more cell death (MacKenzie A, 2001, Netea et al., 2009, Ward et al., 2010). This does not seem to be the case with NE as stimulant.

In my work, to rule out cell death as a mechanism of release IL-1β by ECs, especially at 6h, I measured the levels of the cytosolic enzyme LDH in the supernatants, and confirmed that the cells were still viable at the studied time. In addition, apoptosis as a possible pathway for IL-1β was ruled out by measuring caspase-3/7 activity assay in ECs in the presence of NE, suggesting that IL-1β is secreted by active ECs.

Of major interest, there was a significant increase in IL-1β secretion by cytokine-primed ECs at 6h after addition of NE. Since caspase-1 has been identified as the main proteolytic enzyme to play a role in proIL-1β cleavage and secretion in monocytes and macrophages (Dinarello, 2007), I used a specific caspase-1 inhibitor (YVAD-CHO) as a potential means of augmenting IL-1β release. My data shows that caspase-1 appears to be non-essential in ECs for IL-1β cleavage and release by NE. This is at odds with other *in vitro* studies in monocytes (Ward et al., 2010), but in agreement with more recent data from other cell types (Cassel et al., 2014) and *in vivo* models. My findings are also supported by the findings of Guma *et al.*, that suggests the presence of IL-1β in the synovial fluid of caspase-$1^{-/-}$ mice (Guma et al., 2009). Moreover, my data may explain why caspase-1 suppression did not show promise in vascular healing (Chamberlain et al., 2006) or atherosclerosis progression (Menu et al., 2011).

In cell lysates, using immunoblotting, I confirmed that NE efficiently cleaved proIL-1β, which was attenuated in the presence of NEIII but not affected by YVAD-CHO. Cleavage was, therefore, independent of caspase-1, which was confirmed by the absence of caspase-1/inflammasome activation. This finding was at odd from Duewell *et al* who suggested the fundamental role for caspase-1 and NLRP3

inflammasome for IL-1 secretion in macrophages following cholesterol crystals stimulation (Duewell et al., 2010). NE seems to induce the secretion without the need to activate this pathway.

In supernatants harvested at 6h of NE treatment, the prominent isoforms of IL-1β both released and present inside cells were 20kDa, 18kDa and 15kDa in size. The literature states that the inactive IL-1β precursor (31kDa) is cleaved upon secretion to generate the mature carboxyl terminal 17kDa isoform (Hazuda et al., 1990, Dinarello et al., 2012). However, The other cleaved products were detected previously *in vitro (*cell-free) where it has been shown that NE cleaves the purified precursor of IL-1β to generate multiple forms, which are 5-10 fold less bioactive (as confirmed by the luciferase assay) than the 17kDa but are active enough for IL-1 to bind to its receptors and initiate the signalling cascade (Black et al., 1988, Hazuda et al., 1990, Black et al., 1991, Hazuda et al., 1991, Stehlik, 2009). This study is the first to show in intact cells that NE is capable of cleaving proIL-1β at multiple sites. Figure 3.14 shows two cleaving sites for NE distinct than that of caspase-1 and generation of the 20kDa and 18kDa bands as been suggested by the literature.
It remains to be seen whether, individually these bands are bioactive intermediate 'products' in IL-1β processing or if they are distinct isoforms that also occur *in vivo*.

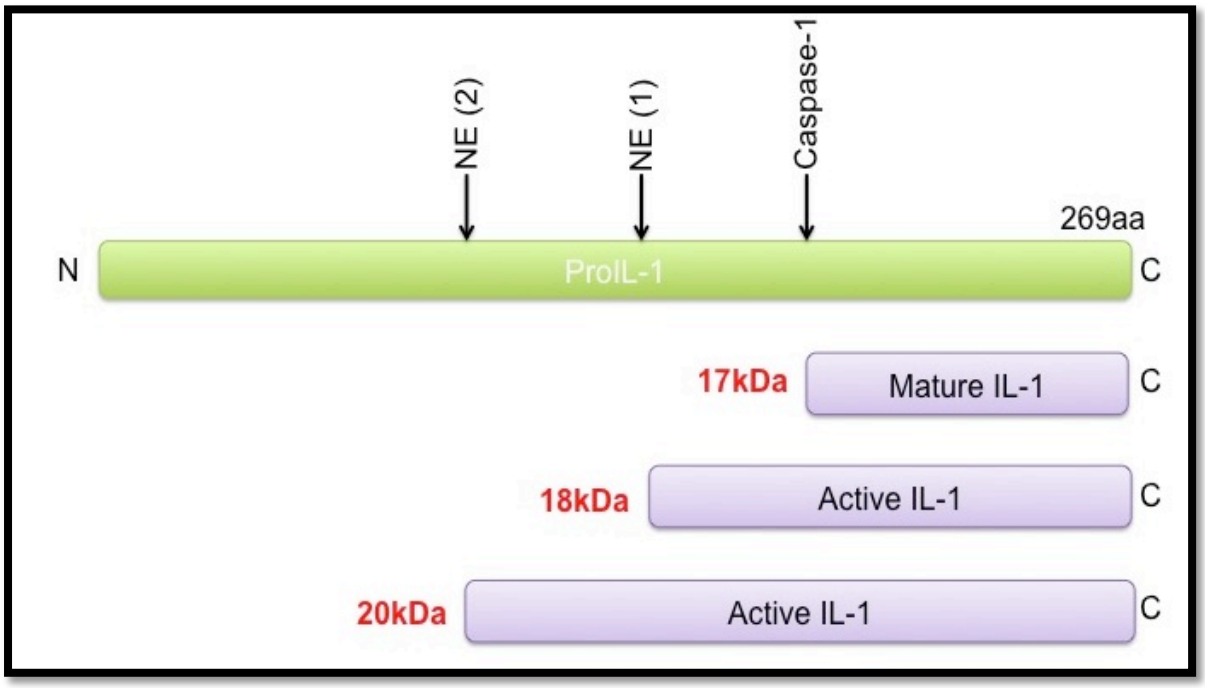

Figure 3.14. Schematic diagram illustrating the site of proIL-1 β cleavage by NE. IL-1 cleavage products were characterised by their sizes using immunoblotting. Mature IL-1β (17kDa) is generated by caspase-1 action at residue 117. NE also cleaves proIL-1 at two main sites distinct than that of caspase-1 to generate 20kDa and 18kDa IL-1β. Permission from (Stehlik, 2009) .

To assess whether the IL-1β released from ECs by NE was bioactive, I used IL-1RI expressing cells (Hela cells) as a system to indirectly measure IL-1β bioactivity in the harvested supernatants from ECs exposed to NE (Kiss-Toth et al., 2000, Wilson et al., 2004, Zheng et al., 2013). I examined the IL-1β bioactivity in EC supernatants using a novel IL-8-luciferase reporter assay (Wilson et al., 2004) and showed that IL-1β in the total supernatants is indeed bioactive to the level of 70% of the assay control (0.1nM IL-1β).

This chapter describes a possible mechanistic role for NE in IL-1β secretion by ECs. These data also suggest that the secretion process in ECs is completely different than other cell types in both the kinetics and the secreted isoforms of IL-1β. Furthermore, caspase-1 and the inflammasome do not seem crucial in this system. This raises the question of how IL-1β is released and whether the secretory pathway is different in ECs from other immune derived cells. In the following chapter I go on to investigate the IL-1 secretory mechanism using flow cytometry, immunoblotting, time lapse, confocal and electron microscopy.

Chapter (4): The Mechanism of IL-1β Secretion from Vascular Endothelium; Microvesicle Shedding and Exosome Release.

OUTLINE:

4.1. Overview of Mechanism of IL-1 Secretion from ECs:

The leaderless IL-1β is proposed to be released by the non-classical pathway of protein secretion (Rubartelli et al., 1990). As a result, several mechanisms for the trafficking of IL-1β into the extracellular space have been suggested, including IL-1β containing microvesicle shedding from monocytic-like cells and exocytosis of secretory lysosomes (Andrei et al., 2004, MacKenzie A, 2001).
However, the mechanism by which ECs secrete IL-1β is enigmatic. Moreover, the site of IL-1 processing inside cells needs further elucidation.

It is crucial to understand the mechanism(s) of release of IL-1β from ECs especially since IL-1β acts at a distance rather than just locally in the vessel wall (Dinarello, 2007). In chapter 3, I presented data on cleavage and release of IL-1 by NE effects and here in chapter 4 I sought to investigate whether NE releases IL-1 from the endothelium via a vesicular mediated mechanism.

4.2. Brief Methods:

In this chapter I study vesicular IL-1 release from ECs treated by NE and I characterise extracellular vesicles (EVs) by flow cytometry, immunoblotting and EM. The site of IL-1 processing was defined using EM and immunofluorescence.
Full detailed methodology used is described in chapter (2).

4.3. NE enhances shedding of MV containing bioactive IL-1:

I sought to determine whether MV shedding occurs in response to NE and whether this is associated with the IL-1β release mechanism in HCAECs.

4.3.1. Microvesicular shedding in response to NE:

Phosphatidylserine (PS) exposure has been associated with MV shedding in monocytes (MacKenzie A, 2001), therefore I used annexin V binding (annexin V-Alexa Fluor 488) as a tool to visualise MV shedding events in live HCAECs.
Small particles (0.1-1μm in diameter, analysed using image J software) were observed separating from the cells in real time using time lapse imaging over the duration of 6h (Figure 4.1A). EC membrane blebbing and MV shedding started as early as 10 minutes of NE incubation and continued to 6h (Figure 4.1A).

To define the size of these MVs and confirm the shedding process, electron microscopic (EM) analysis was performed with ECs treated with NE. This showed that the membrane blebbing and (0.1-1μm) membrane-bound MVs bud outward and shed off the cells treated with NE compared to untreated cells (Figure 4.1B).

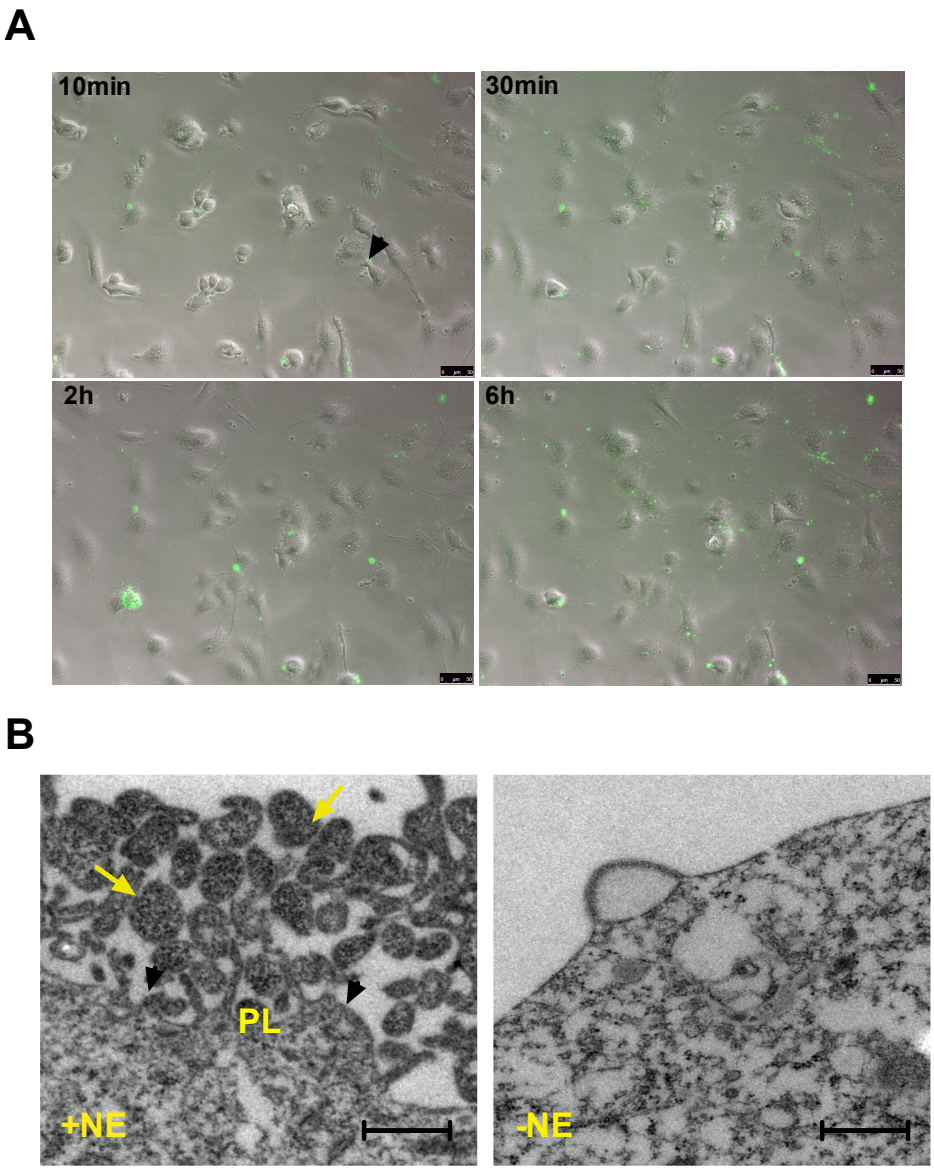

Figure 4.1. Neutrophil elastase activates microvesicle shedding from endothelial cells. HCAECs were left untreated, or treated with cytokines for 48h (IL-1α/TNF-α; 10ng/ml each), and then labelled with annexin-V AlexaFluor®488. Cells were visualised after the addition of 1μg/ml of NE in a heated chamber (5% CO_2 v/v) using time-lapse fluorescent microscope to detect MV release. **A)** Images were captured at 10 minutes, 30 minutes, 2 hours and 6 hours. Arrowheads indicate green fluorescent MVs. Scale bars=50μm. The representative images are from three independent experiments. **B)** Electron micrographs representing images from n=3, confirming the shedding of MVs (yellow arrows) from ECs after NE (+NE) treatment compared to non-NE treated (-NE) cells. Scale bars=200nm. PL indicates plasma membrane (arrowheads).

I then isolated the MVs using a centrifugation gradient (Gyoergy et al., 2011, Jansen et al., 2012) and analysed these using flow cytometry. The flow cytometer was calibrated using a previously published method (Nolan et al., 2008). As a control, I used a population of lysed MVs and gated MVs using a live and dead dye (Figure 4.2A).

I found that there was a significant increase in the number of MVs isolated from ECs following NE treatment compared to controls. The MVs appeared as early as 10 minutes, but the number was significantly increased after 2h of NE stimulation compared to non-NE treated cells (366.3 ± 81.68 vs. 40.67 ± 18.71, respectively, $p<0.01$, $n=3$) (Figure 4.2B). Small numbers of MVs were detected in the media in NE untreated cells (cytokine primed ECs without NE stimulation) at 6h. However, the number was significantly increased following NE treatment (542 ± 97.93 vs. 193 ± 60.79, $p<0.05$, $n=3$). NE inhibition, using NEIII, effectively attenuated this MV formation and shedding, whereas caspase-1 inhibition had no significant effect (Figure 4.2C).

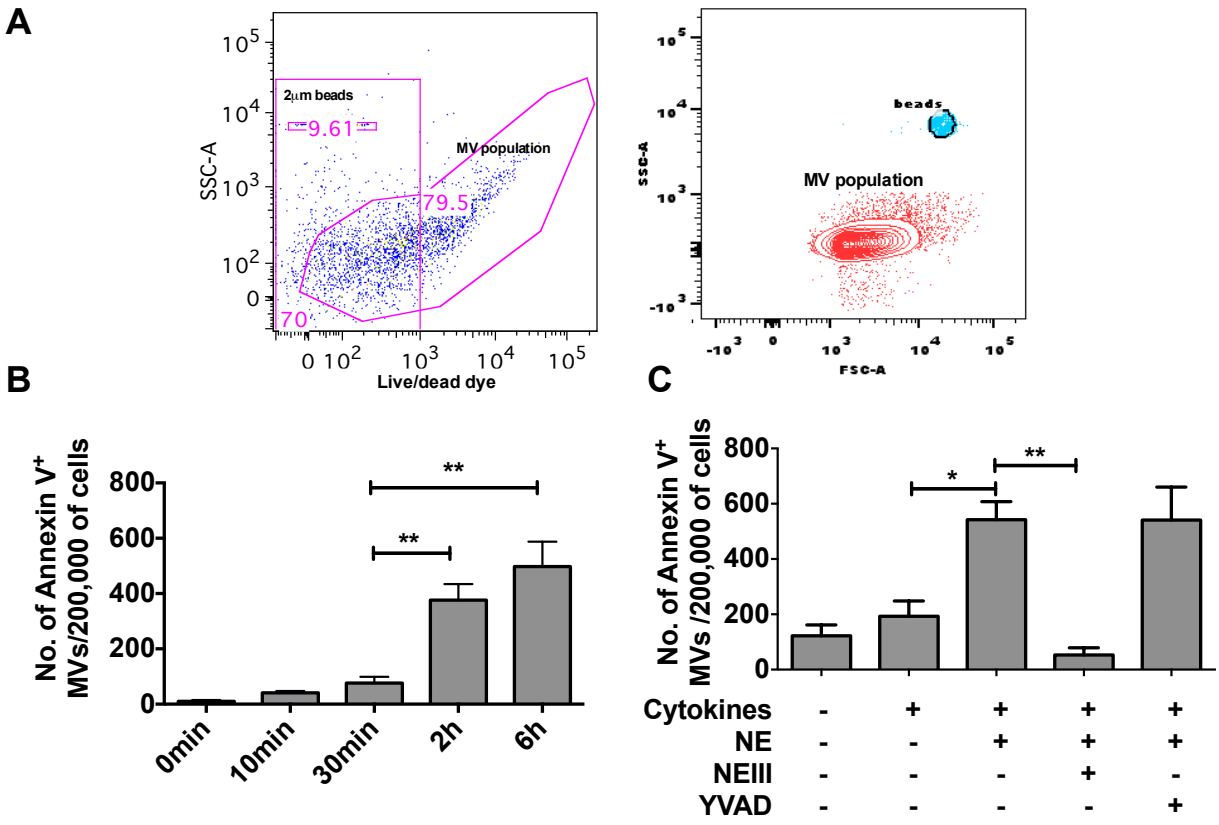

Figure 4.2. Characterisation of MV released in response to NE, using flow cytometry. A) MVs were isolated and stained with annexin V PE-CY7 as described in Chapter 2**.** A population of MVs were gated using a live and dead dye and analysis of MVs (red) using Accu Count Beads (SPHERO, 2µm) (blue) shows they are within the 2µm size limits. **B)** Graph illustrating increasing numbers of MVs over time, following NE treatment. Data are mean ± SEM, n=3, **p<0.01. **C)** Graph showing a significant increase in MV number in NE treated cells compared to untreated controls. MV shedding is reduced following NE inhibition, but not after caspase-1 inhibition. Analysis was performed by Flow Jo software from three independent experiments, expressed as mean ± SEM. Statistical significance was tested using One-way ANOVA followed by Tukey's post- test, *p<0.05, **p<0.01.

4.3.2. Microvesicle contents:

Having identified NE as 1) an effective stimulus for IL-1β release and 2) causing MV release from HCAECs, I next investigated whether the released MVs contain IL-1β. MV shedding was tested using immunogold EM. In addition, isolated MVs (by centrifugation from freshly collected EC supernatants) were analysed for their IL-1β content using immunoblotting.

Using immunogold EM, I detected immunogold labelled (20nm gold particles) IL-1β in MVs (0.1-0.2µm diameter), in NE treated cells (Figure 4.3A, +NE). No IL-1β was detected in ECs that were not treated with NE (Figure 4.3A, -NE).

MVs were collected at 30 minutes, 2h and 6h of NE treatment to study the kinetics of IL-1β content within them (Figure 4.3B). MVs from unstimulated cells contained no IL-1β, contrasting with MVs from cytokine-primed cells, which contained proIL-1β (31kDa) at all time-points (Figure 4.3B).
However, in MVs isolated from the supernatants of NE treated cells, cleavage of the 31kDa IL-1β isoform to approximately 20kDa-19kDa was observed as early as 30 minutes (Figure 4.3B, upper blot) with further cleavage to the 18 and 15kDa isoforms at 6h (Figure 4.3B, lower blot).

Treatment of cells with NEIII, but not YVAD-CHO, abolished the cleavage of proIL-1β in these MVs, confirming that these bands are the result of direct NE activity.
MVs were also assessed for caspase-1 and NRLP3 content. Active caspase-1 p20/p10 and NLRP-3 were not detected in MVs isolated from cells treated with either NE or NE and YVAD-CHO together (data not shown), indicating that intra-vesicular cleavage of proIL-1β is independent of caspase-1 activation.

Investigation of the supernatant of ECs after removal of the MVs showed very little IL-1β is present in the supernatant alone (at the 6h time point), under each experimental condition (Figure 4.3C). These findings suggest that IL-1β is released by a MV mechanism specifically and that either NE cleaves the released proIL-1β inside MVs or NE treated cells continually generate more MVs containing IL-1β as a route of secretion.

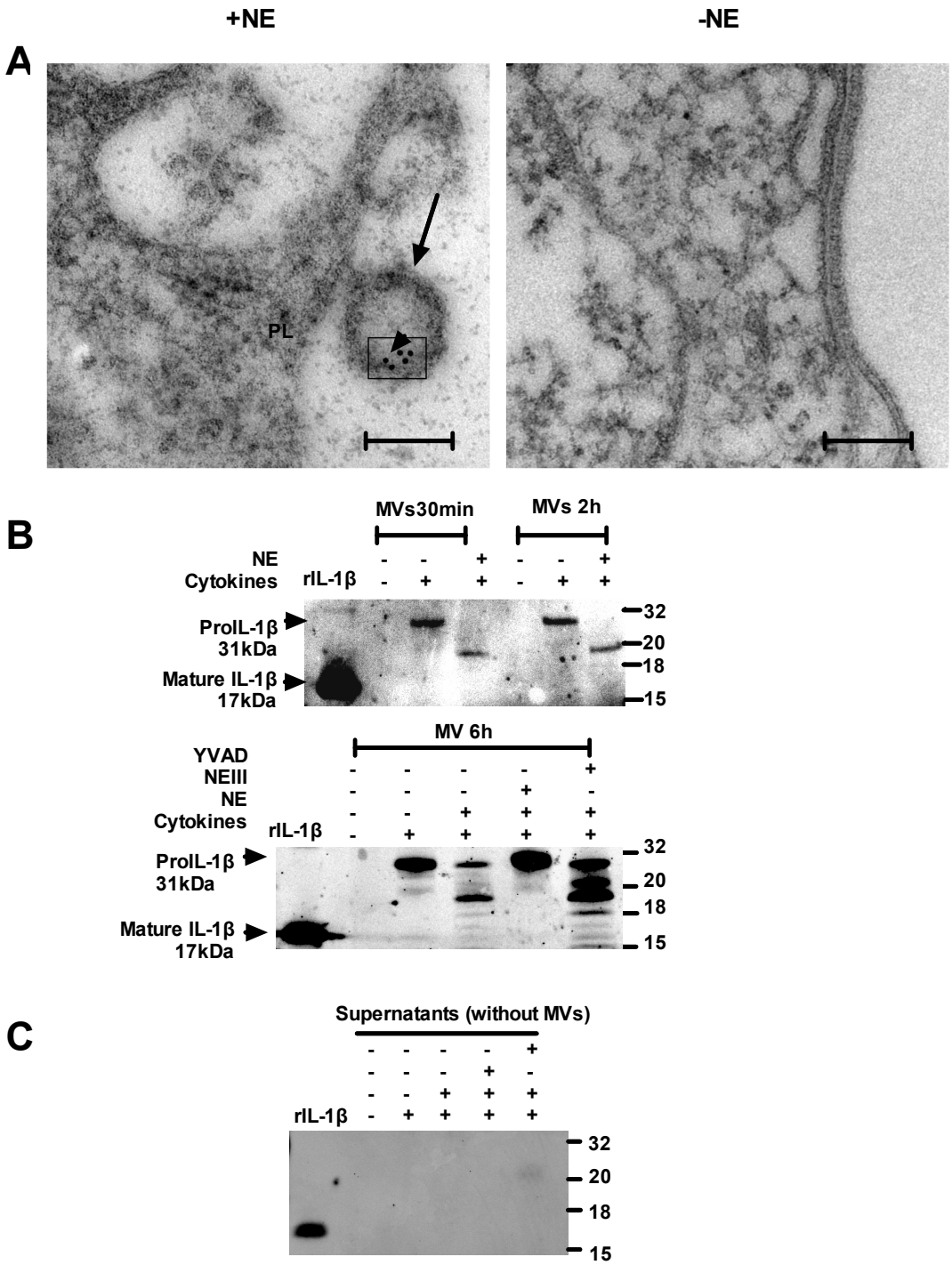

Figure 4.3. MVs containing IL-1β are shed from HCAECs after cytokine priming and NE treatment. A) Immunoelectron microscopical analysis of IL-1β in ECs with or without NE treatment. Anti-IL-1β conjugated immunogold (20-nm gold particles, arrowhead, the area within the box) confirm the presence of IL-1β in the MVs (0.2μm, arrow) released from the plasma membrane (PL) of ECs. Scale bar=0.2μm. **B)** Detection of IL-1β in MVs but not in supernatants without MVs **(C)** by immunoblotting. Fresh supernatants were harvested from cytokine-primed HCAECs and incubated with NE (1μg/ml) or NE and NEIII (500μM) or YVAD-CHO (50μM) for 30min, 2h and 6h. MVs were isolated from supernatants by centrifugation at 20,000g after pelleting the cellular debri and lysing them using 1% v/v Triton-X100/PBS. Equal protein concentrations (15-20μg) were loaded in each lane. Recombinant IL-1β (rIL-1β; 20μg) was used as a positive control (17kDa). Data are representative of n=4. **C)** Supernatants, with MVs removed, loaded under the same conditions, showing the absence of IL-1β in the supernatants.

4.3.3. Microvesicle shedding is preceded by cytosolic Ca^{2+} changes:

In secretory cells, MV shedding has been linked with a transient increase in intracellular calcium $[Ca^{2+}]_i$ (Rodriguez et al., 1997) and this increase in the cytosolic Ca^{2+} has been linked to IL-1β MV secretion in monocytes (Andrei et al., 1999).

Therefore, to look in further detail into the mechanism by which NE induces MV shedding from ECs, I studied cytosolic calcium changes in relation to NE activation. Using a Ca^{2+} sensitive fluorometric dye, I sought to assess the role of $[Ca^{2+}]_i$ in MV formation and release in response to NE, performing experiments in the presence or absence of exogenous calcium.

In this experiment, $[Ca^{2+}]_i$ is released from intracellular stores during an initial stimulation/treatment in Ca^{2+} free media, and application of $CaCl_2$ during the second phase, allows Ca^{2+} influx into ECs. In Ca^{2+}-free media, there is a slight but non-significant increase in cytosolic Ca^{2+} levels in NE treated cells compared to untreated cells after NE stimulation (Figure 4.4A). However, $[Ca^{2+}]_i$ was significantly increased in NE stimulated cells after the addition of $CaCl_2$ compared to unstimulated and cytokine-primed cells (Figure 4.4B). This finding suggests that NE treatment increases free $[Ca^{2+}]_i$ by promoting Ca^{2+} influx into ECs.

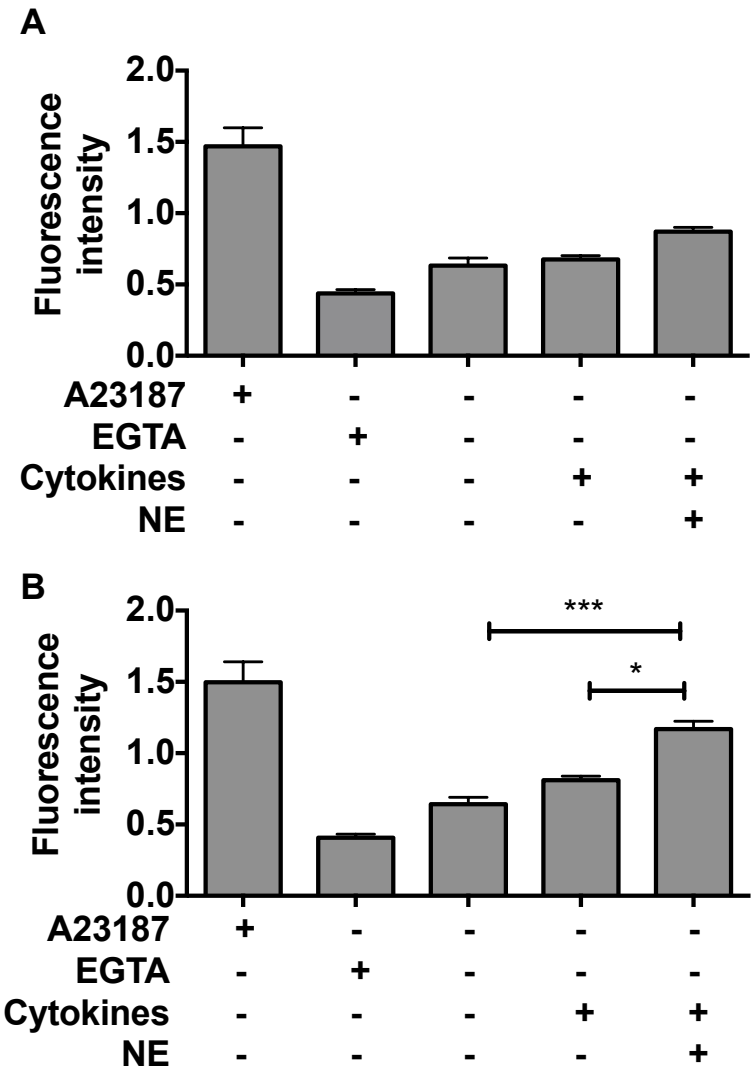

Figure 4.4. NE effects on endothelial $[Ca^{2+}]_i$. HCAECs were assayed for changes in cytosolic-free Ca^{2+} in response to the indicated conditions. **A)** No significant change in cytosolic Ca^{2+} -free media. **B)** The fluorescent intensity in intracellular calcium changes after 5 minutes of NE stimulation, in the presence of 1mM of $CaCl_2$. Ionophore A2347; 10μM and EGTA; 6mM were used as positive and negative controls, respectively. Data are from six independent experiments, mean ± SEM, analysed by One-way ANOVA and Tukey's post-test, *p<0.05, ***p<0.001.

4.4. Released exosomes and their roles in IL-1 secretion:

Having identified MVs as a secretory route for IL-1β from ECs due to the actions of NE, I sought to determine if there were any additional routes for the leaderless cytokine. In the literature, and as discussed in chapter 1, section 1.4.4, different mechanisms can be proposed in the same cell type. For example, in platelets, it has been suggested two populations of EVs are released, including MVs and exosomes containing vWF (Heijnen et al., 1998).

In my first experiments, I examined the location of IL-1β in ECs using EM. ECs were treated with NE or left untreated for 2h and then cells were immunogold labelled with anti-IL-1β (20nm conjugated gold particles). The cells were then observed using EM to detect if there was any IL-1β outside MVs.

ECs, after 2h of NE treatment, released 100nm average sized exosomes from their plasma membrane (Figure 4.5, left panel) and these exosomes were rich in IL-1β as evident by detection of 20nm-conjugated gold particles labelled IL-1β (Figure 4.5, right panel).

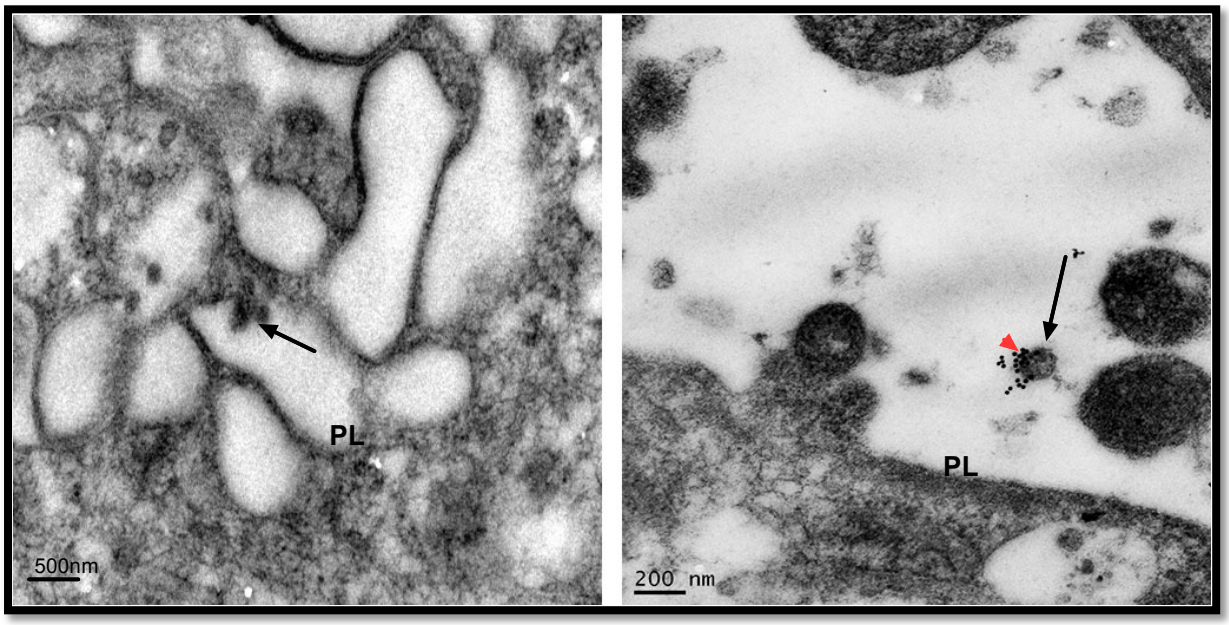

Figure 4.5. Released exosomes as an additional secretory route for IL-1β. Immunoelectrographs showing exosome release (arrow) from the plasma membrane (PL) of ECs activated by NE for 2h. The exosomes are positively immunolabelled with anti-IL-1β (red arrowhead; 20-nm gold particles).

4.5. Extracellular vesicles (EV) express LAMP-1:

The EM clearly suggested that some exosomes contained high levels of IL-1β. To confirm the potential role of the endolysosomes (a possible source of exosomes) in IL-1β secretion by NE, EVs were also tested for the presence or absence of LAMP-1 protein using western blot. LAMP-1 is a commonly used marker to identify endolysosomes within cells.

EVs isolated from NE-treated cells using centrifugation gradient expressed LAMP-1 and this was attenuated in the presence of NEIII (Figure 4.6A). However, there were no significant changes in LAMP-1 expression in the cell lysates (Figure 4.6B). These data suggest that EVs are derived from endolysosmes and may be released via an endolysosomal mediated mechanism.

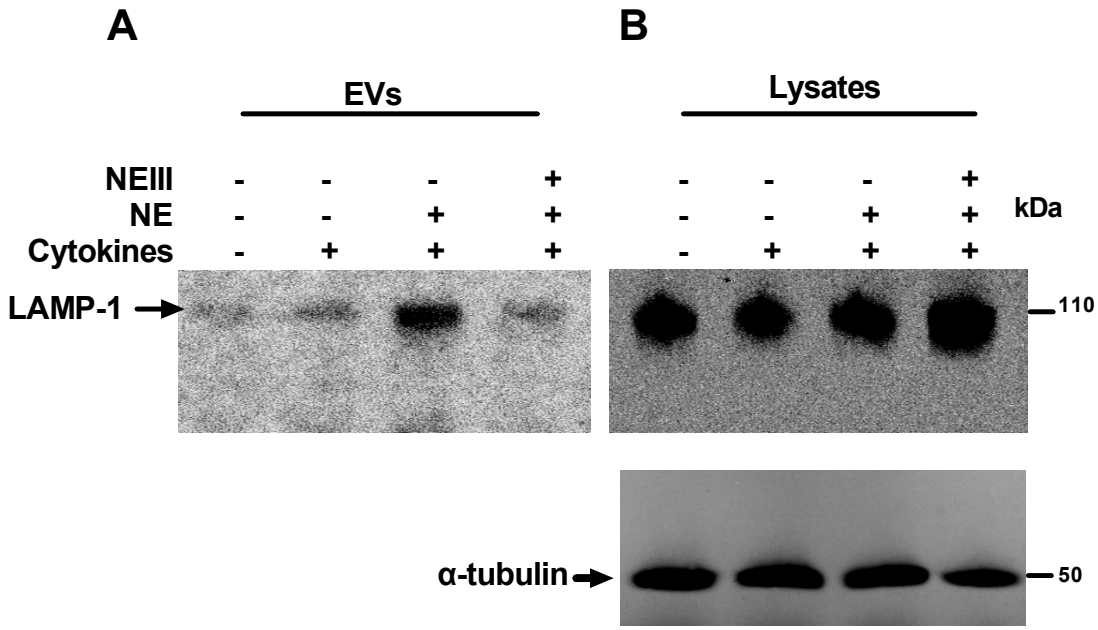

Figure 4.6. NE induces IL-1β release by an endolysosomal mechanism. HCAECs, treated for 48h with cytokines or left untreated were incubated with NE for 6h then EVs **(A)** and cell lysates **(B)** were analysed using Western blotting for LAMP-1. 12-20μg proteins were loaded per lane and α-tubulin was used as a loading control. The blot represents three independently performed experiments.

4.6. The cellular distribution of IL-1β and mechanism of secretion:

Inside cells, the site of IL-1β processing is still an area of controversy. In many cell types, it is postulated that IL-1β might be processed in an endolysosomal compartment (Andrei et al 2004). However, how IL-1β is sequestered into compartments and whether it is processed in ECs in endolysosomes is relatively unclear.

4.6.1. IL-1β colocalisation with LAMP-1:

Having shown that EVs released from ECs express LAMP-1, I next asked if IL-1β is processed within endolysosomes. For further elucidation of the mechanisms of IL-1β

release by EVs, ECs +/- NE treatment were immunofluorescently stained for LAMP-1 (a late endolysosomal marker) and IL-1β, detected by confocal microscopy.

No staining was detected for IL-1β in unstimulated ECs (data not shown). Cytokine-primed ECs showed both IL-1β and LAMP-1 positive staining, but without co-localisation, suggesting a wide distribution of IL-1β throughout the cytoplasm (Figure 4.7A).
However, in ECs incubated with NE for increasing times, IL-1β clearly co-localised with LAMP-1. Indeed, following a 2h stimulation of EC with NE, the majority of IL-1β was co-localised with LAMP-1 (Figure 4.7A, B).

These results suggest a direct role of endolysosomes in IL-1β processing and secretion induced by NE.

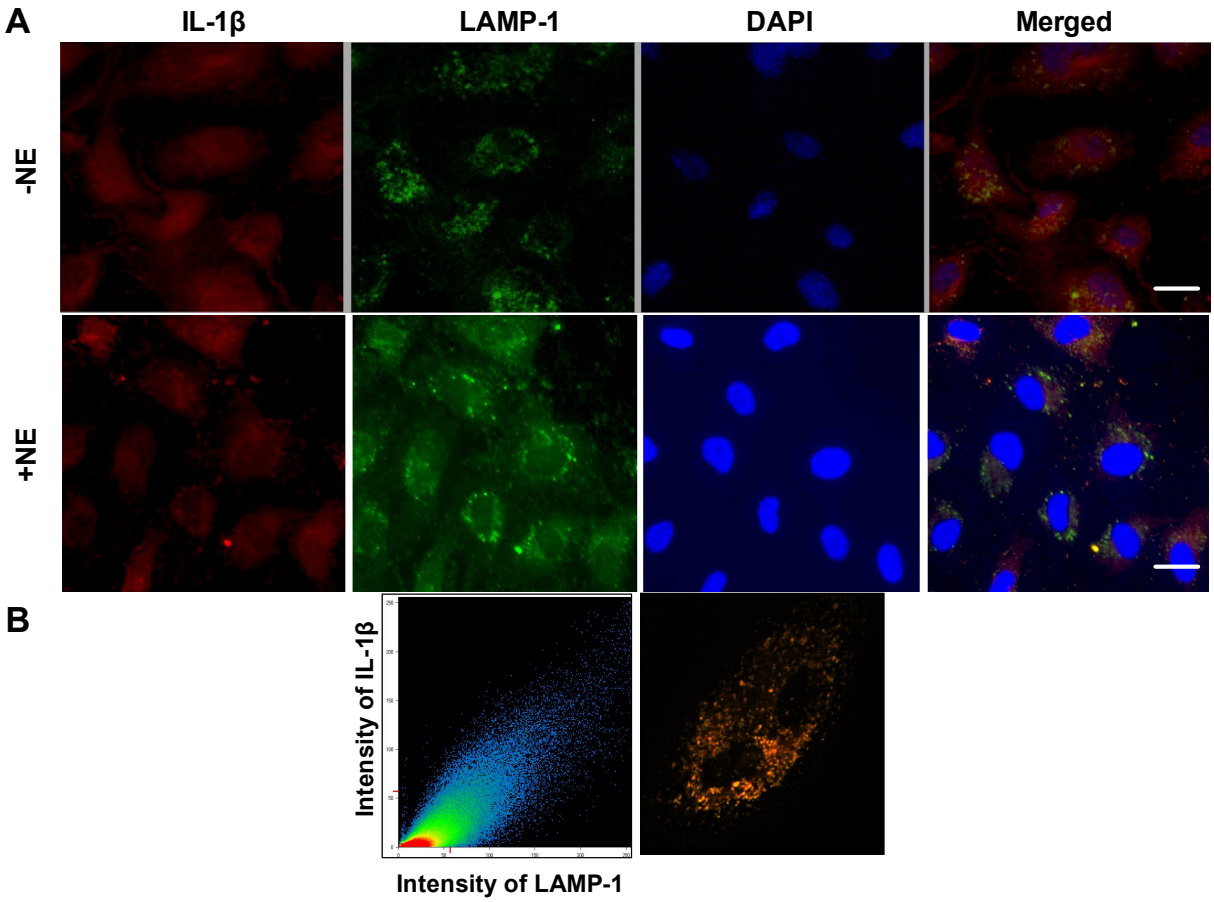

Figure 4.7. Cellular distribution of IL-1β in ECs after NE stimulation. HCAECs in 8-chamber slides were treated or left untreated with cytokines (TNF-α/IL-1α; 10ng/ml each) then the media was replaced with NE (1μg/ml) containing serum free media over 2h. Cells were then immunostained for IL-1β (red) and LAMP-1 (green). **A)** High resolution images of cytokine-primed cells, without NE (-NE), or treated with NE (+NE) for 2hr. Scale bars=10μm. Data are analysed by fluorescent confocal microscopy. The presented images are representative of fluorescent images from four independent experiments. **B)** A Scatter plot representing a high colocalisation between IL-1β and LAMP-1 in ECs after NE activation at the 2 h time-point.

4.6.2. Subcellular distribution and localisation of IL-1:

To study further the ultrastructure of the endolysosomes, I used EM to examine ECs treated with NE and compared to untreated cells.

In unstimulated ECs, no observed MVBs were detected (Figure 4.8A, -NE). However, after 2h, MVBs (>200μm in size) were detected primarily in a close proximity to the plasma membrane in NE-activated ECs compared to unstimulated ECs (Figure 4.8B, arrow, +NE). The MVBs were classically filled with intraluminal vesicles (ILV) or exosomes (30-100nm) (Figure 4.8B, arrowhead, +NE).

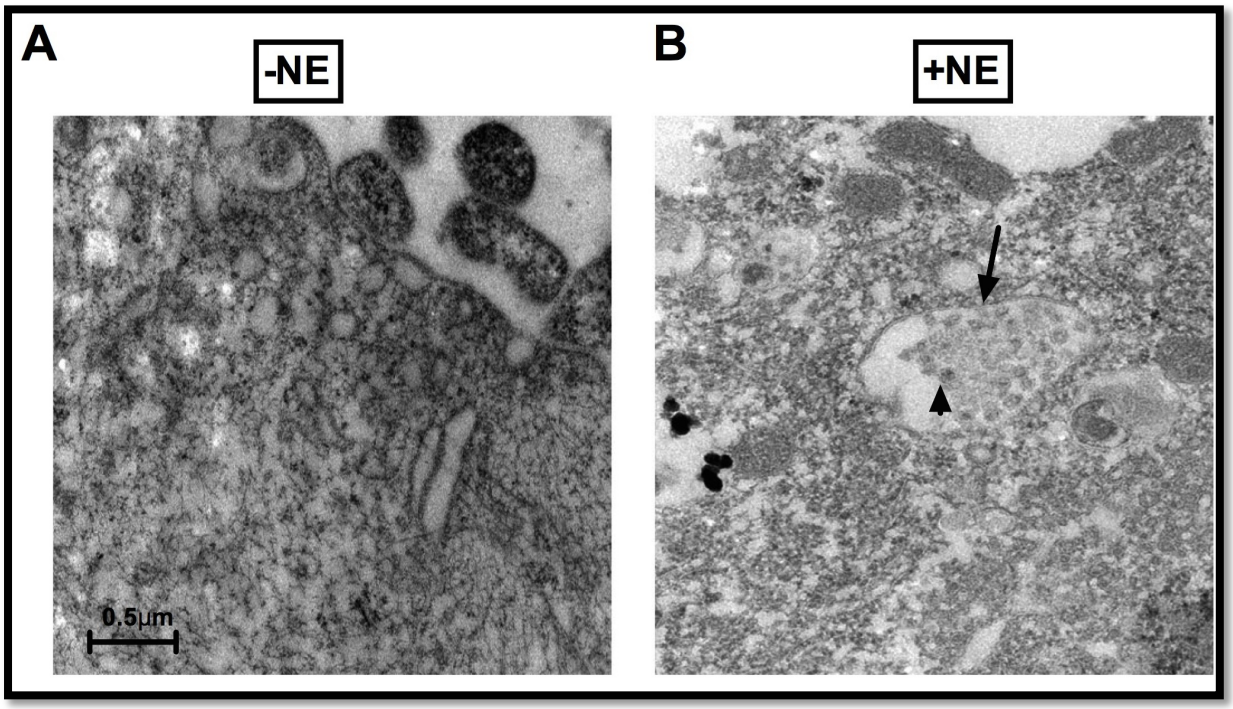

Figure 4.8. NE enhances MVB formation inside ECs. ECs +/- NE analysed using TEM. **A)** Electromicrographs of NE untreated ECs (-NE) and **(B)** NE-treated cells (+NE) showing multivesicular bodies (MVBs, arrow) compared to NE-untreated controls (-NE). In NE-treated ECs, the MVBs (arrow) are filled with exosomes (30-100nm; arrowhead) in close proximity of the plasma membrane. The images are representative from three independent experiments. Scale bar=0.5µm.

Next I wanted to ask the question of whether MVBs contain IL-1β and thus confirming the previous immunofluorescent findings and suggesting that the MVBs are the site of IL-1 processing. To do this, EM sections from ECs-primed first with cytokines, and then stimulated with NE for 30-2h, were immuno-labelled with IL-1β conjugated gold particles (20nm).

Interestingly, inside ECs, IL-1β conjugated gold particles were detected within two distinct sets of intracellular vesicles at different incubation time-points with NE.

At 30min of NE incubation, IL-1 was primarily detected within membrane bound vesicles with an average size of less than 200nm (Figure 4.9, left panel). That was associated with membrane blebbing and MV shedding containing IL-1 (Figure 4.9, right panel).

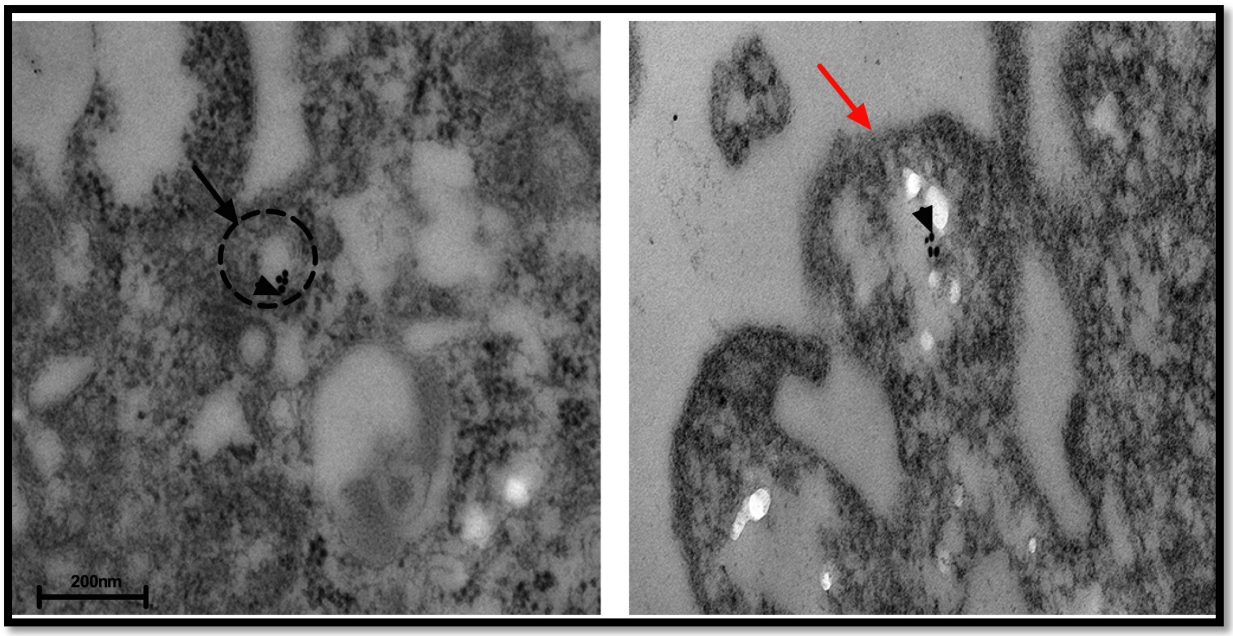

Figure 4.9 Sites of early IL-1 processing. Immunogold EM images for 30min NE treated ECs showing IL-1 (arrowhead) within 200nm vesicles inside ECs (arrow, dotted circle, left panel). Right panel electron micrograph showing membrane blebs (red arrow) containing IL-1 (20nm gold particles; arrowhead). The representative images are from three independent experiments. Scale bar=0.2μm.

However, after 2h incubation with NE, EC IL-1 was only observed within the MVBs (Figure 4.10).

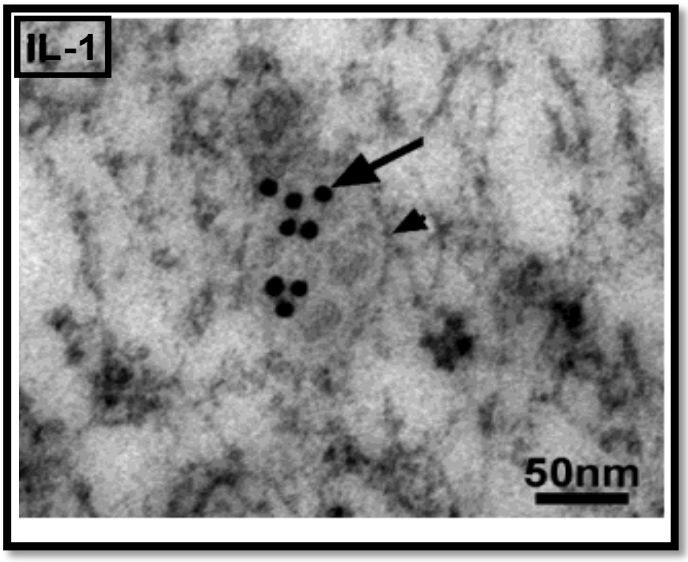

Figure 4.10. IL-1 is detected in the preterminal endolysosomes. Electron micrograph representing 3 independent experiments of ECs treated with NE for 2h and immunogold labelled for IL-1β (20nm-conjugated gold particles). Arrow indicates IL-1 labelled gold particles whereas the arrowhead represents MVBs.

To confirm the endolysosomal nature of these MVBs, EC-activated by NE were also stained for LAMP-1. Unsurprisingly, a sequence of MVBs within the ECs starting from the perinuclear area to the plasma membrane was clearly detected and they were positive for LAMP-1 (10nm-conjugated gold particles) (Figure 4.11).

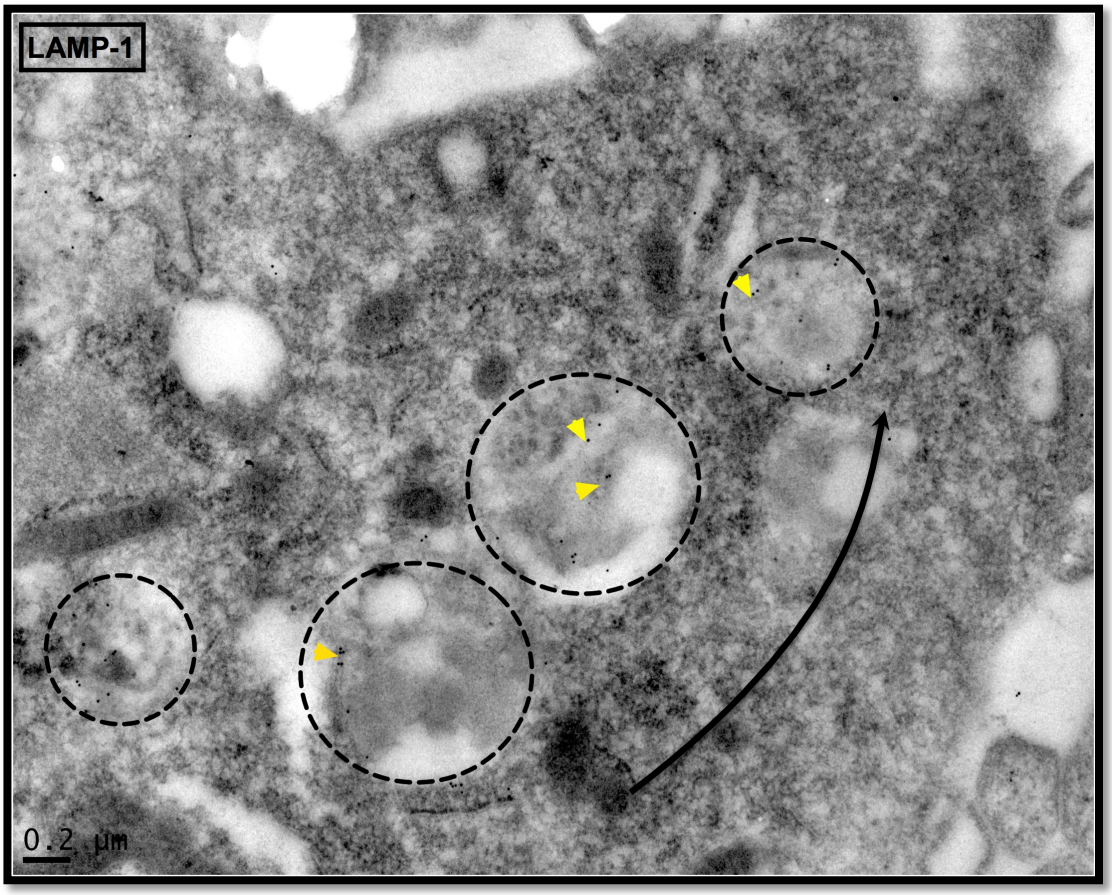

Figure 4.11. Immunoelectron analysis of LAMP-1 in ECs after NE treatment for 2h. The MVBs (dotted circles) are positively immunolabelled with anti-LAMP-1 (yellow arrowheads; 10-nm gold particles). Scale bar=0.2µm. Arrow indicates the direction of MVBs starting from perinuclear area toward cell surface. The electron micrograph is a representative of three independent experiments.

4.7. NE is detected inside ECs and colocalised with LAMP-1:

To follow the fate of NE in activated ECs, I used Alexa-Fluor 647-labelled NE and performed immunofluorescence staining. After permeabilisation, I also labelled the internal endolysosomes with LAMP-1.

NE was detected inside cells and co-localised with LAMP-1, confirming that NE

inside ECs may directly cleave IL-1 within MVBs (Figure 4.12A).

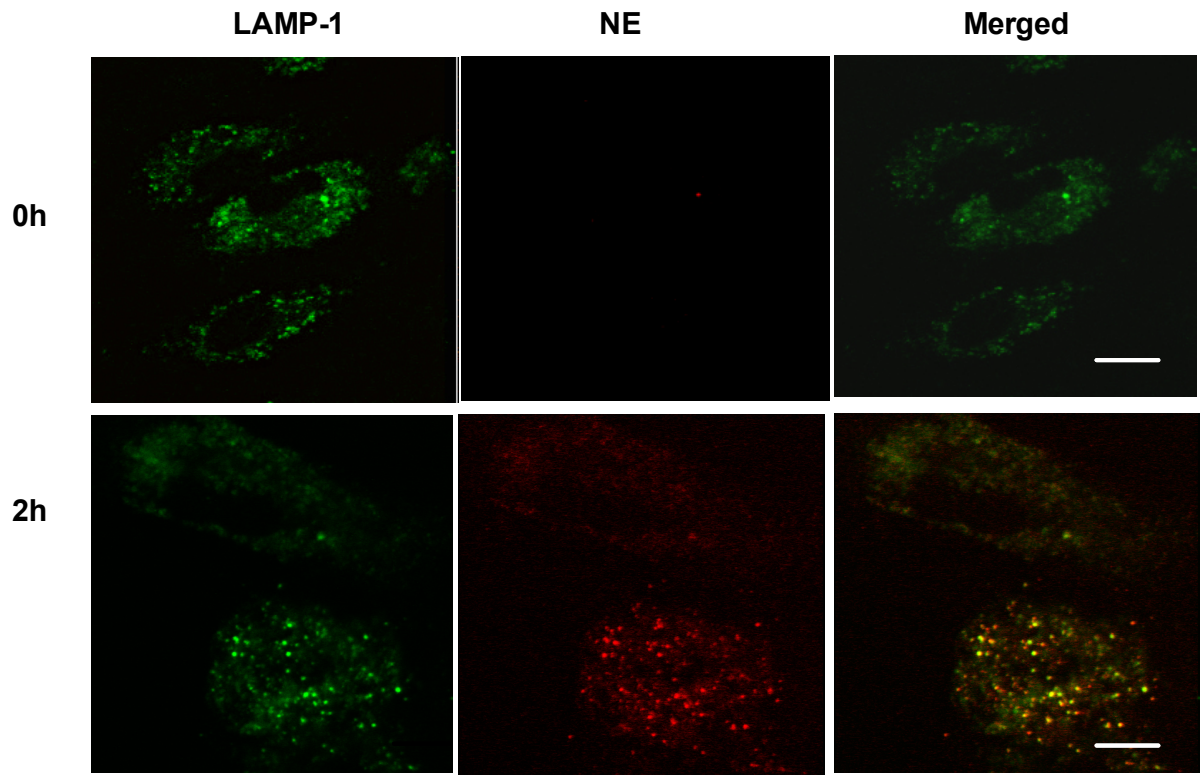

Figure 4.12. NE is detected inside ECs. Confocal images showing LAMP-1 and NE in primed ECs after NE treatment. HCAECs were incubated +/- Alexa Fluor 647-labelled (1µg/ml) NE for 2h in serum free media before washing in PBS and colocalisation performed using an antibody against LAMP-1. Confocal images were analysed using Zeiss image and image j software, scale bars=10µm.

4.8. Formation of endosomes is important for IL-1 secretion:

To confirm that the released IL-1β in EVs was indeed mediated by an endolysosomal mechanism, I evaluated the effect of bafilomycin A1 (BAF1), a lysosomal V/ATPase inhibitor (Drose and Altendorf, 1997), on IL-1β secretion by NE using ELISA and immunoblotting.

As shown in figure 4.13A, treatment of ECs with BAF1 (50nM) before the addition of NE for 6 hours largely decreased IL-1β levels in the supernatants compared to NE treated cells without BAF1 (56.44 ± 20.2 vs. 131.8 ± 23.97pg/ml, respectively, $p<0.05$, $n=4$). In cell lysates, the cleavage of proIL-1β by NE was attenuated in the presence of BAF1 (Figure 4.13B), confirming that the endolysosomes are essential for IL-1 processing by NE in ECs.

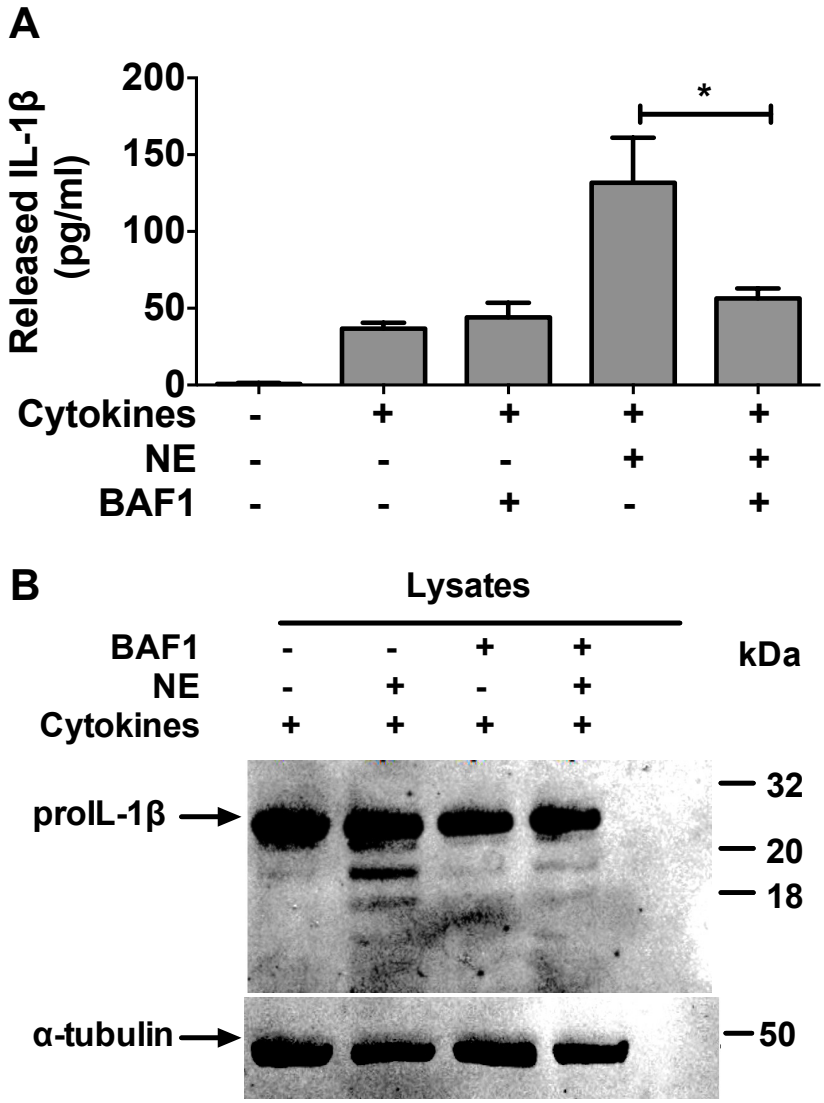

Figure 4.13. Formation of endosomes is an important for endothelial IL-1 secretion. A) ELISA measuring IL-1β released in conditioned media of HCAECs primed with cytokines (TNF-α/IL-1α; 10ng/ml) ± NE (1μg/ml)/ ± BAF1 (50nM) after 6h of stimulation. Experiments are n=3 and data are mean ± SEM. Data are analysed by One-way ANOVA followed by Tukey's post-test, *p<0.05. **B)** Western blot analysis of lysates harvested from primed HCAECs activated with NE ± BAF1 (50nM) for 6h, 20μg protein loaded per lane and α-tubulin was used as a loading control. The blot is representative of three independent experiments.

4.9. Detection of NE with IL-1β in the endothelium of mature atherosclerotic plaques:

Finally, I asked whether NE could be detected in atherosclerotic plaques in mice to ascertain if NE could contribute to local IL-1β generation.

Using immunostaining, only in well-developed atheromatous lesions of ApoE$^{-/-}$ mice fed a high fat for 12 weeks, IL-1β was detected, predominantly in ECs (Figure 4.14A, left panel).

Interestingly, in these lesions, NE also appeared to be expressed in the luminal endothelium (Figure 4.14B) and in the same location as that of vWF-positive stained ECs (Figure 4.14C).

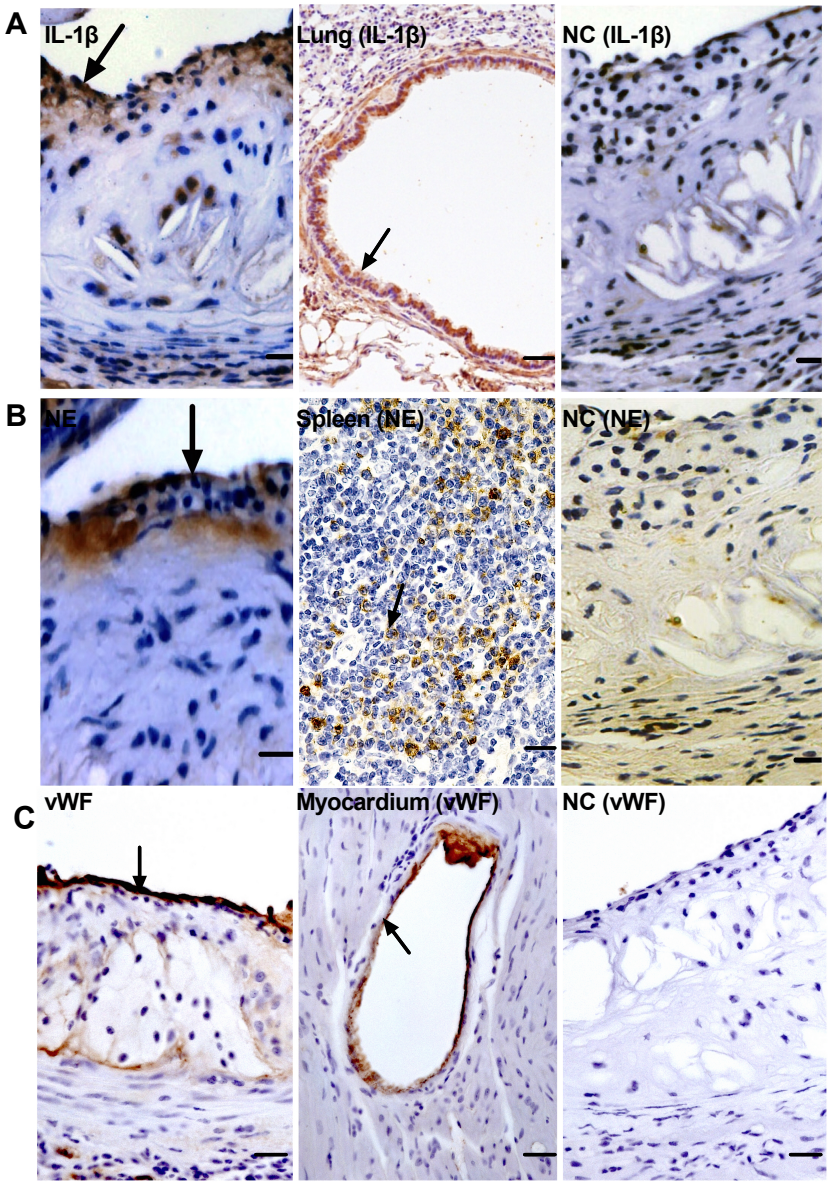

Figure 4.14. NE is detected with IL-1β in serial atherosclerotic sections in the endothelium. A) and B) Immunohistochemical detection of NE and IL-1β in the luminal endothelium of mouse atherosclerotic plaques. Paraffin embedded aortic sinuses from ApoE$^{-/-}$ mice fed high fat diet for 12 weeks were stained with primary antibodies as indicated. Specificity of staining is confirmed by no primary negative control (primary antibodies were replaced by PBS). Scale bars=200μm. **C)** vWF is detected in a sequential section of aortic atherosclerosis. NE positivity was detected predominantly in the endothelium (top panel; arrows). IL-1β positive endothelium (top left panel) was also detected. The bottom panels show vWF stained endothelium. Middle panels are positive control tissues, including lung, spleen and myocardium for their respective antibodies. Images are representative of histology data obtained from a total of 6 animals. Scale bars=100-200μm.

4.10. Summary:

The data in this chapter demonstrate that ECs release MVs/exosomes in response to NE. The data also lead to a range of mechanisms by which EVs are released, as summarised below:

1. MV shedding from ECs treated with NE is time dependent and independent of caspase-1 activation.
2. The MVs are only secreted from ECs activated by NE, and contain IL-1β.
3. The shedding process is preceded by a transient, yet significant, increase in cytosolic calcium levels.
4. Within ECs, IL-1β is diffusely distributed in cytokine-primed ECs. However, following NE treatment, the majority of IL-1β is clearly co-localised with LAMP-1.
5. The co-localisation between LAMP-1 and IL-1β increases with increasing time incubation with NE.
6. Immunoblotting analysis of EVs isolated from NE treated cells shows expression of LAMP-1.
7. The secretion of IL-1β is significantly attenuated in ECs pretreated with BAF1 and followed by NE.
8. MVBs were only detected in ECs treated with NE, and exosome release was observed using EM.
9. The MVBs express LAMP-1, and contain IL-1β.
10. The endothelium also releases exosomes containing IL-1 after a 2h incubation with NE.
11. NE was detected within the vascular endothelium of matured plaque and appears to colocalise with IL-1β.

4.11. Discussion:

The mechanism of IL-1β secretion by the endothelium is still an enigma and the mysteries of its secretion have long intrigued scientists. In the previous chapter, I showed that ECs release IL-1β after NE activation and that the secretion is time and dose dependent. NE selectively cleaves proIL-1β and this is independent of caspase-1. However, relatively little is known about the mechanism for the trafficking of IL-1β into the extracellular environment.

The release of extracellular vesicles has increasingly been recognised as an avenue for delivering various cellular signals or bioactive factors (Bianco et al., 2005). Virtually all cells are able to release microvesicles from their plasma membranes (MacKenzie A, 2001) or exosomes from exocytosis of multivesicular bodies (Ghossoub et al., 2014). Therefore, I investigated MV/exosome release in response to NE.

Using time-lapse microscopy, I found that HCAECs generate Annexin V^+ MVs in response to NE. The generation was monitored for 6h and showed that the shedding process starts early after NE application (within 5-10 minutes post-NE activation). Membrane flipping and PS exposure were only observed in the MVs. This finding is in agreement with MacKenzie and collaegues who have described this phenomenon in monocytes (MacKenzie A, 2001).
PS exposure also occurs as a result of apoptosis (Naito et al., 1997) but in the previous chapter, I showed that caspase-3/7 activity used as a tool to measure apoptosis (Sebbagh et al., 2001) did not increase with NE treatment (section3.1.5). Moreover, in apoptosis, the PS flipping is a generalised process along all of the outer surface of the plasma membrane (Majno and Joris, 1995, Winn and Harlan, 2005), yet in NE treated cells, I observed the PS flipping only prior to the shedding and localised to MVs.

There is no general consensus on the method of MVs isolation and using a centrifugation gradient to separate MVs from exosomes (van der Pol et al., 2012) relies on the differences in sizes only (within the 200nm range) and one cannot avoid contamination and degradation of vesicles. The gold standard technique, therefore,

to describe MV size and characterisation is still via electron microscopy. As a result, I examined ECs treated with NE using EM and showed membrane tethering and 0.1-1μm MVs separated from ECs, confirming my earlier light microscopy data.

To study the kinetics and the number of the MVs released in a greater detail, I used a standardised MV isolation and quantification technique (Robert et al., 2009), and I showed a significant increase in MVs isolated from NE-treated ECs. To confirm that the MV shedding is a direct action of NE rather than indirect may be via an intermediate product, NE inhibitor was added and the shedding was indeed attenuated. Strikingly, this NE induced MV shedding was independent of caspase-1. This was most clearly seen when YVAD-CHO was added to HCAECs followed by NE treatment, a protocol that caused markedly greater generation of Annexin V^+ MVs. This finding contrasts with previous investigations on immune cells in terms of their caspase-1 dependency of MVs shedding (Keller et al., 2008). However, to date, there have been no other investigations on caspae-1 dependency of MV shedding in ECs. MV shedding from ECs has been recently proposed as a mechanism for other non-classical protein secretion (Betapudi et al., 2013).

My data showed that IL-1β is present in the MVs but not in MV-free supernatants, confirming that IL-1β is released only in MVs. This is in line with the Mackenzie *et al* study demonstrating that monocytes secrete IL-1β by MV shedding (2001). The immunogold EM further confirms this finding by the detection of IL-1β immunogold labelled particles within the MVs. To my knowledge, in ECs, this is the first study to detect IL-1 using immunogold EM within MVs.

Interestingly, MVs did not contain caspase-1 or any components of the inflammasome, suggesting that caspase-1 activation is not fundamental in this setting. The processing of proIL-1β has long been linked with caspase-1 activation in immune cells (Netea et al., 2009). A controversy, however, has arisen more recently in other cell types such as RAW264.7 macrophages where caspase-1 does not seem to be crucial (Pelegrin et al., 2008).
Although a few studies have begun to investigate NE-mediated IL-1β secretion in renal (Schreiber et al., 2012) and pulmonary (Couillin et al., 2009) inflammatory

diseases, my data are the first to propose a direct effect of NE on IL-1β release and to link this to a MV shedding mechanism.
MVs have previously been shown to express a number of cell surface markers such as MHC-II (Qu et al., 2009), but due to time constraints, I was not able to carry out a detailed biochemical analysis to fully characterise other microvesicle surface markers.

The mechanism by which MVs are released is poorly understood. Several studies indicate that cytokine-containing MV shedding and membrane tethering occurs by a calcium dependent mechanism. Removal of extracellular calcium has been shown to decrease the shedding of the MVs by microglia (Bianco et al., 2005) and THP-1 monocytes (MacKenzie A, 2001). Moreover, a common biogenesis has linked endolysosomes with Ca^{2+} regulated vesicle secretion with an increase in intracellular calcium associated with IL-1β secretion (Qu et al., 2007).

I demonstrated that NE transiently increased $[Ca^{2+}]_i$ to a maximum after the addition of exogenous Ca^{2+}. Furthermore, it has been shown that removal of extracellular calcium ameliorated IL-1 secretion (Rodriguez et al., 1997), suggesting that NE mobilised $[Ca^{2+}]_i$ mainly by influx of extracellular Ca^{2+} and thus it may enhance IL-1β secretion. This is in agreement with recent studies demonstrating that extracellular Ca^{2+} is required for IL-1β secretion (Wang et al., 2011a). However, how cytosolic calcium changes induce an assembly of IL-1 and MV shedding are still to be fully clarified.

The site of IL-1β processing in ECs is relatively unknown. Although the processing and the release of IL-1β occur rapidly and possibly concurrently, it has been previously postulated that the cleavage of proIL-1β and mature IL-1β secretion are relatively unrelated (Galliher-Beckley et al., 2013, Netea et al., 2009). However, this speculation remains hypothetical because, due to the technical limitations, the presence of IL-1β in different compartments within the cells coupled with mature IL-1β secretion had not been documented prior to my work.

This chapter investigated the cellular distribution of IL-1β and whether the subcellular location of IL-1β changed following NE incubation and related the location to a late

endolysosomal marker; LAMP-1. Recent immunofluorescence staining data in murine macrophages has shown that IL-1β does not co-localise with LAMP-1 in macrophages (Brough and Rothwell, 2007). This finding is consistent with my data in ECs prior to NE treatment (cytokine-primed cells) where IL-1β appears to be diffusely distributed within the cytoplasm. Most interestingly, 2h after NE treatment, IL-1β is co-localised with LAMP-1, strongly suggesting that IL-1β compartmentalisation may be acutely induced by this stimulus and destined for maturation by a regulated transport.

Another important outcome of this study is the proposed mechanism of the role of the endolysosomes in IL-1β release from ECs. Endolysosomal markers were evident in EVs isolated from NE-treated ECs and the detection of IL-1β in LAMP-1 positive EVs after 2h is a novel finding.
The co-localisation of IL-1β and LAMP-1 and the unique appearance of LAMP-1^{+ve} EVs after NE stimulation of primed EC, suggests that NE may trigger a signalling pathway that allows processing to occur in secretory endolysosomes. It is possible that proIL-1β travels to a endolysosomes (Andrei et al., 1999) to be processed to the mature form before being packaged into extracellular vesicles expressing LAMP-1 for the secretion.

Additionally, I showed that release of IL-1β is significantly attenuated by BAF1. BAF1 is known to inhibit endolysosome formation by preventing fusion of the late endosomes to lysosomes, in addition to inhibiting vacuolar H+/ATPase (Yamamoto et al., 1998). An interesting finding by (Rubartelli et al., 1990) suggests that IL-1β secretion is ameliorated by agents that prevent endocytosis, confirming that endosomes may play a key role in the secretion of IL-1β. My work is supportive of this concept. Since MVBs were detected only following NE treatment of ECs, this suggests that the MVBs are a part of the secretory pathway of IL-1β. However, how IL-1β is recognised and gets into these vesicles compared with the entry of other cytosolic proteins, is still to be investigated.

The EM data also confirmed the presence of another population of extracellular vesicles with 30-100nm in size, fitting the classical morphological features of exosomes. Interestingly, exosome release was only observed at later time points,

alongside shedding of MVs, suggesting a continuum mechanism by which ECs release their IL-1β contents.
The secretory pathway described here (early MV shedding and then late exosome release) has been used to describe the secretion of other non-classical proteins such as epidermal growth factor (EGF) (White et al., 2006). Since a motif common among those proteins has not yet been identified, it is possible that they undergo some sort of posttranslational modifications in an 'assemblosome' directing those proteins for their selective export. Other possible roles, involving heat shock proteins, have also not yet been completely defined (Piper and Katzmann, 2007), and their expression in exosomes could also be postulated.

Given the continued prominence and topicality of IL-1 in the progression of atherosclerosis (Seropian et al., 2014), I studied the expression of NE *in vivo* in a recognised atherosclerosis preparation: aortic root plaques taken from ApoE$^{-/-}$ mice fed a high fat diet for 12 weeks. Although previous work has detected NE in aortic aneurysms (Rao et al., 1996) and in carotid plaques (Dollery et al., 2003), my study is the very first to investigate NE distribution in experimental atherosclerosis.

Significantly, NE was detected in the endothelium and sub-endothelial cells of atherosclerotic plaques, and was detected alongside IL-1β. The antibody used for these studies recognises both proIL-1β and mature forms, and I show some cellular localisation with NE, in support of my data that NE activates and promotes secretion of IL-1β. The detection of NE within the endothelium has been demonstrated in carotid atherosclerosis (Dollery et al., 2003), however, no other publications linked endothelial IL-1 expression with NE.

The mechanism of IL-1β secretion from ECs has been an intriguing and unresolved question in IL-1β bio-physiology over decades. Using different experimental systems in this study, I provide, for the first time, direct evidence for the involvement of NE in MV/exosome release from ECs containing mature IL-1β. The proposed mechanism of IL-1β secretion from ECs by NE is summarised in figure 4.15.

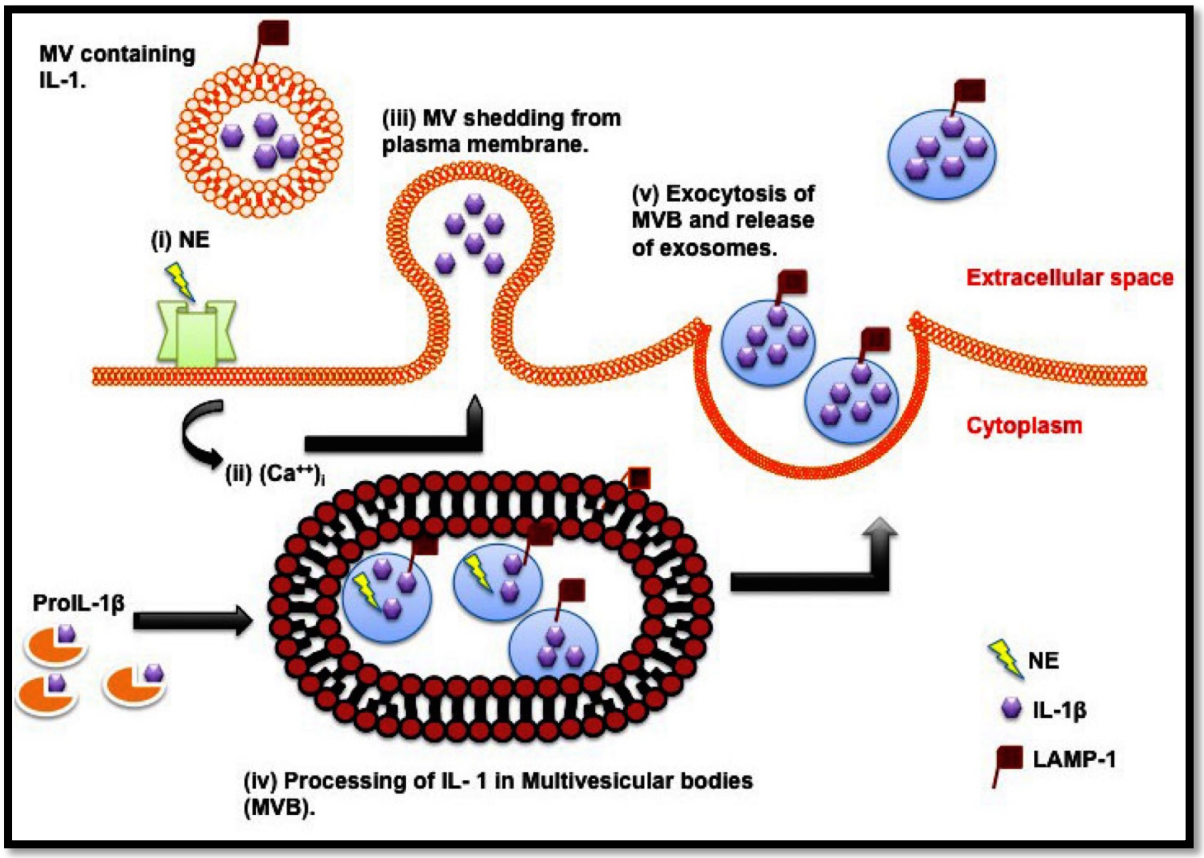

Figure 4.15. Schematic of mechanism of IL-1β secretion from ECs by NE. (i) NE is released by circulating cells at the site of atheroma and transported by endocytosis inside the diseased endothelium (primed by inflammation). **(ii)** An increase in intracellular calcium due to NE effects leads to remodelling of cell membrane and vesiculation **(iii)** which, in turn, facilitates shedding of MVs containing IL-1β. **(iv)** Inside ECs**,** NE enters secretory endolysosomes (multivesicular bodies; MVBs) and cleaves proIL-1β within. MVBs also fuse to plasma membrane and released exosomes containing IL-1 **(v)**.

In conclusion, I have significantly added to and cemented the emerging role of NE in IL-1 induced inflammation. I suggest a novel mechanism for NE-mediated IL-1 secretion by ECs, namely pro-IL-1 processing in the secretory endolysosomes and packaging of mature IL-1 within MVs/exosomes for release into the extracellular environment. NE and IL-1β are detected *in vivo* in the setting of atherosclerosis within endothelium in atheromatous plaques.

My findings have a wider application for a better understanding of the role of other important proteases with prominent non-proteolytic and possibly signalling roles, such as azurocidin, proteinase 3 and Cathepsin G, and provide other avenues for therapeutic targets to limit the influence of interleukin-1.

Future perspectives for that with possible therapeutic implications are discussed in depth in the general discussion chapter (8) of this thesis.

Chapter (5) Omega-3 Fatty Acid effects in Experimental Atherosclerosis.

OUTLINE:

5.1. Overview of n3FA effects in Experimental Atherosclerosis:

A strong body of evidence has demonstrated that hypertension and dyslipidaemia are the underlying risk factors for atherosclerosis (Drazner, 2011). Hypertension is a major health care concern due to its prevalence among the population and its devastating complications; namely stroke and IHD (Lewington et al., 2002).
Despite major advances in the disease management, substantially high-risk IHD patients are fuelling intensive investigation into pharmaco-modulation of the underlying disease and hypertension (Wang et al., 2011b, Vasan et al., 2001). In the search for a novel therapeutic intervention, the rising importance of inflammatory biomarkers and immune body system involvement in the disease is of major importance.

Dietary control for atherosclerosis and hypertension has also been a focus of intensive investigation (Burr et al., 1989). Epidemiological and prospective clinical studies have suggested remarkable effects of n3FAs in the reduction of cardiovascular mortality rates among IHD patients (Yates et al., 2014). These effects have been ascribed to the improvement of the cardiovascular risk profiles, resulting in disease prevention (Marchioli et al., 2002).

N3FAs, especially DHA and ALA are shown to be anti-atherogenic (Hall, 2009), with multiple underlying mechanisms (Thies et al., 2003, Cawood et al., 2010). Recent evidence indicates that n3FAs may have anti-inflammatory (Calder, 2012, Yates et al., 2011), anti-lipidemic (Mozaffarian and Wu, 2011), anti-arrhythmic (Yates et al., 2014) and blood pressure lowering effects (Miller et al., 2014). Moreover, strong evidence suggests that n3FAs are a positive dietary intervention in various inflammatory diseases such as RA and inflammatory bowel disease (IBD) (Goldberg and Katz, 2007). However, the data in IHD and hypertension are less robust.

Clinical trials in post-MI patients have suggested that n3FA may exert a possible therapeutic effect, particularly on sudden cardiac death and the risk of re-infarction (Vedin et al., 2008), although this effect has not been shown unequivocally nor has any link been demonstrated with atherosclerotic plaque stabilisation. Moreover, recent meta-analysis studies did not support the protective roles of n3FAs on

cardiovascular mortality (Rizos et al., 2012, Kwak et al., 2012). In addition, there is limited information from large randomised studies on n3FA effects, particularly in atherosclerosis and patients without MI.

Some uncertainty also exists regarding the optimum dose required to provide the maximum beneficial effects of n3FAs (Saravanan et al., 2010). Although some population-based studies have suggested that a low dose of n3FAs (<1g/day) is enough to provide favourable impacts of n3FAs on IHD (Marchioli et al., 2002), the most recent case-control study has demonstrated the opposite (Rauch et al., 2010). This area of uncertainty needs further investigation. Moreover, the underlying molecular mechanism of action of n3FAs has yet to be extensively studied.
I hypothesised that omega-3 fatty acids; ALA and DHA, preferentially decrease high fat diet induced atherosclerosis and high blood pressure in ApoE$^{-/-}$ mice.

5.2. Brief Methods:

The detailed methods for this section are described in chapter 2. Briefly:
Male ApoE$^{-/-}$ mice (8 weeks of age) were divided into one of three groups: control group fed Western diet alone, DHA treated group fed DHA (300mg/kg/day) with Western diet, and ALA group fed ALA (300mg/kg/day) with Western diet. All groups were fed for 12 weeks. During the study, the mice were monitored daily for their food intake and wellbeing, blood pressure and weekly for their body weight changes. Echocardiogram was performed on mice at baseline and again just before the termination of the study. By the end of study, the freshly collected blood by cardiac puncture was analysed for red blood cell (RBC) indices and plasma lipid profiles
The extent of atherosclerosis was assessed in the whole aortae, cross-sectional aortic sinus and brachiocephalic sections.
Data are expressed as mean ± SEM and analysed using prism software (Version 6, GraphPad, San Diego, CA). Blood pressure and body weight data were analysed by Two-way ANOVA followed by Tukey's post-test as described by (Hoorn et al., 2011). For two-group comparisons, data were analysed by an unpaired Student's t test for normally distributed data whereas for multiple comparisons, data were analysed by One-way ANOVA and Tukey's post-test. A level of $p<0.05$ indicated statistical significance.

5.3. DHA but not ALA Reduces High fat-diet Induced Hypertension and high Left Ventricular Mass (LVM) in ApoE$^{-/-}$ mice:

Given that DHA is the major type of PUFAs in fish and fish oil, and ALA is the predominant type of omega-3 fatty acid in vegetable oil and plant derived food (Mozaffarian and Wu, 2011), I investigated the differential effects of these two main types of fatty acids on atherosclerosis and hypertension in an experimental model.

This section outlines the observed effects of ALA or DHA feeding on high blood pressure and left ventricular function in ApoE$^{-/-}$ mice.

5.3.1. Food intake and body weight changes:

In order to study the effects of n3FA supplementation, atherosclerosis was augmented in ApoE$^{-/-}$ mice using the HFD (21% fat w/w).

To ensure that the mice fed n3FAs received the same concentration of ALA or DHA on each individual day of feeding, the free fatty acids were mixed with jelly at equal and final concentrations of 300mg/kg/day, and the mice were monitored daily to make sure they consumed all the n3FAs.

The method of using jelly to deliver drugs to mice was published recently by (West et al., 2014) and has shown to be an efficient way to deliver drugs to mice at the required concentrations.

The mice were trained for at least one week to eat jelly alone, and then the jelly containing free fatty acids (DHA or ALA) was given to the mice along with the HFD. For the duration of the study, all the mice ate the jelly containing DHA or ALA without missing a single dose, and throughout the period of the feeding, there was no significant difference in the jelly containing fatty acid intake among the studied groups. Importantly, both ALA and DHA were well tolerated, and no major side effects were observed.

In addition to their daily intake, the mice were monitored weekly for body weight changes. There was no significant change in the body weights between the ApoE$^{-/-}$ mice receiving HFD (control) and the ApoE$^{-/-}$ mice on HFD supplemented with ALA (Figure 5.1). However, in the DHA fed group, there was a slight, yet non-significant, slower body weight gain throughout the duration of the study. By the end of the

study, these DHA-receiving mice showed a statistically significant reduction in their body weight compared to the ALA fed group (29.88 ± 1.89 in DHA group vs. 33.79 ± 5.038g in the ALA group, $p<0.05$, at week 12) (Figure 5.1).

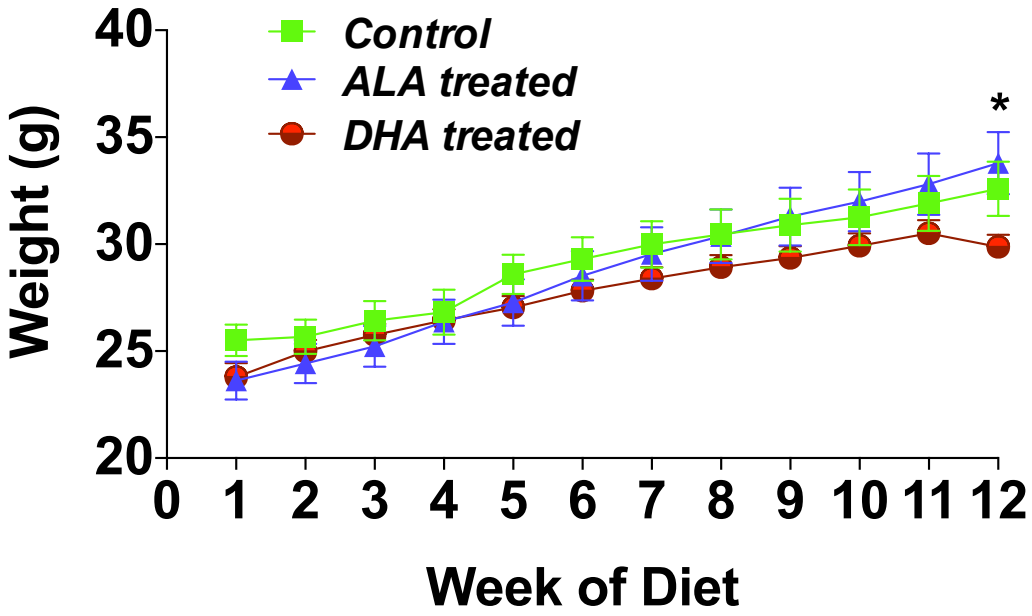

Figure 5.1. Body weight changes in response to n3FA feeding. Male ApoE$^{-/-}$ mice were fed HFD (Western-type diet) alone or HFD and docosahexaenoic acid (DHA) or α-linolenic acid (ALA) (300mg/kg/day) (n=12/group) and body weight in grams were recorded weekly. Graph shows a significant reduction in the body weight among the DHA group compared to ALA group at week 12. Data are mean ± SEM, analysed by 2-way ANOVA followed by Tukey's test, *$p<0.05$.

5.3.2. Blood pressure changes in response to fatty acid feeding:

To study the anti-hypertensive properties of DHA and ALA in the atherosclerotic mouse model, the three groups of mice were monitored for changes in their blood pressure weekly for the duration of the study.

The mean values of systolic (SBP), diastolic (DBP) and mean (MBP) blood pressure (BP) in each group at the baseline (before initiating feeding), and weekly for 12 weeks, are shown in figure 5.2A-C. The mean individual blood pressure in the mice fluctuated from week to week. However, overall trends among the studied groups showed that the DHA group had a significant drop in their blood pressure (SBP, DBP and MBP) compared to the control and the ALA groups.

Between the control and ALA groups, the SBP, DBP and MBP showed an incremental, yet non-significant, difference in blood pressure at any given week. At baseline, the SBP between the two groups was 115.3 ± 3.21mmHg in the control group and 110.1 ± 7.849mmHg in the ALA supplemented mice. Subsequently the SBP rose to reach 159.7 ± 2.842mmHg vs. 157.8 ± 7.274mmHg, in control and ALA groups, respectively, at week 12 (Figure 5.2A). The DBP and the MBP in the two groups follow the same trend (Figure 5.2B & C).

By comparison to controls, the SBP, DBP and MBP were remarkably lower in the DHA fed mice. At baseline, the SBP in both groups (control vs. DHA) was 115.3 ± 3.21 vs.109.3 ± 8.88mmHg, p=ns. Subsequently, there was a rise in the SBP between the two groups until the mice reached week 5 of feeding, when the SBP significantly declined in DHA fed mice compared to the control group (132.3 ± 9.18 vs. 146.6 ± 3.563mmHg, respectively, $p<0.05$). The SBP continued to drop in the DHA fed group until the study ended. At week 12, the SBP reached an average of 119.5 ± 7.33mmHg in the DHA group compared to 159.7 ± 2.482mmHg, $p<0.001$ in the control mice (Figure 5.2B).

The DBP in the DHA fed mice compared to the control group also significantly decreased in the former compared to the latter group (75 ± 14.65 vs. 100.5 ± 7.549, respectively, $p<0.01$) (Figure 5.2B & C).

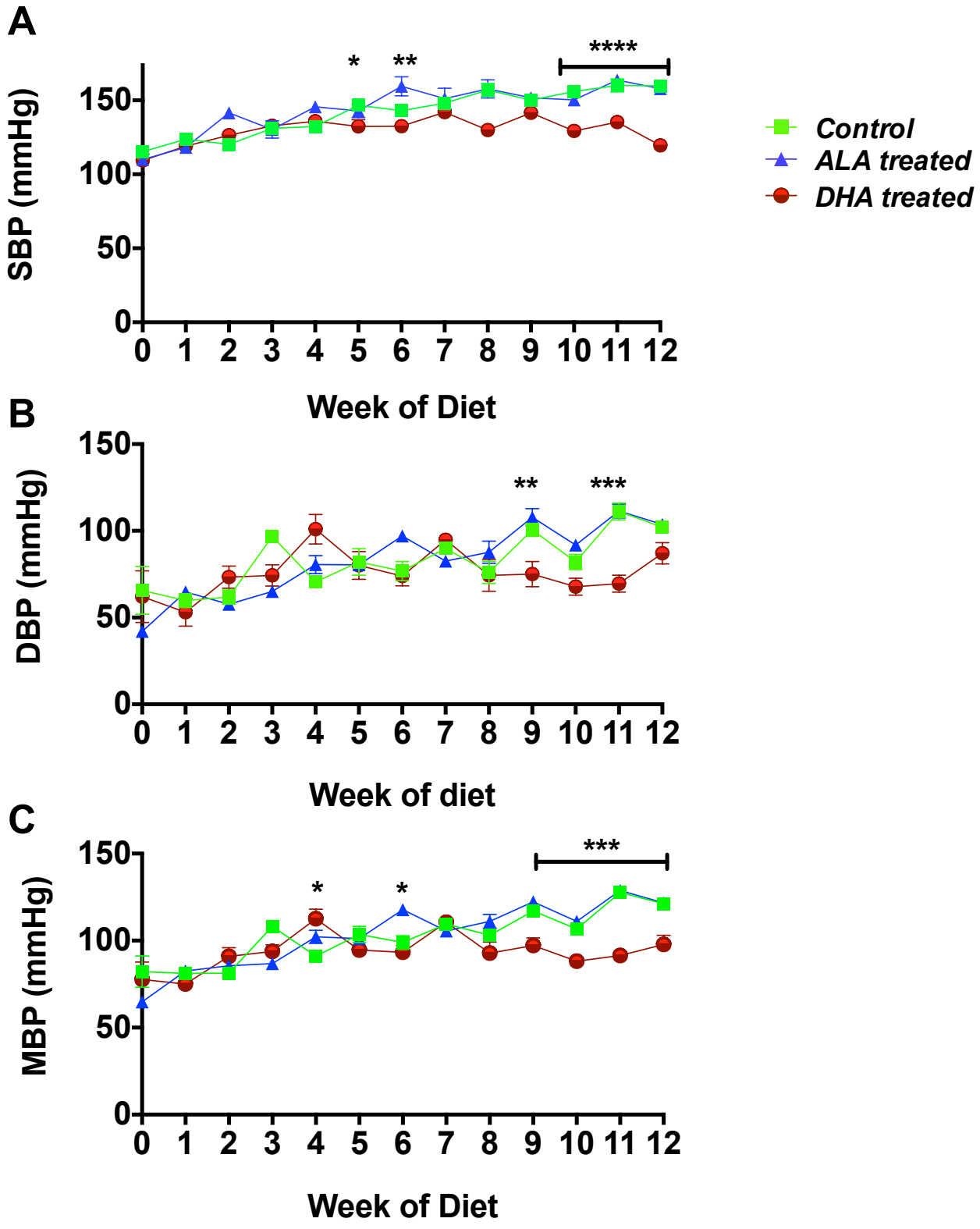

Figure 5.2. DHA but not ALA attenuates HFD induced hypertension in ApoE$^{-/-}$mice. A) Systolic (SBP), **(B)** diastolic (DBP) and **(C)** Mean (MBP) blood pressure were measured in ApoE$^{-/-}$ mice fed either HFD alone (control) or HFD and DHA (DHA treated) or ALA (ALA treated) for 12 weeks (n=4/group) using a tail cuff (see methods for details). All data are expressed as mean ± SEM, analysed by 2-way ANOVA and Tukey's post-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.3.3. Left ventricular mass and function in response to fatty acid feeding:

Despite aggressive medical treatments, the majority of IHD patients develop left ventricular (LV) dysfunction and heart failure (HF) (Nichols et al., 2014). In addition to myocardial ischemia and infarction, pressure overload and systemic hypertension are contributing factors for LV dysfunction (Carabello et al., 1992).

The changes in blood pressure of mice in response to fatty acid feeding led me to investigate the possibility that there would be consequent changes in cardiac function.

Because no significant differences in the ALA fed animals were seen, in terms of their blood pressure response compared to controls, changes in LV wall thickness and function were only recorded in the DHA and the control groups. Echocardiographic measurements were analysed at week 0 (baseline) and at week 12 (+12 weeks) in these two groups.

Echocardiographic findings showed changes in LV fractional shortening (LVFS) and ejection fraction (LVEF) in the DHA mice compared to control animals after 12 weeks. At baseline, the average %LVFS in the control group was 43.83 ± 3.180%, n=4 which was reduced after 12 weeks of high fat diet feeding (35.41 ± 3.129%, n=4), although this was not significant (Figure 5.3A).

In the DHA fed animals, LVFS was significantly increased after 12 weeks compared to the basal levels (45.90 ± 6.05 vs. 28.42 ± 6.25%, $p<0.05$, respectively) (Figure 5.3A).

The %LVEF follows a similar trend to that of LVFS in the control group after 12 weeks of feeding (Figure 5.3B). In the DHA fed group, %LVEF increases after 12 weeks of DHA feeding, although this was not statistically significant compared to the baseline levels (74.98 ± 6.091% after 12 weeks vs. 54.74 ± 10.38% at baseline) (Figure 5.3B).

In comparison to the basal levels, the cardiac output (COP) did not show any significant differences between the control and DHA groups after 12 weeks of feeding (22.24 ± 4.912 in the control group vs. 27.56 ± 10.58 ml/min in the DHA fed mice, figure 5.3C).

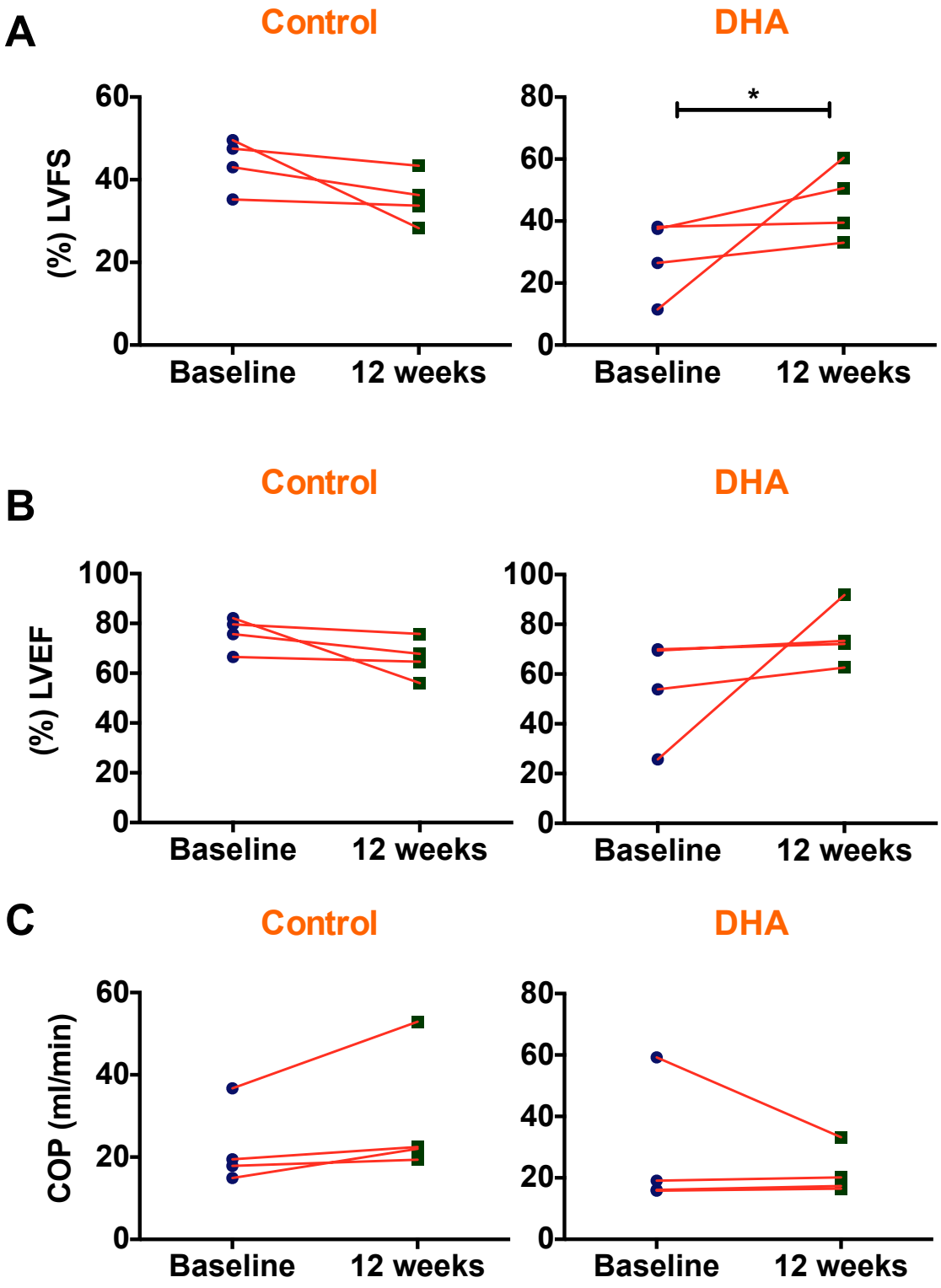

Figure 5.3. Left ventricular function in response to n3FAs. Effect of 12 weeks of feeding of HFD alone (control) or HFD and DHA (300mg/kg/day) on **(A)** Left ventricular fractional shortening (%LVFS), **(B)** left ventricular ejection fraction (%LVEF) and **(C)** cardiac out put (COP). Data were measured in anaesthetised mice with the help of Mrs Nadine Arnold. Data are presented as mean ± SEM, (n=4/group), analysed by unpaired Student's t test, *p<0.05.

Cardiac enlargement and LV hypertrophy (LVH) is one of the observed features among hypertensive patients and is considered an alarming sign among poorly controlled hypertension in IHD patients (Drazner, 2011). Therefore, in my study, I sought to determine if there is a LVH in response to HFD feeding, and if there is any improvement in the LVH, correlated with the observed reduction in BP, in DHA fed animals.

LVH was assessed using echocardiography by direct measurements of LV cavity diameter (internal diameter during diastole; LVIDd) and corrected LV mass (LVM), both at baseline and after 12 weeks of dietary supplementation with DHA (see chapter 2 for the detailed methods).

In the control group, there was no significant reduction in LVIDd after 12 weeks of HFD feeding compared to the baseline diameter (2.963 ± 0.158 vs. 3.195 ± 0.235mm, respectively, n=4) (Figure 5.4A). Likewise, in the DHA supplemented group, there was no significant difference in the LVIDd after 12 weeks of the supplementation compared to the baseline (2.703 ± 3.978 vs. 2.963 ± 0.1481mm, respectively, n=4, Figure 5.4A), nor was there a significant difference or to the control group (2.703 ± 0.3978 vs. 2.963 ± 0.1583, n=4, respectively) (Figure 5.4A).

Within the control group, LVM significantly increased after 12 weeks of HFD compared to the baseline levels (191.2 ± 23.63 vs. 114.1 ± 22.02mg, respectively, n=4, $p<0.05$) (Figure 5.4B), suggesting LV hypertrophy in response to HFD feeding.
In the DHA fed mice, there were no significant differences in the LVM at week 12 compared to baseline measurements (114.5 ± 6.655mg vs. 108.3 ± 2.208mg) (Figure 5.4B). However, in comparison to the control group, LVM significantly decreased in the DHA group after 12 weeks (191.2 ± 23.63mg vs. 114.5 ± 6.655mg, n=4, $p<0.05$) (Figure 5.4C).

Collectively, these data suggest that DHA supplementation protects against LVH induced by HFD feeding in ApoE$^{-/-}$ mice, secondary to the high blood pressure.

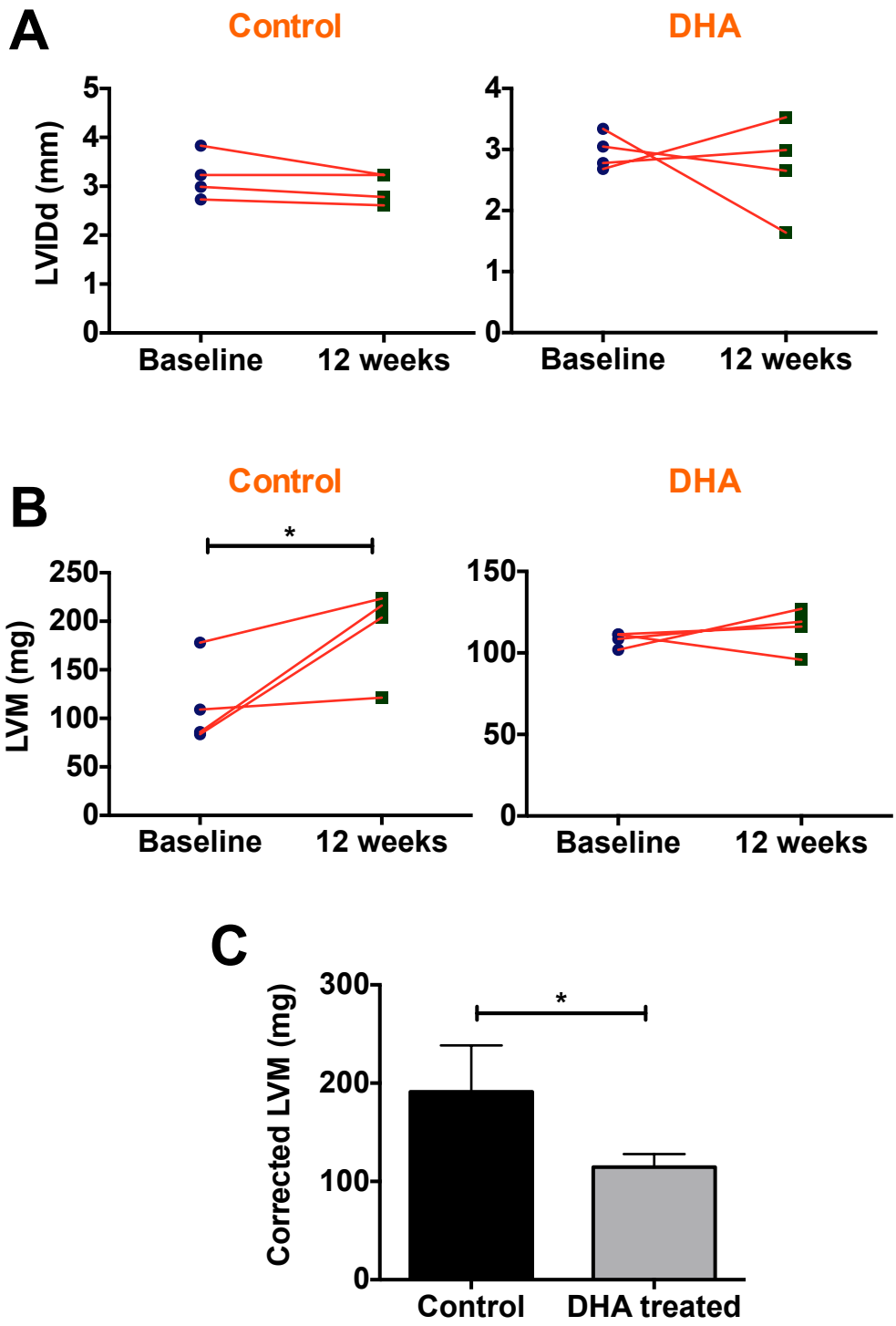

Figure 5.4. DHA decreased LVH induced by HFD. Effect of 12 week feeding of HFD alone (control) or HFD and DHA (300mg/kg/day) on: **A)** LV wall and cavity dimensions (LVIDd, mm) and **(B & C)** corrected Left ventricular mass (LVM) calculated by echocardiography. Measurements were performed by the help of Mrs Nadine Arnold. Data are mean ± SEM, n=4 mice/group, and analysed by unpaired Student's t test (*p<0.05).

5.4. Erythrocyte membrane and fatty acid changes in response to DHA and ALA feeding:

The fatty acid intake into the body by the mice was further confirmed by the changes in the fatty acid composition in the red blood cell (RBC) membranes. RBC omega-3 compositions and indices were measured by BioLab, London, UK, in pooled blood samples (pooled samples from 4 mice per group) (detailed methods in chapter 2).

As expected, the amount of DHA in the DHA fed mice was increased (average of 38µmol/L), with no changes in the other fatty acids levels, compared to control or ALA fed mice (Figure 5.5A). Surprisingly, however, consumption of ALA did not increase ALA levels in the RBCs of ALA-fed mice compared to control and DHA fed mice. However, there was a slight increase in eicosapentaenoic acid (20:5n-3, EPA) concentration (approximately 12µmol/L) compared to the control and DHA fed mice (3µmol/L and 10µmol/L, respectively). Changes in eicosatetraenoic acid (20:4n-3, ETA) levels were not different between the three groups.

The red blood cell omega-3 index has been linked in previous clinical studies with MI risks in IHD patients (Salisbury et al., 2012). High omega-3 index ((DHA+EPA / Total RBC fatty acids) X 100) has also been considered as an independent risk factor for plaque stability (Harris, 2007).

Therefore, I measured the omega-3 index (%) in the RBCs isolated from the three groups of mice. Interestingly, the RBC omega-3 index in DHA fed mice increased with an average of 1.3% compared to the ALA fed mice (0.7%). In the control mice, the detected index was 1% (Figure 5.5B).

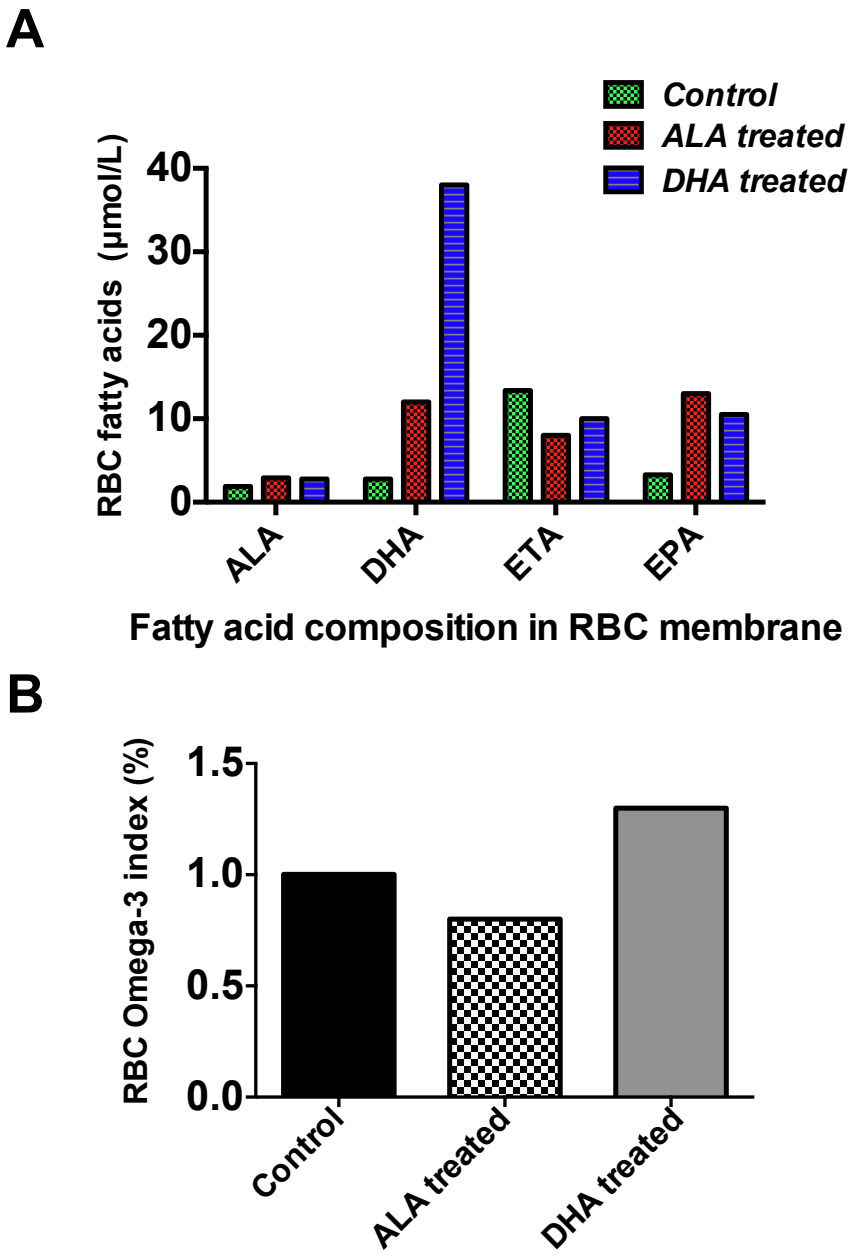

Figure 5.5. Fatty acid composition in RBCs of ApoE$^{-/-}$ mice fed HFD alone or HFD and ALA or DHA for 12 weeks. A) Fatty acid composition (µmol/L) in the RBC membrane (pooled blood from 4 mice per group). **B)** RBC omega-3 index (%) among the studied groups from pooled blood (4 mice per group). ALA indicates alpha-linolenic acid (18:3n-3), DHA; docosahexaenoic acid (22:6n-3), ETA; eicosatetraenoic acid (20:4n-3), EPA; eicosapentaenoic acid (20:5n-3).

5.5. DHA but not ALA reduces high fat diet induced atherosclerosis in ApoE$^{-/-}$ mice.

Having shown that BP and LV function change in response to the fatty acid feeding, atherosclerotic lesion formation in ApoE$^{-/-}$ mice fed HFD alone or HFD and DHA or ALA for 12 weeks was examined at three different sites in the vascular tree.

5.5.1. Proximal aortic atherosclerotic development in response to fatty acid feeding:

There was no significant difference in atherosclerotic lesion burden in the aortic sinuses among the three experimental groups after 12 weeks of diet consumption (Figure 5.6A & B).

Of note, using histological staining, the lesion in the proximal aortae after 12 weeks of feeding the mice HFD alone did not seem different than that in ALA mice (Figure 5.6A). However, aortic lesions in DHA fed mice showed, by eyes, phenotypic changes that were different than those in the controls e.g. the lesions seem to be less cellular than that of the controls (Figure 5.6A), even though quantitative analysis demonstrated similar results in atherosclerotic disease burden among all studied groups (Figure 5.6B).

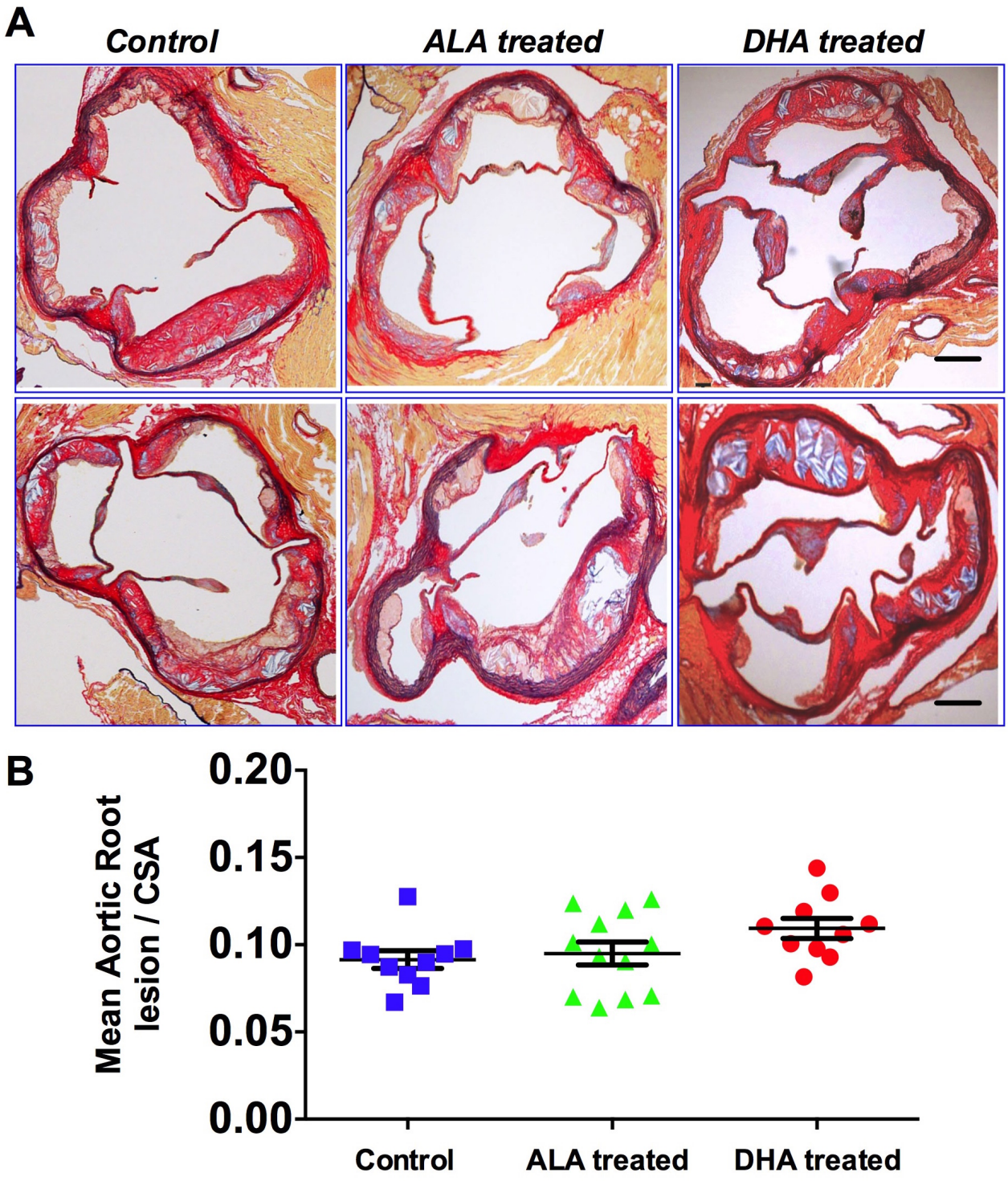

Figure 5.6. DHA & ALA supplementation has no effect on HFD induced atherosclerosis in the aortic roots of ApoE$^{-/-}$ mice. Starting at the age of 8 weeks, male mice were fed a Western diet (HFD) alone (control) or HFD and jelly containing ALA (ALA treated) or DHA (DHA treated) (300mg/kg/day each), daily over a 12-week duration. **A)** Representative stained aortic roots with alcian blue & elastic van Gieson (AB/EVG) of ApoE$^{-/-}$ fed HFD alone or HFD and DHA, or ALA over 12 weeks (n=12/group). Scale bars=100µm. **B)** Data analysed using NIS-Elements microscopy (Nikon, USA) representing mean ± SEM from 12 mice per group (n=10-11), analysed by One-way ANOVA, followed by Tukey's multiple comparison test.

5.5.2. Distal vessel atherosclerotic lesion formation in response to fatty acid feeding:

5.5.2A) Brachiocephalic atheromatous lesions:

Lesions in brachiocephalic arteries are usually small in size and show early phenotypic changes that are different from the complex lesions seen in the aortic sinuses. In addition, the lesions tend to be segmental and tend to occur at the areas of disturbed blood flow (Peng et al., 2009). Therefore, I was interested to examine this vascular bed and compare the effects of the two types of free fatty acids on brachiocephalic atheroma.

The atherosclerotic lesions in brachiocephalic vessels was analysed histologically and showed less atheromatous lesions in the DHA, compared to the ALA and the control, groups (Figure 5.7A).

Atherosclerotic plaque in the brachiocephalic arteries was quantified by measuring the ratio between the lesions to the total surface areas, and showed no significant difference between the control and ALA fed mice (0.303 ± 0.034 vs. 0.289 ± 0.01839, respectively). However, the lesion ratio was significantly lower in the DHA fed mice compared to the controls (0.1295 ± 0.01664 vs. 0.303 ± 0.034, respectively, $p<0.001$) (Figure 5.7B).

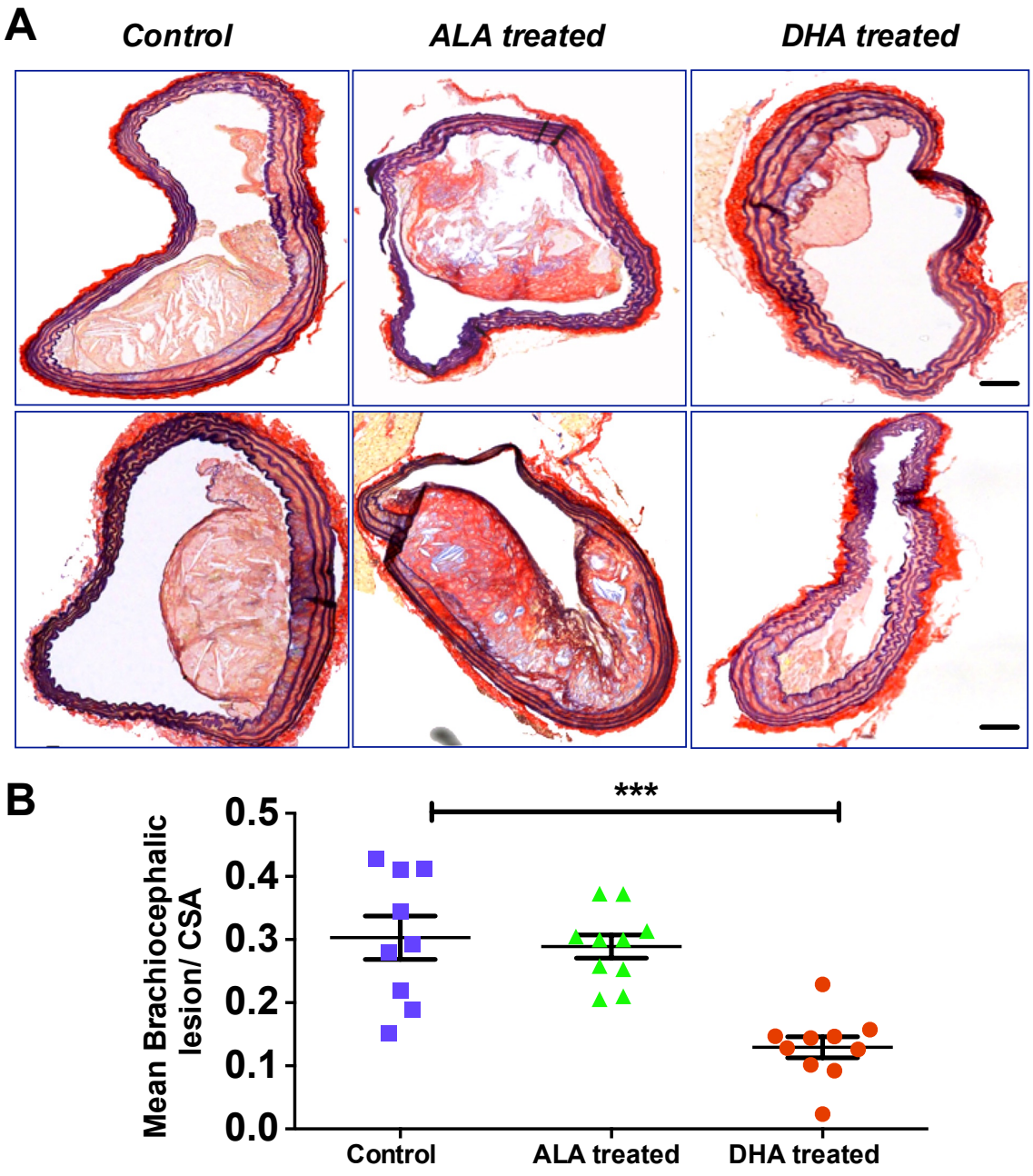

Figure 5.7. Atherosclerosis is ameliorated in the distal vessels by DHA supplementation but not ALA. Male ApoE$^{-/-}$ mice fed HFD alone or HFD plus ALA or DHA (300mg/kg/day each) daily for 12 weeks. At the end of the study brachiocephalic arteries were harvested and stained for alcian blue/elastic van Gieson stain. **A)** Representative photomicrographs (scale bars=100μm). **B)** Lesion areas analysed using NIS Elements software and calculated as mean lesion area to total cross sectional area (CSA). Data are expressed as mean ± SEM, and analysed by One-way ANOVA and Tukey's post-test, n=9-10/group, ***p<0.001.

5.5.2B) Whole & differential aortic atheroma:

To further investigate the differential response to DHA in the different vascular beds, I examined the atheromatous plaques in the whole aortae (from the aortic

infundibulum of the heart to the aortic orifice of the diaphragm) and the different segments (aortic arch and descending parts) of the aorta.

The atherosclerosis in the whole aortae of the studied groups was calculated as % of lesion area to the total aortic surface area.

ALA had no effect on atherosclerotic area, with no significant difference to that of the control group (4.931 ± 0.3662 vs. 6.373 ± 0.637%, respectively) (Figure 5.8 A & B). However, in DHA fed mice, there was approx. 30-40% decrease in the total aortic atherosclerotic area ($p<0.001$) (Figure 5.8 A & B). On examination of the different areas of aortae, this DHA effect was found to be concentrated in the descending aortic plaque: no effect of DHA supplementation was observed in the proximal aorta (aortic arch) (Figure 5.8C). However, DHA significantly attenuated the descending aortic plaque area by an average of 30% ($p<0.05$) (Figure 5.8D).

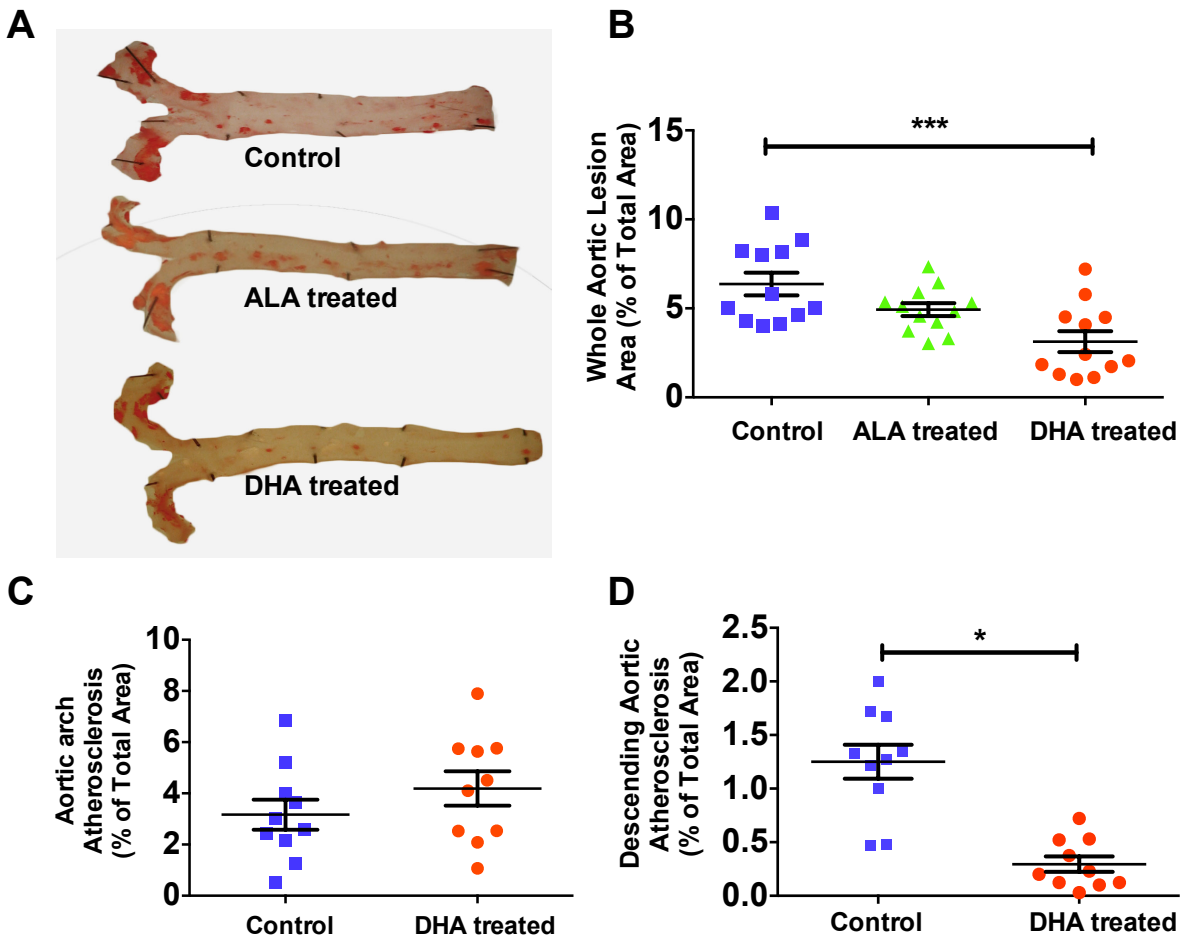

Figure 5.8. Differential effects of DHA feeding on different lesion areas of aortae. A) Representative *en face* morphometric images of the total aortic lesion area and **(B)** calculated whole aortic atherosclerosis (% of the total surface area), showing significant reduction in the total lesion formation in the DHA group compared to the control group. Data are mean ± SEM, analysed by One-way ANOVA and Tukey's post-test, ***p<0.001, n=11-12/group. **C) & (D)** Lesion areas in aortic arch and descending parts of aorta, respectively, in ApoE$^{-/-}$ mice receiving HFD alone or HFD and DHA over a 12 weeks duration and showing a significant reduction in atheroma in the distal part of aorta, n=10 per group. Data are mean ± SEM, analysed by unpaired Student's t test, *p<0.05.

5.6. DHA effects on atherosclerotic plaque composition:

Since no significant difference in atherosclerosis of mice fed ALA, subsequent studies will focus entirely upon DHA effects in the DHA treated mice and compare that with the control mice.

To elucidate the differences in the composition of the atheroma in response to DHA feeding, I investigated collagen and smooth muscle content in the plaques. The aortic root and brachiocephalic atheroma was stained for collagen using martius scarlet blue (MSB) and for smooth muscle actin using immunohistochemistry (IHC-P).

5.6.1. Collagen content:

Aortic roots from the two groups (control vs. DHA fed mice) were analysed for their collagen content. Collagen in aortic roots and is shown as a bright blue stain using MSB stain (Figure 5.9 A).

High power magnification showed lesions from both DHA and control groups had widely distributed collagen fibres in the intimal area, predominately located in the cap and the shoulders of the lesions (Figure 5.9A).

DHA-fed mice appeared to have less collagen in the atheromatous cap compared to controls. Quantification of the percentage of total collagen in both groups, however, did not show any significant difference (0.4096 ± 0.2226% in control vs. 0.4211 ± 0.03956% in DHA) (Figure 5.9B).

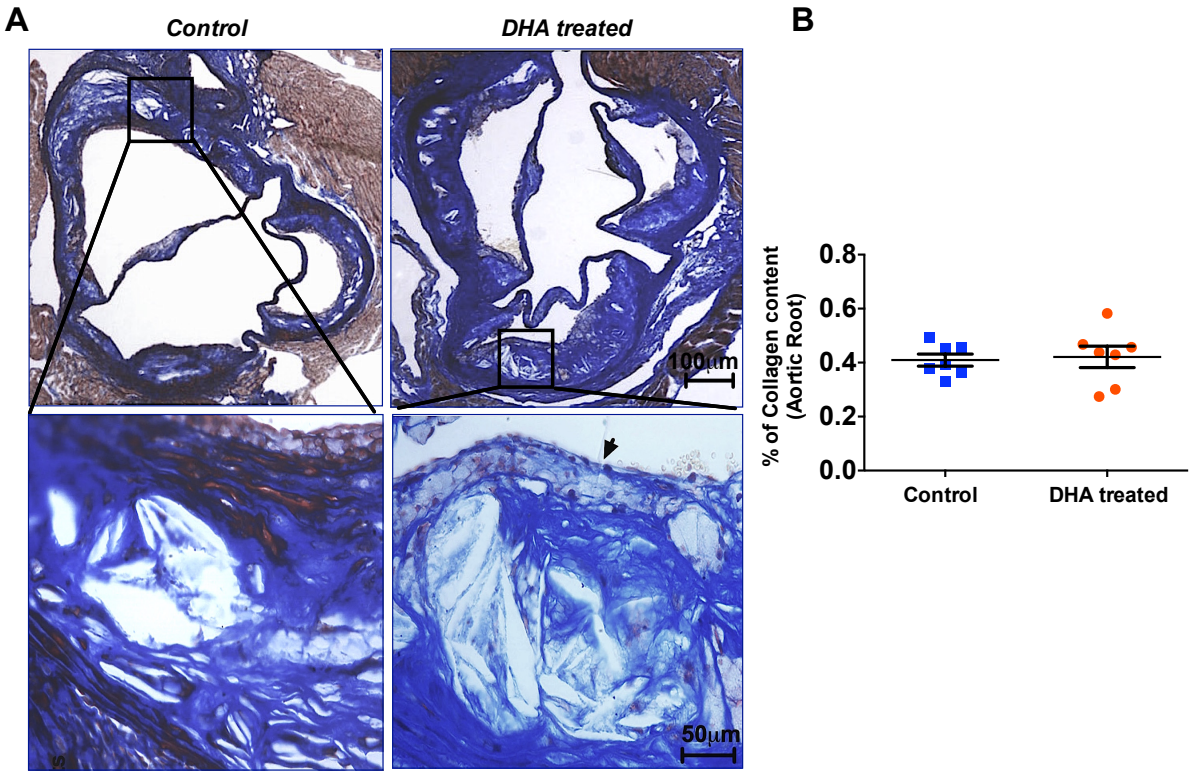

Figure 5.9. Collagen content in aortic root of studied groups. Representative photomicrographs of aortic roots **(A)** of ApoE$^{-/-}$ mice fed with HFD alone or HFD and DHA for 12 weeks, stained for martius scarlet blue. Collagen stains as a bright blue. Scale bars=100µm and 50µm, arrowheads indicate the cap**. B)** Quantification of collagen content between the two studied groups within the aortic roots, measured as % of the total lesion area. Data are shown as mean ± SEM, n=7/group, analysed by unpaired Student's t test, p=ns.

The collagen content in the lesions of the brachiocephalic arteries followed similar trends, also without any significant difference (Figure 5.10 A & B).

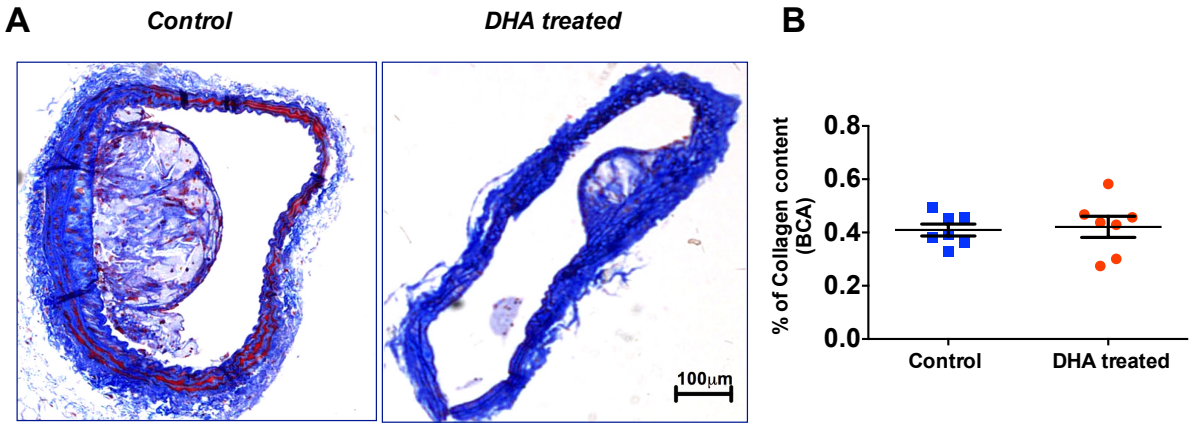

Figure 5.10. Collagen content in brachiocephalic arteries of studied groups. Representative photomicrographs of brachiocephalic (BCA) **(A)** of ApoE$^{-/-}$ mice fed with HFD alone or HFD and DHA for 12 weeks, stained for martius scarlet blue. Collagen stains as a bright blue. Scale bars=100µm. **B)** Quantification of collagen content between the two studied groups within BCA measured as % of the total lesion area. Data are shown as mean ± SEM, n=7/group, analysed by unpaired Student's t test, p=ns.

5.6.2. Smooth muscle actin content:

Since no observed effects for DHA feeding on the collagen content, I next asked whether DHA alters SMA content in the plaques and thus would explain the atheroprotective effects of DHA. Using immunohistochemical staining, α-smooth muscle actin (SMA) was quantified in the lesions of the aortic roots.

Positive (Brown) staining was observed in atheromatous plaque of both control and DHA-fed groups (Figure 5.11A). SMA was detected in the media and in very small amounts in the cap of the lesions (Figure 5.11A).

There was no significant change in the SMA content of the lesion between the two groups (11.36 ± 2.949% in control vs. 20.39 ± 2.704% in DHA) (Figure 5.11B).

SMA protein was also examined in the whole aortae using immunoblotting. There was no significant difference between the two groups in the whole aortic SMA (Figure 5.11C), confirming the aortic root findings and suggesting that DHA has no effect on SMA content in different aortic atherosclerotic parts.

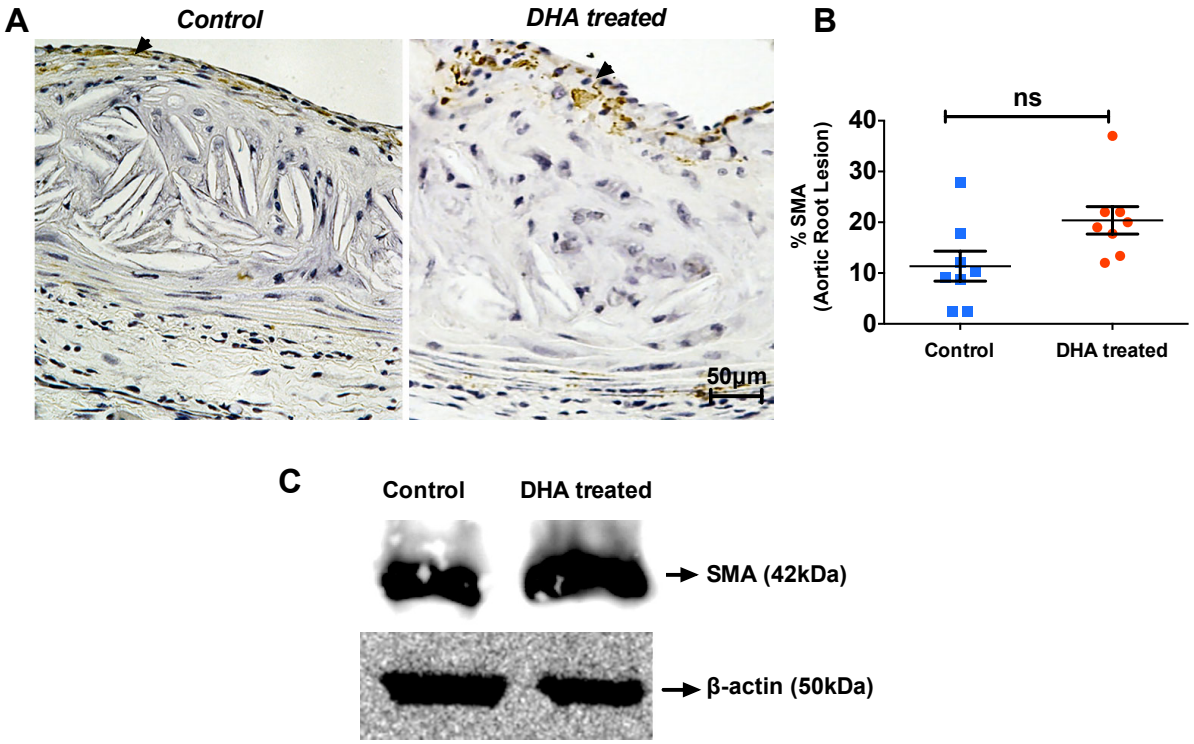

Figure 5.11. Smooth muscle actin distribution in aortic atherosclerotic plaques of ApoE$^{-/-}$ mice in response to DHA feeding. Male ApoE$^{-/-}$ mice were fed HFD alone or HFD and DHA (300mg/kg/day) for 12 weeks. **A)** α-Smooth muscle actin (SMA) content, identified using immunohistochemistry (brown stain, arrowheads), within the plaques. Scale bar=50µm. **B)** Graph showing percentage of positive SMA area to the total lesion area, n=8/group. Data are shown as mean ± SEM and analysed by unpaired Student's t test. **C)** A representative immunoblot from n=4/group mouse aortae showing no differences in SMA protein expression between the groups, protein concentration=34µg/ lane.

5.7. DHA effects on plasma dyslipidaemia:

Having identified that DHA has no effects on atherosclerotic collagen and SMA, I wanted to investigate whether DHA decreases atherosclerosis by an anti-lipidemic effect.

The mean values for plasma lipid and lipoprotein levels at the end of the study (week 12) for DHA-fed and control mice are shown in figure 5.12. Between the two groups, there was no statistical difference in TC (28.39 ± 1.751mmol/L in DHA-fed vs. 26.62 ± 1.299 mmol/L in control) (Figure 5.12A).

Plasma TG levels, however, were significantly higher in the DHA fed animals compared to control (3.622 ± 0.2929 vs. 2.31 ± 0.2501mmol/L, respectively, $p<0.01$) (Figure 5.12B). There were no significant changes in the LDL-C levels between the

two groups (control vs. DHA; 20.89 ± 1.359 vs. 23.33 ± 1.794mmol/L, respectively) (Figure 5.12C); however, the HDL/cholesterol ratio significantly increased in the DHA supplemented mice compared to control mice (10.77 ± 1.858 vs. 6.63 ± 1.0247mmol/L, respectively, $p<0.05$) (Figure 5.12D).

DHA has been shown to have a blood glucose lowering effect (Woodman et al., 2002), therefore, I measured the plasma glucose levels between the studied mice. There was no significant difference in the glucose levels between the control and the DHA groups (10.61 ± 1.410 vs. 11.2 ± 1.531mmol/L, respectively) (Figure 5.12E).

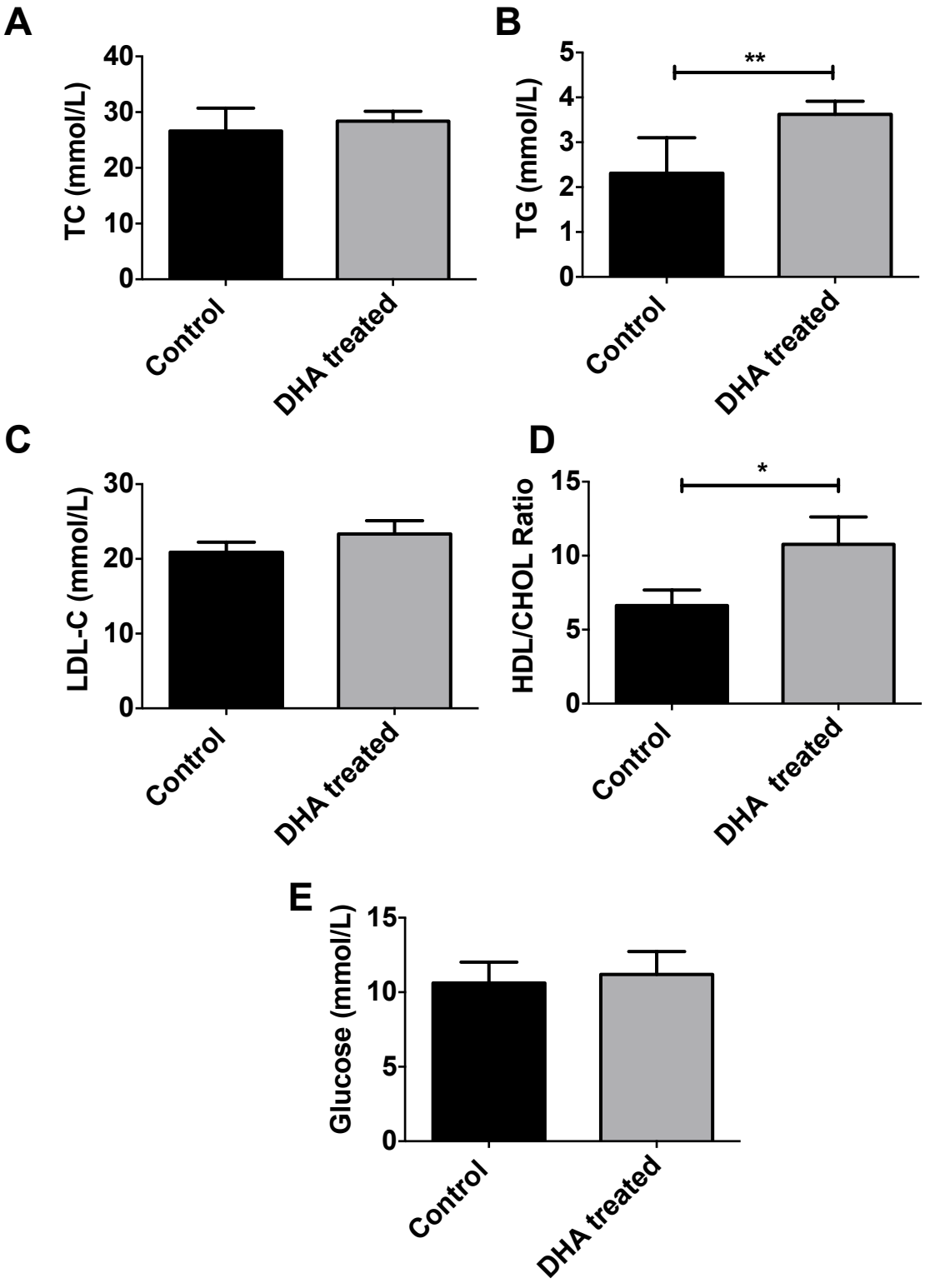

Figure 5.12. DHA supplementation improves diet-induced dyslipidaemia. Male ApoE$^{-/-}$ mice (8 weeks of age) were fed either HFD alone or HFD and DHA (300mg/kg/day) daily for 12 weeks. Plasma samples were collected after 12 weeks by cardiac puncture. **A)** Plasma total cholesterol (TC), **(B)** total triglycerides (TG), **(C)** Low density lipoprotein cholesterol (LDL-C) (mmol/L), **(D)** high density lipoprotein/total cholesterol (HDL/CHOL) ratio, and **(E)** plasma glucose levels (mmol/L) were measured at the Department of Clinical Chemistry (Royal Hallamshire Hospital, Sheffield, UK), n=10/group. Data are shown as mean ± SEM, analysed by unpaired Student's t test, *p<0.05, **p<0.01.

5.8. Summary:

The work presented in this chapter sheds light on the defined DHA effects as being anti-atherogenic and anti-hypertensive in ApoE$^{-/-}$ mice. Results were obtained following 12 weeks of feeding and are summarised below:

1. DHA feeding attenuates blood pressure, mainly systolic, in ApoE$^{-/-}$ mice fed a HFD.
2. ALA supplementation did not show any significant effects on blood pressure or atherosclerosis in ApoE$^{-/-}$ mice fed HFD.
3. DHA supplementation in ApoE$^{-/-}$ mice was associated with an increase in RBC omega-3 index and DHA compositions in the RBC membranes.
4. DHA fed mice had a significant attenuation in atheroma formation, predominantly in the distal parts of aorta, but not in the proximal aorta.
5. In addition, lesion area was significantly reduced in brachiocephalic vessels in the DHA supplemented mice but not the ALA fed mice.
6. The aortic root lesion of the DHA fed mice showed no significant difference in the total amount of collagen content, but the distribution of collagen showed there was less in the cap.
7. SMA content in the aortic roots showed no significant difference between the DHA fed and the control mice.
8. DHA fed mice had a significant increase in their plasma TG and HDL/TC ratio compared to the control mice, but no significant change in the plasma TC, LDL, and glucose levels.

5.9. Discussion:

Omega-3 fatty acids, particularly docosahexaenoic acid (the major type of n3FA in fish oil) and α-linolenic acid (the most common land-based type of n3FAs), are mostly studied in research and have been shown to play an important role in preventive medicine (Winnik et al., 2011, Vedtofte et al., 2011).

To date, studies have reported that diet supplemented with n3FAs lead to increased incorporation of the n3FAs in the plasma lipoproteins with an elevation in plasma ALA, DHA and EPA (Leeson et al., 2002), that is associated with lower rates of plaque vulnerability (Kashiyama et al., 2011, Deckelbaum, 2010, Cawood et al., 2010). In clinical studies, DHA itself has been mostly mixed with EPA or given as fish oil, and thus its individual effects remain obscured. ALA may have a cardio-protective role, yet the overall evidence is mixed and remains inconclusive (Brouwer et al., 2004, Kromhout et al., 2010, Baylin et al., 2003).

Therefore, the atheroprotective roles of DHA and ALA have been questioned for a long time, yet not extensively studied. Several animal models of atherosclerosis have investigated the anti-atherogenic effects of n3FAs (Brown et al., 2012), but these studies did not directly define the differential effects of DHA and ALA on atherosclerosis. In addition, little information is known, especially on whether n3FAs can correct or modulate the progression of atherosclerosis.

I elected to study DHA over EPA as DHA is more commonly investigated for its antihypertensive effects (Stanley et al., 2013) and there is an endogenous conversion for DHA to EPA occurring mainly in the liver (Mozaffarian and Wu, 2011).

To my knowledge, this is the first study seeking to define the anti-atherogenic and antihypertensive effects of the two main types of n3FAs in experimental models of atherosclerosis.

This study is also the first to show different vascular bed responses to n3FA supplementation, and to link this to blood pressure modulation and inflammation. The consistency of the findings across the previous publications (Miller et al., 2014) enhances the generality of my findings and suggest a novel explanation of why, in clinical trials, n3FAs shown mixed results.

My primary hypothesis was that consumption of high concentrations of DHA and ALA are inversely associated with high fat diet induced atherosclerosis and hypertension.

To study the effects of DHA and ALA on atherosclerosis, I chose ApoE$^{-/-}$ mice, as these are one of the most routinely used animal models (Jawien et al., 2004). Apolipoprotein E is a gene responsible for the metabolic clearance of triglyceride rich lipoproteins from the circulation (Mahley and Ji, 1999) and the genetic deletion of this gene leads to elevation of the plasma levels of the triglycerides (Zhang et al., 1992). A high fat diet enriched in cholesterol was also used in this study to augment atherosclerosis and induce hypertension in these mice.
It has been shown that feeding of ApoE$^{-/-}$ mice for 12 weeks with a Western-type diet provides a complex lesion of atherosclerosis with different stages of atheroma similar to that detected in humans with an elevation of the systemic blood pressure (Getz and Reardon, 2006, Chamberlain et al., 2009). Therefore, to study the antihypertensive mechanism of action of DHA and ALA and investigate the vasculature for atherosclerosis, I fed these mice a Western-type high fat diet for 12 weeks.

In my study, body weight gain remained unaffected in the three groups of mice throughout the period of the study; however, the body weight was significantly attenuated at the end of the study in the DHA supplemented mice. This correlates with clinical findings that suggests a significant weight reduction in obese patients supplemented with FO (Kris-Etherton et al., 2002) and supports the importance of the chronic supplementation of DHA. Likewise, the finding that ALA has no effect on body weight gain in experimental animals is also consistent with previous studies (Petrik et al., 2000).

Previous evidence indicates that n3FAs have a blood pressure lowering effect in both normotensive (Sanders et al., 1981, Ayer et al., 2009) and hypertensive individuals (Wang et al., 2010). Additionally, studies using different animal models have shown that fish oil have indisputably a generalised blood pressure lowering effect (Chen et al., 1996). However, the individual anti-hypertensive effects of DHA

and ALA have been less studied in mice. In addition, it's unclear which type of n3FA in the fish oil has the prominent hypotensive properties.

My data suggest that DHA profoundly decreased systolic blood pressure, with a significant attenuation in the diastolic and thus, the mean blood pressure. My finding reinforces all the previous interventional studies (Sagara et al., 2011, Miller et al., 2014) and is in agreement with (Mori TA, 1999) who reported that DHA supplementation significantly attenuated 24h ambulatory blood pressure in hypercholesterolemic patients. Moreover, in animal based studies, it has been demonstrated that DHA reduces SBP in spontaneous hypertensive rats (Engler et al., 2003, Encarnacion et al., 2008) and aldosterone induced hypertension in dogs (Stanley et al., 2013). However, my study is the first to elucidate the hypotensive effects of DHA in experimental mouse model of atherosclerosis.

By contrast, ALA supplementation did not show any significant impact on blood pressure in ApoE$^{-/-}$ mice. ALA as an anti-hypertensive agent has been studied in a limited number of studies (Paschos et al., 2007). My finding is at odds with previous clinical studies that demonstrate the hypotensive effects of ALA in hypertensive individuals (Wang et al., 2010). A possible explanation is that mice may respond differently to ALA than humans. That is, mice are deficient in the enzyme that is responsible for the endogenous metabolism of ALA (Barcelo-Coblijn and Murphy, 2009).

A recent study has shown an independent clear association between LVM and atherosclerotic coronary disease in IHD patients (Kishi et al 2013). Moreover, previous publications have suggested similar findings in different subsets of patients and they support the potential role of higher LVM (an indicator for LVH) as a possible predictor of adverse coronary events in atherosclerosis and, thus, IHD (Kishi et al., 2013). In the present study, therefore, I studied the effects of DHA supplementation on HFD-induced LVH. Interestingly, DHA supplementation significantly attenuated LVM after 12 weeks of HFD feeding. My finding has recently supported by Morin and colleagues who have revealed a significant amelioration in LVM in rats fed HFD for 8 weeks, supplemented with DHA (Morin et al., 2015), suggesting a potential role for DHA in preventing LVH. However, the underlying mechanism is relatively unclear.

I used red blood cell omega-3 fatty acid compositions as an indicator of a successful feeding of fatty acids in the mice. Although no dietary correlation between omega-3 RBCs and n3FAs intake has been reported, the omega-3 contents in the RBC membranes are strongly correlated with the dietary feeding. In previous publications, it has been reported that in patients with MI who frequently consumed marine-derived food or on n3FA supplements had significantly higher levels of omega-3 levels in RBC membranes (Salisbury et al., 2011).

Consistent with all previous publications (Friesen and Innis, 2010, Sands et al., 2005, Brown et al., 1991), I showed that DHA fed mice had higher levels of DHA content in RBC membranes. By contrast, ALA fed mice did not show any significant increase in ALA or DHA content in RBCs. ALA, an essential fatty acid, needs to be converted in the body tissues by a desaturase enzyme into DHA and EPA (Gerster, 1998). It is possible that the mice are either deficient in this enzyme (Salem et al., 1996) or that the desaturation process of ALA in the mice is less efficient than that in the humans (Demar et al., 2008).
That may explain why in this study, feeding mice ALA did not offer any atheroprotection. This finding is at odds with a study by Winnik and colleagues (Winnik et al., 2011), where a reduction in atherosclerosis in $ApoE^{-/-}$ mice fed flaxseed oil containing ALA for 16 weeks was seen, especially at aortic roots.

In patients, the omega-3 index is used as a surrogate marker for prognosis of CAD, where patients with lower index <4% have a poor prognosis, 4-8% an intermediate and >8% a good prognosis (Harris et al., 2013). However, my work is the first to measure the index in mice on HFD for 12 weeks. In addition, in mice the cut off values for omega-3 indices (as those defined in humans) have not yet been studied.

A novel finding of the present study is that DHA supplementation significantly ameliorated atherosclerosis, mainly in the distal parts of the aorta and brachiocephalic arteries. The differential atherosclerotic responses for DHA between the aortic sinus and brachiocephalic vessels has been studied before by (Peng et al., 2009) who showed a reduction in the brachiocephalic lesions without effect on aortic atherosclerosis in $LDLR^{-/-}$ mice, but they did not propose any mechanism for such observations.

The finding of significant attenuation of total aortic lesion by DHA effects has not been published before. However, Wan *et al*., 2010 have suggested a significant reduction in aortic atherosclerosis of mice that have high plasma levels of n3FAs. Unravelling the mechanism(s) by which DHA decreases distal vessel atheroma and whether this reduction also applies to the hypotensive effects will require further elucidation. Possibilities include flow-mediated effects (i.e. disturbed blood flow in the proximal vessels may mask DHA effects on atherosclerosis); dysregulated signalling, or perhaps because DHA acts effectively on the early stages of atherosclerosis (distal segments harbour early atheroma than the complex lesion in the proximal segments).

The local anti-inflammatory effect of DHA has been associated with a thickening in the fibrous cap and thus more stable plaque formation (Libby et al., 2014). However, this was not the case in my study, especially in the aortic sinus lesions and I showed that DHA had no effect on SMA or collagen content. In light of previous work demonstrating that DHA suppresses SMC proliferation (Pakala et al., 1999, Yates et al., 2014), the mechanism of atheroprotection mediated by DHA may be quite complicated.

Much of the focus in clinical studies was on the lipid lowering effects of n3FAs (Harris, 1996, Kris-Etherton et al., 2002); however, in animal based research the data are controversial (Chang et al., 2010). For example, Brown and colleagues have demonstrated that n3FAs did not decrease plasma cholesterol in ApoE$^{-/-}$ mice (Brown et al., 2012). By contrast, a recent study has documented the lipid lowering effect of DHA in rats (Morin et al., 2015). Therefore, I sought to determine whether DHA reduces the plasma lipid profiles, namely TC, LDL and HDL in ApoE$^{-/-}$ mice.

Interestingly, DHA supplementation increased triglycerides and the HDL/cholesterol ratio without any significant impact on the total cholesterol or LDL-C levels. The finding of high levels of triglycerides in DHA fed mice was striking, and opposite to the clinical studies that showed that DHA decreases, instead of increases, TG (Mozaffarian and Wu, 2011). One explanation for this is that apolipoprotein E may be crucial for the triglyceride lowering effect of DHA and thus in ApoE$^{-/-}$ mice that lack apolipoprotein E, this leads to an increase rather than decrease in TG (Wan et al.,

2010). This explanation has been put forward by Brown *et al*., (2012) where they showed a remarkable reduction in TG levels upon DHA feeding in $LDLR^{-/-}$mice but not $ApoE^{-/-}$ mice.

Additionally, the hypertriglyceridemia detected in the DHA fed mice may intervene upon the response of aortic sinus atherosclerosis to DHA. That is, high TG may contribute to atherosclerosis and this may explain my findings of a lack of response observed in the proximal lesions, although I did not find any evidence to support this. Additional investigations are needed to determine the mechanism(s) of these observations and if similar responses are seen with patients.

Overall my data supports the notion of the atheroprotective effects of DHA. I speculate that DHA decreases the main mediators (pro-inflammatory cytokines) that orchestrate inflammation in atherosclerosis and thus reduces the recruitment of the inflammatory cells into the plaque, which subsequently attenuates plaque formation. Consequently, the attenuated lesion formation, with less inflammatory burden and consequent reduced vascular reactivity, could explain the mechanism of action of DHA in this setting, which is discussed in the next chapters.

Chapter (6) Biomechanical factors and DHA athero-protective effects.

OUTLINE:

6.1. Overview of Biomechanical factors and DHA atheroprotection:

Plaques prone to rupture is the underlying pathology observed in CAD (Falk et al., 1995). While it is accepted that the fate of the complex atherosclerosis is influenced by the size of the necrotic core and fibrous cap's thickness (Reininger et al., 2010, Schaar et al., 2004), it also depends on different haemodynamic parameters, including shear stress (Richardson, 2002, Tabas et al., 2015).

Although the risk factors for atherosclerosis, including hypertension and DM are systemic in nature (Fauci, 2008), the localised nature of atheromatous plaque has been appreciated since the earliest days of cardiovascular research. The predilection sites of the lesions are normally branching arteries where the blood flows slowly and in a nonlinear fashion, resulting in low or oscillatory shear stress (Caro et al., 1971).

Wall shear stress (WSS), a biomechanical fractional force induced by blood flow, is an important determinant of endothelial function (Cunningham and Gotlieb, 2005). Endothelial cells sense the changes in the WSS using mechanoreceptors (Tzima et al., 2005) and thus trigger release of multiple inflammatory mediators, including IL-1, that perpetrate inflammation and atherosclerosis (Wentzel et al., 2012).

Although no general consensus has been reached, it is accepted that the fast flow of blood in straight vessels generates protective high shear stress (HSS). Slow flowing blood in the inner curvature of the arch of aorta or upstream of stenotic atheromatous plaque is usually unidirectional and the shear levels are in a low amplitude (low shear stress; LSS) (Giannoglou et al., 2010). Emerging evidence has linked LSS with vulnerable plaque and MI (Cheng et al., 2006). Moreover, oscillatory shear stress, or index (OSI), involves changes in both amplitude and direction of blood flow, and is usually at the sites of bifurcation and downstream of stenosis, with high OSI also being associated with plaque instability (Giannoglou et al., 2010).

In the previous chapter, I demonstrated that DHA selectively decreases distal atherosclerosis in ApoE$^{-/-}$ mice and that this was associated with a remarkable reduction in arterial blood pressure. Recent evidence has suggested a close link between the rise in arterial blood pressure and aortic stiffness (Bryant et al., 2015), resulting in changes of aortic blood flow, direction and pattern (Hashimoto and Ito, 2015). However, the possibility of a change in the flow patterns being a mechanism

of the DHA protective effects is still obscure. Therefore, I hypothesise that the observed favourable changes in the ApoE$^{-/-}$ mice fed DHA are attributable to altered WSS in the distal vasculature.

My aim was to investigate the distal athero-protective effects of DHA and link these with altered WSS patterns.

6.2. Brief Methods:

Detailed Methods are discussed in chapter 2. In briefly:

To investigate effects of DHA on WSS, aortic blood flow mean velocity was assessed using pulsed wave doppler (PWD).

Computational fluid dynamics (CFD) was used to map wall shear stress (WSS) and oscillatory blood flow in different parts of aorta (Time averaged WSS over the cardiac cycle). The analysis was performed by Dr. Torsten Schenkel; Department of Engineering & Mathematics, Sheffield Hallam University, UK.

6.3. DHA effects on shear stress:

Changes in WSS magnitude and direction were calculated using changes in the diameter of the vessel wall and the blood flow velocity using doppler sonography (Figure 6.1A). To compute WSS geometry and examine the differences in WSS patterns, the velocity profiles over the cardiac cycle at the aortic roots were investigated.

6.3.1. DHA effects on aortic blood flow:

After 12 weeks of feeding, the mean velocity of aortic blood flow was assessed in the two experimental groups (Figure 6.1A & B). In the control group, there was an increase in % of the mean velocity at the aortic roots with an average increase of 20 ± 5%, n=4, compared to baseline (before start of high fat diet). However, following DHA feeding, there was a significant reduction in the mean aortic velocity by 15 ± 9% compared to controls at 12 weeks ($p<0.05$, n=4/group) (Figure 6.1B).

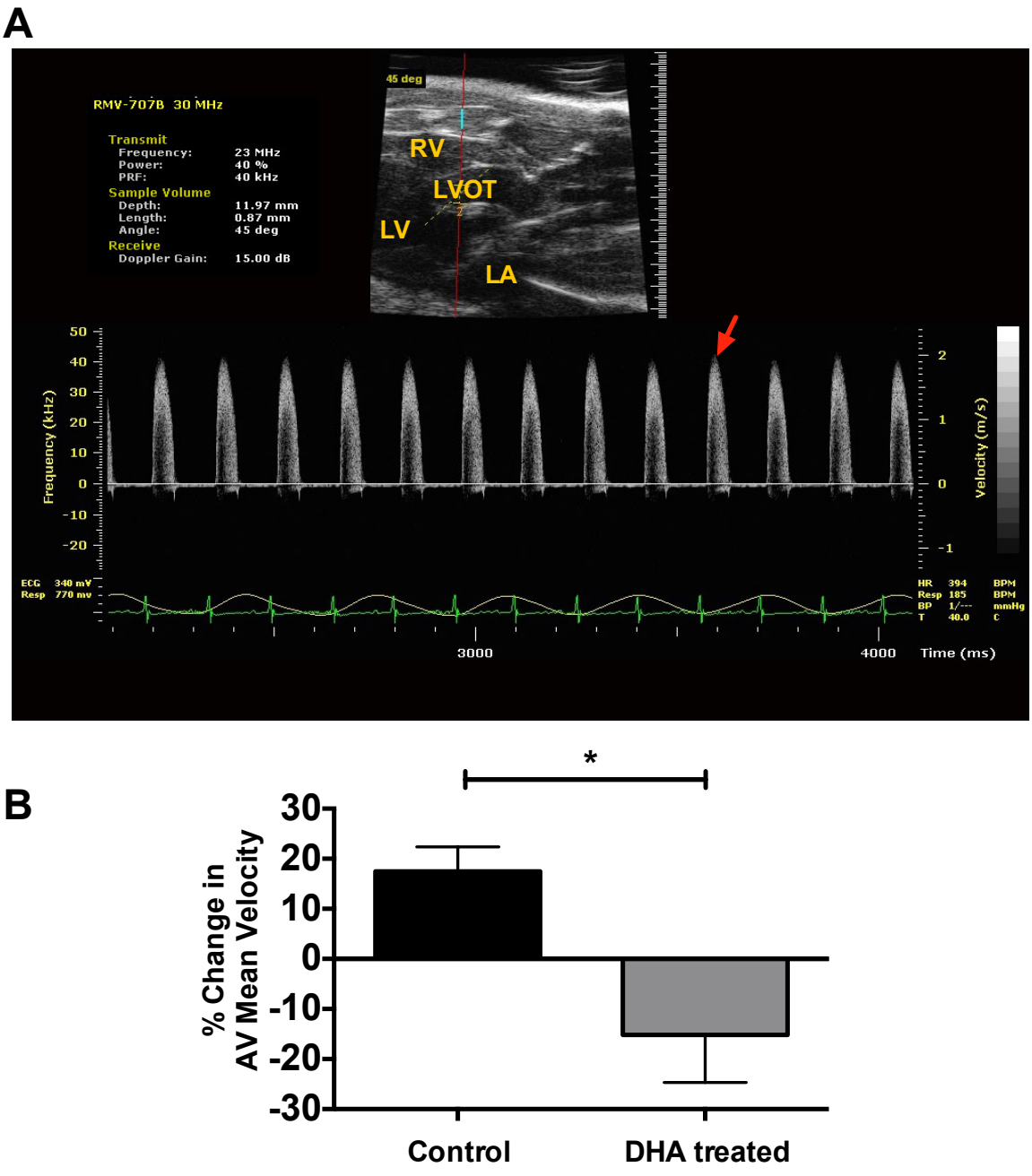

Figure 6.1. DHA significantly prevents the rise in aortic blood mean velocity. Male ApoE$^{-/-}$ mice were fed HFD alone or HFD plus DHA (300mg/kg/day each) daily for 12 weeks. At the end of the study pulse wave doppler was recorded in anaesthetised animals. **A)** Apical long axis view showing left ventricular outflow tract (LVOT) where blood velocity was measured over the cardiac cycle. LV & RV indicates left and right ventricles, respectively whereas LA indicates left atrium. The analysis was performed using ultrasound-imaging system (Vevo 770®, Visual Sonics, Toronto, Canada) where doppler shift frequencies were translated into velocities plotted as white points (red arrow) and generated a flow profile. The x-axis demonstrates the time (ms) whereas the y-axis indicates velocity in m/s. **B)** Echocardiographic analysis showing that exposure to high fat diet for 12 weeks increased flow velocity in aortic roots (average increase was 20 ± 5%) whereas exposure to a high fat, DHA rich diet led to a reduction in flow velocity (average decrease was -15 ± 9%)**.** Data are mean ± SEM, n=4/group, analysed by unpaired Student's t test, *p<0.05.

6.3.2. DHA effects on amplitudes of shear stress in the aortic arch:

Computed WSS patterns were plotted in the inner and outer curvatures of the aortic arch.

CFD simulations were performed using time-varying waveforms from control versus DHA treated mice as inlet boundary conditions and using a single representative geometry as described by (Van Doormaal et al., 2012). The kinetic changes in the WSS magnitude were plotted over the cardiac cycle and for distinct anatomical positions.

Figure 6.2 (top panel) shows the kinetics data on WSS as plotted over a 0.25s period of the cardiac cycle. At baseline, the magnitude of WSS reached a transit peak, which was around (30 Pa). After 12 weeks of feeding with DHA, this magnitude was slightly raised, approximately (33 Pa), before it rapidly declined (Figure 6.2). By contrast, in controls, the WSS magnitude was relatively higher than in the DHA treated group, with a transit peak of 40 Pa before it rapidly declined (Figure 6.2).

Figure 6.2 (lower panel) shows in graphical form the WSS maps in each of the experimental conditions (control vs. DHA treated animals). It can be seen that, in the inner curvature, the shear stress is relatively low in the DHA treated group compared to controls, yet not considerably different to the shear levels at baselines.

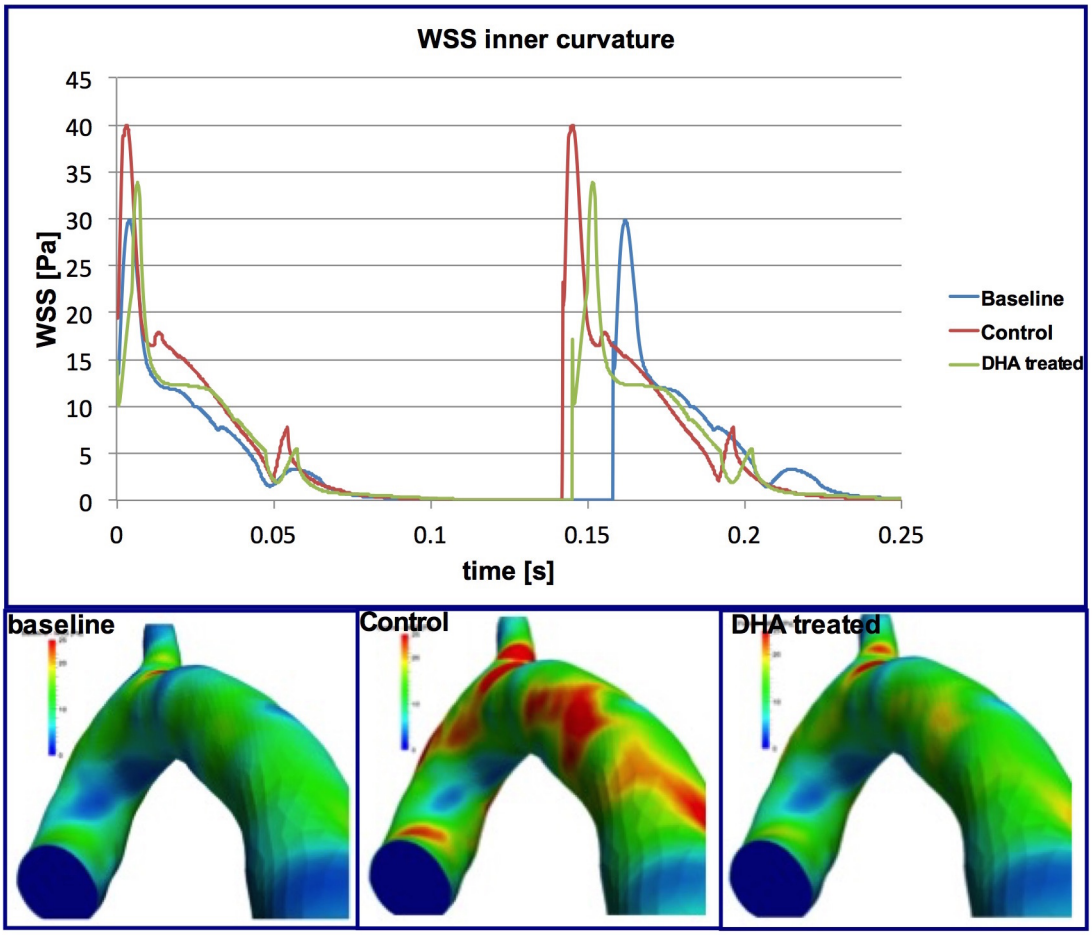

Figure 6.2. DHA effects on WSS amplitude in the inner curvature of the aortic arch. Starting at the age of 8 weeks, male mice were fed Western diet (HFD) alone (control) or HFD and DHA (DHA treated) (300mg/kg/day each), daily over a 12-week duration. Relative WSS distribution in the inner curvature of the arch of aorta was analysed using CFD at baseline and after 12 weeks of feeding, and show relatively lower shear stress in DHA treated mice compared to controls (n=4/group).

In the outer aortic curvature, the trends in the two experimental groups were similar (Figure 6.3). The peak WSS was relatively higher in the controls compared to the DHA and the baseline levels (Figure 6.3).

Additionally, the peak WSS was relatively higher in the inner (Figure 6.2) compared to the outer curvature for most of the cardiac cycle (Figure 6.3).
These observations were also clearly seen in the mapping time averaged WSS (Figure 6.3, lower panel).

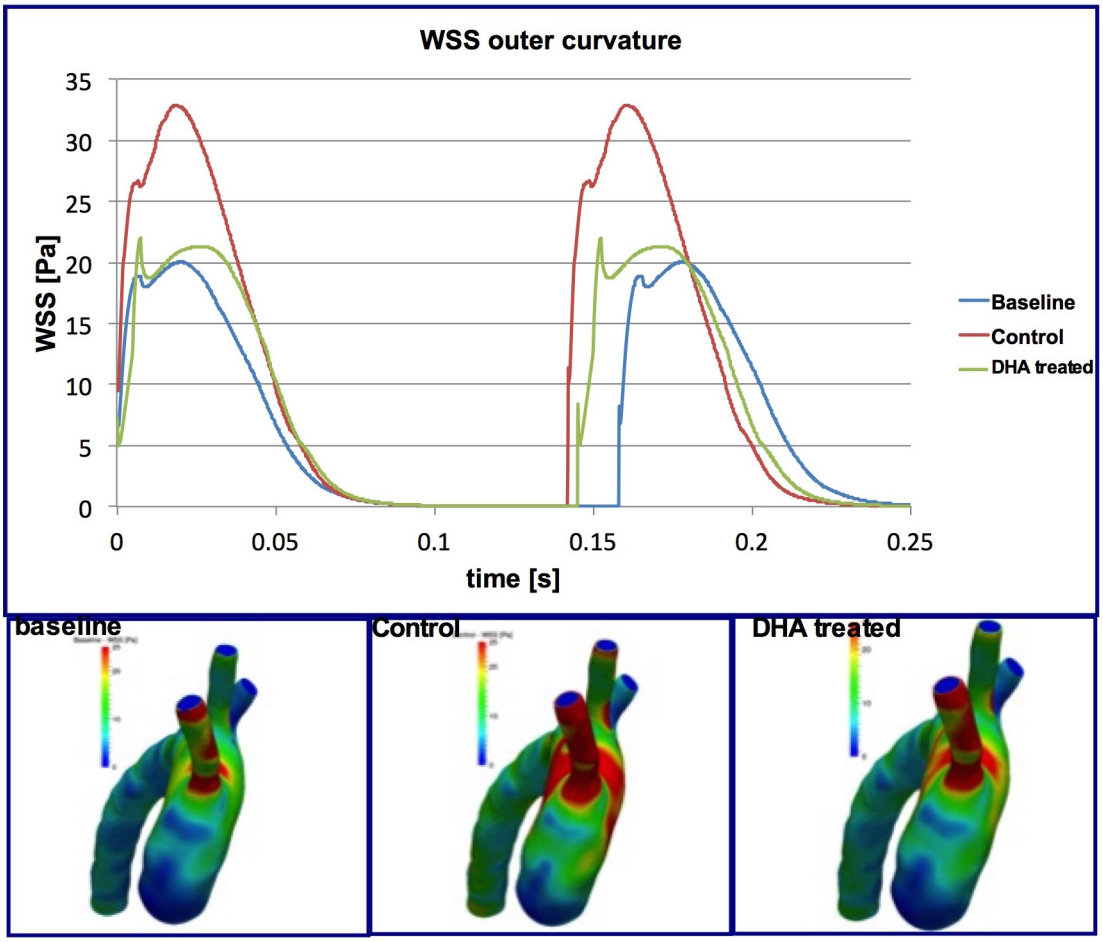

Figure 6.3. DHA effects on WSS amplitude in the outer curvature of the aortic arch. Starting at the age of 8 weeks, male mice were fed Western diet (HFD) alone (control) or HFD and DHA (DHA treated) (300mg/kg/day each), daily over a 12-week duration. Relative WSS distribution in the outer curvature of the arch of aorta was analysed using CFD at baseline and after 12 weeks of feeding, and show relatively lower shear stress in DHA treated mice compared to controls (n=4/group).

6.4. DHA lowers oscillatory shear stress associated with decreased atherosclerosis:

OSI distribution under the applied flow and boundary conditions within the aorta between the control and DHA treated groups was also assessed using CFD. The oscillation in the flow at the inner and outer curvatures of the aortic arch is shown in figure (6.4).

In the controls, OSI was slightly higher, predominantly in the outer aortic curvature, compared to baseline (Figure 6.4). By contrast, DHA fed animals showed a no obvious difference in OSI compared to both controls and baseline (Figure 6.4).

Generally, OSI in the aortic arch follows the same trend as that of WSS magnitude, and was higher in the inner curvature compared to the outer aortic curvature.

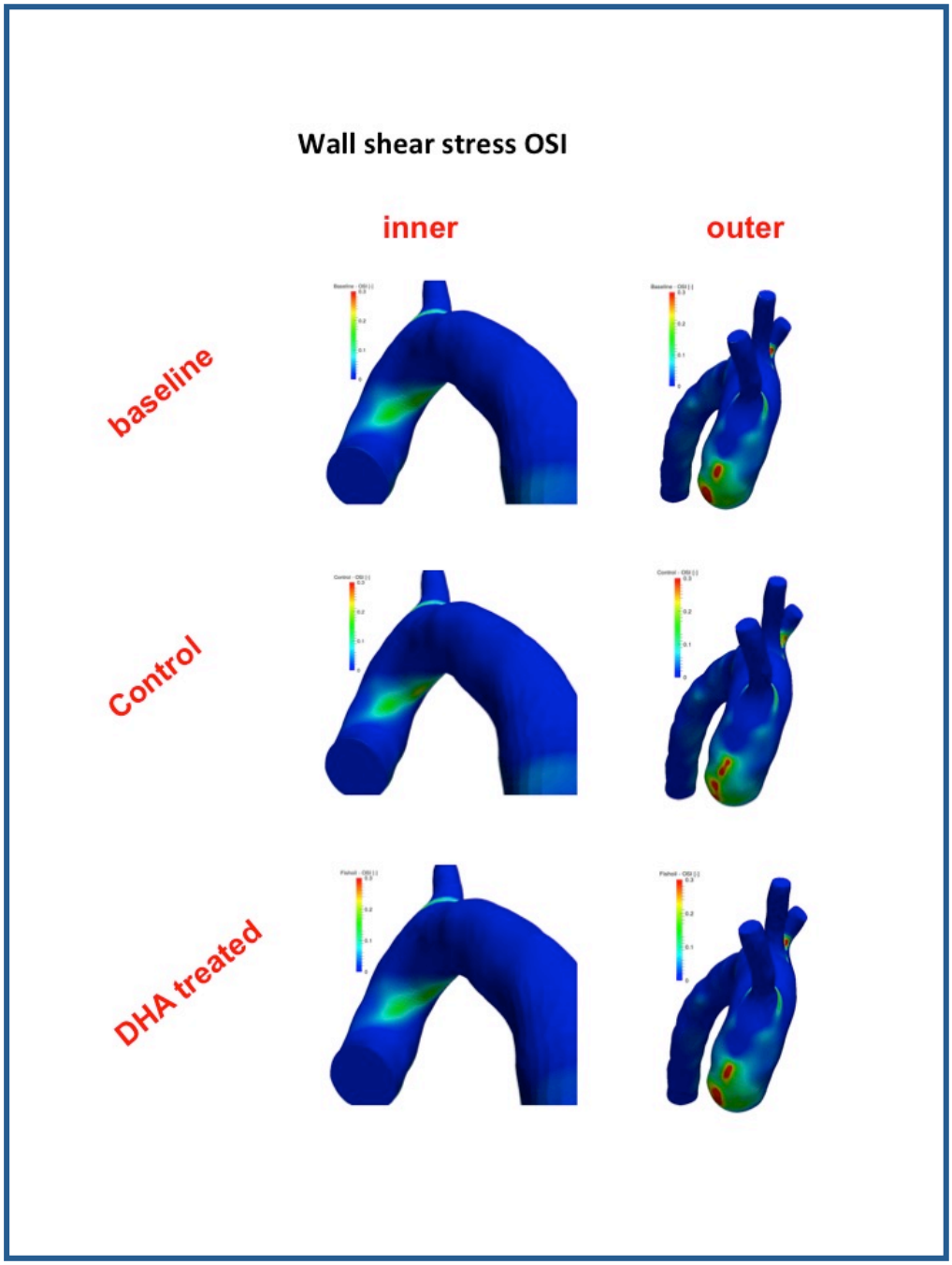

Figure 6.4. DHA effects on WSS direction and oscillation in the aortic arch. Starting at the age of 8 weeks, male mice were fed Western diet (HFD) alone (control) or HFD and DHA (DHA treated) (300mg/kg/day each), daily over a 12-week duration. Relative oscillatory shear index (OSI) in the outer and inner curvatures of the arch of aorta was analysed using CFD at baseline and after 12 weeks of feeding, and show relatively lower OSI in DHA treated mice compared to controls (n=4/group).

OSI was also assessed in the distal aorta between the control and the DHA fed groups. In the descending aorta, the OSI remarkably decreased in the DHA fed mice compared to controls, predominantly at the sites of the aortic branches (Figure 6.5, arrows).

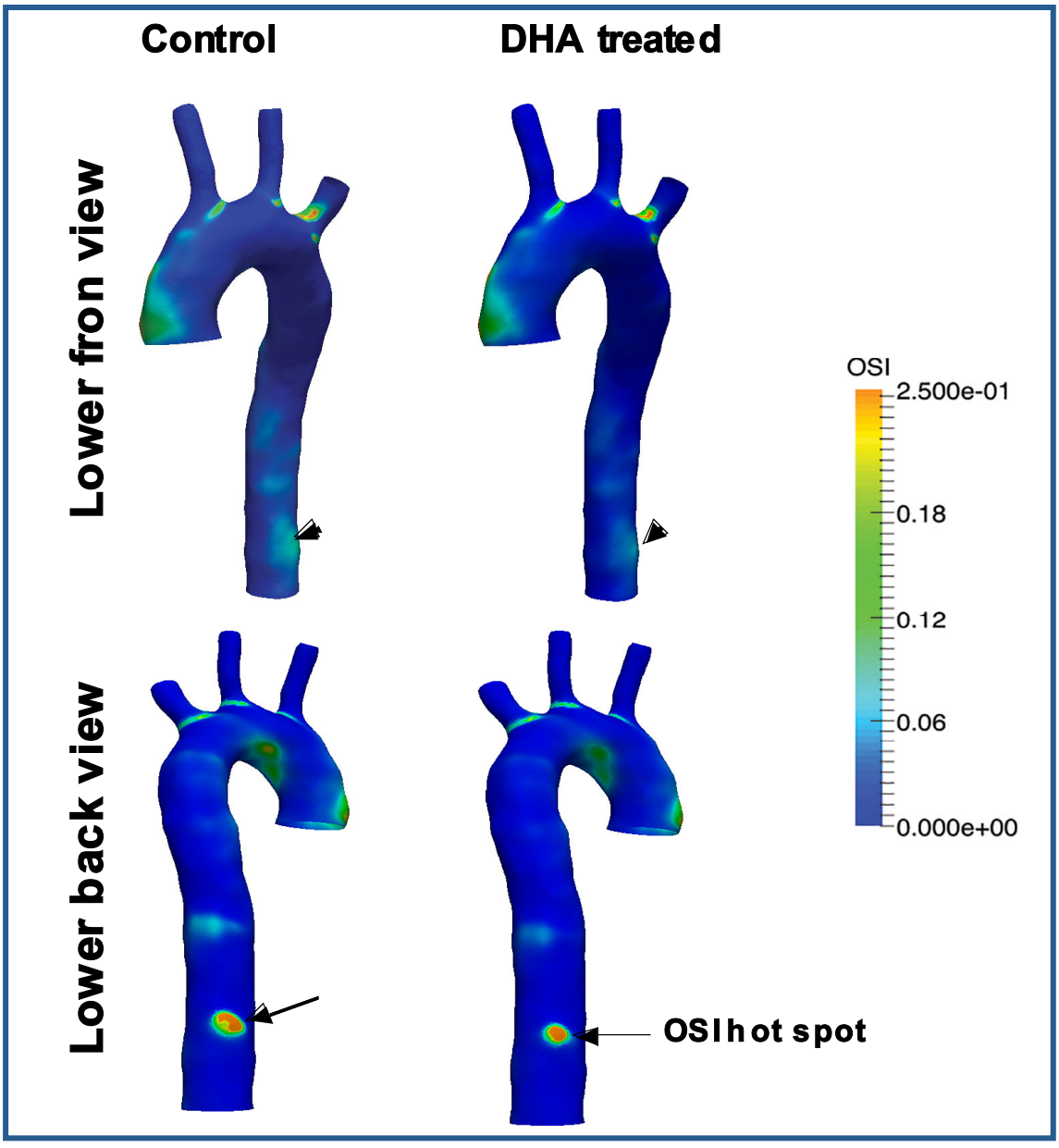

Figure 6.5. DHA effects on WSS direction and oscillation in the descending aorta. Starting at the age of 8 weeks, male mice were fed Western diet (HFD) alone (control) or HFD and DHA (DHA treated) (300mg/kg/day each), daily over a 12-week duration. Relative oscillatory shear index (OSI) in the descending part of aorta was analysed using CFD at baseline and after 12 weeks of feeding, and show relatively lower OSI in DHA treated mice compared to controls (n=4/group), particularly at aortic branches (OSI hot spot, arrow).

6.5. Summary:

The mechanical changes described herein have provided a key new insight into the mechanism of action of DHA. The novelty of the work showed in this chapter lies in investigating, for the first time, *in vivo,* the relationship between WSS and DHA using the applied CFD technique. The important findings are summarised below:

1. High fat diet feeding results in a significant increase in the aortic mean velocity of blood flow after 12 weeks.
2. DHA significantly decreases blood flow aortic mean velocity and prevents the rise in the velocity induced by HFD.
3. The changes in the aortic mean velocity of blood flow were applied as boundary conditions to map WSS using CFD and existing anatomical geometries in the public domain.
4. The peak in the WSS magnitude is higher in the inner compared to the outer curvatures of the aortic arch.
5. In the controls, the peak WSS is relatively higher after 12 weeks compared to baseline at both curvatures of the aortic arch.
6. DHA decreases the peak in WSS in the proximal aorta.
7. However, in the distal aorta, OSI significantly decreases, predominantly at the aortic hot spot (the site of aortic branches).

6.6. Discussion:

WSS is thought to be the main physical cause of endothelial dysfunction that contributes to pathophysiological states, including atherosclerosis and plaque instability (Cheng et al., 2006). The possible role that WSS may play in endothelial dysfunction was first postulated when observing the occurrence of the earliest lesions in a non-random pattern, characteristically at certain regions of the vascular wall, closely linked to the local changes in the haemodynamic forces (Ku et al., 1985).

In chapter 5, I showed that DHA decreased distal atherosclerosis without any significant effect on the proximal lesions. Therefore, in this, I sought to determine whether DHA altered fluid flow e.g. WSS magnitude and blood flow direction in the distal aorta, thus protecting it against distal atherosclerosis. My study demonstrated that DHA significantly altered distal aortic blood flow by decreasing flow velocity and reducing OSI with minimal effects upon the magnitude of WSS.

Using simulation techniques, WSS patterns in the proximal and the distal aortae were compared. Previous studies have used idealised changes in the velocities at the aortic roots to generate aortic arch geometry (Van Doormaal et al., 2012). Therefore, these geometries were used to detect the changes in the mean aortic velocity, comparing the effect following high fat feeding in the presence or absence of DHA.

Interestingly, HFD feeding induced a remarkable increase in the aortic blood velocity. This rise in the blood velocity profile was prevented by the feeding of DHA. The changes in the aortic mean velocity in response to DHA are novel and have not been published before, however, these may be linked to my previous findings on LV function, notably the reduction in blood pressure and LVM that occur with DHA supplementation (Chapter 5).

There are a number of possible explanations. It could be that HFD induces LVH secondary to an increase in systemic vascular resistance and hypertension. Therefore, and secondary to, stretching in the ventricular wall (Costanzo, 2007), the LV contracts forcibly to increase blood flow and hence aortic blood velocity (Devereux et al., 1983).

Perhaps, following DHA feeding, DHA prevents the rise in the blood pressure

induced by HFD and protects against LVH and, therefore, blood flow and the aortic velocity were maintained at low levels compared to animals fed HFD alone.

The changes in the flow and aortic velocities were applied as boundary conditions to map WSS at the aorta (Hartley et al., 1997). This was to study the observed differential effects of DHA on atherosclerosis in different parts of the aorta (the main finding of the previous chapter). There are natural sites for shear stress induced atherogenesis in the vasculature (Curfs et al., 2004), and in experimental studies, the aortic arch has received particular attention (Van Doormaal et al., 2012). Therefore, in this thesis, WSS magnitude and direction were examined at the inner and outer curvatures of the aorta.

To my surprise, HFD feeding alone increased WSS magnitude mainly at the inner curvature of the aortic arch whereas DHA had a minimal effect. The protective nature of the HSS has been demonstrated in human and animal based studies (Buchanan et al., 1999, Pedersen et al., 1999). In deed, previous reports have stated that atherosclerosis preferentially develops at the LSS areas (Pedersen et al., 1999, Davies et al., 2002). My data suggest that DHA may not have a large effect on WSS magnitude in the proximal aorta. This may help to explain the finding that DHA did not decrease the atheroma at the aortic arch (as discussed in chapter 5).

Alternatively, several observational studies have suggested that the patterns in the increase or the decrease in the WSS, rather than the individual amplitude, that is critical. That is, there is a compensated vascular wall remodeling secondary to shear levels that occurs in atheroma. For example, surgical ligation of the left common carotid artery in rabbits induced HSS initially and then, 12 weeks post-surgery, there was a compensated expansion in the arterial wall that militates the HSS (Di Stefano et al., 1998). Therefore, it is possible that compensated vascular remodeling is impaired after 12 weeks of feeding with the HFD (control), and this is why the WSS magnitude remained relatively high at the end of this study. However, in the DHA group, the compensated mechanism is maintained, resulting, in a drop in blood pressure that was associated with lower shear levels.

The underlying mechanism for such adaptive remodelling secondary to the shear

levels is relatively unknown. However, studies show that a number of anti-inflammatory triggers may play a role here, including an increase in NO bioavailability and enhanced eNOS expression (Yu et al., 2006). Although all vascular wall cells play a role in maintaining the arterial remodelling, healthy endothelium seems to play a prominent role. The endothelial surface is consistently exposed to various physical and proinflammatory factors and thus has the ability to sense and express various vasoactive/vasodilators that regulate the vascular tone and vascular remodelling (Gibbons and Dzau, 1994). The possible molecular mechanism by which DHA may act is discussed in the next chapter.

Importantly, and by comparing the direction of WSS in the DHA versus the control groups (as shown in the flow maps), distal oscillatory shear stress is considerably low. The direction of blood flow is an important determinant of the vascular function and the degree of flow oscillation (OSI) is typically enhanced at atheroprone compared to atheroprotected sites (Ku et al., 1985, Zhang et al., 2012). One new hypothesis is emerging from my work is that DHA may decrease the oscillation in the flow, predominantly at the sites of distal aortic branches and, hence, prevent the formation of atheroma at those regions (Figure 6.6).

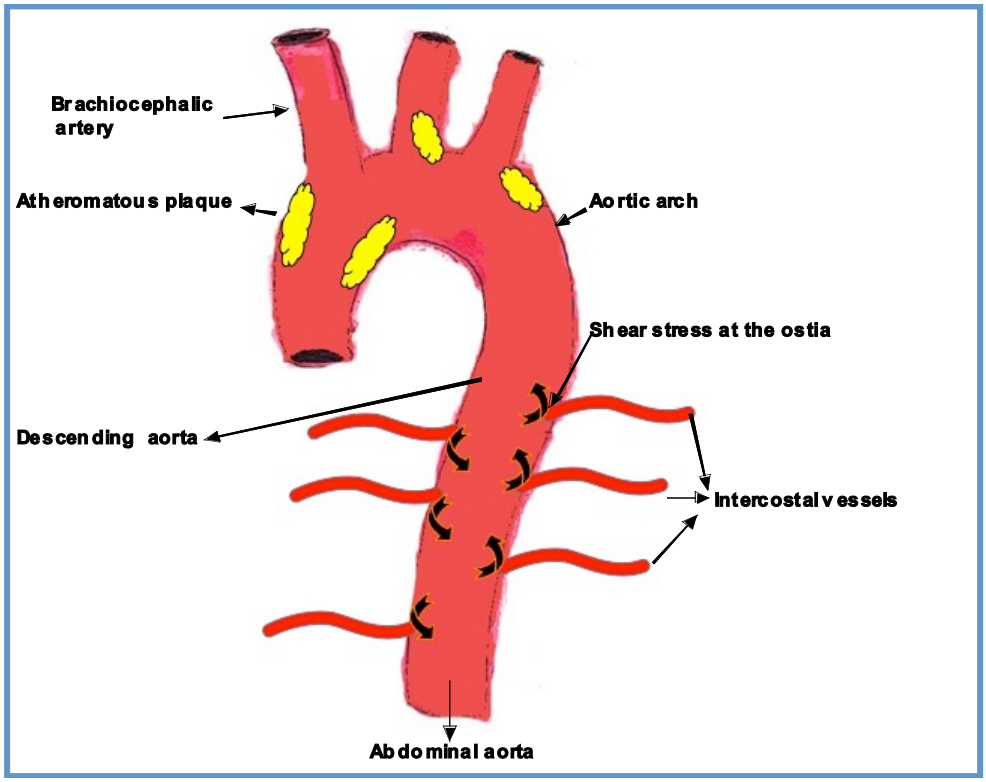

Figure 6.6. Schematic drawing illustrates a longitudinal section of the aorta. Atherosclerosis (yellow shadows) preferentially develops at the aortic curvatures and the sites of intercostal branches (ostia). The role of DHA as an atheroprotective agent typically plays out at the distal vasculature. DHA feeding has no effect on atherosclerosis at the aortic curvatures. However, after 12 weeks of feeding, it induces local changes in the formation of atherosclerosis mainly at the site of the intercostal branches of the descending aorta. This modulation results in low OSI at the ostia.

In conclusion, large vessels of ApoE$^{-/-}$ mice have been extensively studied in atherosclerosis, notably the proximal aorta and WSS changes (Van Doormaal et al., 2012). However, the changes in WSS direction in the distal aorta and in conjunction with atherosclerosis progression have not been well characterised in this animal model.

Therefore, this chapter has provided new proposed insights for the pattern of WSS in the distal aorta and has linked that with the athero-protective roles of DHA.

Chapter (7) The Mechanism of action of DHA in Experimental Atherosclerosis.

OUTLINE:

7.1. Overview of the Mechanism of action of DHA in Experimental Atherosclerosis:

Patients with MI (the disease manifestation of atherosclerosis) are usually prescribed a various combination of drugs that have different functions to control the complex pathophysiology of the disease (Libby et al., 2014, Greenland et al., 2003).
Dietary interventions and increased fish derived food intake have been increasingly proposed as an attractive strategy for preventing most aspects of the acute syndrome, including obesity (Lorente-Cebrian et al., 2013), hypertension (Mori TA, 1999, Sagara et al., 2011, Vandongen et al., 1993), and type 2 DM (Woodman et al., 2002). Nevertheless, research studies on n3FAs did not specify the exact role these free fatty acids could play on atherosclerosis, nor did they specify the underlying mechanism.

In the previous chapters (5 and 6), I showed that DHA significantly decreases distal atherosclerosis and that this effect is associated with a significant drop in blood pressure and an increase in the HDL/TC ratio. In addition, I demonstrated that DHA acts locally at the vessel wall and alters WSS oscillation.

Arterial inflammation has been demonstrated in patients with hypertension (Payne et al., 2010). Therefore, in this current chapter, I aim to explore whether DHA exerts its distal local effects by modulating local vascular inflammation.
I aimed to elucidate whether DHA could modulate vascular contractility via an IL-1β mediated mechanism, thus contributing to blood pressure regulation and WSS modulation in experimental atherosclerosis.
This chapter also sheds a new light into the molecular mechanism of DHA in HFD-induced hypertension and links that to the observed haemodynamic modulation and plaque stabilisation.

7.2. Brief Methods.

For full details on the experimental animals, immunostaining, and immunoblotting techniques used in this chapter, an expanded method section is shown in chapter 2.

7.3. *Effect of DHA on plasma proinflammatory mediators:*

In vitro studies have suggested that DHA decreases a number of proinflammatory mediators, including IL-8 (Wann et al., 2011). To study the anti-inflammatory effects of DHA and to investigate the mechanism by which that DHA decreases distal atherosclerosis, I measured plasma levels of different proinflammatory cytokines, including TNF-α, IL-1α, IL-1β, MCP-1, IL-8, and RANTES using a CBA.

Between the two experimental groups, there was no significant difference in TNF-α levels. Plasma levels of TNF-α in the control group were 25.86 ± 2.481pg/ml whereas the levels in the DHA treated animals were 26.63 ± 3.107pg/ml, p=ns, n=8/group (Figure 7.1A).

An original hypothesis of this thesis was that DHA induces its anti-atherosclerotic effects via an IL-1 dependent mechanism. Therefore, plasma levels of IL-1α and IL-1β were assessed. An unexpected finding is that, in the DHA treated group, levels of IL-1α (17.31 ± 1.446pg/ml) (Figure 7.1B) and IL-1β (13.72 ± 1.572pg/ml) (Figure 7.1C) were not significantly affected by DHA supplementation compared to the control group (15.41 ± 2.666pg/ml for IL-1α (Figure 7.1B) and 14.10 ± 2.210pg/ml for IL-1β (Figure 7.1C), n=8, p=ns).

However, mice that were fed DHA had lower plasma levels of IL-8 than the controls (18.57 ± 1.319 vs. 44.03 ± 5.652pg/ml, respectively, $p<0.01$, n=8) (Figure 7.1D). Likewise, levels of MCP-1 (79.28 ± 7.020 vs. 164.7 ± 36.31pg/ml, respectively, $p<0.05$, n=8) (Figure 7.1E) and RANTES (118.6 ± 6.114 vs. 146.0 ± 5.265pg/ml, respectively, $p<0.01$,n=8) (Figure 7.1F), were significantly lower in the DHA fed mice than the controls, suggesting that the anti-atherogenic effects of DHA may not directly be mediated by (soluble) IL-1 but instead by decreasing other plasma proinflammatory mediators.

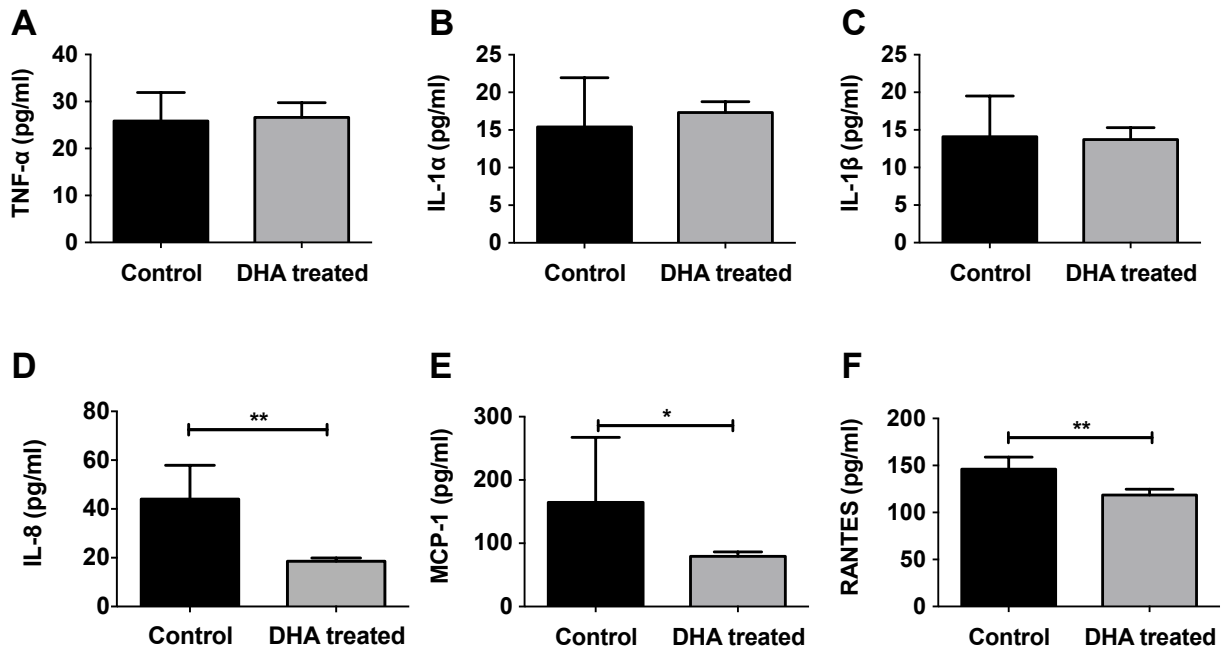

Figure 7.1. DHA supplementation attenuates plasma proinflammatory profiles. Starting at the age of 8 weeks, male ApoE$^{-/-}$ mice were fed either HFD alone or HFD and DHA (300mg/kg/day) daily. Following 12 weeks of diet, freshly isolated plasma was analysed using CBA for TNF-α **(A)**, IL-1α **(B)**, IL-1β **(C),** IL-8 **(D)**, MCP-1 **(E)** and RANTES **(F)** in pg/ml, (n=8-10 per group). Data are expressed as mean ± SEM, analysed by unpaired Student's t test, *p<0.05, **p<0.01.

7.4. *Local anti-inflammatory/ anti-atheromatous effects of DHA:*

I then wished to evaluate the DHA protective effect that attenuating local vascular inflammation had on atherosclerosis. To study the local signalling pathway of DHA, aortic roots from the DHA supplemented mice and the controls were immunostained for IL-1α, IL-1β, IL-1ra and TLR4.

7.4.1. The effect of DHA on IL-1α expression in aortic atherosclerosis:

Positive staining for IL-1α was detected in the plaque area (brown stained cells), predominantly at the intimal-medial junction (Figure 7.2A).

To ensure staining was specific for IL-1α, several additional control experiments were conducted (positive and negative controls). I used lung sections from male ApoE$^{-/-}$ mice fed HFD for 12 weeks as a positive control to confirm the specificity of the binding of the antibody to IL-1α antigen (Figure 7.2A). To further confirm the specificity of antibody binding in the atherosclerotic lesions, a negative control (replacing the primary antibody with PBS) was used. This did not show any positive staining, indicating that my staining for IL-1α was indeed specific (Figure 7.2A).

Consistent with the plasma CBA findings, semi-quantification for the IL-1α^{+ve} cells in aortic plaques showed no statistical significant difference between the two experimental groups (Figure 7.2B).

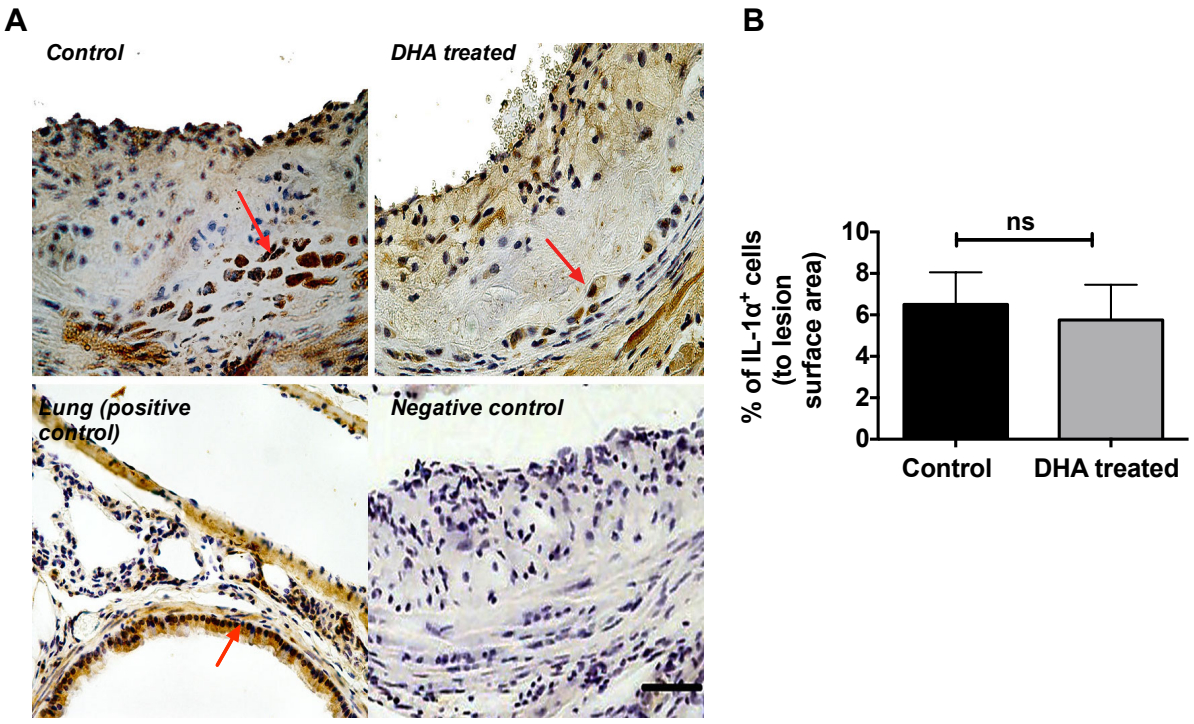

Figure 7.2. The effect of DHA on IL-1α distribution in aortic atherosclerosis. Male ApoE$^{-/-}$ mice (8 weeks of age) were fed HFD alone (control) or HFD and DHA (300mg/kg/day) daily for 12 weeks (DHA treated). **A)** Aortic roots from control or DHA fed animals were stained for IL-1α (red arrows). Lung sections were also stained at the same time for IL-1α and used as a positive control (red arrow, IL-1α positive cells). Negative control sections were assessed by replacing of the primary antibody with PBS. **B)** Semi-quantification of IL-1α positive cells in the plaque area showing no significant difference between the two experimental groups. Data are expressed as mean ± SEM, analysed by unpaired Student's t test, n=6/group. Scale bars=20μm.

7.4.2. The effect of DHA on IL-1β expression in aortic atherosclerosis:

Similar to IL-1α, positive IL-1β staining was also detected in aortic atherosclerosis in both groups (control and DHA supplemented) (Figure 7.3). However, the staining pattern differed to staining for IL-1α in that IL-1β positive cells were predominantly observed in the endothelium and the neointima (Figure 7.3A).

Positive and negative controls were again used to confirm the specificity of the antibody (Figure 7.3A). Importantly, semi-quantification of the IL-1β positive area (%

of the total lesion area) showed no significant difference between the two groups (Figure 7.3B). However, there was a significant reduction in endothelial IL-1β expression in the DHA fed mice compared to controls (8.87 ± 3.76 vs. 37.33 ± 4.48%, $p<0.05$, n=8, respectively) (Figure 7.3C).

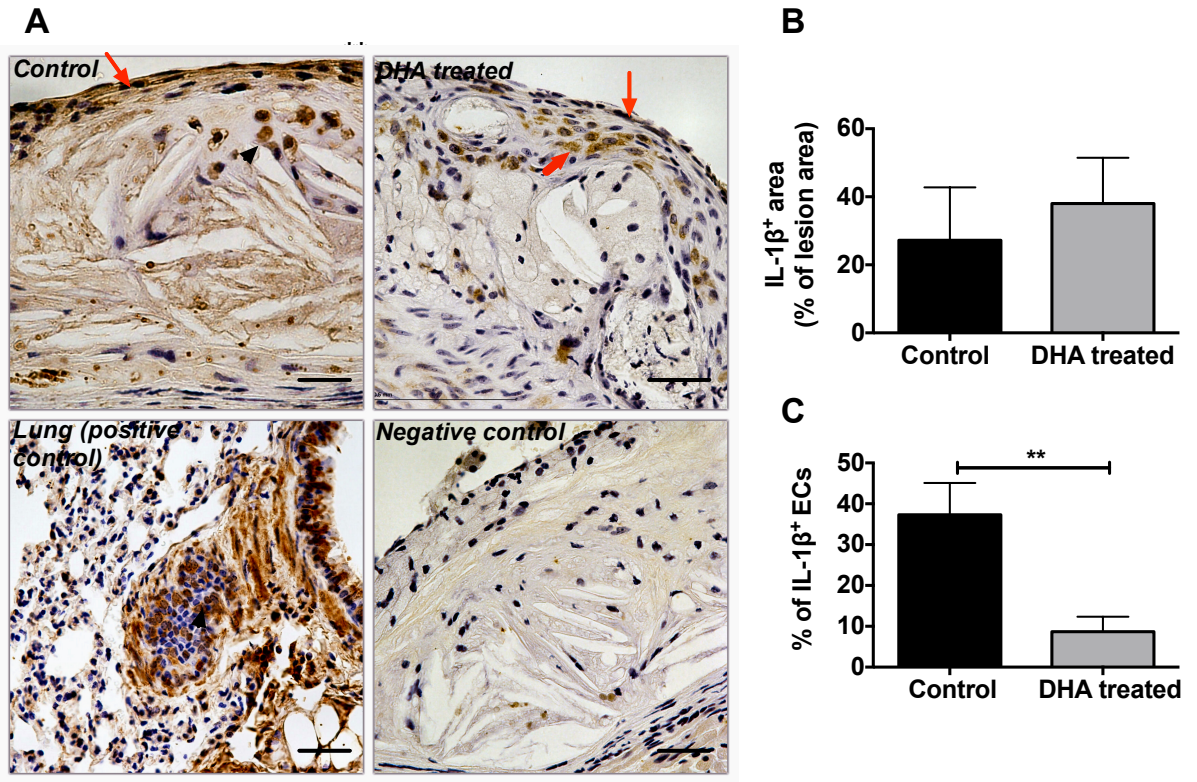

Figure 7.3. Interleukin-1β distribution in aortic atherosclerosis in response to DHA feeding. A) Representative images showing the distribution of IL-1β positive staining in the lesion area of aortic sinuses of male ApoE$^{-/-}$ mice fed HFD alone or HFD and DHA (300mg/kg/day) over 12 weeks. Arrows indicate IL-1β positive cells. **B)** Graphical representation of the total IL-1β distribution within the aortic roots as a % of lesion area and (**C)** of IL-1β positive endothelial cells (ECs) between the two groups (control vs. DHA supplemented groups). Image analysis was performed using NIS-Elements software and data are represented as mean ± SEM, n=8/group, Student's t tests indicate a significant difference with **p<0.01. Scale bars=20-50μm.

To ensure that the IL-1α and IL-1β antibodies did not cross-react, I performed immunoblotting for the respective recombinant proteins. Blots loaded with 20μg/mL of both recombinant IL-1β (rIL-1β) and recombinant IL-1α (rIL-1α) were probed with each antibody (Figure 7.4). The IL-1β antibody detected 17kDa rIL-1β, as expected, but no band was observed for rIL-1α (Figure 7.4A). In contrast, when the membrane was incubated with the anti-IL-1α antibody, the only detected band was observed at

18kDa for IL-1α; no bands for IL-1β protein were detected (Figure 7.4B), confirming the specificity of the two antibodies used in this study.

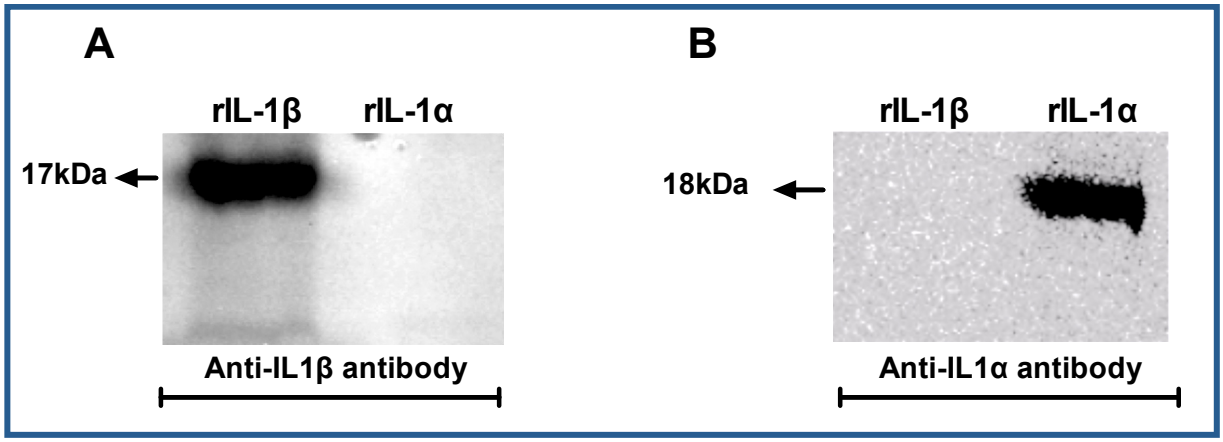

Figure 7.4. Western blot illustrating the specificity of IL-1α and IL-1β antibodies. Recombinant IL-1β (rIL-1β) and IL-1α (rIL-1α) were loaded at equal concentrations (20μg/ml each) and immunoblotted for IL-1β and IL-1α. **A)** A representative blot from n=3 experiments showing 17kDa IL-1β band when anti-IL1β antibody was used. **B)** A representative immunoblot for IL-1α from n=3 independent experiments.

7.4.3. The effect of DHA on IL-1ra expression in aortic atherosclerosis:

Aortic atherosclerotic sections from mice treated with DHA or controls were assessed for IL-1ra using immunohistochemistry (Figure 7.5). IL-1ra (brown stain) was detected in both groups predominantly in the endothelium, subendothelial cells and few scattered cells in the adventitia (Figure 7.5A).

Renal sections from male ApoE$^{-/-}$ mice fed HFD for 12 weeks were immunostained as positive controls, and show positive staining in the renal glomerular cells, as expected. Aortic sections used as negative controls by replacing the primary antibody with PBS, did not show any positive staining (Figure 7.5A).

Semi-quantification of the total IL-1ra in the plaque area did not show any significant difference between the two groups (Figure 7.5B). However, there was a significant increase in IL-1ra expression in the endothelium in the DHA treated compared to control groups (9.75 ± 1.315 vs. 3.5 ± 1.041%, respectively, $p<0.01$, n=6) (Figure 7.5C).

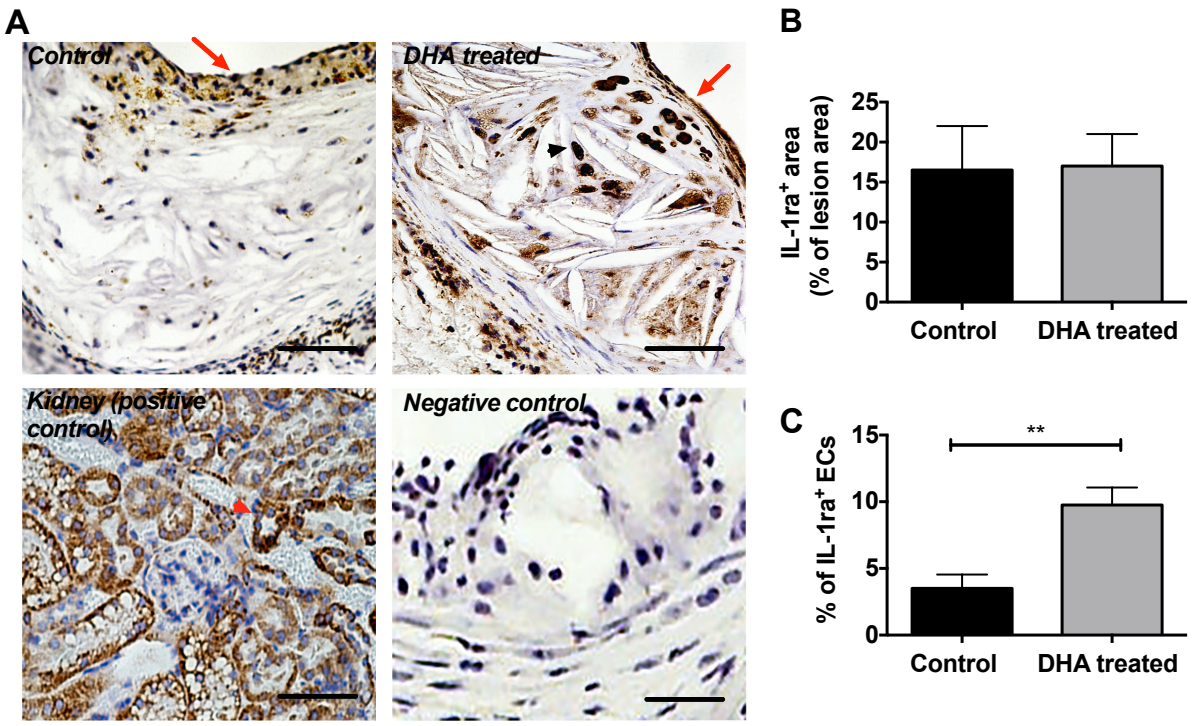

Figure 7.5. Interleukin-1ra distribution in aortic atherosclerosis in response to DHA feeding. A) Representative images showing the distribution of IL-1ra positive staining in aortic sinuses of ApoE$^{-/-}$ mice fed HFD alone or HFD and DHA (300mg/kg/day) over 12 weeks. Arrows indicate of IL-1ra positive stains. **B)** Graphical representation of the amount of IL-1ra within the aortic roots and **(C)** number of EC expressing IL-1ra, measured semi-quantitatively as a % of total number of cells. Analysis were performed using NIS-Elements software and data are represented as mean ± SEM, n=6, Student's t-tests indicate a significant difference with **p<0.01. Scale bars=20μm.

7.4.4. The effect of DHA on TLR4 expression in aortic atherosclerosis:

Having shown that DHA decreased expression of endothelial IL-1β ad increased IL-1ra, I sought to investigate the effects of DHA on TLR4 expression.

TLR4 protein was assessed in the two experimental groups using both immunohistochemistry and immunoblotting (Figure 7.6).

In the aortic roots, TLR4 was widely expressed in the plaque area, predominantly in the endothelium (Figure 7.6A). Semi-quantification showed a slight yet a non-significant reduction in TLR4 expression in the plaque area of DHA treated (10.5 ± 0.56%) compared to control groups (12.5 ± 1.61%, n=6) (Figure 7.6B).

However, immunoblotting confirmed that DHA feeding significantly decreased TLR4 levels in the whole aortae compared to controls (0.39 ± 0.050 vs. 0.628 ± 0.014, n=4, p<0.01) (Figure 7.6C & D).

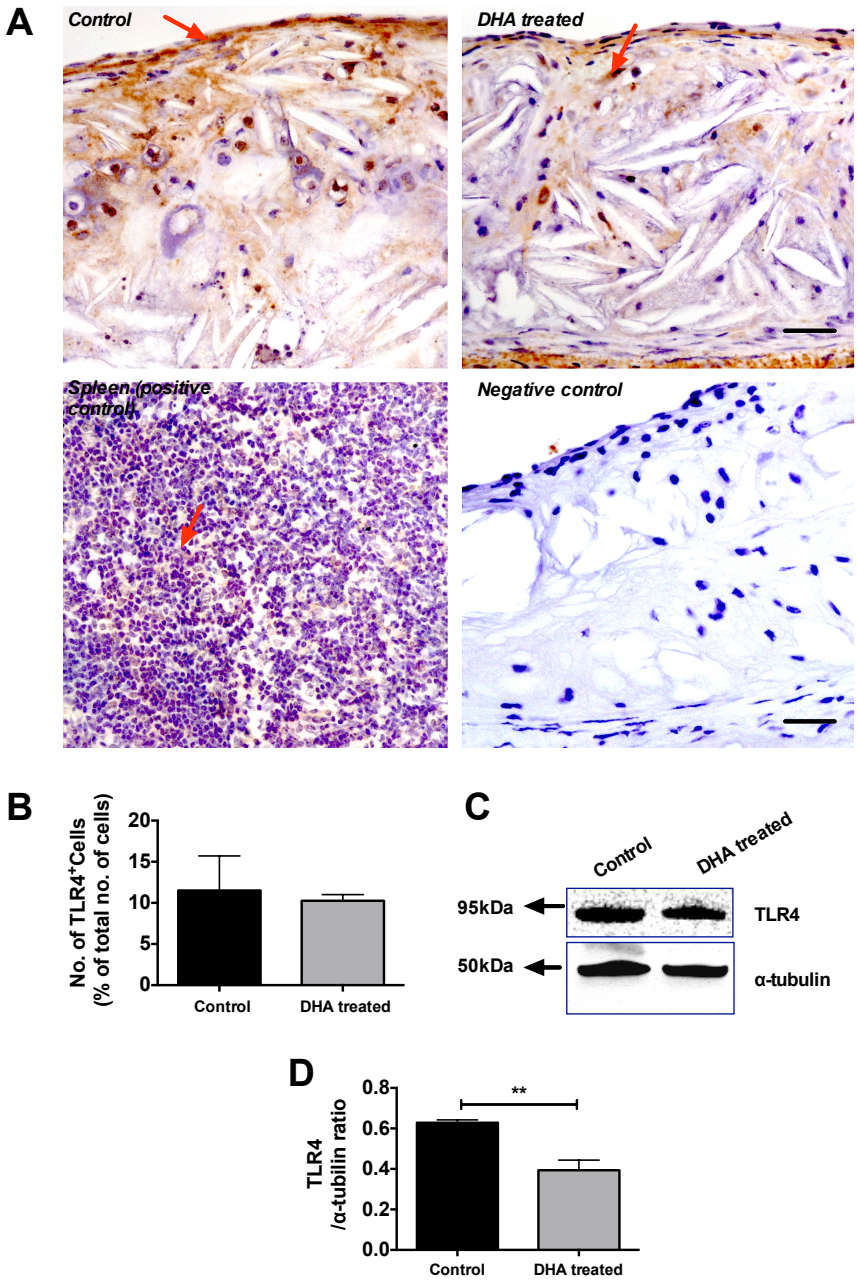

Figure 7.6. TLR4 distribution in aortic atherosclerosis in response to DHA feeding. A) Representative images showing TLR4 expression in aortic sinuses of ApoE$^{-/-}$ mice fed HFD alone or HFD and DHA (300mg/kg/day) over 12 weeks. Arrows indicate TLR4 positive staining. **B)** Graphical representation of semi-quantitative analysis of the amount of TLR4 as a % of total number of cells. Analysis was performed using NIS-Elements software. **C)** Representative Immunoblot of TLR4 protein (95kDa) in the whole aorta of both groups of mice after 12 weeks of feeding, α-tubulin was used as a loading control. **D)** Densitometric analysis of TLR4 in the whole aortae (n=4). Data are represented as mean ± SEM, Student's t-test, **p<0.01. Scale bars=20μm.

7.5. Local effects of DHA on mac-3 expression:

Having established that DHA feeding decreases plasma levels of MCP-1 and IL-8, I sought to explore whether there are any changes in inflammatory cell infiltration into the atheromatous plaque.

I previously showed that DHA supplementation reduced plaque size (approx. 30-40% in the whole aortae, Chapter 5). Therefore, this immunohistochemical analysis aimed to provide further insights on the impact of DHA on plaque composition in terms of macrophage infiltration.

The biomarker mac-3 was selected to identify mac-3^{+ve} macrophages. Mac-3 positive staining was calculated relative to % of total lesion surface area.

Mac-3 positive cells were predominantly detected into the sub-endothelial area of the lesion of both groups of mice (Figure 7.7A).
However, there is a significant amelioration in mac-3^{+ve} macrophages in aortic root lesions of DHA treated group compared to controls (46.98 ± 11.3 vs. 15.53 ± 5.142%, $p<0.05$, n=6/group) (Figure 7.7B), which is consistent with the lower plasma biomarkers and the lesion burden in the DHA treated group.

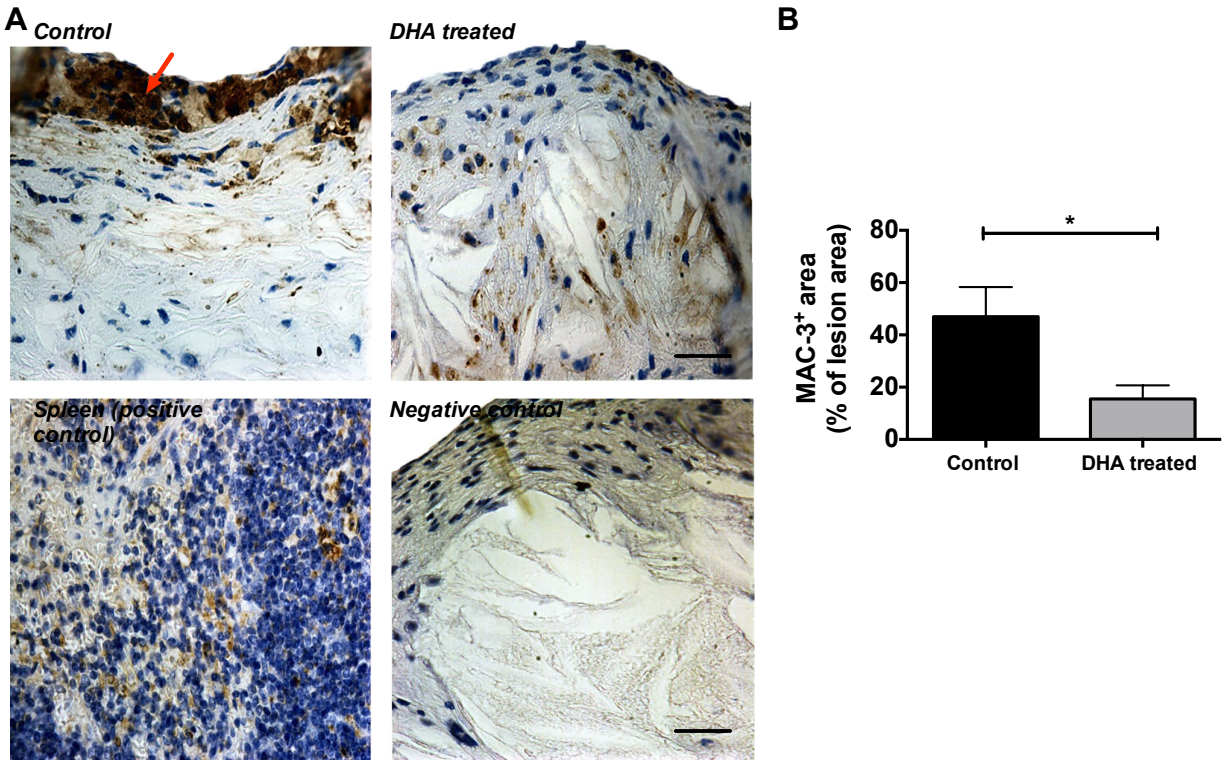

Figure 7.7. Mac-3 distribution in aortic atherosclerosis in response to DHA feeding. A) Representative images of the distribution of mac-3 positive staining in the aortic sinuses of ApoE$^{-/-}$ mice fed HFD alone or HFD and DHA (300mg/kg/day) over 12 weeks. Splenic sections from ApoE$^{-/-}$ fed HFD for 12 weeks were used as a positive control, and aortic sections stained with no primary antibody were used as negative controls. **B)** Graphical representation of the mac-3+ve area as a % of total lesion area. Analysis was performed using NIS-Elements software and data are represented as mean ± SEM, n=6, Student's t-test, *p<0.05. Scale bars=20μm.

7.6. The local effects of DHA on eNOS expression:

WSS at physiological levels is one of the most powerful stimulants for NO production in the healthy endothelium, which is regulated by eNOS (Lam et al., 2006).

In the previous chapter (6), I showed that DHA decreased OSI in the distal aorta; therefore, in this current chapter I sought to determine whether this effect is mediated by eNOS.

The relative expression of eNOS in aortic atherosclerosis of DHA fed-mice versus controls was evaluated using immunohistochemistry and immunoblotting (Figure 7.8). Sections of spleen were used as positive controls (Figure 7.8A, left lower panel), and aortic root sections of male ApoE$^{-/-}$ mice fed HFD for 12 weeks, omitting the primary antibody during staining, were used as negative controls, to ensure specificity of staining (Figure 7.8A, right lower panel).

eNOS was primarily detected in the atheromatous plaque of mice fed with DHA, in the endothelium and some inflammatory cells (Figure 7.8A, upper panels). There was a significant increase in eNOS expression in the DHA treated group compared to controls (0.098 ± 0.02 vs. 0.004 ± 0.003, respectively, $p<0.05$, n=6) (Figure 7.8B).

Immunoblotting confirmed these immunohistochemistry findings with a substantial up-regulation of 140kDa eNOS in the DHA treated group compared to controls (HFD controls) (Figure 7.8C).

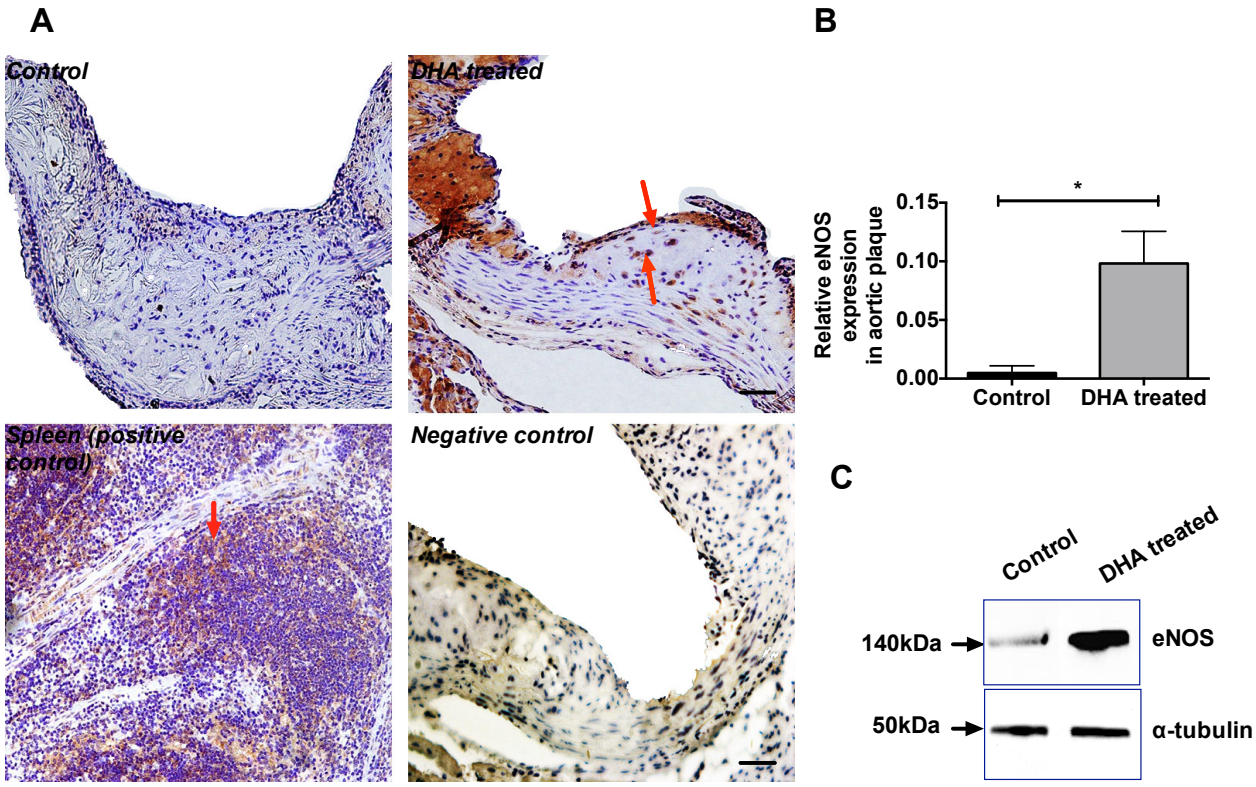

Figure 7.8. eNOS distribution in aortic atherosclerosis in response to DHA feeding. A) Representative images of the distribution of eNOS positive staining in aortic sinuses of ApoE$^{-/-}$ mice fed HFD alone or HFD and DHA (300mg/kg/day) over 12 weeks. Red arrows indicate eNOS positive staining. **B)** Quantification of the relative eNOS expression in control (HFD control) and DHA treated animals, to total lesion area. Data are represented as mean ± SEM, n=6, Student's t-test, *$p<0.05$, indicates a significant difference. Scale bars=100μm. **C)** Representative immunoblotting image of eNOS protein (140kDa) in the whole aortae of each group of mice (n=4/group), fed for 12 weeks, using α-tubulin as a loading control.

7.7. Summary:

This chapter suggests a molecular mechanism of action of DHA in experimental atherosclerosis. To my knowledge, this is the first study to report the protective effects of DHA in relation to HFD induced atherosclerosis in ApoE$^{-/-}$ mice and a local IL-1 induced pathology.

The presented data are summarised below:

1. DHA supplementation has no effect on plasma levels of TNF-α.
2. Although no significant effect was observed on soluble plasma levels of IL-1α and IL-1β, a significant reduction in plasma levels of IL-8, MCP-1 and RANTES following DHA feeding in the mice after 12 weeks was seen.
3. Additionally, DHA significantly decreases mac-3 positive cells in the atheromatous plaque.
4. DHA-fed animals had no significant difference in IL-1α expression in aortic root atherosclerosis compared to controls.
5. However, endothelial IL-1β was significantly reduced in DHA supplemented mice and compared to controls.
6. DHA-fed animals had a significant increase in IL-1ra expression in the endothelium of aortic atherosclerosis.
7. DHA feeding had no effect on TLR4 expression in the proximal aorta but it significantly decreased TLR4 levels in the whole aortae compared to controls.
8. In aortic atherosclerosis, eNOS is significantly increased in the DHA fed mice compared to controls.

7.8. Discussion:

One of the objectives of any future atherosclerotic therapy is to stop progression of any pre-existing lesions and stabilise the atheromatous plaque by changing its lipid and cellular compositions (Libby et al., 2014). IL-1β, produced by the dysfunctional endothelium (Gutierrez et al., 2013), plays a crucial role in the development of atherosclerosis (Insull, 2009, Tamaru et al., 1998). Therefore, the current trend is to therapeutically control the disease by manipulation of local IL-1 production (Dinarello, 2005).

This current chapter investigated the possible anti-inflammatory effects of DHA upon endothelial IL-1 production *in vivo*.

To study the anti-inflammatory effects of DHA, plasma levels of different cytokines were assessed in DHA treated and control groups of mice. Surprisingly, DHA supplementation in mice had no effect on the plasma levels of IL-1 and TNF-α. My finding is at odds with previous *in vitro* studies (Lo et al., 1999). Human based studies have suggested that supplementing the diet of young male volunteers with 1.85g of DHA in fish oil significantly ameliorated IL-1β production in LPS-stimulated monocytes (Endres et al., 1989). Additionally, Vijay-Kumar *et al* have reported that monocytes harvested from mice fed fish oil containing DHA produced less IL-1β compared to controls (Vijay-Kumar et al., 2011).

The discrepancy between my data and these published results could be due to the possibility that DHA may have no effect on the plasma levels of the soluble IL-1 but instead it may decrease local production of IL-1β in inflammatory cells. This is supported by (Denes A., 2012) who suggested that administration of an anti-IL-1β antibody to ApoE$^{-/-}$ mice decreased tissue IL-1β expression but it had no effect on plasma IL-1 β levels.

Another explanation derives from chapter 4 of this thesis where I showed that IL-1β, especially when endothelial cell derived, is released via protected vesicles. The assay used in this chapter (CBA) may not be sensitive enough to measure the vesicular IL-1β in mouse plasma. The gold standard technique used to isolate and quantify EVs is flow cytometry (Robert et al., 2009). However, there is no general agreement on the markers that could be used to detect EVs in mouse plasma,

especially those derived from ECs (Dignat-George and Boulanger, 2011). Therefore, due to these technical limitations, detection of EVs containing IL-1β in mouse plasma was not performed.

Interestingly, DHA-fed mice had a significant reduction in the other plasma biomarkers that act as chemoattractants, including IL-8, MCP-1 and RANTES compared to controls whose production can be induced by IL-1 (Libby et al., 2009). Previous animal based studies reported no significant correlation between DHA and these plasma cytokines (Calder, 2006). However, most of these studies were small and entirely focused on EPA (the other prominent n3FAs in the FO), with little attention was given to DHA effects. I was able to demonstrate that DHA supplementation led to a reduction in these plasma proinflammatory mediators in experimental atherosclerosis. My findings agree well with data from *in vitro* studies, particularly from cultured cells, such as monocytes and ECs (Kelley et al., 1999, Wann et al., 2011). Based on these findings, I conclude that the atheroprotective effect of DHA might be due to reduced levels of these plasma chemoattractants.

The cellular mechanism of action by which DHA decreases these markers remains speculative. Evidence has suggested that endothelial cells treated with n3FAs, notably DHA, inhibits expression of a number of IL-1 induced cytokines such as IL-8 and MCP-1 (De Caterina and Massaro, 2005, De Caterina R, 1994). With this in mind, I hypothesise that DHA exerts the protective effects by a local suppression of IL-1.

While a role of IL-1R signalling has already been established in experimental studies of atherosclerosis (Chamberlain et al., 2009, Devlin et al., 2002), the important role by which the IL-1 cytokines (IL-1α or IL-1β) may play in atherosclerosis *in vivo* is somewhat contradictory. Some authors stated that IL-1α rather than IL-1β plays a key role in inflammation in response to local lipid overload (Freigang et al, 2013). Moreover, a recent work by Lugrin and colleagues has demonstrated that IL-1α$^{-/-}$ mice have less post-ischaemic inflammation, leading to their conclusion that IL-1β has no role in this process (Lugrin et al., 2015). However, other studies have demonstrated that IL-1β is main culprit in atherosclerosis (Dinarello, 2011a). Therefore, I sought to explore whether expression of one or both these cytokines (IL-

1α and IL-1β) is affected by DHA feeding and thus whether they are involved in the DHA signalling pathway.

In atheromatous plaques and in both experimental groups, IL-1α protein was mainly expressed in cells that present at the junction between the media and the intima. Unexpectedly, mice fed DHA had no significant difference in IL-1α expression in their aortic plaque compared to controls, implicating the participation of an alternative IL-1α independent pathway in the observed effects of DHA.

These findings prompted me to evaluate whether IL-1β, instead of IL-1α was the molecular target of DHA in this model. Recent studies support a direct involvement of the leaderless cytokine IL-1β in hypertension (Boesen et al., 2008, Chamberlain et al., 2009), post-MI LV remodelling (Abbate et al., 2010) and atherosclerosis (Kirii et al., 2003). Therefore, I examined IL-1β distribution in aortic plaque using immunohistochemistry.
In contrast to IL-1α positive cells, IL-1β protein was strongly expressed in the endothelium, whereas it was not detected in the media and was only weakly detected in the shoulders of the plaques of both groups. In atherosclerosis, the relative expression of the two cytokines within different population of cells is relatively unknown. Therefore, my findings suggest that IL-1α and IL-1β may play different roles in the pathogenesis of the disease.

Despite no changes in the plasma levels of IL-1β, I observed a clear decrease in endothelial IL-1β expression in aortic atherosclerosis of DHA fed animals, suggesting an instrumental role for DHA in the IL-1β mediated process, consistent with previous findings implicating DHA in IL-1β inflammation (Vijay-Kumar et al., 2011). The primary cellular of origin of IL-1β in atherosclerosis is unclear. However, Galea and colleagues have shown that in coronary atherosclerotic plaques of IHD patients, IL-1β is predominantly expressed in relatively large amounts in the endothelium (Galea et al., 1996). Therefore, my results of selective inhibition of endothelial IL-1β by dietary supplementation with DHA make ECs a possible therapeutic target for modulating IL-1β in atherogenesis.

Animal based studies suggest that levels of IL-1β in atherosclerosis (Galea et al., 1996, Dewberry et al., 2008, Chamberlain et al., 2009) and in hypertension (Dalekos GN, 1997) are increased with a down-regulation of IL-1ra. Therefore, in the present study, I sought to determine whether DHA has any effects on IL-1ra levels and whether TLR4 is involved in this pathway.

The anti-inflammatory effects of n3FAs, including DHA has long been documented (Calder, 2009, Richard et al., 2009, Lu et al., 2011), however, this study is the first that links DHA feeding in mice with the increase in IL-1ra expression in atheromatous plaque. IL-1ra has been given as a treatment to ApoE$^{-/-}$ mice where it effectively inhibited fatty streak formation (Elhage et al., 1998). Moreover, IL-1ra has been introduced in clinical trials as a possible therapeutic intervention in MI patients with established atherosclerosis and hypertension (Morton AC, 2014). However, limitations of its long term use, secondary to the compliance of the patients, has long been a big issue in the field (Dinarello, 2005). IL-1ra is known to be expressed by the endothelium of the coronary vessels (Dewberry et al., 2000). Furthermore, compelling evidence suggests that the balance between IL-1 and IL-1ra determines the fate of the overall inflammatory conditions (Arend, 2002). Therefore, my findings of the up-regulation of IL-1ra and the down-regulation of IL-1β in the endothelium suggest a dual plausible therapeutic role for DHA in modulating vascular disease.

The molecular signalling pathway(s) by which DHA decreases endothelial IL-1β is relatively unclear. I elected to study local TLR4 expression since TLR4 is one of the main pathways by which IL-1 is produced/released by inflammatory cells (Xu et al., 2001). Additionally, enhanced expression of TLR4 has recently been demonstrated in accelerated atherosclerosis (Edfeldt et al., 2002, Higashimori et al., 2011) and hypertensive vascular disease (Sollinger et al., 2014). To my surprise, DHA had no significant effect on TLR4 distribution/expression in proximal aorta of mice fed DHA for 12 weeks compared to controls. However, in the whole aortae, levels of TLR4 were significantly ameliorated in the DHA compared to control groups. This is in agreement with a recent study reporting the inhibition of TLR4 in fish oil (FO) fed mice, predominately in peritoneal macrophages challenged with LPS (Vijay-Kumar et al., 2011) and confirms TLR4 as a molecular target of DHA.

To further explore the anti-inflammatory roles of DHA, I investigated macrophage distribution in the presence or absence of DHA. Macrophages represent approximately of 30-40% of the atherosclerotic mass (Weber and Noels, 2011). Therefore, the importance of macrophage modulation in atherosclerosis is now well appreciated in order to reduce atherosclerosis (Mantovani et al., 2009). The finding of macrophage reduction in aortic plaques of mice supplemented with DHA is in line with a recent study by Gladine and colleagues who demonstrated a decrease in mac-3^{+ve} cells in aortic sinuses of $LDLR^{-/-}$ mice fed DHA (Gladine et al., 2014). My finding may suggest that DHA decreases atherosclerosis by decreasing macrophage infiltration, which, in part, may be due to the reduction in the production of plasma chemoattractants.

Endothelial cells express various atheroprotective molecules, including eNOS and enhanced NO bioavailability in response to HSS (Chatzizisis et al., 2007). A published study by Chamberlain *et al* 2009, has suggested that IL-1 suppression in $ApoE^{-/-}$ mice fed HFD enhanced NO bioavailability by an eNOS dependent mechanism. Therefore, I hypothesised that DHA mediates the WSS effects by enhancing expression of eNOS in the endothelium. Significantly, I found that mice fed DHA express much higher eNOS levels in the endothelium of atheromatous plaque and compared to controls. The increase in expression of eNOS with DHA supplementation has been studied in various *in vitro* and *in vivo* studies (Yates et al., 2014). However, the novelty of my results lies in the detection of eNOS within the endothelium of atherosclerotic plaque of experimental atherosclerosis in particular.

In conclusion, this chapter provides with evidence that regular supplementation of DHA in the diet of mice decreases local IL-1β production and that is associated with distal athero-modulation and local flow changes. While there is enough evidence that DHA is atheroprotective in CAD (Virtanen et al., 2014), understanding the underlying mechanism in even more detail may lead to a novel therapy.

Chapter (8) General Discussion and Future Perspectives.

OUTLINE:

8.1. Overview of General Discussion and Future Perspectives:

In this thesis, IL-1α and IL-1β received particular attention, because of their critical roles in the processes of inflammation and lipid accumulation (Tsimikas et al., 2014). The complexity of IL-1β action has been extensively studied and has led to surprising discoveries that help in our understanding, especially in the field of the cell biology and the drug therapy. Subsequently, blocking of IL-1 activity has been studied in order to control a number of chronic inflammatory diseases, including MI (Ikonomidis et al., 2014). Consequently, after almost two decades of pre-clinical studies, an on-going phase 3 clinical trial, Cankinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), is testing whether specifically blocking IL-1β will reduce the incidence of thrombotic events in patients after MI that remain at high risk due to the underlying inflammation (Ridker PM., 2011). However, an important question regarding the mechanism by which IL-1 is having an effect, and its cellular origin in atherosclerosis has yet to be clear.

An alternative strategy to control atherosclerosis using PUFAs such as n3FA has also been extensively investigated (Adolfo Reza-Albarran, 2013). Recent data suggest that DHA supplementation has the potential to reduce blood pressure in hypertensive animal models (Morin et al., 2015), yet the precise molecular mechanism of action of DHA remains elusive. DHA has broad anti-inflammatory effects, and whether it may also have some inhibitory effects on IL-1 dependent inflammation needs further elucidation.

This chapter summarises the main findings of the *in vitro* and *in vivo* experiments that were conducted in this thesis, to test the hypotheses that IL-1 release from endothelial cells is mediated by NE and that DHA acts via an IL-1 related mechanism. The plausible limitations and the future clinical perspectives of this research are also discussed, before drawing relevant conclusions.

8.2. The mechanism of IL-1 secretion by NE action:

IL-1β is produced as an active isoform in response to inflammation (Church et al., 2008), yet the site of bioactive IL-1β processing within the cell and the mechanism of secretion have been an area of uncertainty.

IL-1 processing is a rate-limiting step that differs in different cell types. For example, in monocytes, only approx. 20-25% of proIL-1β is processed and secreted, a process tightly controlled by an activation caspase-1/inflammasome complex (Asgari et al., 2013). By contrast, the majority of the processed IL-1 within pulmonary derived monocytes infected with chlamydia was released by a caspase-1 independent mechanism (Laudisi F., 2013). In ECs, whether IL-1 is secreted by either a caspase-1 dependent or independent pathway has yet to be studied.

Another issue is that the different kinetics of IL-1β secretion in different cells. While THP-1 monocytes require only a one "hit" stimulus such as LPS for both IL-1β production and release (Martinon et al., 2004), other cell types, including blood monocytes and alveolar macrophages, need an additional "hit" stimulus, eg. ATP, to induce the secretion process (Wewers and Herzyk, 1989).
In contrast, ECs have completely different kinetics for IL-1β secretion, and ATP seems to play no obvious role. Previous work by Ward *et al* has shown that IL-1β is released from activated co-cultures of ECs and monocytes, at greater levels than from monocytes or ECs alone, and that an unidentified monocyte-derived mediator significantly contributed to this response (Ward et al., 2009a).
Therefore, significant attention has been paid to how IL-1β is released from ECs (Warner et al., 1987, Libby et al., 1985, Schonbeck et al., 1997), although no consistent results have been published.

8.2.1. Summary of the findings of the in vitro study:

In this thesis, I showed that ECs only secrete bioactive IL-1β after NE activation. NE cleaved proIL-1β within ECs and released IL-1β without caspase-1 activation. NE also entered ECs within LAMP-1^{+ve} MVBs and enhanced EV release containing active IL-1.
Thus, I propose a new mechanistic role for NE, by which approximately 30% of the processed IL-1β is secreted from ECs, entirely dependent upon NE activation and independent of caspase-1 (Chapter 3). I postulate that NE is the mediator released from monocytes described by Ward *et al* (2009), and would be the natural source of NE *in vivo,* although future work to confirm this is warranted.

NE is present in aortic aneurysms (Rao et al., 1996) and carotid plaques (Dollery et al., 2003) and plays a direct role in IL-1β processing *in vitro* (Black et al., 1991). However, its effects on IL-1β secretion from cultured ECs are relatively unknown. Therefore, my finding, together with the pre-existing literature, offers a novel role for NE in the pathogenesis of endothelial IL-1 dependent inflammation. It also suggests, for the first time, that inflamed ECs can indeed release remarkably high levels of IL-1, which thus further supports the findings of Galea *et al* 1996 in that the endothelium of coronary arteries can be considered a major source for IL-1.

The mechanism by which IL-1β is exported outside cells has so far proven to be inconclusive. In this thesis (Chapter 4) I demonstrated that ECs release two distinct populations of EVs in response to NE activation (a population derived from the plasma membrane; microvesicles (0.1-1μm) and a population derived from internal vesicles; exosomes (30-100nm)).
The detection of bioactive IL-1β within these vesicles offers a novel explanation for the distal endocrine actions of IL-1. In addition, several lines of evidence have indicated that endothelial derived EVs may be implicated in the pathogenesis of atherosclerosis (Dignat-George and Boulanger, 2011, Combes et al., 1999, Jansen et al., 2013, Jimenez et al., 2003, Leroyer et al., 2007). However, the precise mechanism remains unknown. Therefore, it is tempting to speculate that my finding could explain, in part, the involvement of endothelial derived EVs in atherogenesis by releasing IL-1 at the vascular wall and, thus, may offer a future novel therapeutic target.

Intracellularly, the site of IL-1 processing is still unclear. ProIL-1β is detected within the cytosol of monocytes. However, mature IL-1β was only isolated from vesicles resistant to trypsin degradation and not the cytosol (Rubartelli et al 1990). The integrity and characteristics of these vesicles are relatively unknown. In 1990, Andrei *et al* were the first to detect the mature form of IL-1β within LAMP-1^{+ve} vesicles, in LPS activated monocytes. Subsequent studies using different experimental models have suggested a diversity of proposals to investigate the nature of these organelles (Rabouille et al., 2012). In addition, a body of evidence has indicated that translocation of proIL-1β from the nucleus to the cytoplasm may involve intravesiclar compartments such as MVBs (Andrei et al., 1999).

I was able to detect MVBs containing IL-1β in ECs only following NE treatment (Chapter 4). Since these MVBs were not detected in cells that did not produce IL-1β (Qu et al., 2007), this confirms that MVBs are a part of the secretory pathway of IL-1β. Future studies, including the mechanism by which MVBs are formed, which may help in understanding the complexity of the secretory system of IL-1, are required.

In the present study, NE was detected inside the cells within LAMP-1^{+ve} compartments and appeared to colocalise with IL-1β within the endothelium of atherosclerotic plaques (Chapter 4). Therefore in atherosclerosis, I proposed that circulating NE, released from inflammatory cells (Korkmaz et al., 2007), is assimilated into atherosclerotic plaque during their passage either through the vascular lumen or via the vasa vasorum (Leclercq et al., 2007), and NE directly cleaves IL-1 within the endothelium. This proposal remains to be clarified, and a causal connection between IL-1β and NE needs to be confirmed.

8.2.2. Proposed mechanism of action of NE in atherogenesis:

This study has provided important insights into the mechanisms leading to NE/IL-1 inflammatory effects in atherosclerosis. In summary (Figure 8.1), my data suggest the following sequence of events: Following recruitment of inflammatory cells to the site of atherosclerosis (i), these cells release NE and possibly other signalling proteases such as CG and PPR-3 (ii). These proteases initiate a cascade of changes in the inflamed endothelium, including membrane vesiculation and MV shedding (iii). MVs containing bioactive IL-1 pinch off from the ECs and activate other ECs and possibly inflammatory cells in the area (iv). With the persistence of chronic inflammation and thus the exposure of ECs to NE, NE enters ECs and evokes exosomal release enriched in IL-1 (v).

In a broader sense, these findings offer important implications for targeting NE to control IL-1 secretion and thus eliminate IL-1 responses in its local environment.

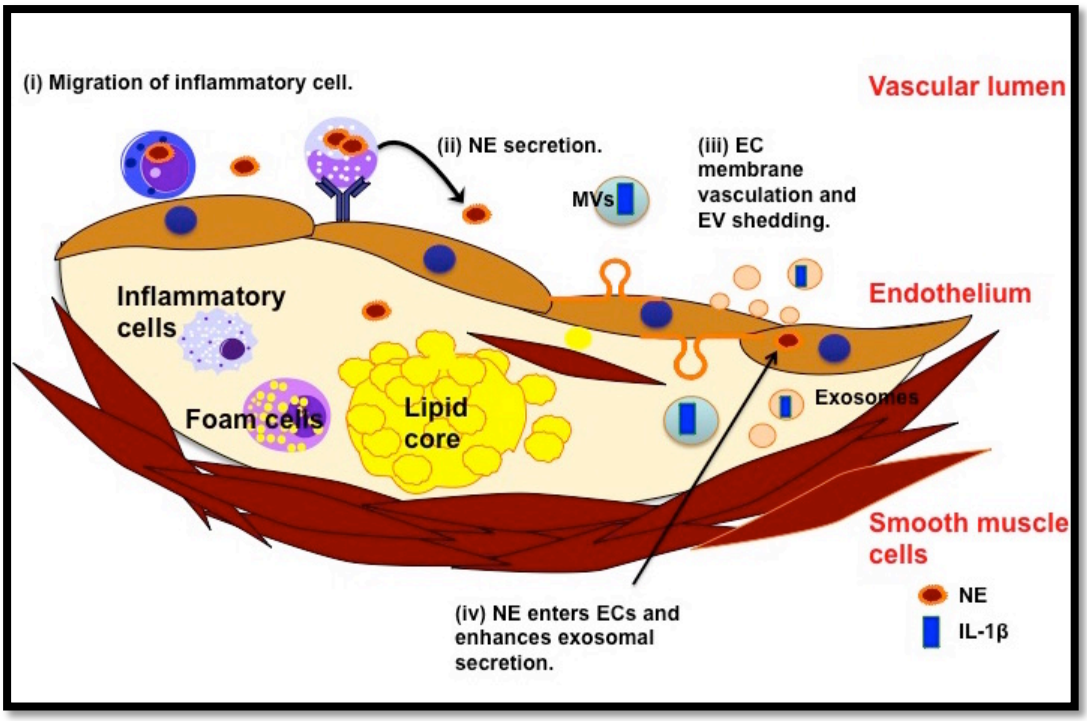

Figure.8.1. Schematic diagram illustrating the mechanism of NE as an important mediator for IL-1 induced injury in atherosclerotic plaque. Dysfunctional endothelium expresses a number of adhesion molecules, enhancing inflammatory cell migration and degranulation, resulting in NE secretion. Subsequently, NE stimulates local IL-1 secretion directly from EC.

8.2.3. Limitations of the in vitro study:

The *in vitro* study has several limitations. The use of cultured ECs offers some technical limitations to the conclusions that may be drawn from data obtained, including the present work. There are many arguments, both against and supporting the use of cultured cells to address any pathologically related hypothesis.

In atherosclerosis, ECs have long been known to interact with a multitude of cell types, but the molecular mechanisms involved has not been explored (Muller, 2015, Tabas et al., 2015). The loss of this kind of interaction between different types of cells within the heterogeneous lesion is considered as one of the greatest challenges of any *in vitro* study. For example, in culture, unstimulated ECs do not tend to produce any IL-1, but when incubated with different combinations of cytokines (that may be secreted by different inflammatory cells in the plaques) the cells became inflamed and endogenously express IL-1. However whether the cells react similarly in atheromatous plaque and whether they are exposed to the same types of cytokines could not be answered.

In addition, it is unknown whether the response of the vascular wall endothelium to NE would be the same as that observed in ECs cultured in dishes.
The time duration and concentrations of NE that were tested in the present study are another limitation. Under physiological conditions and within the vascular wall, the endothelium may be exposed to NE, but whether this exposure would be in the same concentrations as those tested *in vitro* needs further elucidation. In addition, taking into the account the chronicity of the disease, 6h stimulation of ECs with NE may not mimic the duration that vascular ECs may be exposed to NE. NE was detected *ex vivo* within the shoulders of carotid atheroma (Dollery et al., 2003); therefore, it is inevitable that the endothelium is in contact with NE for longer time periods.

In vitro, NE is very unstable and can be deactivated by the presence of NE inhibitors in the serum (Henriksen and Sallenave, 2008), therefore, the study was conducted using serum free media. Since ECs *in vitro* could not survive in the serum free conditions (King et al., 2003), longer incubations were not considered.
Therefore, when interpreting my findings, it is crucial to take into account that this research has been conducted *in vitro* and so any conclusions drawn may be not directly translated into the human lesions. However, given the challenges posed by culturing ECs and the co-incubation with NE, the advantages of cell culture in defining the role of NE in IL-1 production far outweighs any potential drawbacks, without the need to conduct the research on living animals. For example, culturing of ECs enables the involvement of NE in shedding and secretion of different populations of IL-1-containing EVs to be detected, and the kinetics of the secretion defined, something that cannot be explained by conducting *in vivo* studies.

The use of purified NE (commercially provided) may offer some pathophysiological limitations and thus be another limitation of the project. That is, NE is detected in variable amounts in neutrophils and monocytes (Takahashi et al., 1988b). This current research did not address which type of these cells is the primary source of NE in the plaque area. Thus, the pattern of endothelial IL-1 secretion in the plaque area may be more complex than it has been observed in this study.

Another limitation in this study is the bioactivity assay used to measure the activity of the released IL-1 into the supernatants of ECs. This assay did not recognise which of

the secreted intermediate products of IL-1β (15, 18 and 20kDa) is the bioactive isoform. Although the bioactivity of these cleaved products has been tested before *in vitro* (Hazuda et al., 1990, Black et al., 1991), measuring the activity of individual cleaved products within the supernatants could be of great interest as it may provide a further insight into the biological importance of different isoforms of IL-1. Since the scope of the project is on measuring whether NE induces bioactive IL-1 secretion, I chose not to pursue this area any further.

Flow cytometry is a technique commonly used for in research EV characterisation and quantification (van der Pol et al., 2012). However, this technique is not sensitive enough to differentiate exosomes from MVs. The separation of MVs from exosomes using a centrifugation gradient is also inefficient, with a high possibility of having a population of exosomes within the MV fraction. In addition, degradation of EVs as a result of the sequence of centrifugations cannot be avoided.
Another additional problem with flow cytometry is that the markers used to characterise endothelial derived EVs are not specific to endothelial cells. Annexin V is used as a standard marker for EVs it has also been detected in MVs isolated from other cellular types, such as monocytes (MacKenzie A, 2001). Unfortunately, other than E-selectin and vascular endothelial cadherin, which are not widely expressed in endothelial EVs, most of the markers used to identify endothelial derived MVs are not exclusively expressed by the endothelium. For example, CD31 is present on activated platelets, platelets derived MVs, and some subsets of leukocytes (Gyoergy et al., 2011). However, I cultured only ECs and so any released MVs were quantified and expected to be released by ECs.

8.2.4. Future roles for NE in IL-1 dependent inflammation:

The findings that are presented in chapters 3 & 4 of this thesis have raised many future opportunities. The continuation of this research would not only increase in our understanding of IL-1 biology but will also open the door for a new usage for NE in cardiovascular disease.

In immune derived cells, IL-1β precursor is produced in an inactive form that is cleaved upon secretion to generate the mature 17kDa isoform of IL-1β (Thornberry et al., 1992). The lack of activity of proIL-1β was attributed to its inability to bind to

and activate its own receptors. By contrast, the mature IL-1β (17kDa) binds to its receptors with a high affinity in order to trigger a downstream signalling pathway and thus perpetuates inflammation (O'Neill and Dinarello, 2000). However, there are intermediate fragments generated as a result of the proteolytic activity of a number of serine proteases on proIL-1β, including NE, CG, and PR3, and these may have differential activities with different affinities toward IL-1RI (Black et al., 1988).
In this thesis, I showed that NE processes proIL-1β within ECs and this results in the secretion of different isoforms of IL-1β, including the15kDa, 18kDa and 21kDa but not the 17kDa isoform. It is still unproven whether these cleaved intermediate products are identified in patient's blood or whether they have the same affinity to bind to IL-1RI.

Circulating monocytes interact with dysregulated endothelial cells initiating the inflammatory response to injury (Ley, 2015, McEver, 2015), which in the presence of high lipid (Kovacic, 2014) leads to the initiation of atherosclerosis and plaque development within the arterial wall (Bentzon et al., 2014). Ward *et al.,* 2009, have used EC and monocyte co-cultures to investigate the intercellular signalling between these two cell-types and have shown that endothelial cells regulate the production of IL-1β from monocytes, via an unidentified soluble factor, which is not the damage signal, ATP. This production of IL-1β results in a synergistic increase in the production of IL-6 in the endothelial-monocyte co-culture.
In this thesis, I identified NE as a mediator of IL-1β production and processing in human endothelial cells, with evidence that NE is expressed in the endothelium of mouse diseased arteries. Therefore, testing whether the soluble factor released from endothelial cells that regulates IL-1β production in co-cultured monocytes is NE would be very interesting and one of the main research directions in the future.

EVs have been identified to play a key role in intercellular communication and crosstalk between cells in atheromatous plaque (Buendia et al., 2015, Burnier et al., 2009). Although no general agreement has been paid, EVs have different names and types depending on their cellular origin and the differences in their sizes (Gyoergy et al., 2011). In contrast to MVs which are derived from budding of the plasma membrane (0.1-1μm), exosomes (30-100nm) are released by an endolysosomal pathway (Loyer et al., 2014, Sluijter et al., 2014). Therefore, although

both exosomes and MVs have different pathways, they have been increasingly implicated in secretion of different proteins from the same cells, including platelets (Heijnen et al., 1999, Heijnen et al., 1998) and monocytes (van der Pol et al., 2012). In chapter 4, I showed that NE enhances secretion of different types of EVs (MVs and exosomes) specifically derived from ECs. Both contained IL-1 and released it in a time dependent fashion. Because of their different origins and the specific protein compositions (MVs vs. exosomes), these extracellular vesicles may have other functions beside IL-1 secretion. Other complementary cytokines that drive inflammation could be released by a similar pathway from ECs, although this has yet to be proven.

Moreover, in human plasma, different EVs of different cellular origins, predominantly platelet and leukocyte derived, have been isolated and linked to the atherogenesis (Owens and Mackman, 2011, Rautou et al., 2011), whereas endothelial derived EVs are much less studied. Considering the important and versatile roles that the vascular endothelium plays in the disease, isolation and characterisation of endothelial derived EVs in the plasma of patients with MI and linking these with the disease progression may have future prognostic and therapeutic implications. In particular, exosomes may be an attractive therapeutic tool. In cancer therapy, for example, the discovery of exosomes has helped in developing new types for chemotherapy (Grabbe et al., 1991). In atherosclerosis, this new area of interest has been less explored (Sahoo and Losordo, 2014).

Additionally, full characterisation of the prominent type of extracellular vesicles that contain IL-1 in patient plasma, and how these EVs react with their receptors, deserve further investigations. There are several ways that the exosomes/MVs may interact with their cellular targets, for instance, by binding to the plasma membrane, fusion or they may be taken up by cells using the endocytic pathway (Stoorvogel et al., 2002). Therefore, future work on how these vesicles interact with their targets to induce the IL-1 signalling pathway and their other possible physiological targets is required.

I also showed that NE enters ECs in LAMP-1 positive MVBs. Endocytosis and incorporation of NE into the MVBs may require surface binding, internalisation and intracellular signalling. In cancer cells, NE has been shown to get into the cells by

clathrin-dependent endocytosis (Houghton et al., 2010, Gregory et al., 2012). However, little is known about the intracellular trafficking of NE into ECs, especially with the regard to the intracellular routing of NE after its internalisation and delivery to the MVBs.

In addition, the nature of the MVBs inside ECs remains to be established. MVBs formed in certain types of cells play an important role in sorting internalised materials and are thus considered as a part of the endocytic pathway (Piper and Katzmann, 2007). Therefore, it is possible that after internalisation of NE by ECs, NE gets into the MVBs where it cleaves proIL-1β. Further investigations into the mechanism of IL-1 secretion using inhibitors for the endocytic pathway would be of great interest, particularly in defining whether the formation of MVBs is a part of trafficking NE inside the cells.

In the current study and using EM, I observed in NE treated ECs that there are other vesicles containing IL-1 besides the MVBs, mainly in the perinuclear area. This finding has been published before in different IL-1 producing cells (Pond and Watts, 1997; Pond and Watts, 1999; Liu et al., 1998; Turley et al., 2000). Therefore, it is possible that the intracellular trafficking of IL-1 from the perinuclear area to the plasma membrane involve vesicular transport steps, yet the exact characteristics of the intermediate vesicles have yet to be identified.

MVB release their contents by exocytosis (vidal et al 1999) and thus their fusion with the cell surface is likely to be regulated, as shown by platelets and immune derived cells (Nickel and Rabouille, 2009). Exocytosis of exosomes is calcium dependent (Piper and Katzmann, 2007) and may be preceded by changes in syntexins (Ghossoub et al., 2014). It is tempting to speculate that NE causes cytosolic calcium changes and subsequent phosphorylation that triggers syntexin activation.
Recent work by Ghossoub *et al* has suggested that MVB formation and exocytosis of exosomes is mediated by activation of the GTPase ADP ribosylation factor 6 (ARF6), which subsequently increases syntenin exosomal secretion. In light of this, an interesting finding by Zhu and colleagues has suggested that in ECs, calcium dependent ARF6 signalling is implicated in IL-1 mediated responses (Zhu et al.,

2012). Therefore, studying the role of ARF6/syntexin pathway in IL-1 secretion by NE effects is warranted.

NE potentially plays a major role in inflammation, yet its roles in atherogenesis have only been partially understood (Chua and Laurent, 2006). The findings of this current research shed new light on the pathophysiological importance of NE in atherosclerosis. In deed, controlling the activity of NE might help to mitigate IL-1 mediated inflammation, which might slow down the disease progression. NE inhibitors have already been tested in different animal models of pulmonary related diseases (O'Blenes et al., 2000, Zaidi et al., 2002, Zaidi et al., 1999, Sallenave, 2000), although there is a discrepancy between the findings in animal and clinical based research (Alam et al., 2012).

The endogenous inhibitor of NE, elafin, has been detected in coronary atherosclerotic plaques (Alam et al., 2012). In different animal models, elafin has been suggested to attenuate chronic inflammatory progression of several diseases, including atherosclerosis (Henriksen, 2014) and pulmonary hypertension (Zaidi et al., 2002). In addition, it has been shown to have survival benefits in patients with viral myocarditis (Zaidi et al., 1999). Recombinant elafin has low toxicity and it has demonstrated a very good response in different animal studies. By contrast, in clinical studies, due to its short half-life it has been given by continuous intravenous infusion and, thus, this has become one of the biggest limitations to translate NE inhibition by elafin administration into the clinic (Alam et al., 2012).

More recently, a novel oral NE inhibitor AZD9668 “Avelestat” has been introduced and tested in different animal models, including NE-induced lung injury (Stevens et al., 2011). Interestingly, it has been demonstrated that this inhibitor reduced different inflammatory responses in bronchial fluids, including interleukin-1β levels (Stevens et al., 2011).
The safety and tolerability of AZD9668 has already been established in two different healthy volunteer studies (Gunawardena et al., 2013). Subsequently, on-going clinical studies will test the effect of the inhibitor on inflammatory lung diseases (Stockley et al., 2013, Kuna et al., 2012). However, direct evidence from the effect of AZD9668 on vascular wall inflammation and atherosclerosis is relatively lacking.

Continuation of my work by testing the effect of this inhibitor *in vivo* using an experimental model of atherosclerosis would be of particular interest and may help to address the hypothesis of whether inhibition of NE mitigates IL-1 induced atherosclerosis. Studying plaque size and compositions in animals treated by the inhibitor could be performed and thus it may give an insight, for the first time, on the mechanism of action of the inhibitor in cardiovascular medicine.

8.3. The anti-atherosclerotic mechanism of action of DHA:

DHA has mostly been investigated in combination with EPA and using only an exclusive targeted approach, focusing on a particular pathway (Stanley et al., 2013, Matsumoto et al., 2009). The use of such experimental design is, therefore, flawed when interpreting the broad spectrum of actions of DHA (Mozaffarian and Wu, 2011).

8.3.1. Summary of findings of the DHA study:

In this thesis, I showed that DHA decreased distal atherosclerosis and improved plasma dyslipidemia.

It has been postulated that HDL-C prevents LDL-C precipitation into the sub-endothelial space and thus protects against atheroma formation (Martin et al., 2015). Therefore, my findings of increased levels of HDL-C compared to all other cholesterol fractions in the plasma of DHA-fed mice, associated with less atherosclerosis (Chapter 5), suggest the importance of DHA in preventing cholesterol accumulation and plaque formation (Figure 8.2).

In chapter 5, I also investigated the ability of DHA to directly prevent hypertension and LVH induced by feeding a high fat diet. Chronic supplementation with DHA for 12 weeks (human equivalent of 3g/day (Morin et al., 2015)) was found to decrease arterial blood pressure and LVM.

I also observed that DHA feeding had no effect on aortic root atheroma but it preferentially decreased atherosclerosis in the brachiocephalic arteries and the distal aortae. The selective effects of DHA on atherosclerosis formation in different vascular beds suggest that DHA may act through a mechanism separate from the changes in the plasma cholesterol. My findings corroborate observations in patients that lesions tend to occur at certain sites of the vasculature.

That led me to the hypothesis that DHA may decrease distal atherosclerosis by flow-mediated mechanism(s). In Chapter 6, I studied the differences in WSS amplitude and direction in different parts of the aortic wall. Interestingly, in the proximal aorta, WSS was not significantly affected by DHA supplementation. However, DHA decreased OSI in the descending aortae. My finding is the first to associate the differential effects of DHA on atherosclerosis with the changes in WSS oscillation.

De Caterina *et al* have demonstrated a reduction in E-selectin and VCAM-1 expression induced by IL-1 in ECs treated with 25μM of DHA (1998). In addition, a recent study by Moron *et al* suggests a reduction in IL-1β levels in plasma of rats supplemented with DHA and HFD over 8 weeks (2015).
However, only a casual connection between the anti-inflammatory effects of DHA and IL-1 expression in the plaque area was made. In Chapter 7, I showed that DHA decreases endothelial IL-1β in aortic atheroma. Chapter 7 also addressed the mechanism by which DHA decreases IL-1β expression in aortic atherosclerosis. I showed that supplementation with DHA significantly decreases TLR4 expression. Activation of the TLR4 has been implicated in various chronic inflammatory diseases, including hypertension (Liang et al., 2013) and atherosclerosis (Xu et al., 2001), through downstream induction of many inflammatory cytokines, including IL-1 (Schoneveld et al., 2005, O'Neill and Dinarello, 2000).

I also demonstrated that DHA decreases mac-3 positive macrophages in aortic plaques (Chapter 7). The recruitment of inflammatory cells and their subsequent accumulation into the plaque area is critical in atherosclerosis development (Ley, 2015). Therefore, the predicted results of lower macrophage numbers in the aortic plaque would explain, in part, the reduced atheroma formation in mice fed DHA. However, this dose not explain the changes in blood pressure and the haemodynamic responses that I observed in the mice supplemented with DHA.

NO is produced by eNOS that has multiple atheroprotective and vasodilator effects (Li and Forstermann, 2000). Li *et al* has postulated that up-regulation of eNOS in the aortae of spontaneous hypertensive rats mediate blood pressure reduction (2006). Moreover, changes in WSS have been demonstrated to regulate eNOS expression in atheromatous plaque (Davis et al., 2004).

In Chapter 7, I tested the hypothesis that DHA supplementation could alter WSS by eNOS mechanism(s). Interestingly, the finding of eNOS up-regulation in DHA fed animals (Figure 8.2) suggests a novel explanation for the local haemodynamic changes observed in the vasculature of these animals.

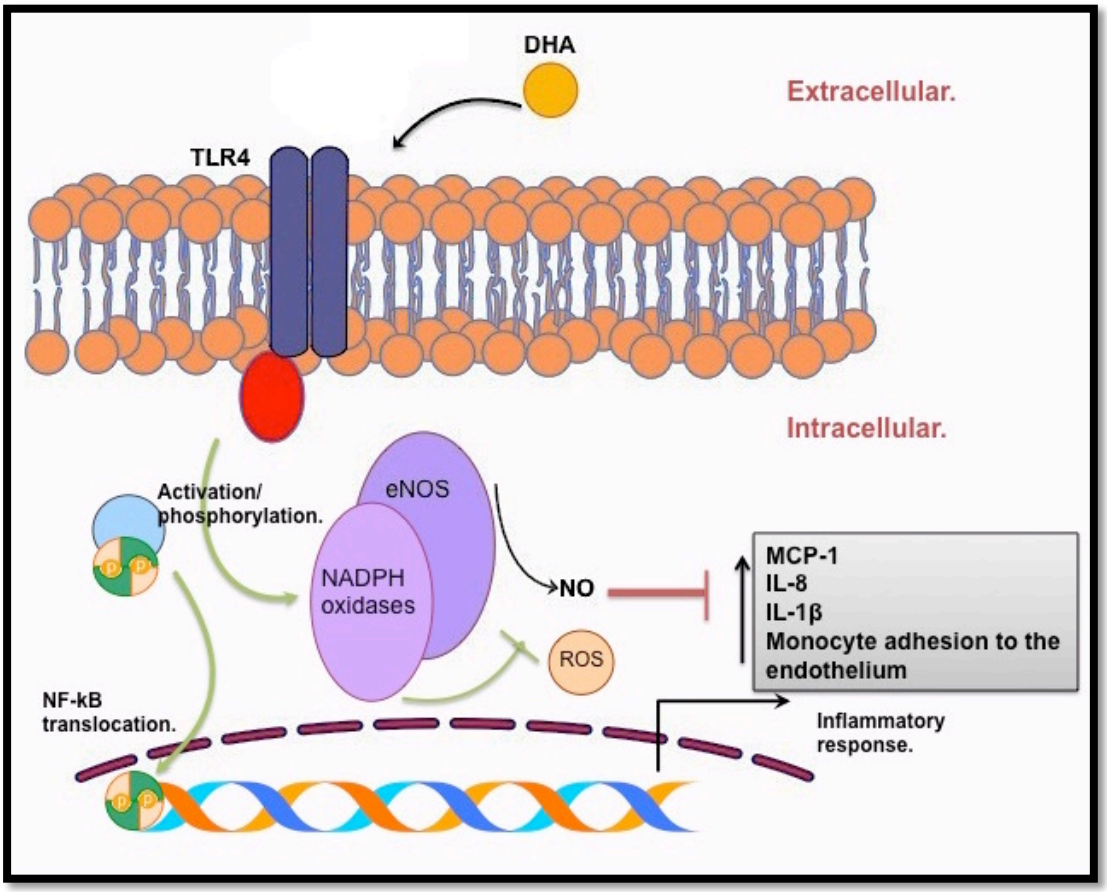

Figure 8.2. Modulation of the TLR4 signalling pathway by DHA in endothelial cells. TLR4 activation triggers a downstream phosphorylation and activation that generates NF-κB translocation into the nucleus, with enhanced production of multiple of proinflammatory mediators, including MCP-1, IL-1, and IL-8. IL-1 also enhances NADPH activation and reactive oxygen species (ROS), which down-regulate eNOS and thus decreases NO bioavailability. However, DHA intervenes with this pathway by decreasing the expression of TLR4 and enhancing eNOS up-regulation.

8.3.2. A proposed mechanism of action of DHA:

In summary, the atheroprotective effects of DHA observed in the ApoE$^{-/-}$ atherosclerotic mouse model may be via either DHA influencing lipoprotein haemostasis or through an anti-inflammatory mechanism(s) or both at the vessel wall. Following development of atherosclerosis, DHA fed mice have pronounced blood pressure protection and shear stress modulation.

My results highlight the importance of choosing the end point to observe the favourable responses of DHA and I believe they provide potential major clinical and therapeutic significance for the role of DHA as anti-atherogenic and/or anti-hypertensive agent.

In a larger context, my results lead me to speculate that DHA selectively decreases distal atherosclerosis by increasing plasma HDL-C, which in turn prevents LDL-C precipitation into the sub-endothelial space. DHA may also directly maintain endothelial function and decrease a number of IL-1β mediated effects. Subsequently, DHA enhances eNOS expression and NO dependent vascular remodelling. Local mechanical forces are then maintained, and the rise in blood flow is prevented with lowering of OSI, which further stabilises the endothelium and reduces plaque formation (Figure 8.3).

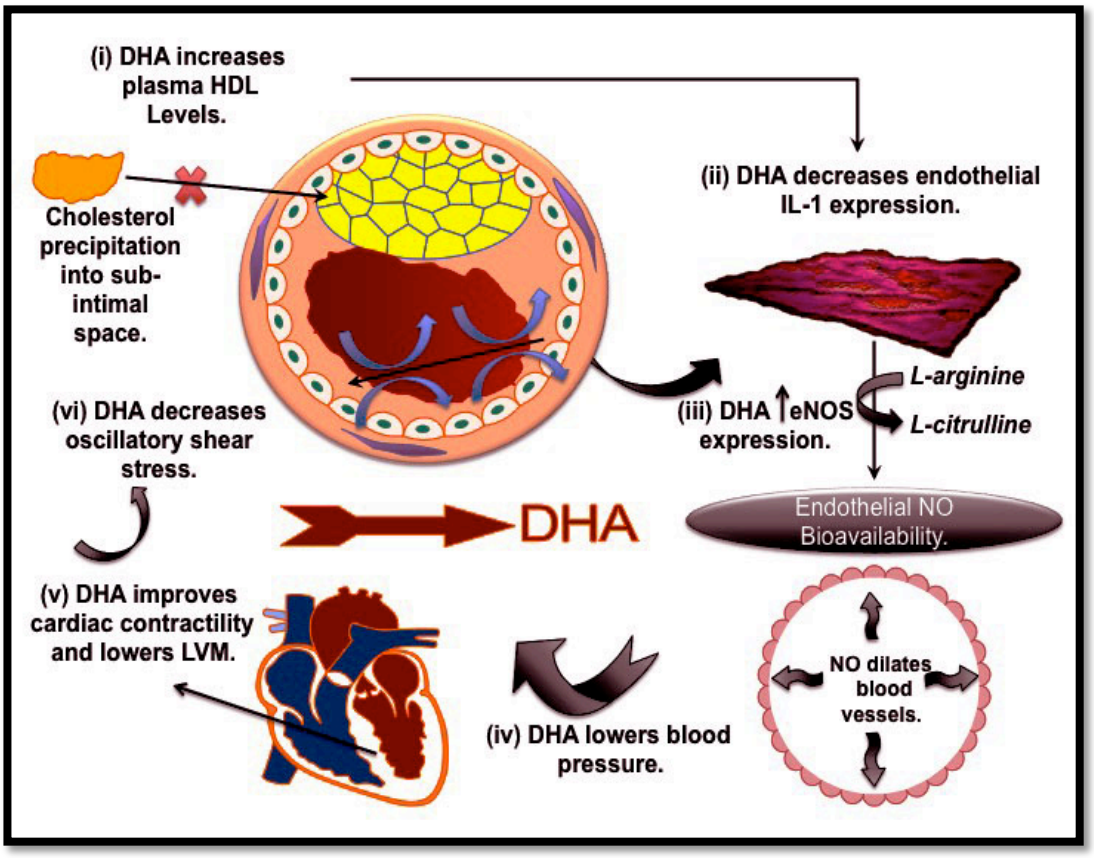

Figure.8.3. The potential therapeutic effects of DHA on vascular wall inflammation and hypertension. (i) Dietary DHA leads to disruption of sub-intimal cholesterol precipitation by increasing HDL. These processes contribute to small atheroma formation and maintain plaque stabilisation. **(ii)** DHA modulates IL-1 expression and, subsequently **(iii)** enhances eNOS activity and NO bioavailability. **(iv)** DHA also lowers arterial blood pressure secondary to vasodilation and **(v)** prevents the increase in LVM induced by HFD. Finally **(vi)** DHA promotes cardiac contractility and aortic blood flow that decreases the oscillation in shear stress and promotes the stability of the atherosclerotic plaque.

8.3.3. Limitations of the DHA study:

The presented DHA study has strengths as well as some limitations. Firstly, I performed the experiment on ApoE$^{-/-}$ mice to study DHA effects on atherosclerosis and hypertension. However, the use of mice to study human pathology has some inherant limitations, which should be considered before any conclusions can be drawn.

There is an argument against using a murine model to study human diseases, including atherosclerosis. Mice do not usually develop atherosclerosis, due to the high protective HDL-C in their plasma. However, by knocking down ApoE receptors, and feeding the mice HFD for 12 weeks, they develop lesions in the vasculature, phenotypically similar to that in humans (Jawien et al., 2004). This fundamental

difference in the instigation of atherosclerosis means that interpreting the findings in this research must be carefully considered, since it was conducted on a nonhuman species and may not directly translate into the clinic.
Despite this, the advantages of using a mouse model, including the ease of genetic manipulation and the ability to perform *ex vivo* assays, far outweigh their potential limitations. Additionally, this mouse model provides a new mechanism of action of DHA in living animals, which could not be offered by *in vitro* studies alone.

Secondly, in this study I measured only one time point (12 weeks) to investigate the anti-hypertensive and anti-atherogenic effects of DHA that may not represent the expected changes that could be seen with chronic supplementation of DHA in patients. Indeed, people may have the disease for a long time and giving DHA supplementation may be required for a significantly long period before observing any changes in the vasculature. To control for this, the animals in my experimental study could be supplemented for longer periods with DHA.
Generally, the findings may not be directly linked to those in IHD patients, but it may help in providing impetus for future clinical studies.

Thirdly, I evaluated the DHA response and did not choose to also evaluate EPA. Previous investigations suggest anti-hypertensive effects are strongly associated with the extent of dietary consumption of DHA and EPA (Wann et al., 2011, Woodman et al., 2002, Vandongen et al., 1993). Therefore, EPA supplementation may be beneficial to atherosclerosis in mice, and this may be by a similar or an alternative systemic anti-inflammatory mechanism(s). Clinically, it is advisable to consume DHA with EPA as both FAs are present in large quantities in FO and both have shown a significant reduction in CVD mortality (Krauss et al., 2000). However, with the time constraints of this project, the high cost of obtaining EPA, and given the knowledge that DHA had been extensively studied in hypertensive patients (Engler et al., 2003) and animal based research (Frenoux et al., 2001, Morin et al., 2015, Stanley et al., 2013), I chose to concentrate this study on investigating DHA. Had cost and time not been a consideration, I would have tested the EPA effects using this model.

Finally, the techniques used in this study may harbour an additional limitation. For example, *en tail cuff* is a standard technique to measure changes in blood pressure in mice. However, this method measures blood pressure in a relatively crude way and the reading in blood pressure of these mice may change from day to day, affected by the condition of the mice, the consistency of handling, time of day when the pressure reading was taken, and general stress level of the mouse at time of reading. To minimise the variations in blood pressure, the mice were trained for a week to familiarise them with the equipment and procedure, kept in a warm, dark constrainer during BP reading, were handled by only one person, and BP readings were taken at the same time every day (within a 2 hour range).

I used *en face* oil red O staining to quantify atherosclerotic burden in the different aortic regions. However, this is a technique used to measure lipid content and may not be sensitive enough to detect the entire size of the lesion, nor give an indication on their composition. The accuracy of the measurements may be limited by any residual fats on the outer surface of the aorta. However, a significant effort was made to remove the residual fats under a dissecting microscope, thus any contaminating positive stain from this is expected to be minimal and would not affect the total quantification. Alternatively, quantification of lesion area using histological analysis of cross sections of the vessels can be used, and are more accurate. However, in a large artery like the aorta where atherosclerosis is usually segmental, the potential of missing an important lesion of interest is possible with cross sectional analysis.

The estimation of the magnitude and direction of WSS is based on echocardiographic data and the change in the velocity profiles of the aorta. One limitation of this is the geometrical variation. The presence of bends or branch points may induce big changes in the WSS pattern and give false results. Therefore, the observational nature of this analysis does not allow us to make definitive conclusions regarding the beneficial effect of DHA on OSI, but it does give an indication on the changes in OSI, which can be linked with anatomical areas where atherosclerosis tends to develop.

8.3.4. The future implications of DHA as an IL-1 suppressor:

The finding of suppression of endothelial IL-1, linked with the atheroprotective and anti-hypertensive roles of DHA, is novel and could be taken further.

An outline of different areas that could be possibly covered in future research is summarised below:

Having shown that DHA mediates a reduction in high blood pressure induced by fat feeding in mice, it would be of great interest to investigate the anti-hypertensive effects of DHA using pharmacologically, more relevant large animal models of hypertension that recapitulate the complex aspects of the disease, before translating the findings into clinical studies. In addition, n3FAs are mostly studied in patients with already established IHD treated with primary PCI (Mozaffarian and Wu, 2011). Therefore, it would be interesting to test its effects directly in a relevant injury model. Murine stenting is an established model of PCI in mice (Chamberlain et al., 2010), and this model could be utilised to investigate whether DHA plays any role in preventing restenosis, which will broaden its application in the cardiovascular field.

Since bioactive IL-1β is found intact within EVs of endothelial, rather than of circulating origin, the decrease in endothelial IL-1 expression during chronic DHA supplementation suggests that DHA suppresses endothelial IL-1 production in mice. Therefore, further studies could be directed at elucidating whether the selective inhibition of endothelial IL-1β has any future therapeutic implications. It has also been suggested that IL-1β$^{-/-}$ mice have reduced atherosclerosis, although whether endothelial IL-1β$^{-/-}$ would decrease blood pressure and atherosclerosis is yet to be investigated. DHA could be used in the endothelial IL-1β$^{-/-}$ model to see if this effect is solely mediated by IL-1β.

In vitro, I showed that NE enhances IL-1β secretion from endothelial cells and, *in vivo,* I demonstrated a reduction of endothelial IL-1β expression with DHA supplementation. A study by (Bates E, 1993) suggested that treatment of HUVECs with DHA prevented neutrophil elastase mediated injury. Therefore, future studies to investigate whether DHA interacts with NE or downstream effects, including IL-1 secretion from the endothelium are warranted.

The regional differences in the lesion in response to DHA feeding were postulated to be secondary to lowering the OSI in distal vasculature. *In vitro* flow models have been used to investigate the effects of WSS on ECs (Huang et al., 2013). Therefore, further confirmation for DHA modulatory effects on WSS in cultured ECs is warranted. Explanted atherosclerotic plaques from different aortic regions, that have been shown to be WSS sensitive (by staining for specific markers) would shed further insights on the mechanism of action of action of DHA.

In patients with IHD, the physiological significance of controlling IL-1beta production in the endothelium versus the neointima is relatively unknown. Although my data support some contributions for DHA in the IL-1 signalling pathway, at least in the vascular endothelium, DHA has been shown to have broad anti-inflammatory roles, including inhibition of TXA2 and PGE2 *in vitro* (Calder, 2006). It is also possible that DHA may mediate the inhibition of endothelial IL-1beta via different molecular pathways. Future studies are needed to fully elucidate the molecular mechanism of action of DHA using different cell types and different modulations.

This thesis has demonstrated, for the first time, that ECs can secrete a large amount of IL-1 in response to NE stimulation. In atherosclerosis, IL-1 is produced by different types of cells, including SMC, macrophages and ECs, and the dominant source for IL-1 production in the plaque area is yet to be elucidated. Whether controlling IL-1 production in these cells would then have an effect on IL-1 production by the other cells is also unknown. Therefore, one of the future directions of this research is to locally suppress IL-1 in ECs and measure IL-1 expression in atherosclerosis.

Chapter (9) General Conclusion.

OUTLINE:

9.1. Conclusion:

Owing to the complexity in the pattern of the disease, modification of atherosclerosis by a single therapeutic agent has so far proven to be impossible. Even if available, life long medications are cost-intensive and may expose patients to serious side effects that may interfere with their compliance. Hence, in addition to lifestyle modifications that should be enforced early, possible curatives for the disease are usually prescribed only when the lesion reaches the later stages of disease. Thus, investigating the mechanism(s) that cause lesion instability and production of mediators that dissolve plaque deserves more future attention.

IL-1 with a wide range of proinflammatory activities has been shown to play different roles in the early and advanced atherosclerosis. Therefore, the finding of NE as a key regulator in IL-1 secretion by ECs and the detection of NE within the atheromatous plaque may help to understand and thus modulate local IL-1 production. Selective inhibition of NE, without affecting other IL-1 favourable responses in the immunity may offer future therapeutic implications.

On the other hand, the finding of reduction of atherosclerosis and hypertension induced in mice fed a HFD, containing DHA, and the suppression of IL-1 selectively in the endothelium offers a novel role for DHA. That may provide an explanation for the observed beneficial effects of n3FAs, notably that DHA contributes to decrease the risk of atherosclerotic changes.

Chapter (10) References.

OUTLINE:

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Appendices.

OUTLINE:

Appendix (I) Chemical Reagent, Company sources and Concentrations

Chemical	Company	Catalogue number	Concentrations
Recombinant Human IL-1α/IL-1F1	R&D Systems, Inc.	200-LA-002	10ng/ml
Elastase	Sigma Aldrich	E8140	1ug/ml
Tumour Necrosis factor-alpha (TNF-α).	Calbiochem	654205	10ng/ml
Interferon gamma (INF-ɣ)	Calbiochem	IF005	100ng/ml
Lipopolysaccharide (LPs) from E.coli	Sigma	O55:B5	1µg/ml
Goat anti-Rabbit IgG (H&L), DyLight^{R} 550 Conjugate	Immunoreagents, Inc	GtxRb-003-D550NSX	1:200
Cathepsin G from human leukocytes	Sigma	C4428	1ug/ml
Caspase-1 inhibitor I **(Ac-Tyr-Val-Ala-Asp-CHO)**	Calbiochem	400010	50uM
DyLight ^{TM} 488 Goat anti-rat IgG	BioLegend	405409	1:200
Calcium Ionophore A23187	Sigma	C7522	10µM
Bafilomycin A1 from Streptomyces griseus	Sigma	B1793	50nM
Calpain inhibitors; PD150606	Santa Cruz	SC-222133	1nM
Calpain inhibitors negative control;	Santa Cruz	SC-222131	1nM

PD 145305			
Active human Neutrophil Elastase full length protein	abcam	Ab91099	1µg/ml
Elastase Inhibitor III	Calbiochem	324745	500uM
Glibynclamide	Sigma	G0639	
(S)-(+)-Campthothecin	Sigma	C9911	10µg/ml
Fibronectin from bovin plasma Albumin	Sigma		5µg/ml
Elastase Substrate I **MeOSuc-Ala-Ala-Pro-Val-pNA**	Calbiochem	324696	100uM
***SIGMAFAST*™ 3,3'-Diaminobenzidine**	Sigma	D4293	-
Recombinant Human IL-1b/IL-1F2	R&D SYSTEMS	201-LB-005	10ng/ml
P38 inhibitors	Promega	SB 203580	1:1000
Albumin from bovine serum	Sigma	A3803-50G	1%.
Sodium pentobarbital	J M Loveridge Ltd		200mg/kg/ip
Hirudin (900 anti-IIa units/mL	Canyon Pharmaceuticals™		5ug/mL
PBS	Oxoid		
Fixative reagents	VWR International Ltd	//	//

Appendix (II) Antibodies used in this study.

Name	Catalogue number or clone	Type	Cell identified	Company	Working Dilutions
TLR4	Ab13556	Rabbit PAb	Monocytes, macrophages, dendritic cells, some Tcells	abcam	1:50 IHC-P 1:1000 WB
IL-1β	MAB201	Mouse Mab	//	R&D Systems	1:1000
IL-1β/IL-1F2	AB-201-NA	Goat Pab	//	R&D Systems	1:100
LAMP-1	D2D11	Rabbit mAb	Lysosomes Marker	Cell signalling	1:100 IF 1:10,000 WB
LAMP-1	Ab24170	Rabbit Pab	Lysosome Marker.	Abcam	1:1000
IL-1 alpha	Ab7632	Rabbit Pab	Monocytes	abcam	1:200 WB, 1:40 IHC-P
Mac-3	550292	Rat IgG1	M3/84 expressing cells	BD Pharmingen	1:100
Caspase-1	Ab17820	Rabbit Pab	Detect p20, p45 subunits	abcam	1:250
PAR2 (SAM11)	sc-13504	Mouse Mab	ECs	Santa Cruz	1:1000
Cathepsin B	Ab33538	Rabbit Pab	Lysosomes	abcam	1:250
Hsp90 alpha	Ab2928	Rabbit Pab	Abundant cellular protein	abcam	1:1000
Antineutrophil Elastase antibody	Ab68672	Rabbit Pab	PMNs	Abcam	1:500

Antineutrophil elastase	M 0752	Mouse Mab	NE	Dako	1:50
Biotinylated antimouse IgG (H+L)	Vector Laboratories	BA-9200			
Anti-IL1RA	Ab124962	Rabbit Mab		abcam	1:100 IHC-P
Smooth muscle actin	M0851	Mouse Mab		Dako	1:100
Alpha-Tubulin	T9026	Mouse Mab	Intracellular tubulin	Sigma	1:1000
NLRP3/NALP3	ALX-804-819	Mouse Mab	Inflammasome	ENZO	
Anti-eNOS	Ab5589	Rabbit Pab	Endothelial plasma membrane	abcam	1:150 IHC-P 1:500 WB
Anti-ARF6	Ab77581	Rabbit Pab	Golgi apparatus, cell membrane, endosomes	abcam	1:50 IMG
Anti-gp91(phox)	611414	Mouse Mab	Macrophages, granulocytes	BD Transduction Laboratories	1:500 WB I:100 IHC-P
Anti-NADPH oxidase 4 (UOTR1B493)	Ab133303	Rabbit Mab	Endothelial cells	abcam	1:500 WB 1:100 IHC-P
Anti-EEA1	610456	Mouse Mab	Early endosmes	BD Transduction Laboratories	1:100 ICC
Anti-CD63	Ab118307	Rabbit Pab	Exosomes	abcam	1:100 ICC 1:50 IMG 1:500 WB
Anti-iNOS	Ab3523	Rabbit Pab	Endothelial, monocytes, epithelial and	abcam	1:100 IHC-P

			alveoli		1:500 WB
Anti-arginase-I	Sc-20150	Rabbit Pab	Monocytes and macrophages	Santa Cruz Biotechnology	1:100 IHC-P 1:500 WB

Abcam, (Cambridge; UK), ENZO Life Sciences (Switzerland). Dako,(Ely, UK), Life technologies (Paisley, UK). Sigma-Aldrach (Poole, UK), BD Biosciences (Oxford, UK). PAb; polyclonal antibody, MAb; monoclonal antibody, WB; western blot, IHC-P; immunohistochemistry, IF; immunofluorescence, ICC; immunocytochemistry.

Appendix (III) Solutions.

Section (1) Media preparation and their supplements:

Materials	Concentration	Source
CGM		
L-Glutamine	2mM	Gibco
Penicillin	100U/ml	Gibco
Streptomycin	100µl/ml	
ECGF	10µg/ml	Tebu-bio
Heparin	90µg/ml	Sigma
FBS	10%	Gibco
NBCS	10%	Gibco
M199 (endotoxin tested)	1%	Gibco
MV2 Media		
FCS	0.02µl/µl	PromoCell
ECGF	0.004µl/µl	PromoCell
Epidermal growth factor (recombinant human)	0.1ng/ml	PromoCell
Heparin	90µg/ml	PromoCell
Basic fibroblast growth factor (recombinant human)	1ng/ml	PromoCell
Hydrocortisone	1µg/ml	PromoCell
MEM Media		
EMEM	500mL	Gibco
Penicillin	100U/ml	Gibco
Streptomycin	100µl/ml	

Amphotercin B	10µg/ml	Tebu-bio
HEPES	10mLl	Sigma
Sodium Bicarb	7.5%	Sigma
DMEM Media		
DMEM	500mL	Gibco
Penicillin	100U/ml	Gibco
Streptomycin	100µg/ml	Gibco
FBS (heat inactivated)	10% (v/v)	Gibco
L-Glutamine	2mM	Gibco

Abbreviations: ***CGM; Complete growth media, ECGF; Endothelial cell growth factor, FBS; Fetal bovine serum, NBCS; New born calf serum, FCS; Fetal calf serum.***

Section (2) Reagent Stocks:

10% Formal Buffered Saline, pH 6.8

Reagents:

• Sodium phosphate, monobasic 119.98g/mol).	4.0gm	(Sigma-S5011,
• Sodium phosphate, dibasic 141.96g/mol).	7.1gm	(BDH 301584L,
• Formaldehyde Ltd, 100mL)	37%	(VWR International
• Distilled water	900mL	

4% w/v Paraformaldehyde solution, pH.7.0

Reagents:

• Paraformaldehyde (PFA)	4.0gm	(BDH 294474L)
• PBS	100mL	
• NaOH (20 drops to dissolve the PFA).		

Oil red O solution.

Reagents:

• Oil red O	0.5gm	(Sigma)
• 1% Isopropanol (v/v)	100mL	(VWR)
• Distilled water	400mL	

NE solution.

Reagents:

- NE was prepared as a 1.2mg/ml stock solution in PBS containing 50% glycerol (v/v) and 20mM sodium acetate (w/v) to prevent spontaneous degradation of the enzyme (Kuwahara et al., 2006). PBS control containing 50% glycerol and 20mM Na acetate was used alongside of the stimulation.

List OF Publications.

The work presented in thesis is based in part, with some unpublished data, on the following original publications:

I) IL-1 BETA SECRETION IN CORONARY VASCULAR ENDOTHELIUM IS MEDIATED BY NEUTROPHIL SERINE PROTEASE AND IS INDEPENDENT OF CASPASE-1.
M Alfaidi, H Wilson, A Burnett, J Chamberlain, S Francis
Heart 99 (suppl 2), A99-A99 (2013).

II) 212 docosahexaenoic acid but not alpha-linolenic acid ameliorates high fat diet induced atherosclerosis and hypertension in APOE-/-mice via IL-1 mechanism
M Alfaidi, J Chamberlain, S Francis
Heart 100 (Suppl 3), A116-A117 (2014).

III) Neutrophil elastase enhances IL-1 beta secretion from coronary endothelium via an endolysosomal mediated mechanism, without caspase-1 activation
M Alfaidi, H Wilson, V Ridger, J Chamberlain, S Francis
ANGIOGENESIS 17 (4), 941-941 (2014).

IV) 192 Dietary Docosahexaenoic Acid Reduced Experimental Atherosclerosis by Inducing Protective Haemodynamic Conditions
M Alfaidi, T Schenkel, P Evans, J Chamberlain, S Francis
Heart 101 (Suppl 4), A107-A107 (2015).

V) Neutrophil Elastase Promotes Interleukin-1β Secretion from Human Coronary Endothelium
M Alfaidi, H Wilson, M Daigneault, A Burnett, V Ridger, J Chamberlain, S Francis
Journal of Biological Chemistry 290 (40), 24067-24078 (2015) *.

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Immunology:

Neutrophil Elastase promotes Interleukin-1 ® secretion from Human Coronary Endothelium

Mabruka Alfaidi, Heather Wilson, Marc Daigneault, Amanda Burnett, Victoria Ridger, Janet Chamberlain and Sheila Francis

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Neutrophil Elastase promotes Interleukin-1β secretion from Human Coronary Endothelium

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Running title: *Neutrophil elastase promotes endothelial IL-1 secretion.*

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Keywords: Interleukin-1β, neutrophil elastase, endothelium, extracellular vesicles, inflammation, atherosclerosis.

Background: The mechanism of IL-1 release from endothelial cells is not fully known. **Results:** Neutrophil elastase causes secretion of bioactive IL-1 from endothelial cells via microvesicles.

Conclusion: A mechanistic link between IL-1 secretion from endothelial cells and neutrophil elastase in atherosclerotic plaques is revealed. **Significance:** Neutrophil elastase could be a potential target for preventing atherosclerosis.

ABSTRACT

The endothelium is critically involved in the pathogenesis of atherosclerosis by producing proinflammatory mediators, including interleukin-1 beta (IL-1β). Coronary arteries from patients with ischaemic heart disease express large amounts of IL-1β in endothelium. However, the mechanism by which endothelial cells (ECs) release IL-1β remains to be elucidated. We investigated neutrophil elastase (NE), a potent serine protease detected in vulnerable areas of human carotid plaques, as a potential 'trigger' for IL- 1β processing and release. This study tested the hypothesis that NE potentiates the processing and release of IL-1β from human coronary endothelium. We found that NE cleaves the pro-isoform of IL-1β in ECs and causes significant secretion of bioactive IL-1β via extracellular vesicles. This release was significantly attenuated by inhibition of neutrophil elastase, but not caspase-1. Transient increases in intracellular Ca^{2+} levels were observed prior to secretion. Inside ECs, and after NE treatment only, IL-1β was detected within LAMP-1 positive multivesicular bodies (MVBs). The released vesicles contained bioactive IL-1β. *In vivo*, in experimental atherosclerosis, NE was detected in mature atherosclerotic plaques, predominantly in the endothelium, alongside IL-1β. This study reveals a novel mechanistic link between NE expression in atherosclerotic plaques and concomitant pro-inflammatory bioactive IL-1β secretion from ECs; this could reveal additional potential anti-IL-1β therapeutic targets and provide further insight into the inflammatory process by which vascular disease develops.

Atherosclerosis is a complex chronic inflammatory disease that involves inflammatory cell recruitment and release of pro-inflammatory cytokines (1). Interleukin (IL)-1β has been implicated in several aspects of vascular inflammation and neointima formation (2). Endothelial cell dysfunction, promoted by IL-1, also plays a central role in atherogenesis, by expression of adhesion molecules and cytokine secretion (3), facilitating leukocyte recruitment and plaque development. The culmination of two decades of pre clinical experimental studies in the IL-1 field has led to the ongoing phase 3 clinical trial Cankinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) which will test whether blocking IL-1β only will reduce incidence of thrombotic events in patients after myocardial infarction that remain at high risk due to ongoing inflammation (4).

It has generally been assumed that IL-1β is produced predominantly by immune derived cells (5). However, we showed in ischemic heart disease (IHD) patients that atherosclerotic coronary arteries synthesise and express significant IL-1β within the endothelium (6) compared to controls. Experimental studies have also indicated that cultured endothelial cells (ECs) synthesize IL-1β in response to different cytokine stimulations, but that the released IL-1β is low and relatively inefficient (7). It is crucial therefore to understand the mechanism(s) of release of IL-1β from ECs especially since IL-1β acts at a distance rather than just locally in the vessel wall.
IL-1β production is a two-step controlled process, requiring an 'initial' stimulus for transcription/translation of proIL-1β (31kDa) which, in turn, is cleaved by an inflammasome- activated caspase-1 (8) ('a second hit') into a biologically active isoform (17KDa), before secretion. The cleavage of proIL-1β is a crucial step and studies in monocytes show that caspase-1 (a cysteine protease) is a cardinal enzyme in this process (9). There is a spectrum of proposed cellular mechanisms responsible for IL-1 secretion in monocytic cells – including rescue from autophagy and subsequent release, release via microvesicles or multivesicular bodies and terminal release (via pores), dependent on cell type and stimulus intensity (10).

In vivo studies have postulated that IL-1β can also be released in the absence of caspase-1 (11) suggesting an alternative, and yet unknown, mechanism by which 'leaderless' IL-1β is secreted. There are other potential enzymes that cleave proIL-1β into its mature form, including the serine proteases (neutrophil elastase, cathepsin G and proteinase 3, (12,13). It is known that in cell- free systems, these serine proteases cleave purified proIL-1β into a biologically active IL-1β *in vitro* at distinct sites to caspase-1 with production of 18kDa and 20kDa isoforms of IL-1β. However, whether, and to what extent, these proteases could contribute to IL-1β release in cells such as ECs is relatively unknown.
Neutrophil elastase (NE) is a potent serine protease that has wide substrate specificity (14, 15). Experimental studies have potentially focused on the destructive nature of NE, but interesting recent data show that NE can provoke a variety of pro-inflammatory responses such as IL-8 release from bronchial epithelium and TGF-β production in bronchial smooth muscle cells (14). Moreover, deletion of NE in mice leads to reduction of serum inflammatory biomarkers such as TNF-α, MCP-1, and IL-1 (16). One study has also demonstrated NE in macrophage rich human atherosclerotic plaque shoulders (17) and it also appears critical in caspase-1 independent IL-1β generation in NE- induced lung (18) and renal injury (19). In this study, we sought to determine whether NE promotes biologically active IL-1β secretion from vascular endothelium. We show that NE stimulation leads to pro-IL-1β

cleavage and increases IL-1β release from coronary artery ECs via a caspase-1 independent, vesicular-release mediated process. Furthermore, we demonstrate that IL-1β is colocalized with NE predominantly in the endothelium in experimental atherosclerosis. This very first demonstration and explanation of active IL-1β release from endothelium potentially provides novel additional strategies for inhibition of IL-1β activity in inflammatory cardiovascular disease.

EXPERIMENTAL PROCEDURES

Human coronary artery endothelial (HCAECs) were purchased from PromoCell (Heidelberg, supplemented cells Germany) and cultured in media according to the manufacturer's recommendations. The cells, at passage 2-5, were seeded into 6 well plates ($2x10^4$ cells/well) and grown at 37^oC/ 5% CO_2 (v/v) until 70% confluent. The first step of stimulation was to up-regulate proIL-1β production by adding cytokines (TNF-α/IL-1α; 10ng/ml each) for 48h as previously described (7). Cells were then washed to remove all traces of stimulating cytokines before the media was replaced with serum free media containing NE (1µg/ml; equating to 60IU). To ensure the stimulating cytokines were completely removed, the final cell wash was tested for presence of IL-1 via ELISA. No cytokines were detected in these washes (data not shown). In some experiments, cells were pre-incubated with NEIII (500µM) (20), caspase-1 inhibitor I (YVAD; 50µM) (21) (8); or BAF1 (50nM) (22) for at least 30 minutes before the addition of NE. At the end of incubations, supernatants were collected and the cells were lysed in ice-cold 1% (v/v) Triton-X100 lysis buffer. Both supernatant and cell lysates were stored at -80^oC until analysis was conducted.

Determination of cell viability: Cell viability was evaluated by Trypan blue dye exclusion and by measurement of lactate dehydrogenase (LDH) levels in conditioned media. LDH detected in cell lysates was used as a positive control for total LDH. Levels of LDH were analysed using CytoTox 96 Non-Radioactive cytotoxicity kit (Promega, USA) according to the manufacturer's instructions.

NE activity: NE activity was measured spectrophotometrically using a highly specific synthetic substrate (Elastase Substrate I, MeOSuc- Ala-Pro-Val-pNA; 100µM) as described in detail (14, 23). Briefly, samples (supernatant and lysate) were added to assay buffer (0.45 Tris-Base and 2M NaCl; pH. 8.0) containing Elastase Substrate I for 6h. The rate of the substrate cleavage was measured using a plate reader (Thermo Scientific) at 410nm.

ELISA for IL-1β: The concentrations of IL-1β (pg/ml) in the supernatants and lysates were quantified by ELISA Quantikine kits (R&D systems) according to manufacturer's recommendations.

Apoptosis: Apoptosis, detected via caspase-3/7 activity, was analysed by Caspase-Glo® 3/7 assay (Promega) according to manufacturer's recommendations.
Western blot analysis for IL-1β processing and release: Samples (lysates and concentrated supernatants using 10k Amicon filter devices (ThermoScientific)) were subjected to Western blotting.

Microvesicle isolation: MV isolation was conducted as described (24). Freshly prepared MVs were used for analysis, to avoid false- positive effects caused by leakage of contents from MVs damaged by freeze-thawing.

Flow cytometry measurement of extracellular vesicles (ECV): A pellet of ECV was resuspended in annexinV-binding buffer and labelled with annexin V PE-Cy7 fluorescence according to the manufacturer's instructions (eBioscience, UK). Events were acquired using LSR II flow cytometer (BD Biosciences) and annexin-V positive ECV were enumerated using Accu Count Beads (SPHERO, 0.2-0.9μm) and analysed using FlowJo software (TreeStar).

Measurements of Intracellular Calcium concentration: HCAECs in 96 well plates at a seeding density of $5x10^3$ were treated with cytokines or left untreated for 48h. Fura-4 was then added to the cells according to manufacturer's instructions (Invitrogen). After washing off the dye, the cells were incubated with or without NE and changes in cytosolic Ca^{2+} were recorded using a plate reader (Thermo Scientific) according to manufacturer's recommendations. EGTA (6mM) and Ionophore A3784 (10μM) were used as controls as previously described (25).

Direct effects of NE on rIL-1β /ProIL-1β:
NE is known to undergo spontaneous autolysis (26) and has a proteolytic activity against many cytokines such as TNF-α (27). For this reason, we tested the effect of NE with the studied concentration on IL-1β/proIL-1β itself. IL-1β standard (R&D) at a concentration of 125pg/ml and proIL-1β standard (R&D) at a concentration of 10000pg/ml were separately mixed with NE (1μg/ml) and kept in the incubator for 30 min, 2h and 6h. The samples were stored at -80^oC and tested by ELISA and western blot.

Detection of extracellular vesicular shedding: HCAECs ($2x10^4$) were plated in LabTek (Fisher) 8-well chamber slides and subjected to the above-mentioned stimulation conditions. AnnexinV-Alexa Fluor 488 (Invitrogen) was then added to the cells at 5μl/well. MV shedding was visualised using image acquisition software (Inverted widefield fluorescence microscope Leica AF6000 Time Lapse) after the addition of NE in a 5% CO_2/ 37^oC (v/v) heated chamber. The images were captured after 15, 30min, 2h and 6h and analysed using Image J software (NIH). MVs (0.1-1μm) were quantified in blinded samples in a random field of cells.

Immunofluorescence: Cells were fixed with 4% w/v paraformaldehyde and permeabilized in 0.3% v/v Triton-X 100. Non-specific binding was blocked for 30min with 5% v/v goat or rabbit serum in 1% w/v BSA prior to sequential incubation of the cells with primary antibodies: anti-goat IL-1β and anti-rabbit LAMP-1 (1:100 dilutions). Alexa fluor 647 and 488 conjugated secondary antibodies were used in 1% w/v BSA (1:200 dilution). The coverslips were washed with PBS and mounted onto glass slides using media containing DAPI. The labelling of NE was conducted as previously described (28) using a Microscale Protein Labelling kit (Molecular Probes) according to manufacturer's instructions. 50μg NE was used for the reaction. Final concentration of Alexa647-NE was 0.1 mg/ml in a volume of 100μl.

Determination of IL-1β biological activity:
The bioactivity of the secreted IL-1β by HCAECs following NE stimulation was assessed using an IL-8 luciferase reporter assay, sensitive to picomolar concentrations of IL-1, as previously described (9); (29); (30). Briefly, HeLa cells ($5x10^3$) were grown to 70% confluence in 96 well plates and transfected with a total of 100ng DNA/well; including 60ng pIL-8-luc (reporter) and 40ng pRL-TK (internal

control). After 24h, transfection efficiency was assessed and cells were then stimulated for 6h with 0.1nM of IL-1β as a control or with harvested supernatants from HCAEC stimulated with NE. IL-1 beta neutralising antibody (1ug/ml, MAB201, R&D Systems) was used in some wells, to prove specificity. Cells were then lysed with passive lysis buffer (Promega) and transferred to white well plates to assay for luminescence intensity using LARII and Stop and Glo reagents (Promega). Luciferase activity was calculated by normalising to the Renilla luminescence measured in each well according to manufacturer's recommendations (Promega).

Conventional and Immunogold Labelling Electron Microscopy: EC pellets (NE or untreated controls) were processed as previously described (31). Thin sections were immunogold labelled with primary antibodies: anti-goat IL-1β and anti- rabbit LAMP-1 (1:50 dilutions each) for 2h at room temperature. After washing the grids, the sections were incubated with immunogold conjugated secondary antibodies (20nm& 10nm gold particles, Agar Scientific, UK) for 2h. Control experiments were performed by using PBS instead of the primary antibodies and all sections were then post-stained with uranyl acetate and lead citrate.

Mice and diets: ApoE$^{-/-}$ male mice were bred in-house at the University of Sheffield. Food and water were given *ad libitum* under a controlled environment (Temp. 22-25oC, humidity 55±5 and 12h light cycle). At 8-10 weeks of age, the mice were housed individually and fed a high fat, Western-type diet (HFD) containing 21% fat, 0.15% cholesterol and 0.296% sodium over a 12- week duration. Special Diet Services, Witham, UK, supplied the diets. This HFD was used specifically to study the diet effects on atherosclerosis as described (32). All animal care and procedures were closely conducted under ASPA 1986, UK and ethically approved by The University of Sheffield Ethics Committee. At the end of the study, the mice were euthanized and proximal aortae were harvested.

Immunohistochemistry: Sections were used for immunohistochemistry as previously described (33).

Statistical analyses: Results are shown as mean ± standard error of the mean (SEM). Analyses were performed using Graphpad Prism version 6.04 (Graphpad). For multiple comparison tests, one way analysis of variance (ANOVA) followed by Tukey's test was performed. Statistical significance was achieved when the *p* value was less than 0.05.

RESULTS

NE promotes IL-1 release in HCAEC: To assess the contribution of NE to IL-1β secretion, cytokine-primed ECs were treated with varying concentrations of NE in serum free media for different time points. As shown in Figure 1A, after 2h of stimulation, NE at 1μg/ml caused significant (10x) release of IL-1β from cytokine-primed cells (198±24.85pg/ml, $p<0.0001$) compared to primed cells without NE (12.1±4.81pg/ml). This release significantly decreased with higher concentrations of NE (2μg/ml) due to a decrease in cell viability (50±10%). Subsequent experiments, therefore, used NE at 1μg/ml to give the highest amount of IL-1β release without a significant increase in cell death (Figure 1B).

After 6h of stimulation, NE caused a significant increase in IL-1β secretion (Figure 1C) compared to cytokine stimulation alone (272.8±50pg/ml vs. 55.5±17.3pg/ml,

p<0.001). No IL-1β was detected in the media of unstimulated cells (Figure 1C).
To specifically confirm that the release was due to NE, the cells were pre-treated with the specific neutrophil elastase inhibitor (NEIII), resulting in a significant attenuation of IL-1β secretion. To determine the involvement of caspase-1, cytokine-primed ECs were pre-treated with YVAD-CHO and then with NE. No significant changes in the secreted levels of IL-1β were seen compared to NE alone (Figure 1C). Thus, NE-mediated IL-1β release in endothelial cells was independent of caspase-1.
In unstimulated EC lysates, we did not detect any IL-1β. However, there was a significant IL-1β production inside cells following treatment with proinflammatory cytokines (Figure 1D). The IL-1β levels in the lysates were not significantly different amongst the groups (Figure. 1D). In addition, NE treatment alone did not provoke IL-1β production in the cells (Figure 1D), suggesting no direct effects of NE on IL-1β generation inside the cells. There was also no effect seen on IL-1β production in cell lysates by NE III or caspase-1 inhibitors (Figure 1D).
To confirm that NE was active over the duration of the study, we measured NE activity using quantitative cleavage of a chromogenic specific substrate and interestingly in the lysates harvested from NE-treated cells this was significantly increased (Figure 1E).
Cell lysis as a mechanism of IL-1β secretion was ruled out by the absence of cytosolic enzyme lactate dehydrogenase (LDH) levels in cell supernatants. There were no significant changes in LDH levels following NE stimulation at 2 and 6h compared to control (Figure 1F). Since IL-1 release could be a feature of cell apoptosis, we investigated caspase-3/7 activation in ECs under our stimulation conditions and showed no significant increase in NE treated cells compared to unstimulated ECs, cytokine stimulated cells, or cells in which NE effects are attenuated using NEIII (Figure 1G). In addition, we used propidium iodide (PI) in conjunction with annexin V (PI/AV) to determine if EC were viable, apoptotic, or necrotic at any timepoint used in this study. We did not detect any significant increase in PI/AV staining in NE treated cells compared to untreated controls (data not shown).
In vitro, some proteases, including NE, have been shown to process proIL-1β into mature IL-1β (13). Thus, as the ELISA does not distinguish between pro- and mature- IL-â, we determined which IL-1β isoforms were present, and their relative respective levels, in cell lysates by immunoblotting. Unsurprisingly, unstimulated HCAEC lysates did not contain any detectable IL- 1β but full-length proIL-1β (31kDa) was seen in cytokine-primed cell lysates (Figure 2Ai). However, after the addition of NE, there was cleavage of the 31kDa pro-form associated with a decrease in the 31kDa proIL-1β in cell lysates (Figure 2Ai) that was NE specific as evidenced by inhibition by NEIII but not YVAD. On addition of NE, 20kD and 17kD IL-1â forms were detected (Figure 2Ai). Again, this was NE specific (Figure 2B). Interestingly, NE induced maturation of IL- 1β that was not associated with procaspase-1 cleavage or alterations in NLRP-3 levels in lysates as detected by Western blot (Figure 2Aii, iii). Nor did we detect p20 (the product of active caspase-1) in cell media, indicating that cleavage and secretion had not occurred. NE is known for its proteolytic properties, therefore, to confirm that NE cleaved the pro-form, but not the mature form of IL-1, recombinant proIL-1β and recombinant mature IL-1β were incubated with NE in a cell- free system. NE cleaved the pro-form of IL-1 *in vitro* (Figure 2C) but not the mature form (Figure 2D, 2E), supporting the hypothesis that NE specifically cleaves the pro-form of IL-1β only. Taken together, these data clearly suggest that NE

increases cytokine-induced IL-1β secretion in ECs via an inflammasome/caspase-1 independent pathway.

NE induced secretion of extracellular vesicles containing bioactive IL-1 from HCAECs: In immune derived cells, protected release of IL-1β (microvesicles, multivesicular bodies (MVB), exosomes) has been proposed as a mechanism for IL-1β trafficking to the extracellular environment (9,34,35). Therefore, we sought to determine whether there was extra-vesicular shedding occurring in response to NE and whether this is associated with IL-1β processing in HCAECs.
Since phosphatidylserine (PS) exposure has been associated with MV shedding in monocytes (9) and EC (24), we used annexin V binding (annexin V-Alexa Fluor 488) as a tool to visualise events in live HCAECs. Small particles (0.1-1μm in diameter) were observed separating from the cells in real time using time lapse imaging over duration of 6h (see video, supplemental material). The first MV generation was at 10 min, with membrane alterations at 30 minutes after NE stimulation with large numbers of MVs observed at later time points (Figure 3). The number of MVs was quantified using flow cytometry and gating for annexin V (Figure 4A). Interestingly, there was a significant increase in the number of MVs isolated from ECs following NE treatment (two to threefold) compared to controls. Importantly, NE inhibition effectively attenuated MV formation and shedding induced by NE and caspase-1 inhibition had no significant effect (Figure 4B).
We next investigated which IL-1β isoforms were inside MVs using immunoblotting. MVs from unstimulated cells contained no IL-1β, and in MVs from cytokine-primed cells (6h), prominent proIL-1β (31kDa) forms were detected (Figure 4C). In MVs isolated from the supernatants of NE treated cells, cleavage of the 31kDa IL-1β isoform to approximately 20kDa- 19kDa was observed from as early as 30 minutes (Figure 4D) with further cleavage to the 18 and 15kDa isoforms after 6h (Figure 4C). Treatment of cells with NEIII abolished any cleavage of proIL- 1β in these MVs, confirming that these bands are the result of direct NE activity. Cleavage of proIL- 1β continued even in the presence of YVAD with more prominent isoforms detected (Figure 4C). MVs were assessed for caspase-1 and NRLP3 content and, interestingly, active caspase-1 p20 and NLRP3 were not detected in MVs isolated from cells treated with NE or NE and YVAD together (data not shown), indicating that intra- vesicle cleavage of proIL-1β is independent of caspase-1 activation. These findings suggest that either NE cleaves the released proIL-1β inside MVs or that NE treated cells continually generate more MVs containing IL-1β as a route of secretion.
We next asked whether the processed IL- 1β released into cell supernatants in vesicles as a result of NE activation, was bioactive. We collected total supernatants (containing MVs) from NE-treated or untreated cells for 6h and applied them to HeLa cells expressing an IL-1RI responsive reporter, and measured IL-8 activity. We compared reporter assay output (IL-8) to media obtained from unstimulated or cytokine- primed EC +/- NE with a positive control (0.1nM recombinant IL-1β) or with an IL-1β neutralising antibody. As shown in figure 4E, supernatants isolated from NE activated ECs significantly increased IL-8 activity compared to unstimulated and cytokine-primed cells and this was completely abrogated by the neutralising antibody. In order to confirm that the bioactivity was due to released IL- 1β and not a result of direct NE effects on HeLa cells, NE (1μg/ml) was added to HeLa cells (as a spike) and this showed no significant IL-8 activation. These data indicate that the IL-1β in the MVs is bioactive.
MVs containing IL-1β were confirmed using immunogold TEM and we detected

0.2μm diameter MVs containing immunogold labelled IL-1β only in NE treated cells (Figure 4F).

Mechanisms of IL-1β release in endothelial HCAEC: To study early MV shedding by NE, intracellular calcium changes were assessed. MV release has been linked with transient changes in intracellular calcium $(Ca^{2+})_i$ in specialised secretory cells (36) and IL-1β secretion in macrophages (34). Using a Ca^{2+} sensitive fluorometric dye, we assessed the role of $(Ca^{2+})_i$ in MV formation and release in response to NE and performed experiments in the presence or absence of exogenous Ca^{2+}. In this experiment, $(Ca^{2+})_i$ is released from intracellular stores during an initial stimulation/treatment in Ca^{2+}free media and application of $CaCl_2$ during the second phase of the protocol, allows Ca^{2+} influx inside ECs. In Ca^{2+} free media, there was a small non-significant increase in cytosolic Ca^{2+} levels in NE treated cells compared to untreated cells after NE stimulation (Figure 5A). However, $(Ca^{2+})_i$ was significantly increased in NE stimulated cells after the addition of $CaCl_2$ compared with unstimulated and cytokine-primed cells (Figure 5B). This finding suggests that NE treatment of EC increases free $(Ca^{2+})_i$ by promoting Ca^{2+} influx into EC and that this calcium influx is associated with MV release.

We detected small MV (approx. 200nm) in response to NE (assessed by flow and electron microscopy), characteristic of exosomes secreted from multivesicular bodies (MVBs) and this led us to study the compartmentalisation of IL-1β in EC (31). HCAECs +/- NE were stained using an immunofluorescence technique for LAMP-1 (a late endolysosomal marker) and IL-1β. Confocal images of cytokine-primed ECs stained for IL-1β and co-stained for LAMP-1 suggested a wide distribution of IL-1β throughout the cytoplasm and no colocalization with LAMP-1 (Figure 5C). Unsurprisingly, no signals were detected for IL-1β in unstimulated ECs (data not shown). However, in ECs incubated with NE for increasing times (30min, 1, 2h), IL-1β was co-localised with LAMP-1 (Figure 5D). Indeed, following a 2h stimulation of EC with NE, the majority of IL-1β was detected in MVBs (Figure 5D).

To confirm that the site of IL-1β processing and secretion was indeed mediated by an endolysosomal mechanism, we evaluated the effect of bafilomycin A1 (BAF1), a lysosomal V/ATPase inhibitor that has been shown to prevent endolysysomal formation (37). Treatment of ECs with BAF1 (50nM) before the addition of NE largely decreased IL-1 levels in the supernatants after 6h, and that was associated with a reduction in pro-IL-1 cleavage in the lysates (Figure 6A, B). We subsequently performed TEM on cytokine- primed HCAECs +/- NE and observed >200nm structures in the cytosol with classical morphological features of multivesicular bodies (MVB) (38). These were detected in close proximity to the plasma membrane in NE- activated ECs but not in unstimulated ECs (Figure 6C, panel i). Interestingly, inside the cells, the majority of IL-1 was detected within the MVBs (Figure 6C, panel ii).

NE is detected in ECs and is colocalized with IL- 1β in the endothelium of mature atherosclerotic plaques: To follow the fate of NE in activated ECs, we used Alexa-Fluor 647-labelled NE and performed immunofluorescence staining. After permeabilization, we also labelled the internal endolysosomes with LAMP-1. Surprisingly, NE was detected inside cells. The enzyme co-localised with LAMP-1 (Figure 7A).

Finally, we asked whether NE could be detected in atherosclerotic plaques in mice to

ascertain if NE could contribute to local IL-1β generation. Only in well-developed atheromatous lesions of ApoE$^{-/-}$ mice fed a high fat for 12 weeks was IL-1β was detected, predominantly in endothelial cells. Interestingly, in these lesions, NE also appeared to be expressed in the luminal endothelium (Figure 7B) and colocalized with IL- 1β-positive stained ECs (Figure 7C).

DISCUSSION

Here we describe for the first time, how coronary artery ECs release IL-1β, which has been a 'holy grail' of endothelial biology for many years. We report that a considerable amount of IL-1β is released from ECs in response to NE via a caspase-1 independent, vesicular pathway. This is supported by the following lines of evidence: 1) Bioactive IL-1β is secreted only in MVs following NE treatment, 2) IL-1β secretion is unaffected by YVAD and occurs without caspase-1/NLRP3 activation and 3) MVB are prevalent in primed NE-treated ECs and contain IL-1β protein. We propose that 'protected release' (10) (i.e. contained within membrane-bound vesicles) of IL-1β is a prevalent mechanism in HCAECs.

The endothelium is fundamental in atherosclerotic plaque development, not only during early lesion development but also later by controlling plaque instability (39). In atherosclerosis, crosstalk between circulating cells such as monocytes and neutrophils and the endothelium can cause ECs to liberate soluble agents perpetuating the cycle of inflammation. Several lines of evidence suggest that IL-1 is an apical cytokine in this process (40) yet its mechanism of release from ECs is largely unknown. Furthermore, the biological pattern of the crosstalk is not completely defined. We hypothesised that NE induces IL-1β secretion from vascular endothelium.

Since caspase-1 has been identified as the main proteolytic enzyme to play a role in proIL-1β cleavage and secretion in monocytes and macrophages (41), we used a specific caspase-1 inhibitor (YVAD-CHO) as a potential means of attenuating IL-1β release. Our data show that caspase-1 appears to be non-essential in EC in this setting for IL-1β cleavage and release by NE. This is at odds with other *in vitro* studies in monocytes (42), but in agreement with more recent data from other cell types (43) and *in vivo* models (44,45). Our findings are also supported by the findings of Guma *et al.* (46), who describe the presence of IL- 1β in the synovial fluid of caspase-1$^{-/-}$ mice. Moreover, our data may explain why caspase-1 suppression did not show promise in vascular healing or atherosclerosis progression (33).

It has been previously shown that IL-1 lacks the signal peptide for directing it to the classical ER-Golgi secretory pathway (31). Therefore, IL-1β release has been proposed to occur by distinct mechanisms, including MV shedding and endolysosomal regulation (10). Strikingly, in HCAEC, NE induced MV shedding occurred independent of caspase-1 activity contrasting with previous investigations on immune cells in terms of their caspase-1 dependency of MVs shedding. We suggest that, at least in HCAEC, NE is able to directly cleave the IL-1β precursor, which is associated with protected release in membrane-bound vesicles. In MVs, the prominent forms of IL-1 released (also present inside cells) were 20kDa, 18kDa and 15kDa. These isoforms have been detected previously *in vitro* (13) and although proposed as 5-10 fold less bioactive than the 17kDa isoform, they are active enough for IL-1 to bind to its receptors and initiate signalling. Our study is the first to show in intact cells that NE is capable of cleaving proIL-1β at multiple sites and that these products are bioactive.

A common biogenesis has linked Ca^{2+} regulated MV shedding and IL-1 secretion

with an increase in intracellular calcium levels (34). We are in agreement with this and demonstrated that NE transiently increased $(Ca^{2+})_i$ to a maximum after addition of exogenous Ca^{2+} suggesting that NE mobilized $(Ca^{2+})_i$, mainly by influx of extracellular Ca^{2+}. The secretory pathway identified here has been used to describe the secretion of other non-classical proteins such as EGF (47). Although a few studies have begun to investigate NE-mediated IL-1β secretion in renal disease and lung ours is the first in cardiovascular inflammation to propose a direct effect of NE on IL-1β release and to link this to a MV release mechanism.

Recent immunofluorescence staining data in murine macrophages has shown that IL-1β does not colocalise with LAMP-1 in macrophages (48). This finding is consistent with our data in EC prior to NE treatment (cytokine-primed cells) where IL-1β appears to be diffusely distributed within the cytoplasm. Significantly, however, 2h after NE treatment, colocalisation increased, strongly suggesting that IL-1β compartmentalisation may be acutely induced by NE and pre-destined for maturation by regulated transport. In ECs primed with cytokines and treated with NE, we observed that MVB with cytosolic IL-1β accumulated within cell invaginations similar to (49). This is consistent with work from Rubartelli *et al* in monocytes (50). Since MVBs were not detected in cells that did not produce IL-1β (51), this strongly suggests that the MVBs are a part of the secretory pathway of IL-1β.

The colocalisation of IL-1β and LAMP-1 suggests that NE may trigger a signalling pathway that allows the IL-1β processing to occur in secretory endolysosomes (MVB). However, the possibility of NE internalisation within ECs, via endocytosis, cannot be ruled out, particularly as we detect enhanced NE activity in NE-treated ECs lysates compared with untreated and cytokine- primed cells. In agreement with others who have shown that NE is internalised by macrophages (52) and tumour cells (28) by active transport, we also detected NE inside EC using confocal imaging. Therefore, it is possible that after internalisation of NE by ECs, NE enters MVBs where it cleaves proIL-1β.

It is possible that NE is also causing release of IL-1â due to a toxicity effect, with NE causing cell damage and leakage of cellular contents. However, at the NE concentration used, this is unlikely to be the case as cell viability was not significantly affected, nor was apoptosis proven. Indeed, IL-1â has been shown to increase caspase 3/7 in cells without inducing apoptosis (53), which is in agreement with our data; NE increases IL-1â levels, which, in turn, affects caspase 3/7, but does not affect apoptosis. We use recombinant NE throughout this study, raising the question of what the natural source of NE would be and whether levels of NE would be enough to activate EC. Previous work by our group has shown IL-â is released from activated co-cultures of ECs and monocytes, at greater levels than from monocytes or ECs alone, and that an unidentified monocyte-derived mediator significantly contributed to this response (54). Taken together with the current findings reported here, we postulate that NE is the mediator released from monocytes and would be the natural source of NE *in vivo*.

Given the continued prominence and topicality of IL-1 in the generation and progression of atherosclerosis (55) we studied the expression of NE *in vivo* in a recognised atherosclerosis preparation: aortic root plaques taken from $ApoE^{-/-}$ mice fed a high fat diet for 12 weeks. Although previous work has detected NE in coronary arteries (6) aortic aneurysms (56) and in carotid plaques (17), our study is the very first to investigate NE distribution in experimental atherosclerosis. Significantly, NE was mainly detected in the endothelium of plaques, and was detected alongside IL-1β. The antibody used for these studies recognises both proIL-1β and mature forms,

and we show a clear cellular colocalization with NE in support of our data that NE activates and promotes secretion of IL-1β. This colocalization of NE and IL-1β suggests that the IL-1â observed is likely to be active. We propose that circulating NE is assimilated into developing plaques from degranulating immune cells during their passage through the main vasculature or via vasa vasorum where these exist. This suggestion remains to be clarified, and a causal connection between IL-1â and NE confirmed, in a future study utilising $NE^{-/-}$ mice.

In conclusion, we significantly add to and cement the emerging role of NE in IL-1 induced inflammation. Further, we suggest a novel mechanism for NE-mediated IL-1 secretion by ECs, namely pro-IL-1 processing in the secretory endolysosomes and packaging of mature bioactive IL-1 within MVs for release into the extracellular environment, as part of a single continuum mechanism (figure 8), which is similar to that previously proposed for other cell types (57). We detect NE and IL-1β *in vivo* in the setting of atherosclerosis within endothelium in atheromatous plaques. The pathophysiological relevance of the detection of vesicular IL-1, particularly derived from endothelium, gives ECs the potential to exert a regulatory influence upon atherogenesis and henceforth to become a possible therapeutic target by modulating IL-1 secretion in the local environment. Our findings have wider application for a better understanding of the role of other important proteases with prominent non-proteolytic and possibly signalling roles such as azurocidin, proteinase 3 and Cathepsin G and provide other avenues for therapeutic targets to limit the influence of interleukin-1.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

MA performed all experiments, analysed the results and contributed to writing the paper. HW, MD, AB, VR all helped design, conduct or analyse experiments involving microvesicles. JC and SF conceived and co-ordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES

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[2]The abbreviations used are: IL-1ra, interleukin-1 receptor antagonist; EC, endothelial cells ; NE, neutrophil elastase; MVs, microvesicles; MVB, multivesicular bodies.

FIGURE LEGENDS

FIGURE 1: NE enhances IL-1β secretion from HCAECs by a caspase-1 independent mechanism. A)
IL-1β is released by HCAECs after 48h stimulation with cytokines (TNF-α/IL-1á; 10ng/ml) followed by NE activation (0.5μg/ml - 2μg/ml) for 2h, measured by ELISA (n=3). **B)** Cell viability (measured by trypan blue) is not significantly reduced following exposure to 1μg/ml NE (n=3). **C)** HCAECs, incubated for 48h +/- cytokines then further incubated for 6h in serum free media +/- NE (1μg/ml), in the presence or absence of inhibitors (NEIII; 500μM & YVAD; 50μM), n=5, shows IL-1β release is increased by NE independent of caspase-1. **D)** Levels of IL-1β in cell lysates is not increased following NE incubation, n=5. **E)** Graph showing increased NE activity in EC lysates treated with NE for 6h compared to unstimulated cells, n=3. **F)** LDH

levels are unchanged following NE treatment, measured in conditioned media or in cell lysates as a total of LDH, n=3. **G)** Caspase-3/7 activity is unchanged in HCAECs following NE treatment. HCAECs in 96 well plates ($2x10^4$ cells/well) incubated +/- cytokines (TNF-α/IL- 1α; 10ng/ml each) for 48h, were subjected to NE (1µg/ml) in serum free media for 6h (n=3). Campthothecin (10µg/ml) was used to induce apoptosis as a positive control. All data are mean ± SEM, analysed by one-way ANOVA with Tukey's multiple comparison multiple test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

FIGURE 2: NE selectively cleaves proIL-1β in primed EC lysates without caspase-1/NLRP3 activation. A) Western blot analysis of cell lysates of primed EC +/- NE, assessed for IL-1β **(i)**, Caspase- 1 **(ii)** and NLRP-3 **(iii)**. The blots are representative of n=3, with α-tubulin levels as loading control. For IL-1â, recombinant IL-1β (rIL-1β; 20µg, 17kDa) was loaded as a positive control and represents the commonly detected mature form, whereas proIL-1β (31kDa) indicates the inactive pro-form. For Caspase- 1, activated THP-1 cell lysates were used as a positive control for the p20 isoform. **B)** Densitometric analysis of 20kDa IL-1â levels (n=3). **C)** Western blot illustrating the cleavage of recombinant proIL-1β (rProIL-1β; 20µg) by NE. rProIL-1â was incubated at 37^oC (5% CO_2; v/v) alone or in the presence of NE (1µg/ml) for 30min, 2h, and 6h. **D)** Western blot for recombinant mature IL-1β (rIL-1â; 20µg) in the presence or absence of NE, shows no cleavage (n=3) and **E)** ELISA for recombinant mature IL-1β shows no difference in levels following NE treatment. Data are mean ± SEM, analysed by one-way ANOVA and Tukey's post-test, *p<0.05, **p<0.01.

FIGURE 3: Neutrophil elastase activates microvesicle shedding from endothelial cells in a time dependent manner. HCAECs were left untreated or treated with cytokines (IL-1α/TNF-α) for 48h and then labelled with annexin-V AlexaFluor®488. After the addition of 1µg/ml of NE, cells were visualized in a heated chamber (5% CO_2 v/v) using a confocal microscope to scan MV release. Images captured after 10 minutes, 30 minutes, 2 hours and 6 hours show an early generation of MVs after 10min of NE stimulation, but more prominent at later time points. Arrowheads indicate fluorescent MVs and arrows represent earliest blebbing in EC treated with NE. Scale bars=50µm; the representative images are from three independent experiments (n=3), and have been digitally altered to remove background fluorescence.

FIGURE 4: NE induces secretion of extracellular vesicles containing bioactive IL-1 from HCAECs A) Flow cytometric characterisation of MV released in response to NE. MVs were isolated and stained with annexin V PE-CY7 as described in the materials and methods**.** Analysis of MVs (red) using Megamex beads (blue) shows they are within the 0.2-0.9ìm size limits. **B)** A significant increase in MV stained with annexin V is seen in NE treated cells compared to untreated controls. Analysis was performed by Flow Jo software, n=3, mean ± SEM, 1-way ANOVA followed by Tukey's post- test, *p<0.05, **p<0.01. **C & D)** Detection of IL-1â in isolated MVs by immunoblotting. Equal amounts of protein (20ìg) were loaded in each lane, with rhIL-1â (20ìg) used as a positive control (17kDa). Data are representative of n=4. **E)** Luciferase assay for measurement of IL-1â bioactivity in HeLa cells exposed to freshly harvested conditioned media (total supernatants from cytokines primed cells (TNF-α/IL-1á; 10ng/ml each +/- NE; 1ìg/ml) or rIL-1â (0.1nM) for 6h +/- anti-IL-1â â (1ìg/ml). Specificity for IL-1â is shown by reduction of IL-8 luciferase detection following incubation with IL-1â neutralising antibody. Data are expressed as mean ± SEM, n=3, analyzed by one way ANOVA followed by Tukey's test, ****p<0.0001. **F)**

Immunoelectron microscopic analysis of IL-1β in ECs +/- NE treatment. Anti-IL-1β conjugated immunogold (20-nm gold particles, arrows) was used to confirm the present of IL-1β in the MVs (0.2μm) released from the plasma membrane of ECs. Scale bars=0.2μm.

FIGURE 5: Mechanisms contributing to IL-1β release in endothelial HCAEC. A & B) HCAECs were assayed for changes in the cytosolic-free Ca^{2+} in response to the indicated conditions. **A)** No significant change in cytosolic Ca^{2+} levels are seen in Ca^{2+}-free media. **B)** The fluorescent intensity of intracellular calcium changes after 5min of NE stimulation, in the presence of $CaCl_2$. Data are n=6, mean ± SEM, analysed by one-way ANOVA and Tukey's post-test, *p<0.05, ***p<0.001. **C)** IL-1â co- localises with LAMP-1 after NE stimulation. Cells were primed with cytokines (TNF- α/IL-1α; 10ng/ml each) followed by incubation +/- NE (1μg/ml) in serum free media over 2h, before immunostaing for IL- 1β (red) and LAMP-1 (green), scale bars; 10μm. **D)** Histogram showing a high correlation between IL-1β and LAMP-1 in ECs after NE activation.

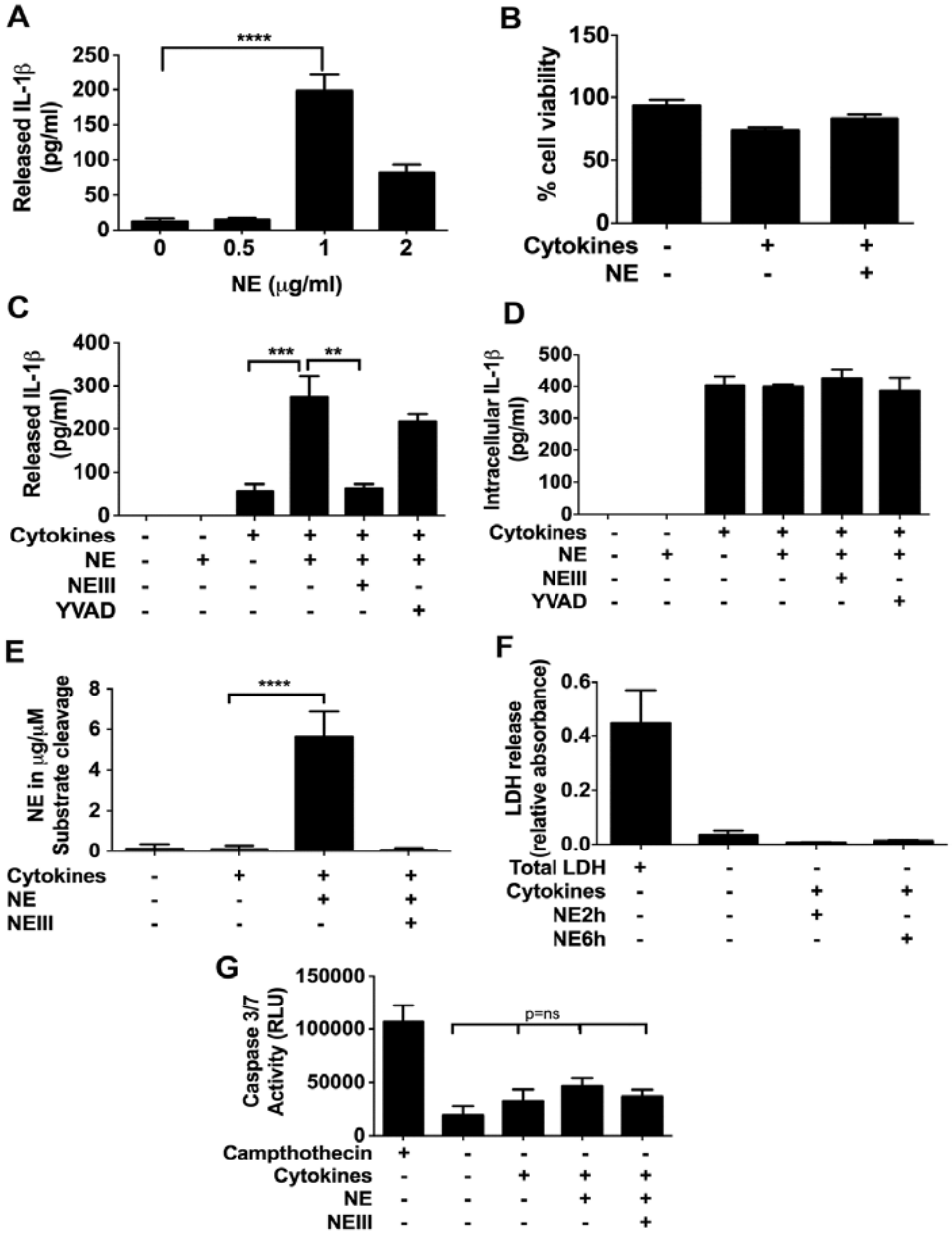
FIGURE 6: NE induces IL-1β release by an endolysosomal dependent mechanism. A) ELISA measuring IL-1â release in conditioned media of HCAECs primed with cytokines (TNF-á/IL-1á; 10ng/ml) ± NE (1ìg/ml) ± BAF1 (50nM) after 6h. Experiments are n=3, and data are mean ± SEM, analysed by 1-way ANOVA followed by Tukey's post-test, *p<0.05. **B)** Western blot analysis of lysates harvested from primed HCAECs activated with NE/±BAF1 (50nM) for 6h, 20ìg protein loaded per lane with á-tubulin used as a loading control. The blot is representative of three independent experiments. **C) MVB characterization**: *i*) EM analysis showing a full appearance of MVB; multivesicular bodies in NE treated cells in the close proximity of plasma membrane. *ii*) Immunolabelling with anti-IL-1â (20-nm gold particles; arrow) shows IL-1â within MVB (arrowhead). Scale bars=50nm

FIGURE 7: NE is detected in ECs and is colocalized with IL-1β in the endothelium of mature atherosclerotic plaques. A) Confocal images showing LAMP-1 and NE in primed ECs after NE treatment. HCAECs were incubated +/- Alexa Fluor 647-labelled (1ìg/ml) NE for 2h in serum free media before washing in PBS and colocalisation performed using an antibody against LAMP-1. Confocal images were analysed using Zeiss image and image j software, scale bars=10ìm. **B)** Immunohistochemical detection of NE and IL-1â in the luminal endothelium of mouse atherosclerotic plaques. Paraffin embedded aortic sinuses from $ApoE^{-/-}$ mice fed high fat diet for 12 weeks were stained with primary antibodies as indicated. Specificity of staining is confirmed by no primary negative control. Scale bars=200ìm. **C)** Colocalisation of IL-1-â, NE and vWF in aortic atherosclerosis. NE positivity was detected predominantly in the endothelium (top right panel; arrows). IL-1â positive endothelium (top left panel) was also detected. The bottom panels show vWF stained endothelium and DAPI for the nuclei. Specificity of staining is confirmed by no primary negative control. Images are representative of histology data obtained from a total of 6 animals. Scale bar = 100ìm.

FIGURE 8. Schematic of the proposed mechanism of IL-1β secretion from ECs by NE. NE is released by circulating cells at the site of atheroma and transported by endocytosis inside the diseased endothelium (primed by inflammation) **(i)**. An increase in calcium, due to NE effects, leads to remodelling of the cell membrane and vesiculation **(ii)**, which in turn facilitates the shedding of MVs containing mature IL-1β **(iii)**. ProIL-1β is upregulated in the inflamed endothelium **(iv)**. NE enters MVBs and cleaves the proIL-1β contained within **(v)**. MVBs also fuse to the plasma membrane and release exosomes containing IL-1 **(vi)**.

Video 1. Typical MV shedding after application of NE to ECs was observed.
Annexin V-AlexaFluor®488 labelling to cell membranes was performed first then NE was applied to the cells and monitored in heated chamber (5% CO_2 v/v) using an Inverted widefield fluorescence microscope Leica AF6000 Time Lapse, as indicated in the materials and methods, for 2h. MVs clearly bud off from ECs starting at 10-15 minutes of NE stimulation and continued for 2h. 2min intervals are shown, 200x magnification was used.

Figure 1: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NEIII
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NEIII
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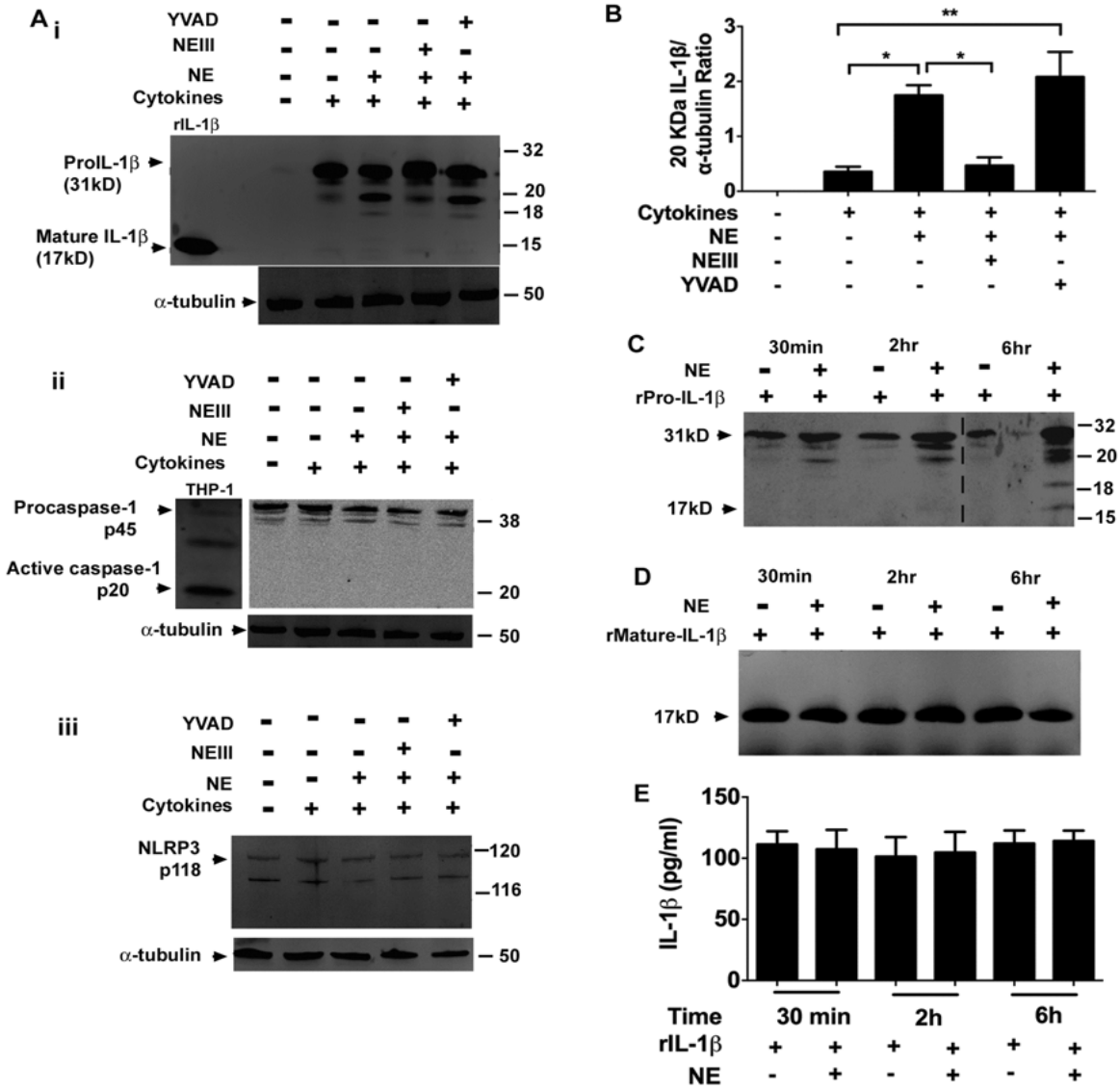
Figure 2:



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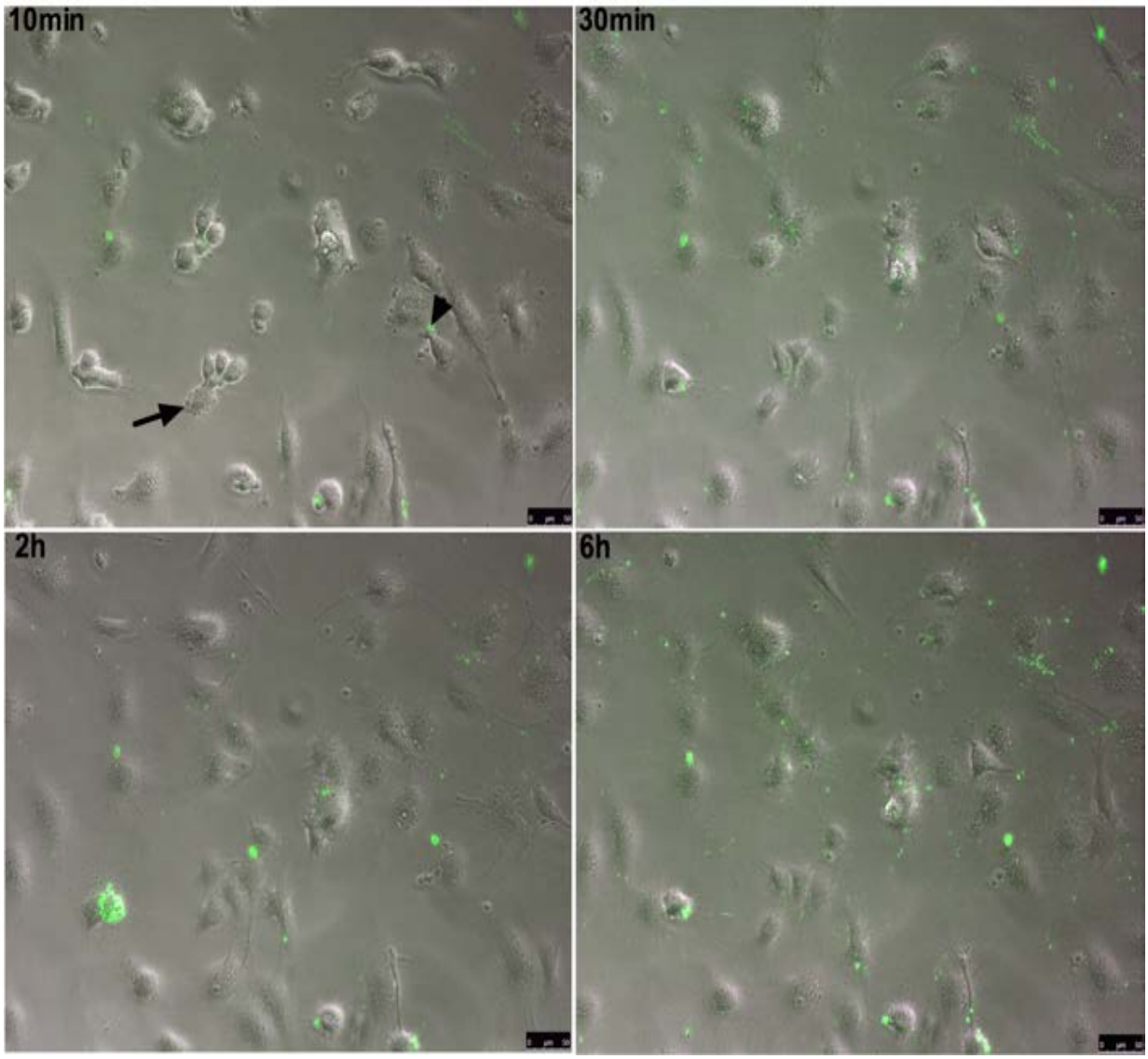

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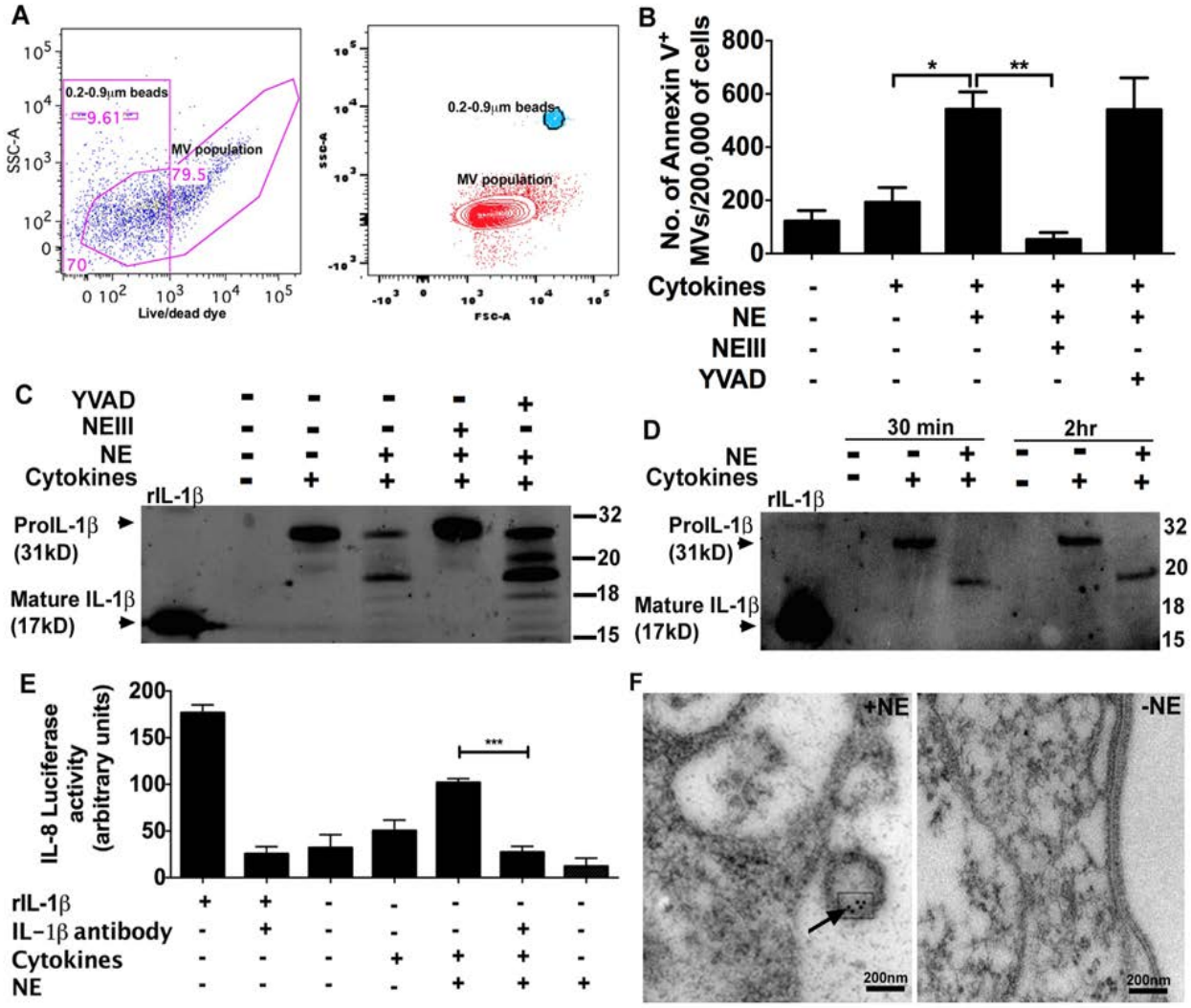

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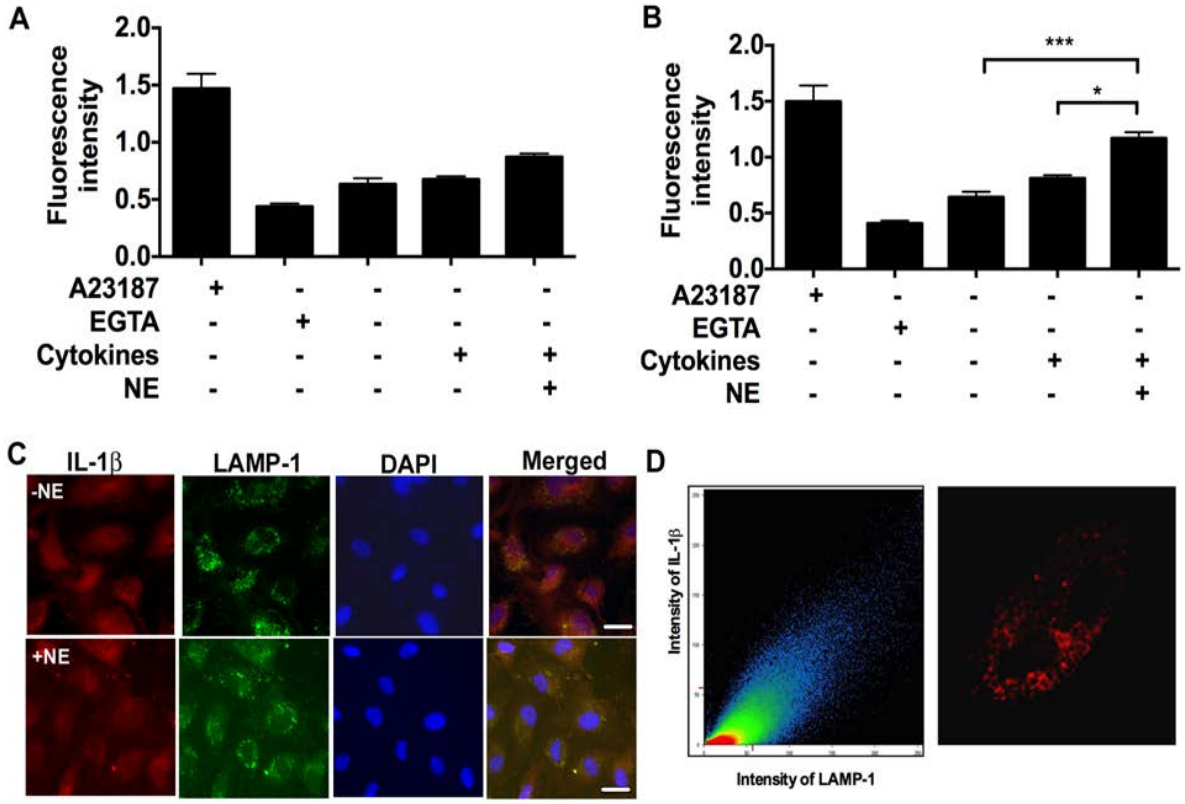
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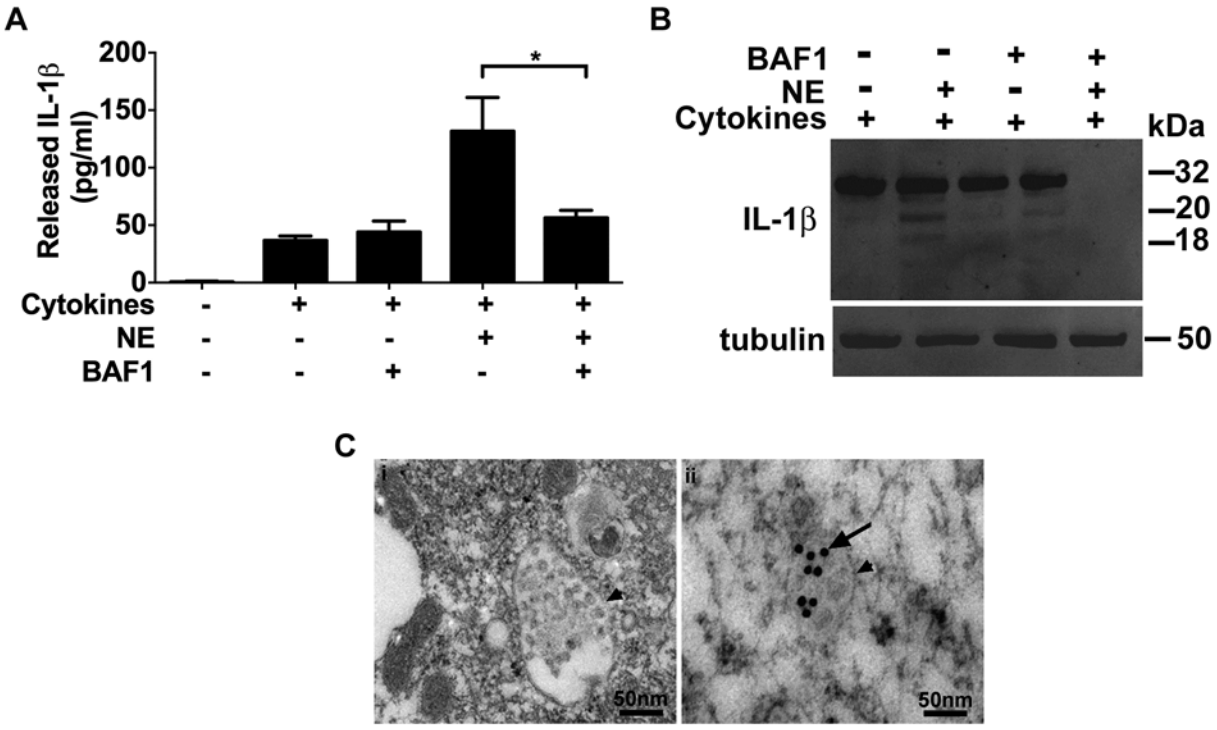
Figure 6.



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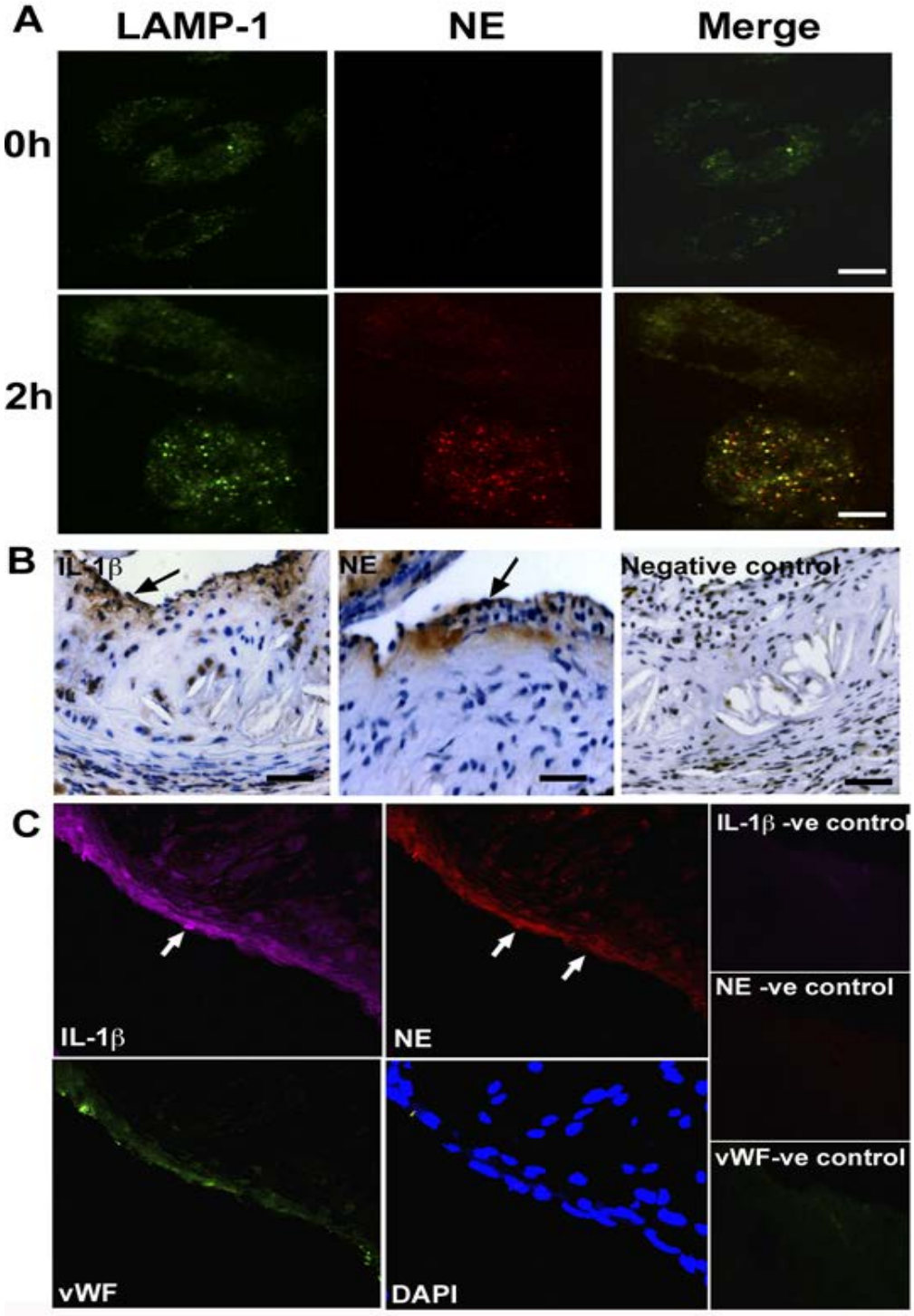
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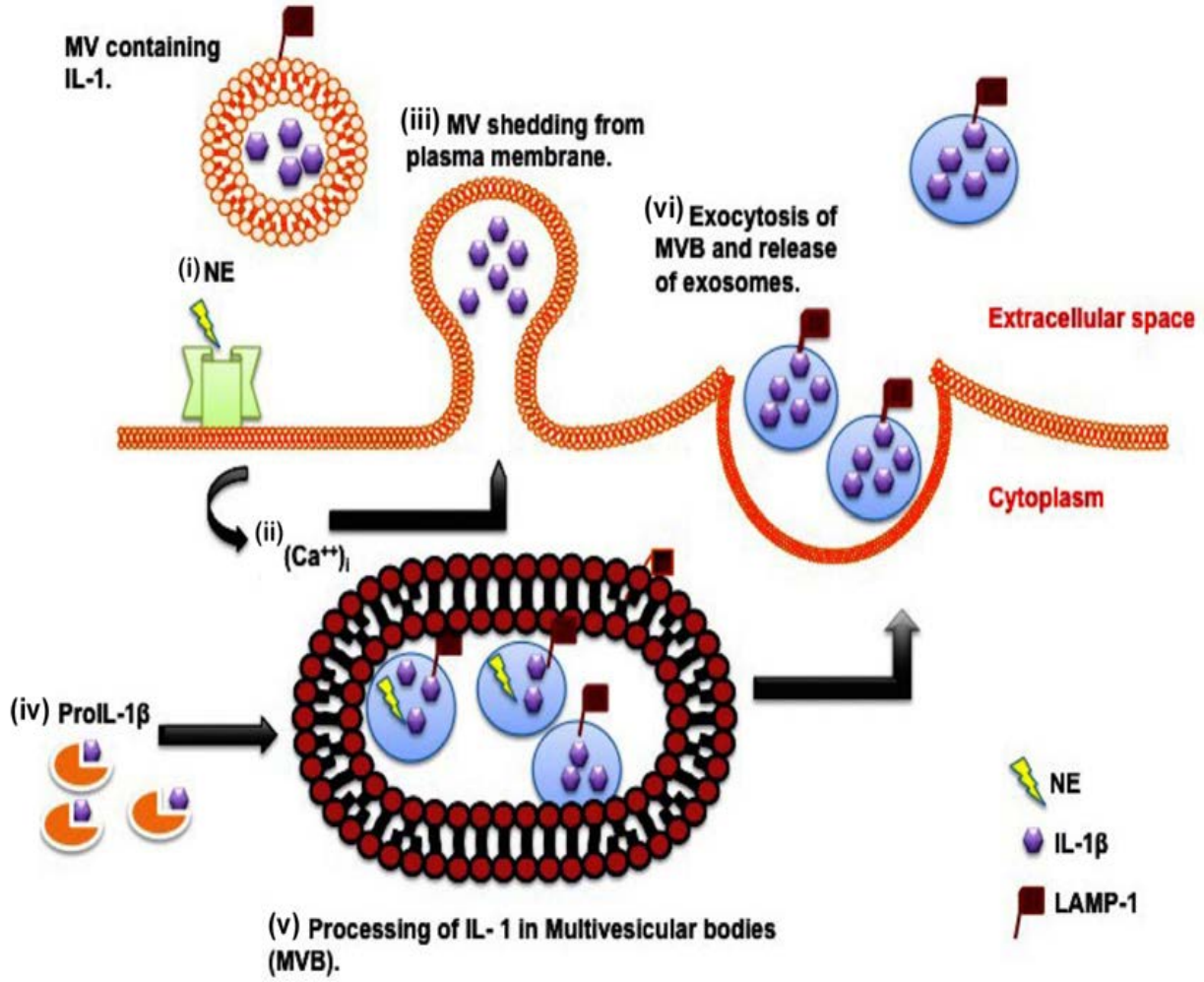
Figure 8



Ref: STH15599NO/NT

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

18 March 2015

Prof Christopher Newman
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Dear Prof Newman,

Change of Investigator

STH ref:	**STH15599**
NIHR CSP ref:	**39601**
REC ref:	**10/H1308/25**
MHRA ref:	**CTA no.: N/A** **EudraCT no.: N/A**
Study title:	**Umbilical Cords for Vascular Cell Research**
Principal Investigator:	**Prof Christopher Newman**
Sponsor:	**STH NHS FT**
Funder:	**British Heart Foundation**
Amendment ref:	**New CI/PI – Prof Newman**

Thank you for submitting the following documents:

Document	*Version/date*
Yorkshire & The Humber REC – Favourable ethical opinion	12 Mar 15
Notice of Substantial Amendment	17 Feb 15
Summary CV of Chief Investigator [Prof Christopher Newman]	No date

These have been reviewed by the Research Department who have no objection to the amendment and can confirm continued NHS permission for the study at STH.

Yours sincerely,

pp

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CC: Charlotte Culver, Sheffield Teaching Hospital NHS Foundation Trust