Observational study of circulating endothelial cell profiles in patients with non-ST-elevation myocardial infarction stratified by plaque erosion or rupture identified by optical coherence tomography: The Plaque Erosion Pilot Study ii (PEPSii)

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ABSTRACT

Introduction

Myocardial infarction (MI) remains a leading global cause of death and disability. There is growing evidence that about 40% of MI cases have plaque erosion due to mass endothelial cell apoptosis and exposure of pro-thrombotic underlying extracellular matrix, in contrast to shearing of endothelial cells during more conventional plaque rupture events. This hypothesis suggests that circulating endothelial cells (CEC) would be higher in MI cases of plaque erosion compared to plaque rupture. Accordingly, I set up a study to measure levels of CEC in patients with non-ST elevation MI (NSTEMI) who underwent plaque morphology evaluation to stratify cases into plaque erosion or rupture.

Methods

This was an observational study which enrolled patients undergoing elective percutaneous coronary intervention (PCI), a feasibility group to establish and refine research methods, and NSTEMI undergoing PCI (cases). Peripheral venous blood samples were obtained to assess CEC and platelet-leukocyte aggregates (PLAs) using flow cytometry. Culprit plaques were imaged using optical coherence tomography (OCT) and independently classified as plaque rupture or erosion. CEC levels in NSTEMI patients were compared between plaque erosion and plaque rupture cases.

Results

Overall, 22 NSTEMI patients and 11 feasibility patients were recruited. Of the NSTEMI patients, 7 did not have plaque morphology, either due to missing data, or inability to agree on a classification. CEC levels, as a percent CD45-ve PBMCs, were significantly higher in the plaque erosion group (17.4%; n=7) compared to the plaque rupture group (9%; n=8: p=0.0012). PLA analysis showed no significant differences between erosion and rupture (p=0.8665).

Conclusion

Higher levels of CEC in the plaque erosion group compared to rupture supports the hypothesis of mass endothelial cell denudation as a causative mechanism. Further research is needed to investigate trigger factors for erosion and to understand if specific therapeutic strategies are needed for these patients.

DEDICATION

To my dear wife Viji, who has been my constant support and encouragement throughout this journey. Your unwavering love and understanding have kept me going, even during the most challenging times.

My beloved cats, Lilly and Daisy, have brought joy and laughter. Your presence has been a source of comfort and companionship, making this journey all the more bearable.

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Publications arising from MD (Res) thus far

Abstract

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Plaque erosion pilot study II (PEPSII): is there an association between circulating endothelial cells and plaque erosion

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List of Abbreviations

ACS	Acute Coronary Syndrome
ADR	Alisdair Ryding
AHA	American Heart Association
BMI	Body mass index
DCB	Drug coated balloon
DES	Drug eluting stent
DICOM	Digital Imaging and Communications in Medicine
CEC	Circulating endothelial cell
CitH3	Citrullinated histone-3
CRP	C-reactive protein
CTA	Computed tomography angiography
EES	Endothelial Shear Stress
Fr	French (scale for the size of catheter, 3Fr = 1mm diameter)
FSC	Forward Scatter
GpIIbIIIa	Glycoprotein IIbIIIa
IFC	Intact fibrous cap
JW	James Wardley
LAD	Left anterior descending (coronary artery)
LCx	Left circumflex (coronary artery)
LV	Left ventricle
MI	Myocardial infarction
NETs	Neutrophil extracellular traps
MACE	Major adverse clinical endpoints
MPO	Myeloperoxidase

NSTE-ACS	Non-ST-segment elevation myocardial infarction
NSTEMI	Non-ST elevation myocardial infarction
OCT	Optical coherence tomography
ORW	Offline review station
PACS	Picture archiving and communication system
PAD4	Peptidyl-arginine-deiminase-4
PBMC	Peripheral blood mononuclear cell
PCAL	Patrick Calvert
PEPS	Plaque Erosion Pilot Study (i)
PEPSii	Plaque Erosion Pilot Study ii
PIS	Patient information sheet
PLAs	Platelet leukocyte aggregates
PMN	Polymorphonuclear leukocytes
POBA	Plain (old) balloon angioplasty
RCA	Right coronary artery
RFC	Ruptured fibrous cap
SEM	Standard error of the mean
SCOT-HEART	Scottish COmputed Tomography of the HEARt Trial
SSC	Side scatter
TIMI	Thrombolysis In Myocardial Infarction (scoring system for coronary blood flow)
ULN	Upper limit of normal
VERDICT	Very early versus deferred invasive evaluation using computerised tomography

1 Introduction

1.1 Myocardial infarction

Myocardial infarction (MI) is a leading cause of death and disability and was estimated to have caused 30% of all worldwide deaths in 2016[1]. MI as a result of acute coronary occlusion leading to ischaemia and myocardial injury is most commonly precipitated by an atherosclerotic plaque disruption, according to the Fourth Universal Definition of Myocardial Infarction (2018), which is defined as Type 1 MI[2]. The most common disruptions leading to atherothrombotic events are plaque rupture (an area of fibrous cap disruption whereby the overlying thrombus is in continuity with the lipid core) or plaque erosion (thrombus confined to the most luminal portion of a fibrous cap in the absence of fissure or rupture after serial sectioning) defined histologically[3].

Plaque rupture events are associated with 60-70% of MI events[3,4] and research into pathological processes involved[4] has led to the development of targeted treatments, including antiplatelet and anticoagulant therapy as well as percutaneous coronary intervention (PCI) to open the blocked artery[5]. While plaque rupture and erosion can lead to Type 1 MI, observations of patients and animal models point to different underlying pathological mechanisms[6,7], opening up alternative targets for treatment.

One of the challenges inherent in the development of targeted therapy has been the inability to discern the two pathologies without a post-mortem. However, developments in advanced intravascular imaging techniques have allowed differentiation of plaque rupture, plaque erosion, and other rarer causes of MI (such as calcific nodules) in patients with acute MI[8,9]. This ability has made it possible to undertake *in vivo* research to help support mechanisms suggested by animal models[10] and develop different treatment strategies between plaque erosion and rupture groups[11,12].

The endothelial cell layer of the arterial intima is the key to diagnosing plaque erosion both histologically[13] and with intracoronary imaging[14]. In their resting state, endothelial cells prevent the adhesion of platelets and the initiation of thrombus formation, even assisting in the breakdown of local clot[15]. Understanding the trilaminar arterial vessel wall helps to understand the initiation and progression of atherosclerosis and how differing disease processes can present as a type 1 MI.

1.2 Arterial structure

The coronary arterial wall can be sectioned histologically into three layers: the adventitia, media and intima. Each layer is separated by a well-demarcated (external and internal) elastic lamina structure (figure 2).

1.2.1 Intima

The Intima comprises a single layer of endothelial cells attached to a basement membrane with a thin underlying layer of an extra-cellular matrix. This single layer of cells separates the luminal contents from the vessel wall[16]. Reviewed in [[17]], these are attached to the extra-cellular matrix via lamin proteins[17].

1.2.2 Media

The media contains circularly arranged multiple layers of smooth muscle cells, which permit changes in blood vessel diameter (vasoconstriction and vasodilatation) regulated by sympathetic vasomotor nerve fibres[16].

1.2.3 Adventitia

The adventitia is the outermost component of the artery and consists of collagen-rich, loose connective tissue and perivascular nerve fibres. A microvasculature within this layer (the *vasa vasorum*) supplies the outer two-thirds of the vessel with nutrients (the rest diffuse in from the lumen)[16] and nitric oxide, which is essential for endothelium-dependant relaxation[18].

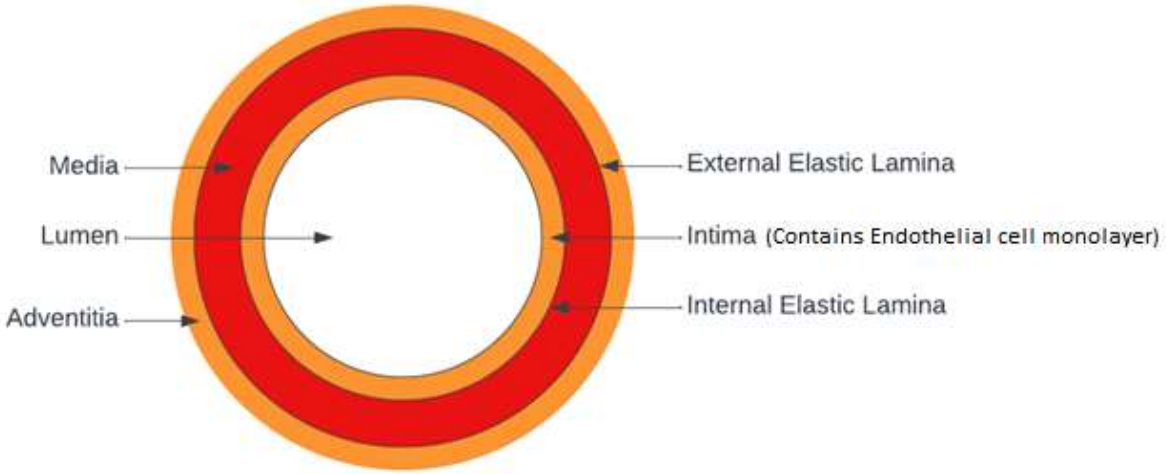

Figure 1 - the trilaminar coronary vessel wall

1.3 Atherosclerosis

The “yellowish thickening in the intima” mentioned earlier in the introduction had been described by Albrecht von Halles in 1755 as *athērōma* (Latin: tumour/mass full of gruel-like matter) to designate the plaque deposited on the innermost layer of systemic artery walls. In 1940, Félix Marchand suggested `atherosclerosis' from the Greek *athéré* (gruel or porridge) and *sclerosis* (hardening) and correlated well with the main components of plaque; the lipid-filled core of atheroma encased with sclerotic or fibrotic cap[19] (figure 2).

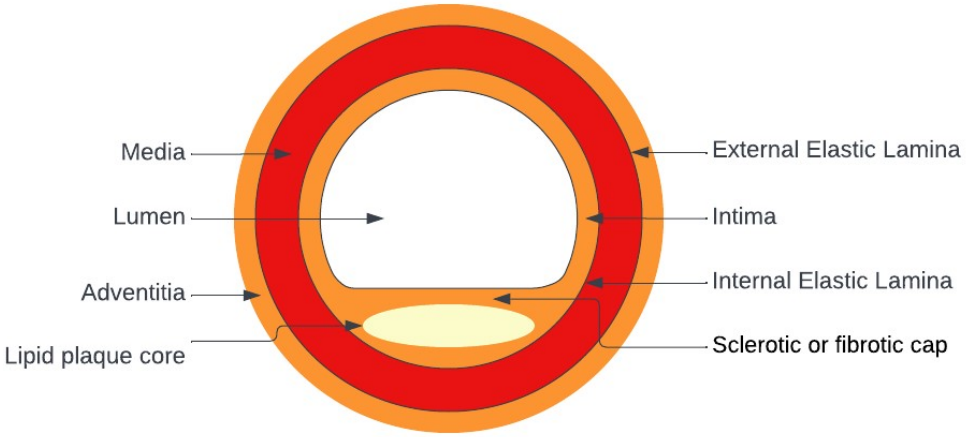

Figure 2 - the main components of atherosclerotic plaque

The development of coronary atheroma is dynamic, with a progression from early lesions to advanced plaques resulting in coronary ischaemia[20]. Systemic risk factors for the growth of atherosclerotic disease include elevated plasma cholesterol, hypertension, diabetes, smoking and male sex[21].

1.4 Classification of atherosclerotic plaque

Through a series of autopsy case series using macroscopic, light and electron microscopic, histochemical, immunohistochemical, and chemical techniques applied to whole-artery segments or tissue samples as well as cell and tissue culture methods, Stary et al. published the 1995 report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association which gave a definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis[22] (figure 3e).

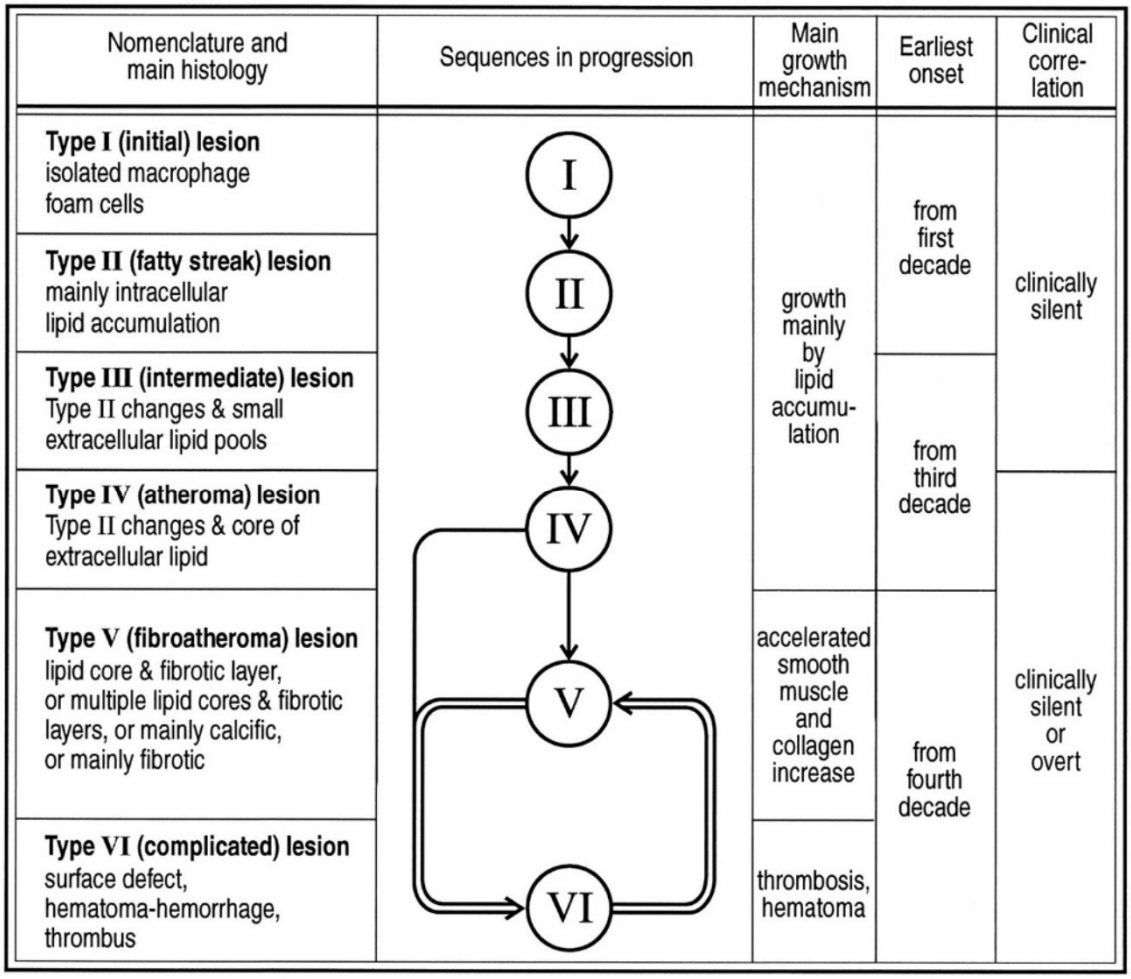

Figure 3 - AHA classification of atherosclerotic lesions

Reproduced with permission from Wolters Kluwer Health, Inc[22].

This schema implied a linear pattern of lesion progression and was difficult to implement clinically. This was also challenged by autopsy case series of sudden cardiac death found plaque rupture in only 60% of lesions with thrombi, with the remaining 40% showing superficial erosion; thrombus confined to the most luminal portion of a fibrous cap in the absence of fissure or rupture after serial sectioning[23] also reproduced in another series demonstrating only 35% of lesions with thrombi failed to show rupture[13]. These (and other) case series struggled to classify plaques through this AHA scheme, so in 2000, Virmani et al. modified this scheme based on the status of the fibrous cap (table 1) with suggestions on how they felt the pathologies could progress (or regress) based on their understanding (figure 4)[3].

	Description	Thrombosis
Non–atherosclerotic lesions		
Intimal thickening	The normal accumulation of Smooth Muscle Cells (SMCs) in the intima in the absence of lipid or macrophage foam cells	Absent
Intimal xanthoma, or "fatty streak"	Luminal accumulation of foam cells without a necrotic core or fibrous cap. Based on animal and human data, such lesions usually regress.	Absent
Progressive atherosclerotic lesions		
Pathological intimal thickening	SMCs in a proteoglycan-rich matrix with areas of extracellular lipid accumulation without necrosis	Absent
Erosion	Luminal thrombosis; plaque same as above	Thrombus mostly mural and infrequently occlusive
Fibrous cap atheroma	Well-formed necrotic core with an overlying fibrous cap	Absent
Erosion	Luminal thrombosis; plaque same as above; no communication of thrombus with necrotic core	Thrombus mostly mural and infrequently occlusive
Thin fibrous cap atheroma	A thin fibrous cap infiltrated by macrophages and lymphocytes with rare SMCs and an underlying necrotic core	Absent; may contain intraplaque haemorrhage/fibrin
Plaque rupture	Fibroatheroma with cap disruption; luminal thrombus communicates with the underlying necrotic core	Thrombus usually occlusive
Calcified nodule	Eruptive nodular calcification with underlying fibrocalcific plaque	Thrombus usually nonocclusive
Fibrocalcific plaque	Collagen-rich plaque with significant stenosis usually contains large areas of calcification with few inflammatory cells; a necrotic core may be present.	Absent

Table 1 – Modification of the AHA classification of atherosclerotic plaque

Reproduced from Virmani et al. [3] *with permission from Wolters Kluwer Health, Inc.*

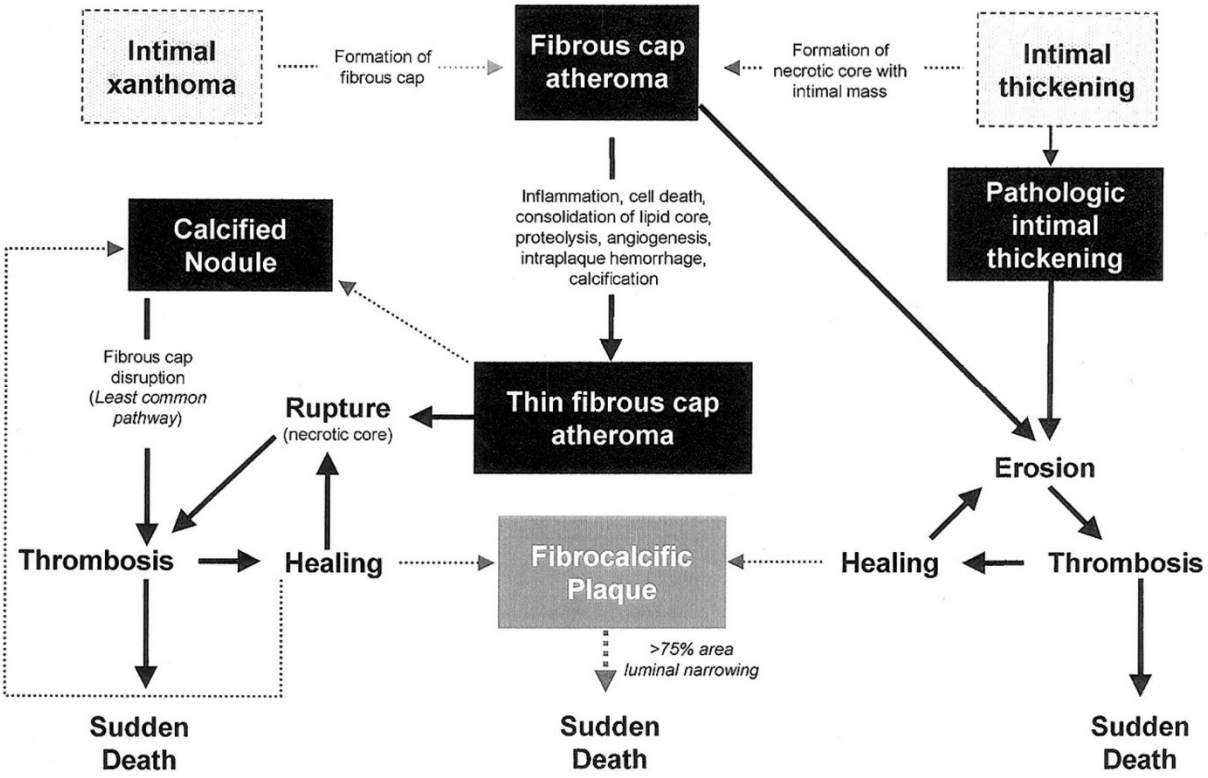

Figure 4 - Virmani et al. simplified scheme for classifying atherosclerotic lesions

Lines (solid and dotted, the latter representing the least-established processes) depict current concepts of how one category may progress to another

Reproduced from Virmani et al.[3] with permission from Wolters Kluwer Health, Inc.

1.4.1 Plaque Rupture

Plaque rupture is a structural defect in the fibrous cap overlying a lipid-rich necrotic core[24]. This occurs at sites of advanced atheroma where the cap is thinned due to the loss of smooth muscle cells and degradation of the extracellular matrix. Evidence suggests that thin caps are prone to rupture[25] due to increased biomechanical strain[26]. Other processes, such as spotty calcification and intra-plaque haemorrhage from fragile *vasa vasorum,* may also be critical.

Macrophages and T-cells weaken the fibrous cap by inducing smooth muscle cell apoptosis and degradation of the extracellular matrix (ECM). This includes changes in collagen type, reduced collagen synthesis, and increased collagen degradation through altered expression of matrix metalloproteases (MMPs) and tissue inhibitor of matrix metalloproteinases (TIMPs)[27–29]. These changes appear to be orchestrated by T-cell-derived cytokines and signals (IL-1, IL-4, TNFα, CD40 ligand and IFNγ) and oxidised LDL[30,31].

Thrombi over plaque rupture are characterised by higher fibrin content than platelets, with immunopositive fibrin areas significantly greater than platelets. Additionally, ruptured plaques show more intense immunoreactivity for tissue factor and C reactive protein (CRP), indicating a more

significant contribution of these factors to thrombus formation in the case of plaque rupture compared to erosion. This suggests that thrombi associated with plaque rupture have a different composition and are more influenced by tissue factor and inflammation, as indicated by CRP levels [32].

1.4.2 Plaque Erosion

Histologically, some lesions leading to fatal, occlusive coronary thrombosis were not due to plaque rupture; the luminal surface was irregular, eroded, and lacked endothelial cells. Plaques associated with this state had a cellular and proteoglycan-rich luminal surface overlying a fibrous and hypocellular body with variable foci of loose collagen containing mononuclear cells[13]. Immunohistochemical staining of these culprit plaques showed that plaque erosion's mechanism may differ from plaque rupture. In plaque rupture, macrophages typically infiltrated the thin fibrous cap and were present at the margins of the rupture site. When present in eroded plaques without lipid core rupture, macrophages were sparsely distributed in the upper layers of the plaque near the luminal surface[13]. Thrombus associated with plaque erosion tends to be dominated by platelets and fibrinogen[32], built on a scaffold of neutrophil extracellular traps[33] (figure 4).

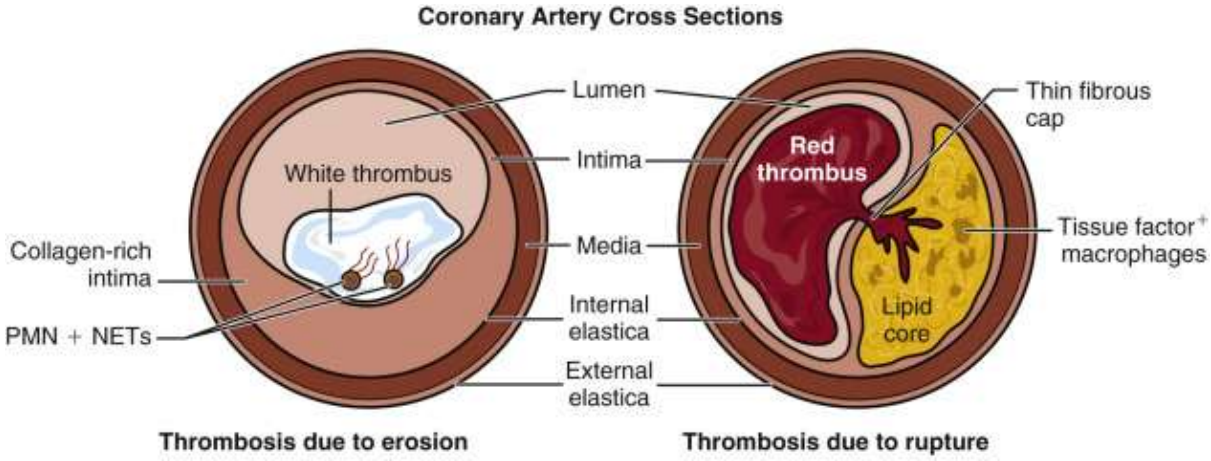

Figure 5 - Histopathological differences between plaque erosion and plaque rupture

PMN – Polymorphonuclear leukocytes

NETs – Neutrophil extracellular traps

Reproduced from Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine – with permission from Elsevier[34]

1.4.2.1 Mechanisms of Plaque Erosion

The mechanisms leading to endothelial denudation and subsequent thrombosis are poorly understood. Current theories are based mainly on histological examination of human coronary artery histology at post-mortem, carotid endarterectomy specimens, and a murine carotid artery injury model that replicates certain features of plaque erosion[35]. Confirmatory evidence in patients with myocardial infarction is sparse but growing [36,37].

Quillard et al. (2015) have proposed a "two-hit" model in which endothelial cell loss is first promoted by chronic endothelial damage through apoptotic stimuli, such as vessel wall shear stress[38] or hyaluronic acid (HA) in the extracellular matrix. This activates toll-like receptor-2 (TLR-2) on endothelial cells, producing endothelial cell apoptosis[39]. Toll-like receptors (TLRs) are pattern-recognition receptors of innate immunity that initiate inflammatory pathways critical in the host; TLR2 detects exogenous microbial components and endogenous ligands. Deletion of TLR2 is athero-protective in mice[40], and *in vivo, it* is downregulated in cells exposed to laminar flow[41].

A second phase of endothelial injury occurs when neutrophils are attracted to areas of endothelial damage. Neutrophils release myeloperoxidase, which may increase endothelial cell toxicity by generating reactive oxygen species[39,42]. Other potential apoptotic stimuli include disturbed blood flow (e.g. increased shear stress at sites of coronary stenosis)[35] and the endothelial cell basement membrane degeneration by MMP 2, 9 and 14[26]. Thrombosis is triggered by endothelial damage exposing the underlying extracellular matrix and tissue factor. Disintegrating endothelial cell microparticles also release procoagulant factors whilst neutrophil-extracellular-traps (NETS)[39] bind tissue factor and act as a nidus for thrombus formation[33].

Hyaluronidases are a family of enzymes that catalyse the degradation of hyaluronic acid (a constituent of the extracellular membrane) and can induce the release of nitric oxide from the coronary endothelium and is hypothesised to have a protective action in myocardial infarction by stimulating the production of endothelium-derived nitric oxide which in turn inhibits leukocyte adhesion to the intima and platelet adhesion and aggregation[43]. A small study has found evidence of significantly increased gene expression of hyaluronidase (HYAL2) and a cell surface HA receptor (CD44v6) in peripheral blood mononuclear cells (PBMC) in patients with plaque erosion compared to those with plaque rupture[44].

Our group has previously investigated the aetiology and possible impact of plaque erosion. The Plaque Erosion Pilot Study (PEPS) was a prospective observational study that took forty ST-segment elevation myocardial infarction patients within <6 hours of chest pain were classified as ruptured fibrous cap (RFC) or intact fibrous cap (IFC) using optical coherence tomography. A cytokine array was used, demonstrating that 12 out of 102 cytokines were differentially expressed between erosion and rupture. Epidermal growth factor and thrombospondin-1 were identified as potential mediators of plaque erosion with high concordance between peripheral and coronary sampling[45]. As the two-hit hypothesis of endothelial cell apoptosis is an attractive explanation for the disappearance of endothelial cells in plaque erosion, we proposed to look for apoptotic CEC differences between plaque erosion and rupture using similar diagnostic techniques to help further understand the mechanism of

endothelial damage in erosion which we believe is different to the rupture process. Such an understanding will open up targets for further treatment of erosions.

1.4.3 Circulating endothelial cells

Endothelial cells from the vascular wall[46] desquamate in response to injury can be detected in blood as CECs (circulating endothelial cells) and are considered to reflect endothelial defects and vascular disruption in a close temporal manner [46–60].

Apoptotic endothelial cells can be identified by flow cytometry as circulating endothelial microparticles (EMPs). These are small membranous fragments (from 0.1 to 1 µm size, CD31+, Annexin V+) originating from activated or apoptotic endothelial cells. EMPs are increased in patients with risk factors for coronary artery disease, stable coronary artery disease, and acute coronary syndromes[61–64], and levels correlate with cardiovascular outcomes, disease severity and endothelial dysfunction. The CECs themselves can be detected by staining for CD146(+), CD45(-), and CD31(+) and are increased when levels in patients immediately post-myocardial infarction were compared with stable CAD patients[51].

Circulating apoptotic EPCs (CD34+, Annexin V+, 7-ADD-) are increased in acute coronary syndromes stress compared to healthy subjects and correlate with the burden of coronary atheroma[65]. Undoubtedly, apoptotic EMPs and apoptotic EPCs are present in both plaque rupture and erosion, but it is not known whether there are any qualitative or quantitative differences.

1.5 Characterisation of erosion by intracoronary imaging

To investigate plaque erosion *in vivo*, it is necessary to have some means of characterising plaque morphology. Early research used a combination of coronary angioscopy for direct plaque visualisation and intravascular ultrasound. An angioscope is a device used to visualise blood vessels (angioscopy) from the inside. It typically consists of a thin, flexible tube (catheter) with a miniature camera at its tip[66]. The catheter is inserted into the blood vessels, allowing real-time imaging of the interior of the vessels. The culprit lesions of ACS can be detected by angioscopy in the angiographically normal segments of coronary arteries and further classify the culprit lesions of ACS as (1) ruptured yellow plaque where its contents protrude into the lumen, mixed with a substantial thrombus (referred to as the plaque rupture group), (2) the presence of mural thrombus over yellow plaque without apparent protrusion (identified as the plaque erosion group), and (3) the absence of yellow plaque or adhering thrombus after reperfusion[67]. The angioscopic appearance of different thrombus types is described in terms of colour and texture. Red thrombus appears as red intraluminal or mural material, while white thrombus is characterised as cotton-like or white mobilised fragmented material[68].

Combining angioscopic data with intravascular ultrasound (IVUS) assessment of the lesion, rupture was defined if they showed intimal thickening, a lipid core, and a localised area of intimal dissection, where communication between the dissected space and the vascular lumen was confirmed; they could be defined as eroded if there was no evidence of dissection or cleft. Such methods are challenging as they are large pieces of equipment and difficult to deliver to the site of a culprit. However, they managed to diagnose rates of plaque rupture in 60% of patients compared to 40% of erosions – similar to previously published autopsy studies[69].

The advent of Optical coherence tomography (OCT), an intravascular imaging modality, has allowed high-resolution arterial wall imaging in the range of 10–20 microns and has allowed a detailed assessment of *in vivo* plaque morphology, characteristics of plaque have been validated by histology[70] and used to assess plaque clinically[71–73]. Despite the high resolution of OCT to perform this optical histology, it cannot directly visualise the endothelial monolayer; as such, the distinction of plaque erosion is one of exclusion with an absence of fibrous cap rupture at the culprit lesion by OCT (surface irregularity at the culprit lesion in the absence of thrombus or attenuation of underlying plaque by thrombus without superficial lipid or calcification immediately proximal or distal to the site of thrombus) suggesting probable plaque erosion, with a more definitive identification when there is presence of attached thrombus overlying an intact and visualised plaque [74]. The terms "red thrombus" and "white thrombus" used in macroscopic evaluation either from microscopy[75] or angioscopy have entered the definitions of thrombus on OCT, given the nature of light attenuation with red thrombi. Intracoronary thrombi are defined as a mass (diameter >250 µm) attached to the luminal surface or floating within the lumen, including red (red blood cell-rich) thrombus, defined by high backscattering and high attenuation or white (platelet-rich) thrombus, defined by homogeneous backscattering with low attenuation[74].

1.6 Endothelial function

The endothelial layer at the luminal boundary provides anticoagulant and antithrombotic properties, preventing the initiation and propagation of coagulation/thrombosis within the vessel in its normal resting state. They express thrombomodulin (binds thrombin promoting the activation of Protein C anticoagulant axis)[76], heparin sulphate (activating antithrombin III to accelerate thrombin inhibition)[77], release tissue factor pathway inhibitor (blocking factor VIIa-TF-factor Xa complex)[78] and annexin V (preventing the binding of coagulation factors)[79]. The surface expression of CD39 (an ecto-ADPase) is responsible for inhibiting platelet function[80, and the generation of nitric oxide and prostacyclin is] accountable for the vaso-active function of arteries[81].

Along with these properties, the resting endothelial cell can express plasminogen activators in response to contact with thrombus[82,83] to inhibit the formation of an evolving clot, which may come in some way to explain why NSTEMI (transient/sub-total occlusions of the coronary artery) are more common than STEMI (where occlusive thrombus is evident). Tissue plasminogen activators are released by the endothelial cell to activate plasminogen into plasmin, which in turn encourages the breakdown of fibrin into degradation products and the breakdown of thrombus[84].

1.6.1 Endothelial cells as initiator/propagators of thrombosis

The activated/apoptotic endothelial cell plays a crucial part in the pathogenesis of plaque erosion and behaves differently; in response to various exogenous factors, they can express membrane phosphatidylserine (which accelerates the rate of activation of factor X by factors VIII & IX)[85], release pre-formed von Willebrand factor favouring the formation of organised clot[86] and express tissue factor increased thrombogenicity of nearby plasma[85,87]. Tissue factor binds to Factor VIIa, acting as a cofactor, activating and improving the catalytic functions of Factor X[88], which then converts prothrombin to thrombin[89].

Additionally, endothelial activation/apoptosis can lead to increased production of plasminogen activator inhibitor-1. This molecule binds tissue plasminogen activator, limiting the amount available at the endothelial cells/local plasma environment's surface and reducing the potential for fibrinolysis[84]. This molecule is also known to be stored in large volumes in platelets and helps make platelet-initiated clot resistant to thrombolysis[90].

1.6.2 Thrombus formation at sides of endothelial damage

Platelets are small discoid anucleate cellular elements in circulation derived from megakaryocytes[91.] They are central to primary haemostasis and repair but also play a role in acute thrombosis following a coronary plaque event[92]. Activation of platelets is mediated primarily through the glycoprotein receptors, which bind collagen in the exposed extracellular matrix[93]. Further glycoprotein (GpIIbIIIa) complexes are then aggregated on the platelet surface, helping further platelet aggregation and endothelial adherence, initially forming a platelet plug (white thrombus)[94]. Platelet agonists, such as ADP through the P2Y12 pathway, and α-granules, which play a crucial role in platelet activation[95], are released by the activated platelet along with synthesised Thromboxane A2, which aids in vasoconstriction and platelet aggregation[96]. When tissue factor (TF) from damaged endothelium binds to factor VII/VIIa, it forms a complex that activates factor X, converting prothrombin to thrombin. Thrombin then cleaves fibrinogen into fibrin, which, along with platelets, includes a web-like structure (red thrombus) to achieve haemostasis. A similar process occurs in myocardial infarction when an

atherosclerotic plaque ruptures, exposing TF and triggering the extrinsic pathway, leading to occlusive thrombus formation and vessel blockage [96].

OCT case series have shown plaque erosion is associated predominantly with white thrombus compared to plaque rupture (96.6% vs 29.2%) in contrast to red thrombus (3.4% vs 70.8% respectively) [97] suggesting that the mechanism of atherothrombosis in plaque erosion is much more driven by platelet activation by collagen rather than including the tissue factor-mediated activation of the clotting cascade.

1.6.3 Platelet/Leucocyte aggregates (PLAs)

Flow cytometry studies have shown that collagen-activated platelets express P-selectin, which mediates the binding of activated platelets to many different types of leukocytes[98]. This is through the glycoprotein pathway (platelets) to the leukocyte integrin Mac-1[99]. PLAs have been investigated as potential biomarkers of many diseases and extensively in cardiovascular pathology, with an increase between patients with symptomatic cardiovascular disease having higher levels than healthy controls[100,101] with the more specific marker of PMA (platelet monocyte interactions) showing higher levels in patients with acute coronary syndromes versus healthy subjects with an associated greater expression of tissue factor[102]. Considering that plaque erosion is associated with higher white thrombus (platelet plugs) compared to the red (tissue factor/clotting cascade mediated) thrombus predominantly seen in rupture[97], and that these PLAs can induce monocyte migration and recruitment into atherosclerotic plaques, resulting in plaque platelet-macrophage aggregates propagating to sustained plaque growth[103] it may be that differential levels of these PLAs can be observed between plaque erosion and rupture.

1.7 The Endothelium in Atherosclerosis

The balance of cell loss and cell replacement maintains the healthy endothelium. Endothelial cell loss may be due to cell death (e.g. apoptosis, anoikis – a subset of apoptosis in endothelial cells when they detach from arterial walls, necrosis) or detachment of viable cells. Replacement is due to the proliferation of differentiated endothelial cells and recruitment of endothelial progenitor cells to sites of injury. In addition, cells adjacent to endothelial cells called pericytes also help to maintain vascular integrity. An imbalance of these processes may be significant in plaque erosion and plaque rupture, but differences in endothelial biology between erosion and rupture have not been studied.

1.7.1 Endothelial cell apoptosis

Endothelial cell apoptosis is an attractive explanation for the disappearance of endothelial cells in plaque erosion[7], for which circumstantial evidence exists. Chemically induced endothelial cell

apoptosis in a rabbit femoral artery model results in vessel thrombosis and a histological appearance like plaque erosion[104]. Experimental findings reproduced trying to model plaque rupture by chemical inducement of endothelial cell apoptosis in atherosclerotic rabbit aorta specimens, resulting in thrombotic lesions without endothelial cell layers but intact fibrous caps [105]. Using an endothelial marker, CD31, Quillard et al. found that clusters of apoptotic endothelial cells were evident in smooth muscle (characteristic of erosion) rich atheromatous plaques in patients who had died after myocardial infarction[39]. In mouse carotid arteries with established intimal lesions tailored to resemble the substrate of human eroded plaques, acute flow perturbation promoted downstream endothelial cell activation, neutrophil accumulation, endothelial cell death and desquamation, and mural thrombosis. Neutrophil loss-of-function limited these findings, as did agonism of Toll-like receptor 2[35].

Various toxins, signalling molecules and cellular stresses can induce apoptosis in cultured endothelial cells and animal models. Factors relevant to the ACS setting include cigarette smoke[106], hypoxia, oxidative stress[107] and disturbed flow[38,108].

1.7.2 Endothelial shear stress

The entirety of the arterial tree is exposed to systemic risk factors for the development of coronary atheroma, yet plaque disease does not appear apparent throughout the whole tree. It has been long observed that atherosclerotic lesions tend to be distributed in areas of arteries with low or turbulent flow [109]. *In vitro,* it has been shown that cultured endothelial cells (from human umbilical vein cords) undergo a low level of apoptosis compared to cells cultured under dynamic flow conditions[110]. Analysis of human carotid artery specimens (taken en bloc from carotid endarterectomies) appears to support this with more apoptotic endothelial cells downstream of stenotic plaques (an area of low shear stress) than upstream[111].

Balloon angioplasty creates an inflammatory reaction resulting in the recruitment of fibroblasts[112]. Rabbits were used to develop an erosion model, with balloon injury to induce a smooth muscle cell-rich neointima (mimicking the plaques found at an erosion site). After three weeks, acute flow restriction was applied (after three weeks) on both these and non-treated arteries. Endothelial detachment, platelet adhesion and neointimal cell apoptosis became evident in the post-stenotic regions of all injured femoral arteries with endothelial detachment with small platelet thrombi at post-stenotic regions without fibrin or occlusive thrombosis[113].

Cell line, *in-vivo,* and *ex-vivo* models demonstrate that flow perturbation can be responsible for some aspects associated with plaque erosion. Under normal conditions, laminar flow endothelial cells are ellipsoid and aligned with the direction of flow[114]. This could provide evidence of mechanisms keeping

the apoptotic cycle in check and that multiple checkpoint failures are required for a culprit lesion to reach clinical significance.

Atherosclerotic plaques predominantly form in regions of low endothelial shear stress (ESS), whereas moderate/physiological and high ESS regions are generally protected[115]. Complex haemodynamic effects occur at vessel bifurcations, creating very high gradients of shear stress; these were modelled using endothelial cells in flow tanks exposed to differential pressures emulating wall shear stress, supraphysiological gradients resulted in a failure of the endothelial cells to align to flow, increased cell proliferation and increased apoptosis[116]. Further evidence for this comes from the 3D reconstruction of vessels with OCT-defined plaque erosion. Yamamoto was able to reconstruct representations to look at thrombus characteristics of patients affected with plaque erosions and found that the size of the thrombus generated from erosion also correlates to the degree of shear stress with thrombus localising in the region between high endothelial shear stress/endothelial shear stress gradient at the throat and high oscillatory shear index/low endothelial shear stress at the distal shoulder of lesions suggesting that the conditions of normal, laminar flow promote an endothelial environment resistant to thrombosis[38].

1.7.3 Neutrophil Extracellular Traps (NETs)

Alongside the innate response of neutrophils to release antimicrobial peptides, lytic enzymes and reactive oxygen species before phagocytosis to clear invading pathogens, neutrophils can also undergo a specialised form of programmed cell death, “NETosis”, in response to inflammatory stimuli. Nuclear material within the neutrophil breaks down before disintegrating intracellular organelles, mixing cytoplasmic and nuclear components. The plasma membrane ruptures to expel a Neutrophil Extracellular Trap (NET) composed of DNA in association with histones, as the most abundant proteins in NET, with granular proteins such as elastase, myeloperoxidase and other cytoplasmic proteins. These structures have anti-bacterial/fungi properties and, in a dose-dependent manner, can directly cause endothelial cell death[117].

The Libby group have a human carotid endarterectomy tissue collection and demonstrated that NETs co-localise in plaques with erosion-prone rather than rupture-prone characteristics. With this knowledge, they could look again at flow perturbation (which, from the earlier work, is associated with endothelial cell apoptosis and erosion[35]), demonstrating NET formation in contact with a disrupted luminal endothelial cell layer[118].

NETs induce endothelial expression of leukocyte adhesion molecules (VCAM-1 and ICAM-1) depending on the NET concentration, resulting in peaks 12 (VCAM-1) to 24 (ICAM-1) hours after the stimulus. These augment the adhesion of monocytes to the activated endothelial cell monolayer. The

mechanism for upregulation of the mRNA to achieve this has evidence for being shared with an upregulation of Tissue Factor mRNA resulting in a peak activity at 6 hours augmenting thrombogenicity. As a result, neutrophils recruited to plaques by activated endothelial cells can release NETs that can propagate and amplify local inflammatory processes, further leading to endothelial damage and escalation of endothelial injury[87].

With this background and observation of evidence for NETs in situ in the thrombus mass of AMI patients, Pertiwi et al. examined specimens from the pathology archives. They compared lesions from intact plaques, plaques with intramural haematoma, thrombosed plaques overlying erosions and thrombosed plaques overlying a rupture using immunohistochemical identification of Neutrophils and NETs[119]. They found coronary atheroma without complication (haemorrhage/erosion/rupture) had sparse neutrophils/NETs. In contrast, the haemorrhagic plaque (without overlying thrombus), eroded or ruptured plaques contained large numbers of myeloperoxidase (MPO) positive neutrophils and anti-CitH3/PAD4 positive (citrullinated histone-3/peptidyl-arginine-deiminase-4) NETs. These appeared to be localised to either the haemorrhage itself or inside the thrombus mass and at the thrombus–intimal plaque interface of both plaques with erosion and rupture. In contrast, parts of the plaque remote from the erosion/rupture site contained only scarce or no NETs[119].

1.8 Treatment of Type 1 MI

Depending on the clinical context, the treatment of type 1 MI is relatively standardised and consists of antiplatelet therapies (to reduce atherothrombosis), anticoagulation (low molecular weight heparins or Fondaparinux following an ACS or unfractionated heparin/Bivalirudin during angioplasty to prevent acute thrombosis with vessel injury), coronary revascularisation (either in the form of fibrinolysis or more commonly angioplasty) and secondary prevention therapies aimed at plaque stabilisation and reducing the risk of endpoint sequalae[120,121].

1.8.1 Coronary Angioplasty

In 1977, Gruentzig presented a novel technique for the treatment of stable atherosclerotic heart disease based on a case series of peripheral angioplasty where balloons were inflated at high pressures within coronary stenoses to modify the lesion and improve coronary blood flow, reporting success rates acutely in >80% of patients[122]. Bare metal stents were developed to combat high restenosis rates following balloon angioplasty. They were used as a bailout for abrupt closure, though they necessitated better medical therapies to prevent stent thromboses. Despite improvements in the technology, re-stenosis rates within stents remained high (~25%) with associated morbidity and mortality, leading to the development of drug-eluting stents coated with anti-proliferative drugs that would elute off the stent, dramatically lowering restenosis rates with successive iterations of design

and are currently the mainstay of treatment for sealing a plaque after an acute coronary syndrome[123]. The advent of drug-coated balloons (a technique involving balloon angioplasty ala Gruentzig, then delivering an antiproliferative drug directly into a lesion using a suitable excipient) has almost brought treatments full circle with the PEPCAD NSTEMI suggesting in patients with NSTEMI, treatment of coronary de novo lesions with DCB was non-inferior to stenting with BMS or DES[124].

1.8.2 Alternative treatment strategies for plaque erosion

Prati et al. reasoned that the coronary stenosis underlying an erosive pathology was not always significant, combined with a lack of vessel wall disruption meant that treatment with medical therapy (in the form of dual antiplatelets) rather than the common practice of deploying an intracoronary stent could be appropriate. They described an observational case series where (after thrombectomy to allow OCT diagnosis) operators had chosen to either treat with medical therapy only or with stent implantation. After a median follow-up of 753 days, target lesion revascularisation was performed in a single patient from the medically managed group. Still, no myocardial infarction, heart failure, or deaths occurred in either group. They postulated that the dual antiplatelet therapy post-thrombectomy prevented further thrombosis within the eroded vessels not treated with a stent, whilst the endothelial layer healed similarly with re-endothelialisation after stent implantation[125].

Building on this, Jia et al. hypothesised "that patients with ACS caused by plaque erosion might be stabilised by effective anti-thrombotic therapy without stent implantation, thereby abrogating stent-related early and late complications" and set about testing this with a prospective study testing the feasibility of this conservative strategy. Of the 458 patients screened with OCT, 103 (22%) had plaque erosion and subsequently, 60 patients were recruited into the study (on core lab analysis, four were found not to meet inclusion criteria). The primary endpoint was the thrombus resolution at one month (following a second angiogram/OCT), completed by 47 (78%). One patient died as a result of melena and hypotension (later explained in a follow-up paper to be a result of a penetrating aortic ulcer) 8 days after their recruitment (angiography was repeated and showed the culprit artery to be patent), another had an intervention to the culprit artery at one month for angiographic stenosis without symptoms nor objective evidence of ischaemia performed at the discretion of the operator[11]. This study suggests that a conservative strategy without stent implantation may be feasible and practical for treating ACS caused by plaque erosion.

Further follow-up at one year was undertaken for this cohort who completed the EROSION study. "Among 53 patients, 49 (92.5%) remained free from MACE for ≤1 year: 3 (5.7%) patients underwent nonurgent revascularisation because of exertional angina, and 1 (1.9%) patient had gastrointestinal bleeding at three months. No cardiac death, recurrent myocardial infarction, or stroke occurred". Of

the 49 MACE-free patients, they could repeat angiography and OCT and showed a further decrease in thrombus volume. Twenty-three patients were completely free of any thrombus (an additional three patients during the one follow-up period compared to 1 month). The authors noted that the 23 patients without residual thrombus had more frequent use of Glycoprotein IIb/IIIa Inhibitors (abciximab, eptifibatide, and tirofiban, which act at the final common pathway of platelet aggregation) during the acute phase and had low thrombus volume at baseline[12]. These one-year follow-up results reinforce the efficacy of the conservative strategy for ACS caused by plaque erosion (albeit in the same cohort). The low incidence of MACE, coupled with a sustained reduction in thrombus volume and the absence of adverse cardiovascular events, underscores the potential benefits of this approach. The observed correlation between the lack of residual thrombus and the use of Glycoprotein IIb/IIIa Inhibitors during the acute phase further supports the importance of tailored anti-thrombotic therapy in optimising patient outcomes.

Four-year data for this cohort has recently been published, dividing the patients into those who had required target lesion revascularisation (the TLR group) and those for whom it was not undertaken (the non-TLR group). The patients in the non-TLR group had more optical coherence tomography (OCT) thrombus reduction from baseline to one month as well as an angiographic improvement of the lumen[126;] these were the patients in the 1month/year follow-up that had more frequent use of Glycoprotein IIb/IIIa Inhibitors as explained above[12]. This four-year data reinforces the durability and effectiveness of the conservative strategy in managing acute coronary syndrome (ACS) caused by plaque erosion. The positive trends in thrombus reduction and angiographic improvement in the non-TLR group, coupled with the earlier association with Glycoprotein IIb/IIIa Inhibitors, underscore the potential benefits of this tailored treatment approach. This suggests that if we pursue this conservative strategy, a more aggressive anti-thrombotic regimen should be considered to reduce the risk of later need for revascularisation.

Souteyrand et al. repeated this observational work by recruiting five patients each into an RFC and IFC group not treated with stent deployment. They used OCT in the acute phase (index angiogram) and at a follow-up (between 1 and 7 months) to understand the repair mechanisms of plaque complications causing acute coronary syndrome. In their series, the margins of the cavity in RFC plaques showed a smooth surface and a morphology similar to that of baseline images (they labelled as a neointima). In contrast, IFC plaques showed a smoothened intimal border and a double layering, indicating an organising thrombus incorporated in the superficial layers of the plaque[73]. The luminal narrowing associated with this could well lead to angina before a clinically relevant myocardial

infarction, as seen in the case series by Wang et al., who found that pre-infarct angina had a lower prevalence of plaque rupture and lipid-rich plaques in culprit lesion[127].

1.8.3 Personalised Medicine

Plaque erosion myocardial infarctions are prevalent in our populations, and whilst the result (coronary occlusion resulting in subsequent morbidity/mortality) can be like that found in plaque rupture, there does appear to be a different pathological process leading up to the two events. This opens exciting lines for the prevention and treatment of these conditions; specifically, does targeting the inflammation and thrombus formation at the time of the event itself benefit our patients as the relative incidence of plaque erosion increases in the statin era (reducing plaque rupture)?

Immunotherapy on patients with coronary artery disease has been postulated to improve cardiovascular outcomes. The Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) involved patients with a history of myocardial infarction and an elevated baseline level of C-reactive protein; the results showed that the risk of recurrent cardiovascular events was lower among those who received canakinumab than among those who received placebo at the risk of a higher incidence of fatal infections. Colchicine is an inexpensive, orally administered, potent anti-inflammatory medication that has also been studied in patients with stable cardiovascular disease[128] and post-MI[129] showing a reduction in cardiovascular endpoints (MI, ischaemia-driven revascularisation, cardiac death) but with no impact on overall death rates. Colchicine is known to reduce the formation of neutrophil extracellular traps[130]. It inhibits leukocyte-platelet aggregation[131], so it may well be that the treatment groups (chronic coronary disease or all-comer myocardial infarctions) are too broad. That categorisation of MI into erosion and rupture may show a group for which this medication is beneficial.

Personalised medicine enables us to tailor our treatments to get the best outcomes for our patients. Focusing on statin therapy and subsequent reduction in plaque rupture events has dramatically benefited our patient cohort[12]. With the advent of prolonged antithrombotic regimes showing a decrease in long-term major adverse cardiac events[132,133] comes another opportunity to further explore the understanding of the erosion mechanisms so targeted treatments may be employed. The Souteyrand data earlier explains neo atheroma formation of a ruptured fibrous plaque early into follow-up (by OCT). Yet, thrombus and endothelium layering can occur in intact fibrous cap myocardial infarction (potentially benefiting from prolonged antiplatelet durations). Clinical tools to help confidently predict the likelihood of a culprit lesion being an erosion have already been tested from observational data (Figure 6)[134].

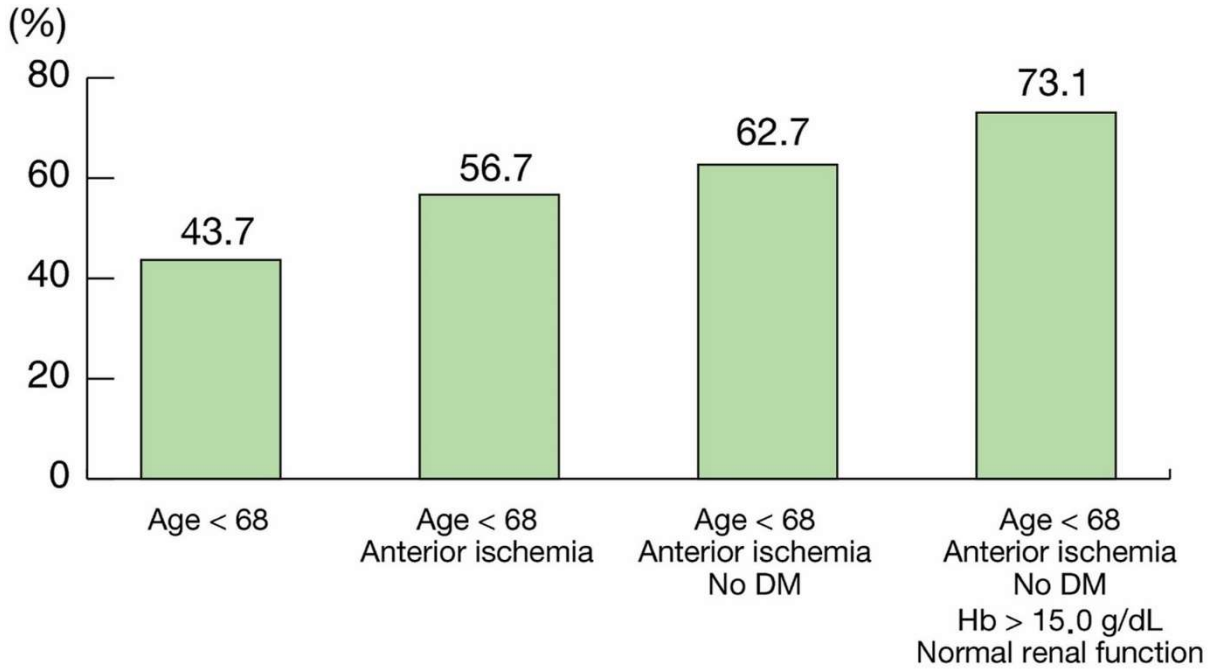

Figure 6 - Probability of plaque erosion based on clinical features

When all five parameters are present in a patient with non–ST–segment–elevation acute coronary syndrome, the probability of plaque erosion increases to 73.1%. DM = Diabetes mellitus

Adapted from Yamamoto E et al. Clinical and Laboratory Predictors for Plaque Erosion in Patients With Acute Coronary Syndromes. J Am Heart Assoc. Open access article under the terms of the Creative Commons Attribution-NonCommercial License[134]

While scoring systems are practical in our clinical assessment, we still need to find a reliable peripheral biomarker of plaque erosion vs plaque rupture to help with our future tailored strategies. Previous prospective studies using proteomics have suggested plasma levels of insulin-like growth factor 1 (IGF1), ferritin family homolog 3 (FERMT3), and collagen type VI α-2 chain (COL6A2) could predict plaque erosion independently and that levels of epidermal growth factor (EGF) and thrombospondin 1 were higher in patients with plaque erosions [36,37,135]. Proteomic arrays offer a robust and high-throughput approach to assess biomarkers, allowing simultaneous screening of many proteins within a sample and offering a comprehensive snapshot of the molecular landscape associated with a particular disease. However, it is essential to acknowledge the challenges of proteomics, such as limited coverage, sensitivity, and specificity variations, as well as difficulties in detecting proteins across a dynamic concentration range. Despite these limitations, proteomic approaches hold promise in identifying potential biomarkers for plaque characterisation. In light of this, these candidates, including IGF1, FERMT3, COL6A2, EGF, and thrombospondin 1, must undergo rigorous testing before commercialisation to ensure their viability as rapid, reliable, and non-invasive stratification biomarkers for distinguishing plaque erosion from plaque rupture in clinical settings.

The thinking behind how these could help develop a paradigm shift in care has already begun, building on the prospective work by Jia et al. and the prolonged antiplatelet duration studies already mentioned (Figure 7) to target invasive assessment of myocardial infarctions to those who would benefit the most from a percutaneous intervention to seal ruptured coronary plaque.

CT Coronary angiography already has defined features of high-risk plaques (positive remodelling, low attenuation plaque, spotty calcification, and the "napkin ring" sign) and was tested in a post hoc exploratory analysis of the SCOT-HEART study (an RCT of outpatients with suspected angina secondary to CAD). They showed in a stable group that adverse coronary plaque characteristics are associated with a tripling of the risk of coronary heart disease death or nonfatal myocardial infarction, with obstructive disease conveying the greatest risk[136]. This was further examined in the NSTEMI population by an arm of the VERDICT trial, which demonstrated a negative predictive value of CTCA to exclude clinically significant CAD of 99% (positive predictive value of 87.9%)[137].

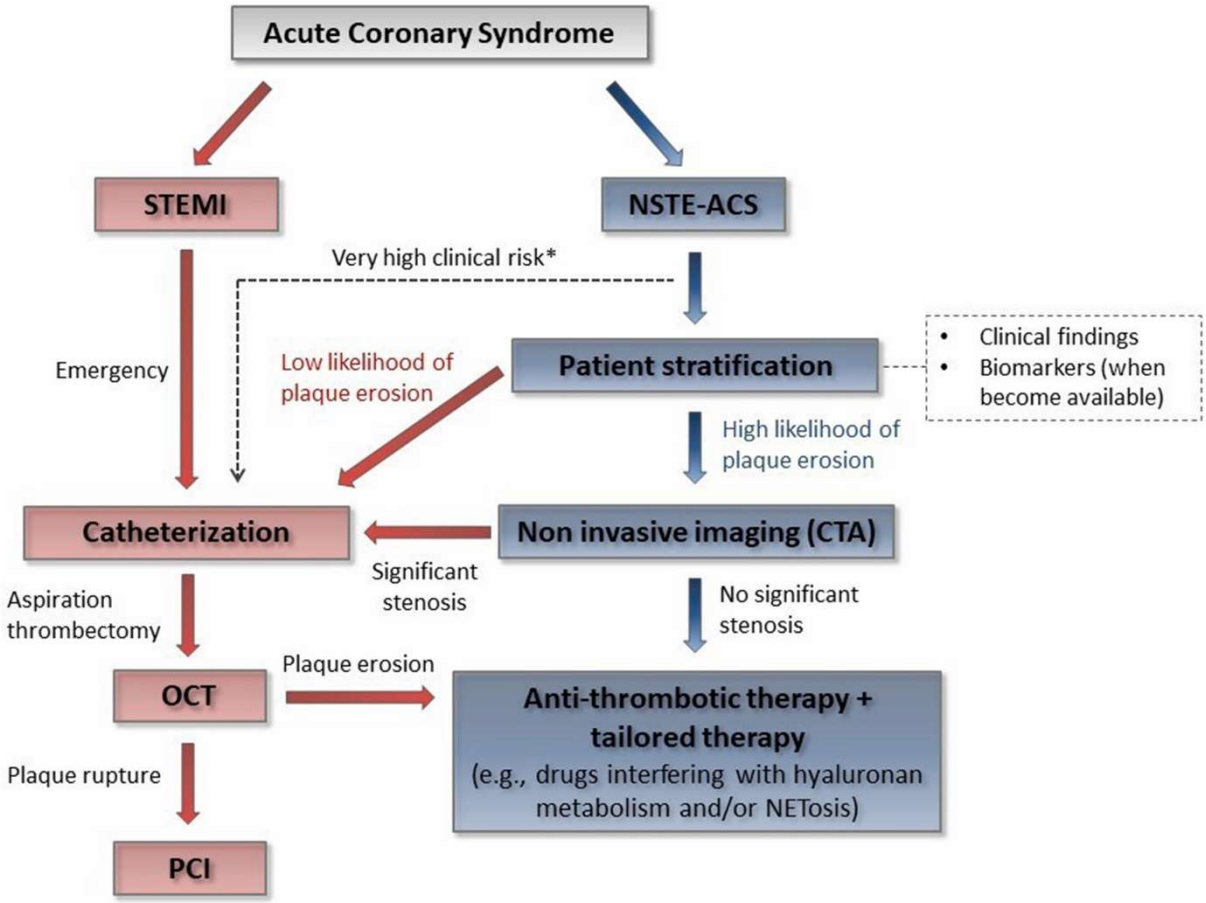

Figure 7 - Proposed future tailing of the treatment of Type 1 MI

STEMI, ST-segment elevation myocardial infarction; NSTE-ACS, non-ST-segment elevation myocardial infarction; CTA, computed tomography angiography; OCT, optical coherence tomography; PCI, percutaneous coronary intervention

Reproduced from Vergallo R, Jang I-K, Crea F. New prediction tools and treatment for ACS patients with plaque erosion. Atherosclerosis. With permission from Elsevier[138]

Such schemes are a promising future direction for a tailored approach to myocardial infarctions, though small hypothesis-generating studies currently drive them.

1.9 Future Research

Further research needs to continue to address the underlying pathology of how erosions occur and then focus on treatments for how each step can benefit our cohorts on top of the current dual antiplatelet/anticoagulation (in the acute MI phase)/beta-blocker/RAAS blockade/statin therapy used as secondary (and indeed even primary) prevention[139].

1.9.1 Translational

Research into the underlying pathological mechanism is vital as it allows for developing new strategies for preventing and treating myocardial infarction. Work to date *in vivo* has focussed on differential cytokine profiles between plaque erosion and plaque rupture patient cohorts[36,37].

As discussed earlier, endothelial cell apoptosis is an attractive explanation for denudating endothelial cells in erosive plaques. However, the evidence is circumstantial and from animal models or post-mortem specimens. I propose to undertake a prospective observational study of endothelial cells using flow cytometry of blood samples from patients undergoing cardiac catheterisation following admission with a Non-ST elevated myocardial infarction and correlate these with findings with plaque morphology to increase our understanding of the endothelium and, ultimately, plaque erosion.

1.10 Hypothesis and Aims

1.10.1 Hypothesis

The primary hypothesis asserts that elevated levels of circulating endothelial cells (CECs) can be detected in the peripheral circulation following plaque erosion compared to plaque rupture, suggesting a distinctive vascular pathology underlying these two mechanisms of acute coronary syndromes.

The selection of CECs for investigation is grounded in their potential to serve as direct indicators of endothelial damage in response to the distinct pathophysiological processes involved in plaque erosion. Flow cytometry has already demonstrated that CEC count in peripheral blood can be determined. These are elevated in MI patients compared to those with stable angina and healthy controls[51]. Plaque erosion consists of the disruption of the endothelial lining, and consequently, CECs are anticipated to be shed into the bloodstream. This choice aligns to capture a real-time and specific marker of endothelial injury in keeping with the proposed mechanism of mass endothelial cell denudation at the site of plaque erosion.

The selection of the 48-hour post-index heart attack timeframe for sample collection aligns with previous studies demonstrating its independence as a reliable marker for acute myocardial infarction[57]. However, to ensure the specificity of our investigation into circulating endothelial cells (CECs) as potential biomarkers for discriminating between plaque erosion and plaque rupture, it is crucial to acknowledge and address potential confounders, particularly those arising from other sources of vascular inflammation. While we have excluded patients with known haematological or inflammatory disorders to mitigate external influences, the possibility of undetected vascular inflammation remains.

1.10.2 Secondary Hypotheses

To measure platelet-monocyte aggregates (known to be raised in MI[101]) and see if there are differences in levels between RFC and IFC as a surrogate of a differential mechanism of atherothrombosis between plaque rupture and plaque erosion myocardial infarctions.

1.10.3 Aims

The primary aim is to provide *in vivo* evidence for the hypothesised mechanism that leads to plaque erosion as observed in the animal and cell flow-tank models.

1.10.3.1 Study Objectives

1. To demonstrate the feasibility and utility of this methodology.

a. To correlate numbers of circulating endothelial cells in peripheral blood samples pre- and post-elective coronary angioplasty.
b. To correlate numbers of circulating endothelial cells in peripheral blood samples between OCT-defined plaque erosion and plaque rupture myocardial infarctions.
c. To correlate platelet/leukocyte aggregates (PLAs) proportions in peripheral blood samples between OCT-defined plaque erosion and plaque rupture myocardial infarctions.

2 Methods

2.1 Study design

This prospective observational study assesses the feasibility of studying the circulating endothelial cell differences between coronary atherosclerotic plaque rupture and plaque erosion with plaque morphology during myocardial infarction by OCT in patients presenting with NSTEMI.

2.2 Ethics

This thesis is based upon the study of patients recruited prospectively into the Plaque Erosion Pilot Study ii (PEPSii) at Norfolk and Norwich University Hospital, UK. The study was given ethical approval by the East of England – Cambridgeshire and Hertfordshire Research Ethics Committee (N° 270706). Subsequently, the study was given full permission for research by the Norfolk and Norwich University Hospitals National Health Service Foundation Trust (Research and Development Reference Number: 270706).

2.3 Patient Recruitment

Two cohorts of patients were recruited for this study. Patients were identified (feasibility group) by prospectively contacting patients due to come in for angioplasty at the Norfolk and Norwich University Hospital and (NSTEMI group) by screening patients admitted under Cardiology.

The feasability group had stable angina and had been scheduled to undergo elective PCI for single-vessel coronary artery disease. Initially, patient recruitment for this group was proposed during pre-assessment clinic visits. Following the COVID-19 pandemic, these pre-assessments happened virtually. The elective waiting list was interrogated for patients listed for PCI before a screening telephone call; patients who gave their consent were posted copies of the patient information sheet (PIS), and recruitment then proceeded as per the study flowchart (figure 9).

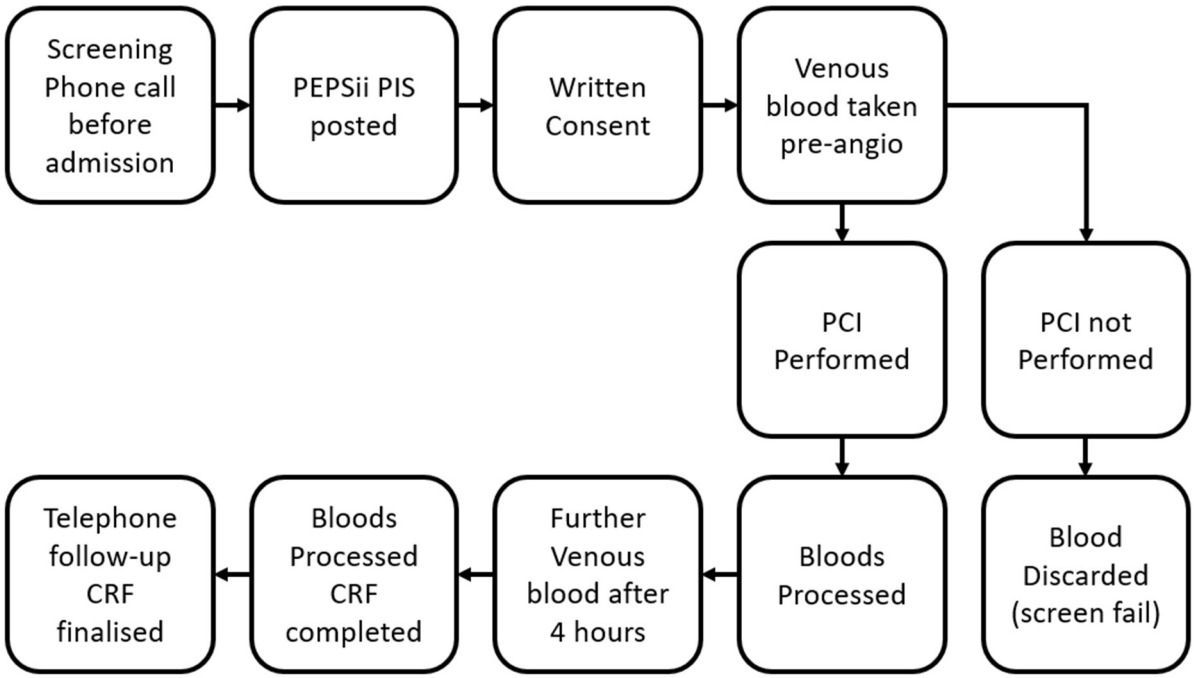

Figure 8 - Feasibility patient group recruitment flowchart

The NSTEMI group patients were recruited following a diagnosis of NSTEMI (episode of chest pain occurring no more than 24 hours before admission with elevation of cardiac troponin I greater than the upper limit of normal (ULN) and new ST-segment depression at least 1 mV and/or T-wave inversion in ≥2 contiguous leads). They were scheduled to undergo invasive angiography ± PCI during their index admission. Study progress then proceeded as per the recruitment flowchart (figure 10).

In this study, care was taken only to recruit patients with a Type 1 MI into the NSTEMI group. This was done by scrutinising all patients diagnosed with an NSTEMI who were referred to the cardiology team for admission. Following assessment by the clinical team and a formal confirmatory diagnosis, I approached them to consider being included in the PEPSii project; at this stage, whilst they read the PIS, I assessed the clinical notes to confirm a diagnosis of Type 1 MI (as per the criteria above) and also took a clinical history. If recruited to the study, patients were classified as screen failures if a non-Type 1 MI cause became apparent during their clinical journey.

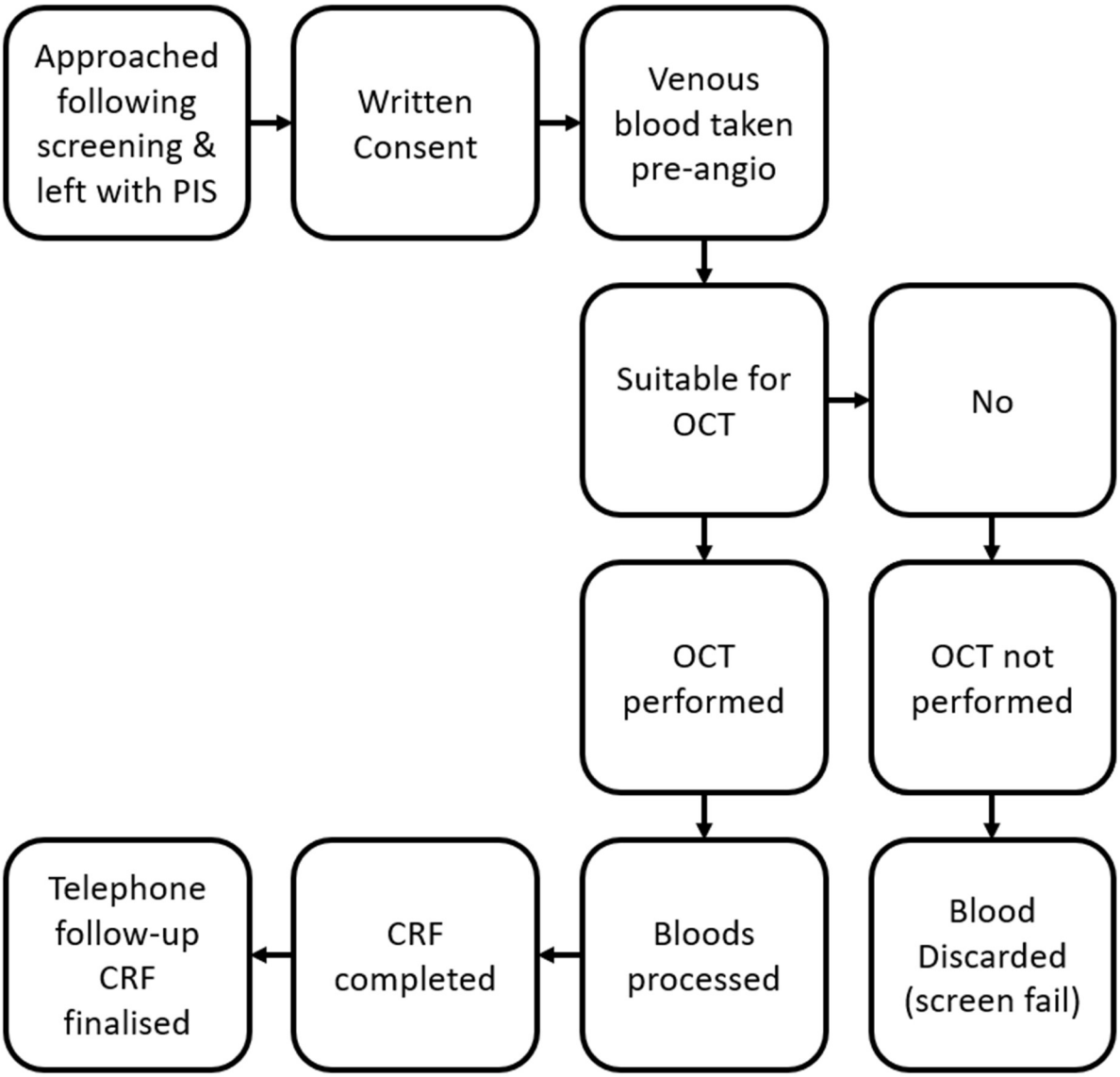

Figure 9 - NSTEMI patient group recruitment flowchart

2.4 Consent

Written study information was provided to eligible NSTEMI patients on admission to the hospital, and ample opportunity was given to discuss the study with a researcher. Written informed consent was sought by a research team member immediately before their coronary angiogram.

We had planned to approach the feasibility group during the pre-operative assessment clinic; with the change in working practices driven by the COVID-19 pandemic, pre-assessment clinics were undertaken by telephone. In response, the feasibility group was initially approached by telephone (after their pre-operative assessment), and study information was sent through the post. Written informed consent was sought by a research team member on admission for their angioplasty procedure.

Copies of the signed, informed written consent forms were given to the patient and filed in the patient's clinical notes and Trial Master File. The original signed consent forms were stored in a study folder in a locked research office. Letters were sent to the patients' General Practitioners outlining the study and confirming the patients' participation once written consent was provided.

2.5 Eligibility

Eligible patients included were those who:

All patients:

1) Could provide informed consent
2) < 75 years old

NSTEMI group:

1) Diagnosis of NSTEMI (episode of chest pain leading to admission with elevation of cardiac troponin I greater than the upper limit of normal and new ST-segment depression at least 1 mV and/or T-wave inversion in ≥2 contiguous leads)
2) Admission to hospital within 24 hours of pain onset
3) Scheduled to undergo invasive angiography ± PCI during index admission

Stable angina (feasibility) group:

1) Scheduled to undergo elective PCI for single-vessel coronary artery disease

<u>The exclusion criteria were:</u>

1) Cardiogenic shock or haemodynamic instability
2) NSTEMI due to stent thrombosis, restenosis, coronary dissection or embolism
3) Previous coronary artery bypass grafting
4) Requirement for mechanical ventilation
5) Requirement for balloon dilatation before OCT
6) Failed (or unsuitable for) OCT examination of the culprit lesion
7) Known severe renal impairment (eGFR <45ml/min/1.73m2)
8) Known haematological or inflammatory disorder
9) Requirement for emergency cardiac surgery

2.5.1 Limitations

Patients aged 75 and above were excluded, as they were less likely to experience a plaque erosion event, more prone to renal dysfunction, at a higher risk of contrast-induced nephropathy (CIN), and

more likely to exhibit tortuosity/calcification, making OCT analysis more challenging. The Research Ethics Committee (REC) panel expressed concerns about the potential impact of excessive contrast leading to CIN (the proposed protocol aimed to exclude patients with an eGFR <30), prompting the implementation of a more stringent set of exclusion criteria.

In the NSTEMI group, previous work[57] had demonstrated detectible levels of CECs <12 hours after admission with acute myocardial infarction, remaining elevated at 48 hours. We restricted patient recruitment to those admitted within 24 hours of the NSTEMI event (to allow a further 24 hours to arrange the angiogram and processing of blood samples within this 48-hour window).

2.6 Sample collection

Once informed consent had been taken for the procedure, peripheral venous blood was taken for all patients; two gold top (clot activator and gel for serum separation) BD Vacutainer® bottles for serum (both to allow the serum to be stored for potential biomarker interrogation and to prevent CEC contamination from venepuncture into the main study bloods), and two lavender top (spray coated k_2EDTA) BD Vacutainer® bottle for flow cytometry which were inverted 5-8 times as per the BD Vacutainer® instructions to process within 30 minutes (maximum 2 hours). In the feasibility group only, a further venous sample was taken 4 hours after intervention (to allow heparinisation to wear off) before discharge. These blood samples were transported to the Tissue Culture Laboratory 1.27 in the Bob Champion Research and Education Building, University of East Anglia, for processing.

NSTEMI patients already had routine emergency blood tests, including a full blood count, urea and electrolytes, liver function tests, CRP, cholesterol and troponin. Feasibility patients would have routinely checked their full blood count, urea, and electrolytes.

2.6.1 Serum processing - gold top bottle

- Confirm clot formation (takes 30-40 minutes)
- Centrifuge at 3000 rpm for 15 minutes at 4°C resulting in 2 visible layers
- 1ml aliquots were taken from the top layer (serum – clear & yellow) into cryovials
- Cryovials were then stored at -80°C

2.6.2 EDTA processing, staining and fixation for flow cytometry - lavender top bottle

- Empty the two lavender top (k_2EDTA) BD Vacutainer® bottles into a suitable tube
    - Double the volume of fluid by adding phosphate-buffered saline (PBS) (12ml) and mix
- Add 8ml of Histopaque into two separate suitable tubes
    - Gently add 12ml of the EDTA blood/PBS mixture (layering technique)

- Centrifuge with a relative centrifugal force (RCF) of 400G for 15 minutes (acceleration & brake off)
- Gently pipette the central leucocyte layer into a suitable tube
    - add 10ml PBS (mix well)
    - centrifuge with an RCF of 400G for 5 minutes (acceleration & brake max)
    - discard fluid and add 10ml PBS (mix well)
    - centrifuge with an RCF of 400G for 5 minutes (acceleration & brake max)
    - discard fluid, leaving a pellet in the bottom of each tube

2.6.2.1 Endothelial cell experiment (fixed cells)

- Reconstitute Zombie viability stain by the addition of 100µl of DMSO (mix well)
    - 2µl aliquots are put in cryovials (each containing two tests) and stored at -20°C
    - Addition of 198µl of PBS defrosts and provides two tests
- Suspend one pellet in 0.9ml of BioLegend wash buffer
    - Transfer 0.4ml into a 1ml tube
    - Add 100µl reconstituted Zombie (no light) and allow to stain for 15 minutes in the fridge
    - Remove from fridge and add
        - add 1µl of Brilliant Violet 510™ anti-human CD146 (mix well)
        - add 1µl of APC/Cyanine7 anti-human CD45 (mix well)
        - add 1µl of APC anti-human CD31 (mix well)
        - then leave in the dark for 15 minutes

2.6.2.2 Platelet/monocyte experiment

- Transfer 0.5ml of cells into a 1ml tube
    - add 1µl of APC/Cyanine7 anti-human CD45 (mix well)
    - add 1µl of APC anti-human CD31 (mix well)
    - add 1µl of FITC anti-human CD41 (mix well)
    - then leave in the dark for 15 minutes

Centrifuge both experiments with an RCF of 400G for 5 minutes (acceleration & brake max)

- Pour off liquid
- Resuspend cell pellet in 0.5ml of 4% formaldehyde to fix
    - Leave in the fridge for 15 minutes to fix
- Add 0.5ml PBS before centrifuging at 400G for 5 minutes

    - Pour off fluid and resuspend cells within 1ml of BioLegend buffer
- Store in the fridge ready for flow cytometry

2.6.3 Limitations

The study employed a single investigator for patient recruitment, sample collection, and processing. Due to the social distancing measures mandated by the COVID-19 pandemic, access to the Tissue Culture laboratory required pre-booking, accommodating various groups within the University. Concurrently, another study necessitated the laboratory to initially process blood samples from patients undergoing elective percutaneous coronary intervention (PCI) and at a subsequent sampling several weeks later. Patients in the feasibility group were predetermined, and the laboratory was reserved for a 6-hour interval to facilitate the processing of both pre-PCI samples and those collected at the 4-hour mark. The optimal post-angioplasty sampling time was uncertain, but for practical reasons, 4 hours was chosen; the entire process, from sample extraction to storing cells in the fridge, typically took around 2 hours. This time point was deemed sufficient since patients undergoing day-case procedures are typically discharged 4 hours post-recovery. This allowed for the heparin effects of the procedure to wear off and prevented discharge delays for patients, with samples taken and case report forms completed just before the patient left the hospital.

2.7 Flow cytometry

The flow cytometer at the Norfolk and Norwich University Hospital is a FACSCanto II Flow Cytometer from BD Biosciences, which has lasers with three excitation energies: 405nm, 488nm and 633nm.

2.7.1 CEC Experiment

Using the Biolegend® Multicolour Panel Selector and the Fluorescence Spectra Analyser[140] and the recommendations of Khan et al.[50] for the quantification of circulating endothelial cells, we designed a flow panel of fluorescence linked antibodies (Figures 10-12) for their detection, Brilliant Violet 510™ anti-human CD146, APC/Cyanine7 anti-human CD45, APC anti-human CD31 – fluorophore-conjugated antibodies previously used to isolate CECs using a flow cytometry method in acute myocardial infarctions[51]. These figures show the wavelength of the excitation laser (solid vertical line), the fluorophore (dotted line) and the emission profile of these fluorescent markers.

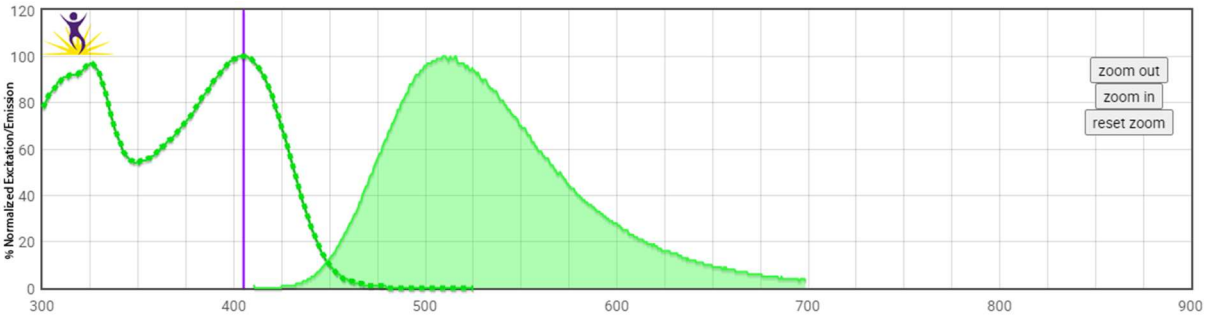

Figure 10 - Brilliant Violet 510™ excitation (405nm laser) and emission energy

Biolegend Spectra Analyzer[140]

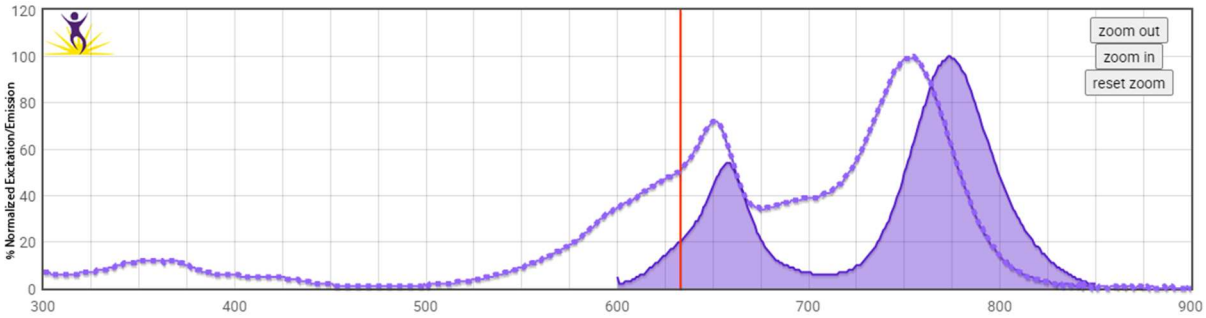

Figure 11 - APC/Cyanine7 excitation (633nm laser) and emission energy

Biolegend Spectra Analyzer[140]

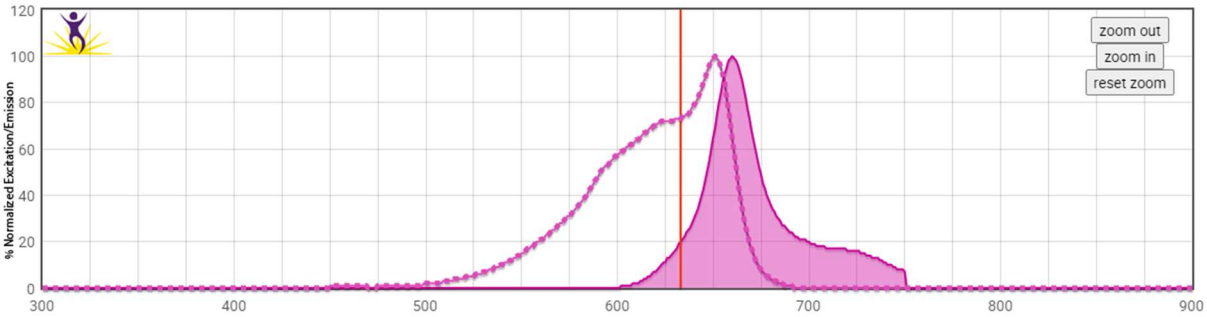

Figure 12 - APC excitation (633nm laser) and emission energy

Biolegend Spectra Analyzer[140]

One of our limitations was that we could not stain for annexin V, as our samples needed to be fixed for block processing on the flow cytometer. Before fixing, we stained with Zombie™ Viability Kit, an amine-reactive (FITC) fluorescent dye that is non-permeant to live cells but permeant to the cells with compromised membranes, which can be used to assess the live vs. dead status of mammalian cells (figure 14).

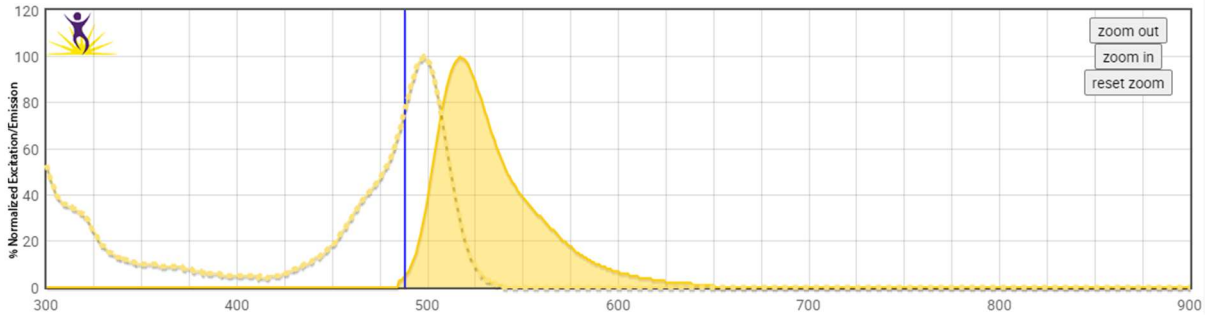

Figure 13 - FITC excitation (633nm laser) and emission energy

Biolegend Spectra Analyzer[140]

When the linked antibodies were used to stain the cells, each cell would have a unique absorption/fluorescence pattern after excitation with the three laser energies to determine which cell was positive for which fluorescent linked antibody.

2.7.2 Platelet/Leucocyte Experiment

Using the Biolegend® Multicolour Panel Selector, the Fluorescence Spectra Analyser[140,] and the antibody fluorophores we had already ordered for the CEC experiment, we produced a flow panel to aid in the quantification of platelet/PBMC aggregates, APC/Cyanine7 anti-human CD45, APC anti-human CD31, FITC anti-human CD41 (figures 11 to 13). We expected to be able to isolate PBMCs from the forward (size) and side (complexity) scatter from the flow data, gate for leucocytes with by CD45 positivity and then identify the proportion of these positive for bound platelets by measuring for CD41.

2.7.3 Calibration of flow cytometry (Gating)

To set up the gating of the flow cytometer, EDTA blood was prepared using the method described earlier; before any staining, the cells in the buffer were split in equal proportions into 10 1ml vials.

2.7.3.1 Endothelial cell experiment

1. Control vial – no stain added
2. 1µl of APC/Cyanine7 anti-human CD45 (mix well)
3. BV510 vial – 1µl of Brilliant Violet 510™ anti-human CD146 (mix well)
4. APC/Cyanine7 vial – 1µl of APC/Cyanine7 anti-human CD45 (mix well)
5. APC vial – 1µl of APC anti-human CD31 (mix well)
6. All vial
    - add 1µl of defrosted Zombie viability stain (mix well)
    - add 1µl of Brilliant Violet 510™ anti-human CD146 (mix well)
    - add 1µl of APC/Cyanine7 anti-human CD45 (mix well)
    - add 1µl of APC anti-human CD31 (mix well)

2.7.3.2 Platelet/Leucocyte Experiment

1. Control vial – no stain added
2. APC/Cyanine7 vial - add 1µl of APC/Cyanine7 anti-human CD45 (mix well)
3. APC vial - add 1µl of APC anti-human CD31 (mix well)
4. FITC vial - add 1µl of FITC anti-human CD41 (mix well)
5. All vial
    - add 1µl of APC/Cyanine7 anti-human CD45 (mix well)
    - add 1µl of APC anti-human CD31 (mix well)
    - add 1µl of FITC anti-human CD41 (mix well)

These were then centrifuged, fixed and resuspended for storage to await flow cytometry as per the methods reported earlier.

2.7.4 Calibration of flow cytometry (FMO)

To help calibrate the findings of the FlowJo software, fluorescence minus one (FMO) control samples were obtained. Samples were stained with all the fluorophores in the panel minus one. They are used to set the upper boundary for the background signal on the omitted label in the flow cytometer settings. Thus, they identify and gate positive populations in the multicolour experiments.

2.7.5 Limitations of flow cytometry

Flow cytometry, a versatile tool in cellular analysis, presents inherent limitations that we strategically addressed in our project. Sample preparation challenges, particularly with poorly dissociating cell types, were mitigated by initial practice on volunteer blood samples (from JW and ADR), which were processed in tandem (paired samples), producing consistent PBMC and CD45 gating results. Whilst the volunteers (JW and ADR) did not have any isolatable CECs in their blood, the next stage was to start recruitment of the feasibility group, where again, the first several patient blood samples were split into two and processed in parallel to check for reproducible results. This deliberate choice allowed us to assess JW's ability to consistently process samples, a crucial step in ensuring the reliability of this method.

The study's design further emphasised sensitivity, a strength of flow cytometry. Using the volunteer blood samples enabled the assessment of the technique's capacity to identify cell populations, especially relevant in the heterogeneous nature of blood samples. Volunteer blood did not show any apparent detectable circulating endothelial cells (CECs) but demonstrated reproducible populations of CD45+ and CD45- cells with parallel processing. This allowed JW to process feasibility group patients, where I could consistently detect CECs (CD45-/CD146+/CD31+). This sensitivity was coupled

with the focus on specificity, achieved by selecting well-characterized antibodies against specific cell markers. By incorporating positive and negative controls (gating and fluorescence minus-one calibrations), I validated staining and instrument performance, enhancing the reliability of the specificity measures.

Reproducibility, a critical aspect of any analytical technique, recognises the potential variations introduced by inconsistent sample handling and instrument settings. To address this, after training with volunteer blood, I started recruiting the feasibility arm of the study, where the technique's capability to isolate circulating endothelial cell (CEC) populations was validated. The parallel analysis of the first four patients ensured consistent and reproducible results to establish the reliability of our findings.

Subjectivity in interpreting flow cytometry data was minimised through standardised analysis protocols and the investigator's (JW) blinding to the OCT diagnosis. By employing consistent gating strategies across analysts and ensuring parallel analyses, we aimed to reduce potential variations in the interpretation of the data. This approach enhanced the overall objectivity and reliability of my conclusions, aligning with the scientific rigour integral to this project.

This project acknowledged the inherent limitations of flow cytometry and strategically employed controls and validation steps to mitigate these challenges. Volunteer blood samples, careful consideration of sensitivity and specificity, and a strong emphasis on reproducibility and standardised analysis protocols collectively contributed to the reliability and validity of the flow cytometry analyses.

2.8 Percutaneous coronary intervention (PCI)

All aspects of the PCI procedure were at the discretion of the consultant cardiologist responsible for the patient's procedure. Diagnostic coronary angiography was performed via the right radial artery or (there was the backup option of) the right femoral artery through a 6 French (Fr) sheath following administration of 1% lignocaine for local anaesthesia. Based on the presenting ECG, coronary angiography was performed using diagnostic 5 Fr catheters to investigate the likely non-culprit artery(s). An appropriate 6 Fr guiding catheter was then used to engage the culprit artery, after which the patient was given weight-adjusted unfractionated heparin (usually starting a bolus of 100units of Heparin per Kg body weight, activated clotting time is measured at 20–30-minute intervals with the aim of therapeutic heparinisation with an activated clotting time around 250) before the culprit lesion was subsequently crossed with a suitable angioplasty wire (0.014 inch) into the distal vessel.

2.9 Optical coherence tomography (OCT)

OCT pullback of the infarct-related artery (IRA) was performed before further intervention on the culprit lesion using an ILUMIEN™ PCI Optimization™ OCT System (St Jude Medical, Inc. Minnesota, USA). This involved the passage of a 2.7 Fr Dragonfly™ OPTIS™ Imaging Catheter (figure 7). Image acquisition is performed by injecting 10-14 ml of x-ray contrast medium into the coronary artery to completely displace the blood and opacify the artery, as would be generally performed during conventional coronary angiography: during this, the optical sensor is automatically withdrawn 50-75mm over 2 seconds within the protective sheath of the catheter. Data was stored digitally, anonymised, and sent to blinded collaborators for interpretation.

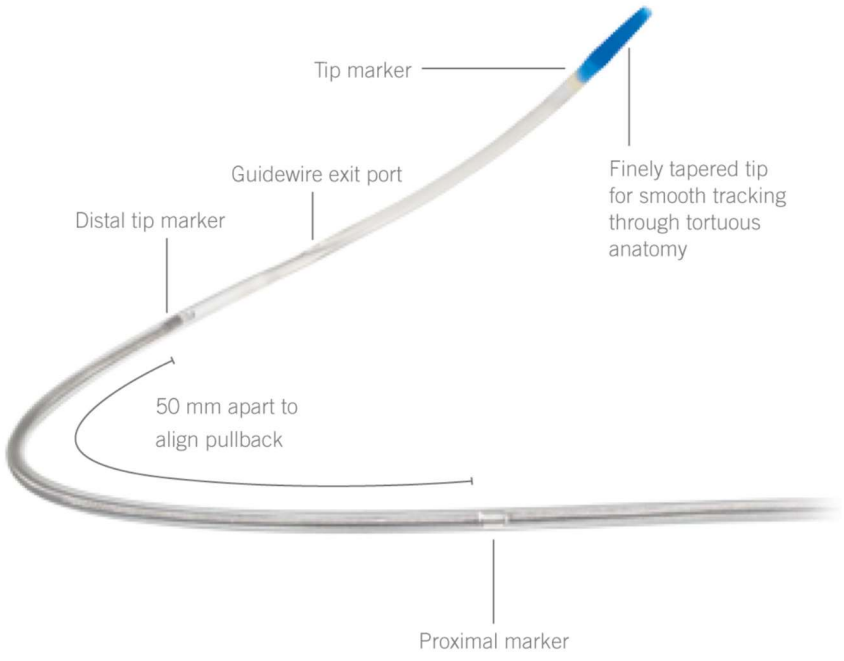

Figure 14 - Dragonfly™ OPTIS™ Imaging Catheter – Abbott Cardiovascular

Patients consented to the risk of trauma to the vessel when this imaging catheter is placed (1 in 1200)[9]; this compares to significant vessel wall disruption as part of the angioplasty procedure (1 in 13)[141].

2.9.1 OCT Definitions of Plaque & Limitations

The development of Optical Coherence Tomography (OCT) criteria for intracoronary plaque assessment has progressed through iterative steps. Initial experiments with excised coronary and aortic specimens showcased OCT's ability to detect microstructural features of atherosclerotic

plaques. This progress extended to animal studies involving swine coronary arteries. Yabushita et al. advanced the correlation between OCT and histology by assessing 357 grossly diseased arterial segments from 90 cadavers post-mortem. The study demonstrated high interobserver and intraobserver agreements for characterising plaque type, coupled with notable sensitivity and specificity compared to histopathological diagnoses[142].

An International Working Group has undertaken a comprehensive examination of coronary plaque diagnosis, leveraging the high resolution offered by OCT images and the widespread commercial availability of the technology with a broad user base. The group has formulated consensus-based standards addressing the use of this technology, the interpretation of OCT images, and the reporting of findings. According to their assessment, plaque rupture (ruptured fibrous cap – RFC) in OCT is characterised by features such as intimal tearing, disruption, or dissection of the cap. This is often associated with OCT-TCFAs and may manifest as a cavity during image acquisition. On the other hand, plaque erosion is indicated by evidence of thrombus, an irregular luminal surface, and the absence of cap rupture in multiple adjacent frames[143]. It is important to note that these binary definitions have limitations, as they may not fully capture the entire spectrum of plaque pathology. As the detection of an endothelial monolayer (i.e., 1–5 µm) is below the resolution of current OCT systems, plaque erosion remains a diagnosis of exclusion *in vivo.* As the criteria lack the precision of the pathology definition of erosion, with limited histopathological correlation, most studies call this entity an intact fibrous cap (IFC)[10,125]. Similarly, most OCT studies report plaque erosion is predominantly accompanied by white thrombus rather than red thrombus. However, this may be that large red thrombi attenuating the OCT light diagnosis of plaque erosion becomes less definite as plaque rupture cannot be excluded[144].

2.10 Baseline demographic/clinical data

Baseline data about the study participants was collected following written consent and stored on a password-protected Excel spreadsheet within the Norfolk and Norwich University Hospital cloud-based computing system. Data included age, gender, past cardiovascular history (previous myocardial infarctions, PCI procedures, or coronary artery bypass grafting, hypertension, hyperlipidaemia, stroke, transient ischaemic attack, peripheral vascular disease), smoking status, diabetes and renal function.

NSTEMI Group only: Clinical data specific to each patient's presentation was also recorded, including time from symptom onset to admission to the hospital (and blood sample) and culprit lesion location. Results of routine inpatient investigations were also recorded, including post-MI echocardiography (left ventricular ejection fraction), full blood count (FBC), renal function, high sensitive troponin I and C-reactive protein (CRP).

2.11 Angiographic and procedural data

Angiographic and procedural data were recorded for each patient participating in the study. The parameters included the number of lesions treated, the infarct-related artery, the presence or absence of multivessel disease (>1 significantly diseased vessel was defined as ≥70% in luminal diameter stenosis), the presence or absence of pre-procedural thrombus, use of glycoprotein IIb/IIIa inhibitors, arterial access route (radial versus femoral), number of stents implanted, the total number of stents implanted per patient, total stent length, use of direct stenting without pre-dilatation, maximum balloon diameter, initial and final TIMI flow, procedural success, reference vessel diameter pre and post-intervention.

2.12 Clinical outcomes

Major adverse cardiac or cerebrovascular events (MACCE), including death, recurrent myocardial infarction (admission with symptoms/ECG changes associated with an acute myocardial infarction along with an elevated blood troponin level), stroke (admission due to neurological symptoms resulting from a lack of blood supply to the brain), target-lesion revascularisation (a revascularisation procedure with repeated stenting, balloon angioplasty or surgical bypass grafting for re-stenosed or occluded culprit target lesion), and stent thrombosis (definite stent thrombosis is defined as occurring when the clinical presentation is consistent with an acute coronary syndrome, and angiographic or pathologic examination with autopsy confirm stent occlusion or thrombus in the stented segment. Angiographic confirmation is the presence of a thrombus during angiography that originates in or from the stent or in the segment 5mm proximal or distal to the stent. Probable stent thrombosis is any unexplained death occurring within the first 30 days that cannot be attributed to other causes. Irrespective of the time after the index procedure, any MI related to the territory of the implanted stent without angiographic confirmation of stent thrombosis can be regarded as probable stent thrombosis in the absence of any other apparent cause) was assessed at one month. This information was ascertained initially through the Trust Patient Administration System database to determine if any unexpected deaths or admissions had occurred, followed by telephone calls by the investigator to the patient directly. Patients were encouraged to notify the research team of concerns or hospital admissions. If information was not obtainable through the sources described above, the patient's GP was contacted to establish more details. For any unexpected deaths in the community, the patient's GP was to be contacted to ascertain more information, including the cause of death written on the death certificate. The electronic records from NNUH are linked to NHS digital; therefore, an up-to-date status on mortality data was available. Adverse events and clinical endpoints were assessed from the time of written consent until the 1-month follow-up was completed.

2.13 OCT Analysis

All OCT data were exported to the DICOM® (Digital Imaging and Communications in Medicine) PACS (picture archiving and communication system) as part of routine clinical records. The original high-resolution OCT data was stored on the stand-alone ILUMIEN™ PCI Optimization™ OCT System cart. A St Jude OCT offline review workstation (ORW) was available to transfer this data for offline review; this machine was archived during the COVID-19 pandemic to make room for a clinical workspace. We intended to transfer the OCT data from the cart to this stand-alone work system (with copies distributed to our collaborators in Royal Papworth Hospital and Bristol Royal Infirmary) for further analysis.

2.13.1 OCT data loss

Unfortunately, a total data loss occurred during routine clinical use of the OCT cart (not a PEPSii recruit) due to human error by a non-study clinical team member. Despite the best efforts of Abbott (who had bought St Jude and provided the OCT equipment) and a specialist data recovery company, we could not restore the high-resolution (raw) OCT data.

After this, the DICOM® backup files were interrogated, and it was found that 2 of the OCT studies had not adequately transferred to the PACS system. AVI files were generated from the DICOM® backup files to allow analysis to be undertaken using previously standardised criteria[9] using a systematic approach to ensure consistency. These were blinded and analysed independently by two operators (James Wardley & Alisdair Ryding); any discrepancies between the two readings were resolved by consensus, with a third blinded operator (Patrick Calvert) analysing these files to help with accuracy. Operators used the following definition criteria:

1. Assess whether the lesion was interpretable
    a. Yes/no
2. OCT images were categorised into RFC, IFC/eroded plaque and undefined
    a. RFC - the presence of a cavity formation in the plaque beginning at the luminal-intimal border with an apparent discontinuity of the thin fibrous cap

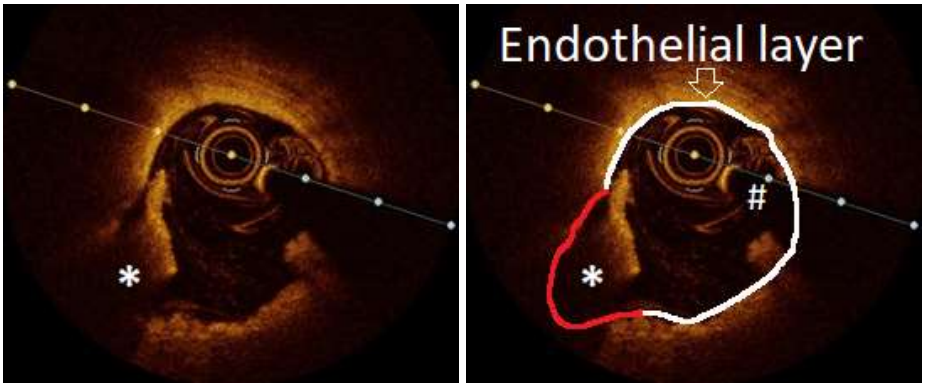

Figure 15 - Example of RFC/ruptured fibrous plaque

*Annotated on the right panel, * red thrombus (blocks OCT laser light) overlying rupture cavity (red), # wire artefact*

b. IFC/eroded plaque - no evidence of cap rupture at the culprit site, in multiple adjacent frames and the presence of thrombus on an irregular luminal surface

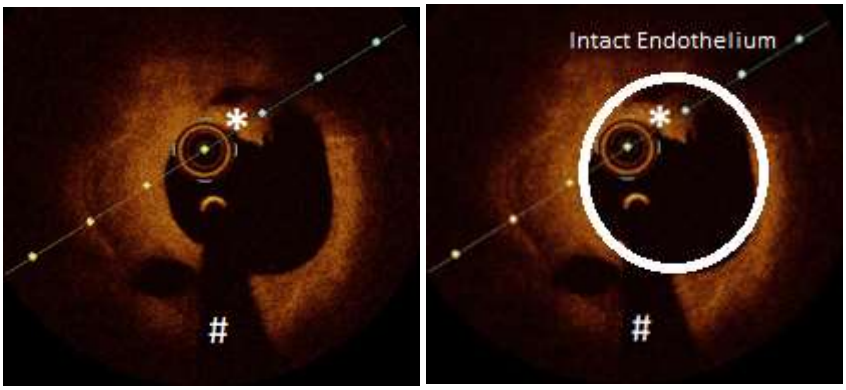

Figure 16 - Example of an IFC/Eroded plaque

*Annotated on the right panel, * presence of white thrombus on an irregular luminal surface without evidence of a ruptured cap, # wire artefact*

c. Undefined - OCT was unable to distinguish the lesion type because where:
    i. an excess of thrombus obscuring the underlying structures or
    ii. images were too unclear to interpret
    iii. loss of OCT data, as mentioned above

3. The presence or absence of a thrombus was recorded
    a. Red thrombus was identified as a high-backscattering protrusion inside the lumen of the artery, with signal-free shadowing in the OCT image
    b. White thrombus was identified as signal-rich, low backscattering structures projecting into the lumen
    c. Mixed encompassed characteristics of both in equal measure

2.13.2 OCT Consensus

OCT DICOM® files were anonymised and then reported by JW and ADR using the above criteria. Before image interpretation, we had agreed that if one investigator said IFC and the other RFC, we would categorise it as undefined. Studies, where consensus was not reached (undefined Vs RFC or IFC), were then sent for blinded analysis by PCAL at Royal Papworth Hospital. JW and ADR were unblinded to this external analysis and re-looked at the images; we had agreed that in circumstances where one could not define the plaque but the external reviewer could contingent with JW or ADR, we would go with the majority view, in circumstances where there was disagreement between JW or ADR on the plaque type then this should be undefined (despite the opinion of the external reviewer).

2.14 Flow Cytometry Analysis

FlowJo 10.8.1 software was used to analyse the raw flow cytometer data. The investigator was blinded to the outcome of the sample (pre or post-angioplasty, erosion or rupture) files, which were loaded into FlowJo and the forward scatter (a measure of the relative cell size) and side scatter (a measurement of the relative complexity of the cell) profiles was used to isolate PBMCs from the raw data.

2.14.1 CEC Experiment

PBMCs were gated against CD45 to exclude leucocytes; CECs were then isolated from this group by gating for CD31 and CD146 before assessment of the live/dead status of the cell, looking at the Zombie stain (figure 17).

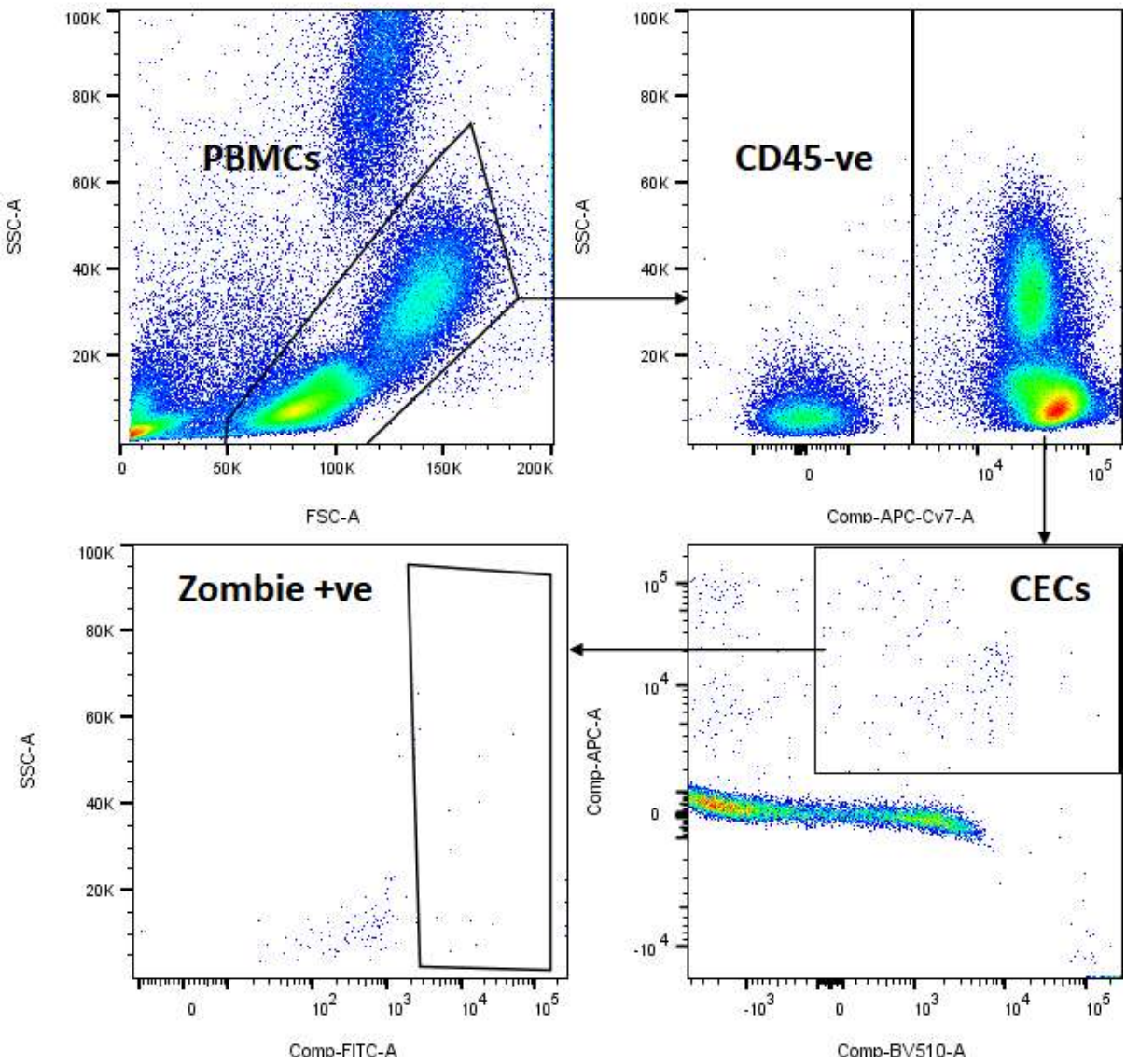

Figure 17 - Detection of CECs

FSC – A (Forward scatter,) SSC – A (Side scatter), APC-Cy7-A (CD45 marker), APC-A (CD31+), BV510 (CD146+), FITC (Zombie)

2.14.2 Platelet/Leucocyte Experiment

FlowJo 10.8.1 software was used to analyse the raw flow cytometer data. The files were loaded into FlowJo, and the forward/side scatter was used to isolate PBMCs; I then gated on CD45 (leucocyte positivity) and then CD31 (platelet positivity) from the raw data (figure 18).

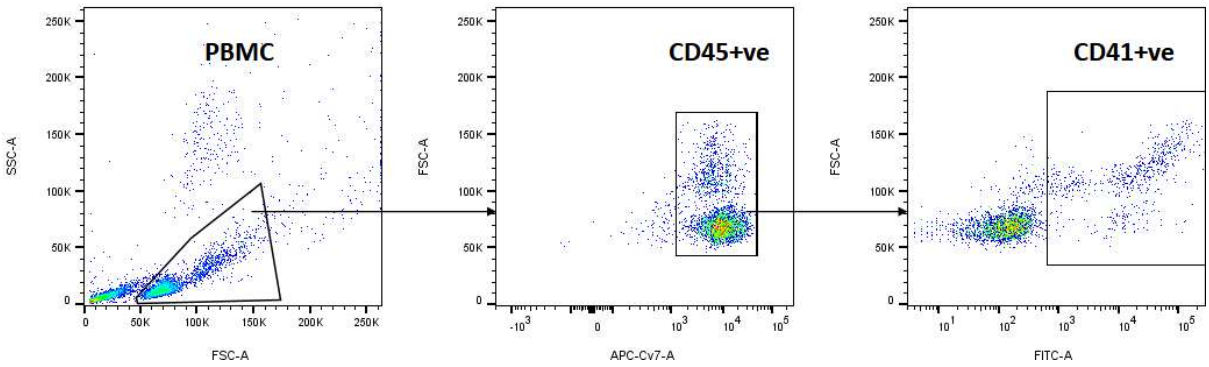

Figure 18 - Detection of Platelet/Leucocyte interactions

FSC – A (Forward scatter,) SSC – A (Side scatter), APC-Cy7-A (CD45 marker), FITC (CD41)

2.15 Statistical Analysis

Continuous variables were described using summary statistics, presenting the mean, minimum, and maximum values. This approach offers a concise representation of central tendency and variability within the dataset. On the other hand, categorical data were presented as counts and relative percentages, providing a comprehensive overview of group distributions.

Comparisons across groups were conducted employing specific statistical tests tailored to the nature of the variables. The non-parametric Wilcoxon rank-sum test was chosen for continuous variables as it is robust in handling non-normally distributed data or situations with a limited sample size. This test allows for reliable between-group comparisons without assuming a normal distribution.

For categorical variables, Fisher's exact test was utilised for groupwise comparisons. This test is particularly suited for situations with small sample sizes or when the assumptions of chi-square tests are not met, ensuring an accurate assessment of associations or differences in categorical outcomes.

All p-values were calculated as two-sided, and statistical significance was predetermined at a threshold of 0.05. This standard significance level helps maintain consistency in hypothesis testing and facilitates the interpretation of results.

Graphical representations of the data were generated using means accompanied by standard deviation bars. This visual depiction aids in illustrating the central tendency and dispersion of continuous variables, enhancing the interpretability of the findings.

Power calculations were deliberately omitted from the study design, considering the pilot nature of the investigation with a limited number of patients. Pragmatic reasons drove the choice of a sample size of 50; the research budget allocated for consumables was £48000, and a bulk discount from Abbott, the manufacturer of the OCT catheters, enabled the purchase of 50 catheters. This approach

ensured the efficient utilisation of available resources and supported the feasibility of this hypothesis generating study within budget constraints.

The statistical analysis was executed using GraphPad Prism 9, a widely recognised software for data analysis and visualisation. Importantly, this analysis was conducted under the supervision of Dr. Rushworth, ensuring methodological rigour and expert oversight throughout the process.

3 Results

3.1 Baseline Clinical and Angiographic Characteristics

Between the 8th of April 2021 and the 18th of January 2022, 11 patients were recruited into the feasibility arm of PEPSii (representing approximately 33% of all potentially eligible patients). 22 patients were recruited into the NSTEMI arm of PEPSii (representing approximately 24% of all potentially eligible patients),

Table 2 - Baseline clinical characteristics

		NSTEMI (22)	Feasibility (11)	p
Age		63.27 (43-78)	62 (52-70)	0.6653
Sex	males	61%	73%	0.4585
Smoker		32% Current 45% Ex 12% Never	9% Current 36% Ex 55% Never	
BMI	kg/m^2	28.9 (24.3-45.8)	32.7 (22.3-52.5)	0.2897
Hypertension		41%	55%	0.4740
Diabetes		14%	27%	0.3539
Hypercholesterolemia		41%	36%	0.8085
Creatinine	umol/L	72.77 (55-105)	81 (53-105)	0.1145
Haemoglobin level	g/L	149.7 (118-186)	144.3 (124-169)	0.3197
Neutrophil count	**10^9/L**	**7.335 (3.1-15.8)**	**4.758 (2.96-6.64)**	**0.0073**
Peak troponin	ng/L	854.2 (50.2->50000)	N/a	
ECG Changes		Anterior 50% Inferior 18% Lateral 5%	N/a	
Radial Access		100%	100%	1
Culprit Vessel		LAD 59% LCx 24% IM 5% RCA 14%	LAD 64% LCx 18% RCA 18%	
Treatment strategy		DES 77% DEB 9% Conservative 14%	DES 73% DEB 27% POBA 9%	

Recruitment into the Feasibility arm occurred whenever the investigator was available, and a hood was available to process the samples (social distancing reduced the number of members of available workspaces). Figure 19 shows the feasibility arm recruitment flowchart.

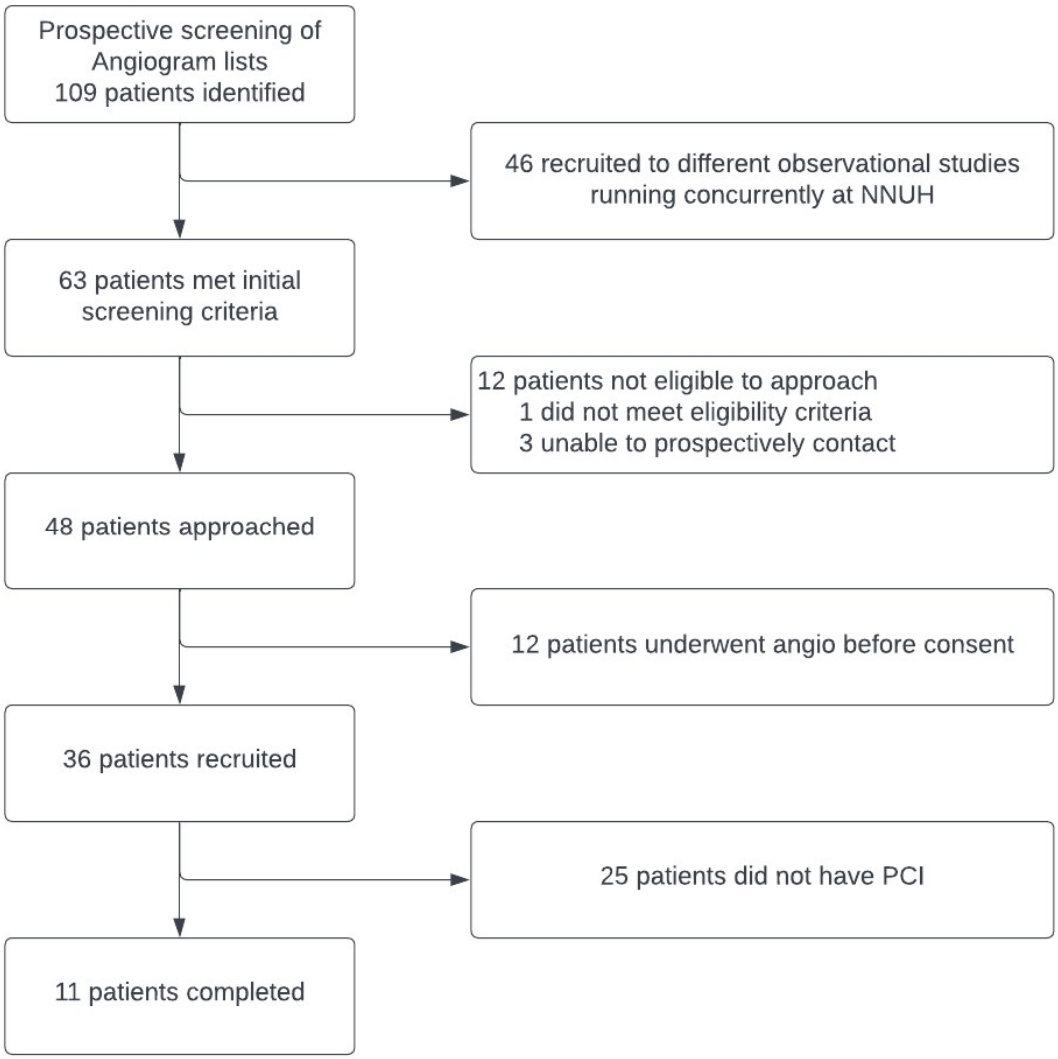

Figure 19 - Study enrolment Feasibility arm

Once the Feasibility arm yielded results, and we were confident that we were able to isolate CECs from blood using flow cytometry, we started to recruit into the NSTEMI arm in all 404 patients who were not eligible for the study (either due to a delayed presentation or lack of availability of lab space, or functionality of the OCT or flow cytometer machines) – figure 20.

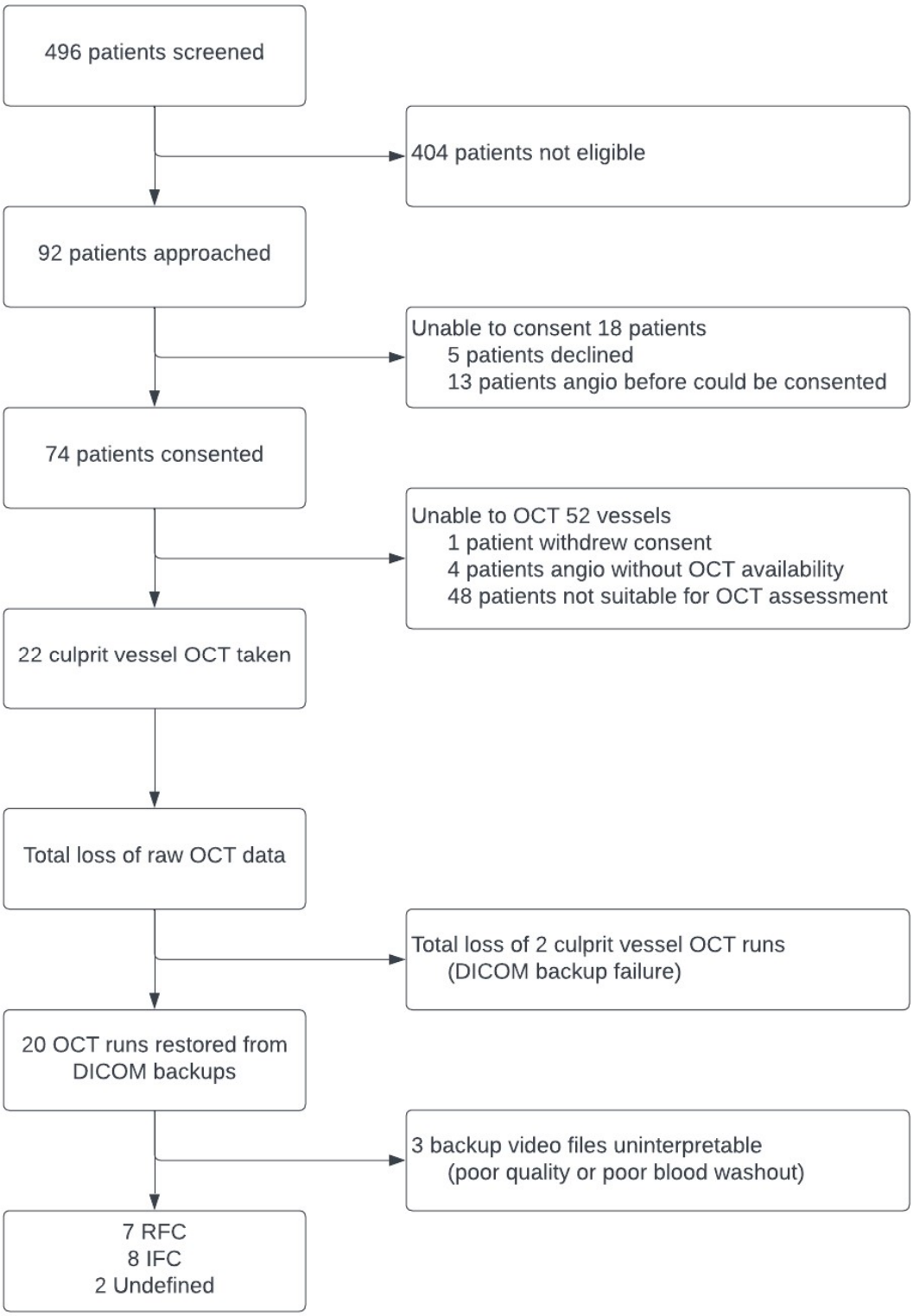

Figure 20 - Study enrolment NSTEMI arm

The OCT files were interpreted using the methods explained in 2.15, looking to determine whether the images were interpretable and to classify them into IFC – 8 patients 36% (OCT defined plaque erosion – example in figure 21A), RFC – 7 patients 32% (OCT defined plaque rupture – example in figure 21B) or undefinable – 7 patients 27% (2 data files lost, 2 OCT uninterpretable due to poor blood clearance, 1 OCT with artefact on the recording throughout, 2 OCTs with no evidence of culprit plaque or thrombus – one of these cases subsequently diagnosed as myocarditis on a cardiac MRI).

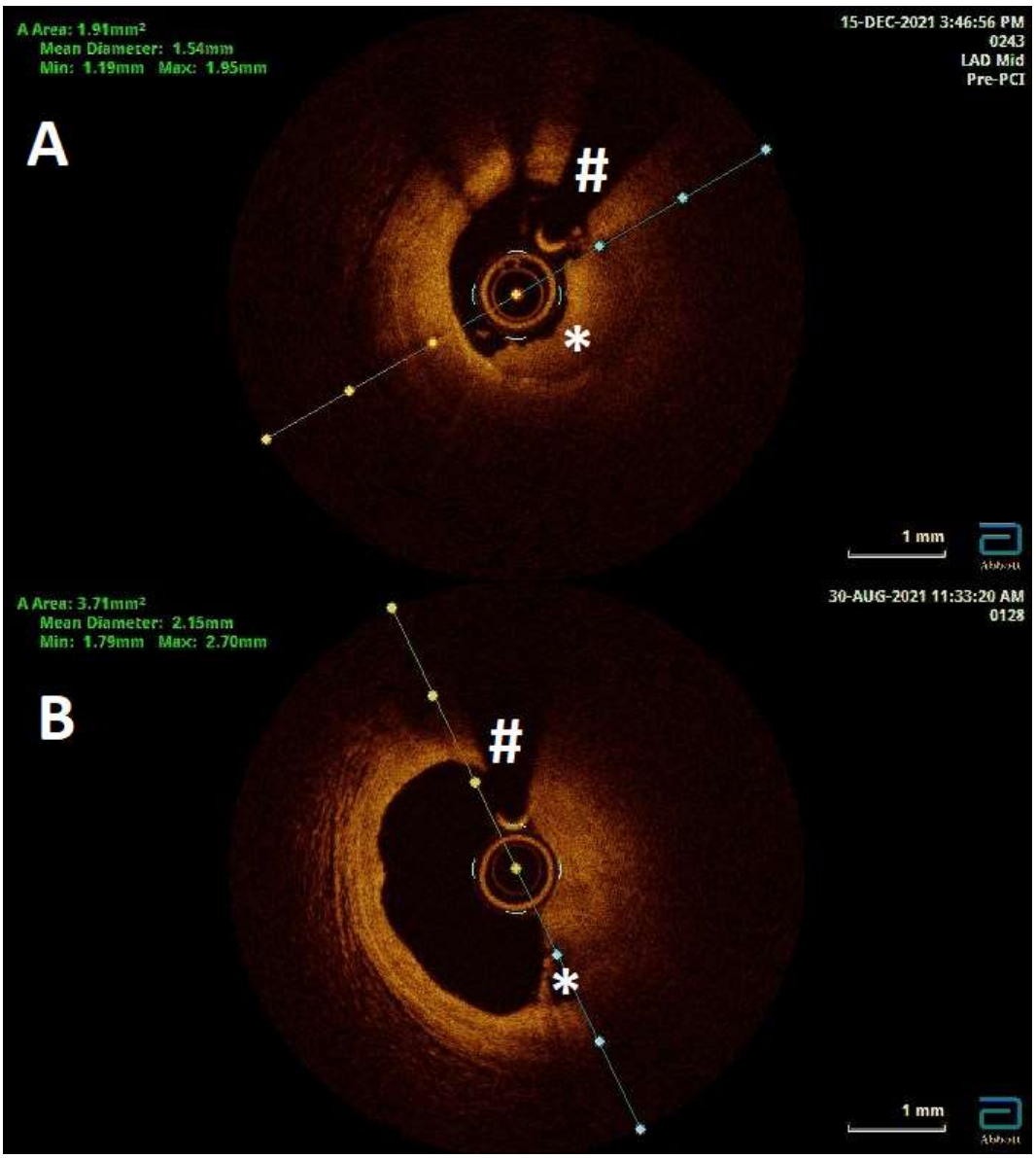

Figure 21 - Examples of IFC and RFC appearances on OCT (enrolled patients)

A - Intact fibrous cap ** thrombus* *# wire artefact*
B - Ruptured fibrous cap ** rupture cavity* *# wire artefact*

The baseline clinical and angiographic criteria were separated into identified IFC (OCT-defined plaque erosion) and RFC (OCT-defined plaque rupture) – table 3.

Table 3 - Baseline clinical characteristics (NSTEMI group)

RFC Ruptured fibrous cap
IFC Intact fibrous cap
BMI Body mass index
CRP C-reactive protein
LV Left ventricle
LAD Left anterior descending (artery)
LCx Left circumflex (artery)
RCA Right Coronary Artery
DES Drug-eluting stent

		RFC (7)	IFC (8)	P value
Age	years	61.7 (43-72)	64.6 (55-78)	0.4117
Sex	males	57%	63%	
Smoking		17% Current 66% Ex-smoker 17% Never smoked	37.5% Current 25% Ex-smoker 37.5% Never smoked	
Hypertension		57%	25%	0.3147
Diabetes		14%	0%	0.4667
Cholesterol	mmol/L	5.0 (3-7)	5.9 (4.4-7.9)	0.2427
BMI	kg/m^2	29.4 (24.6-35)	30.4 (24.4-45.8)	0.9551
Creatinine	umol/L	74.4 (58-91)	67.8 (57-89)	0.2197
Haemoglobin level	g/L	147.6 (127-163.0)	148.8 (137-162)	0.8867
Neutrophil count	$10^9/L$	6.6 (3.1-11.3)	7.3 (4.6-10.3)	0.6126
CRP	mg/L	4.8 (1-21)	4.8 (1-16)	0.8182
Peak troponin	ng/L	2226 (231.9-6627)	11000 (173-50000)	0.9795
LV Ejection Fraction		59% (55-68)	58 (37-67)	0.8435
Target vessel		LAD 66% LCx 17% RCA 17%	LAD 63% LCx 38% RCA 0%	
Treatment		DES 100%	DES 87.5% No treatment 12.5%	
Length of Stent	mm	21.7 (12-44)	20.1 (15-28)	0.8875
Size of Stent	mm	3.1 (2.5-3.5)	3.3 (2.75-4)	0.7582

3.2 OCT Characteristics

Using the methods described in 2.15, OCT files were blindly interrogated, listed as interpretable, and categorised, and comments were made on the presence and type of thrombus (table 4).

Table 4 - Reviewers interpretation of the OCT data

ADR Alisdair Ryding (Reviewer 1)
JW James Wardley (Reviewer 2)
PCAL Patrick Calvert (External Reviewer)
IFC Intact fibrous cap
RFC Ruptured fibrous cap
OCT Optical coherence tomography

	Lesion Interpretable			Lesion Category			Thrombus		
	ADR 1st	JW 2nd	**Consensus**	ADR 1st	JW 2nd	**Consensus**	ADR 1st	JW 2nd	**Consensus**
N004	Yes	Yes	**Yes**	IFC	IFC	**IFC**	White	White	**White**
N007	Yes	Yes	**Yes**	IFC	Undefined	**IFC**	White	None	**White**
	After external review (PCAL), JW agreed with ADR that this was IFC								
N008	Yes	Yes	**Yes**	Undefined	Undefined	**Undefined**	None	None	**None**
N010	Yes	Yes	**Yes**	IFC	IFC	**IFC**	White	White	**White**
N011	No	Yes	**Yes**	N/a	RFC	**RFC**	N/a	Red	**Red**
	After external review (PCAL), ADR agreed with JW that this was RFC								
N018	Yes	Yes	**Yes**	IFC	IFC	**IFC**	White	White	**White**
N024	Yes	Yes	**Yes**	Undefined	RFC	**RFC**	Red	Red	**Red**
	After external review (PCAL), ADR agreed with JW that this was RFC								
N025	No	No	**No**	**Poor washout**					
N026	No	No	**No**	**Lost OCT**					
N027	Yes	Yes	**Yes**	Undefined	Undefined	**Undefined**	None	None	**None**
N028	Yes	Yes	**Yes**	IFC	IFC	**IFC**	White	White	**White**
N029	Yes	Yes	**Yes**	IFC	IFC	**IFC**	White	White	**White**
N030	Yes	Yes	**Yes**	RFC	RFC	**RFC**	Red	Red	**Red**
N031	Yes	Yes	**Yes**	RFC	RFC	**RFC**	Red	Red	**Red**
N032	No	No	**No**	**Poor washout**					
N033	Yes	Yes	**Yes**	RFC	RFC	**RFC**	Red	Red	**Red**
N034	Yes	Yes	**Yes**	Undefined	RFC	**RFC**	Red	Red	**Red**
	After external review (PCAL), ADR agreed with JW that this was RFC								
N035	Yes	Yes	**Yes**	IFC	IFC	**IFC**	White	White	**White**
N036	Yes	Yes	**Yes**	RFC	RFC	**RFC**	Red	Red	**Red**
N037	No	No	**No**	**Lost OCT**					
N038	No	No	**No**	OCT images degraded by artefact					
N039	Yes	Yes	**Yes**	IFC	IFC	**IFC**	White	White	**White**

The following sections (3.2.1-3.2.22), along with the corresponding figures (22-43), show representations of each NSTEMI patient, with the angiogram (A), angiogram lesion of interest highlighted (B) and (where possible) stills of distal vessel, culprit lesion and proximal vessel (C-E).

3.2.1 N004 Angiogram and OCT images

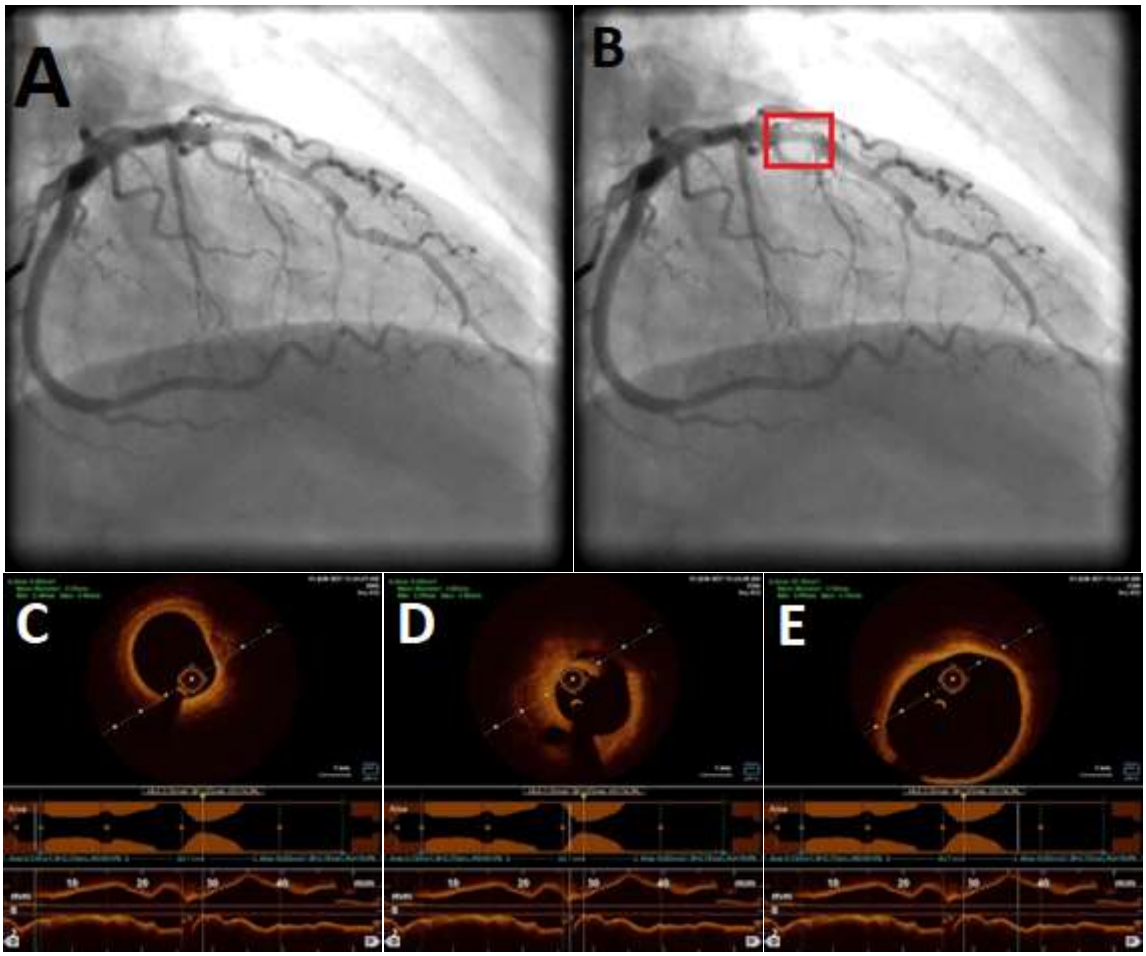

Figure 22 - N004 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 22 demonstrates a lesion within the proximal LAD. The OCT images showed an irregular surface to the endothelium with adherent white thrombus – characterised as an intact fibrous cap (plaque erosion).

3.2.2 N007 Angiogram and OCT images

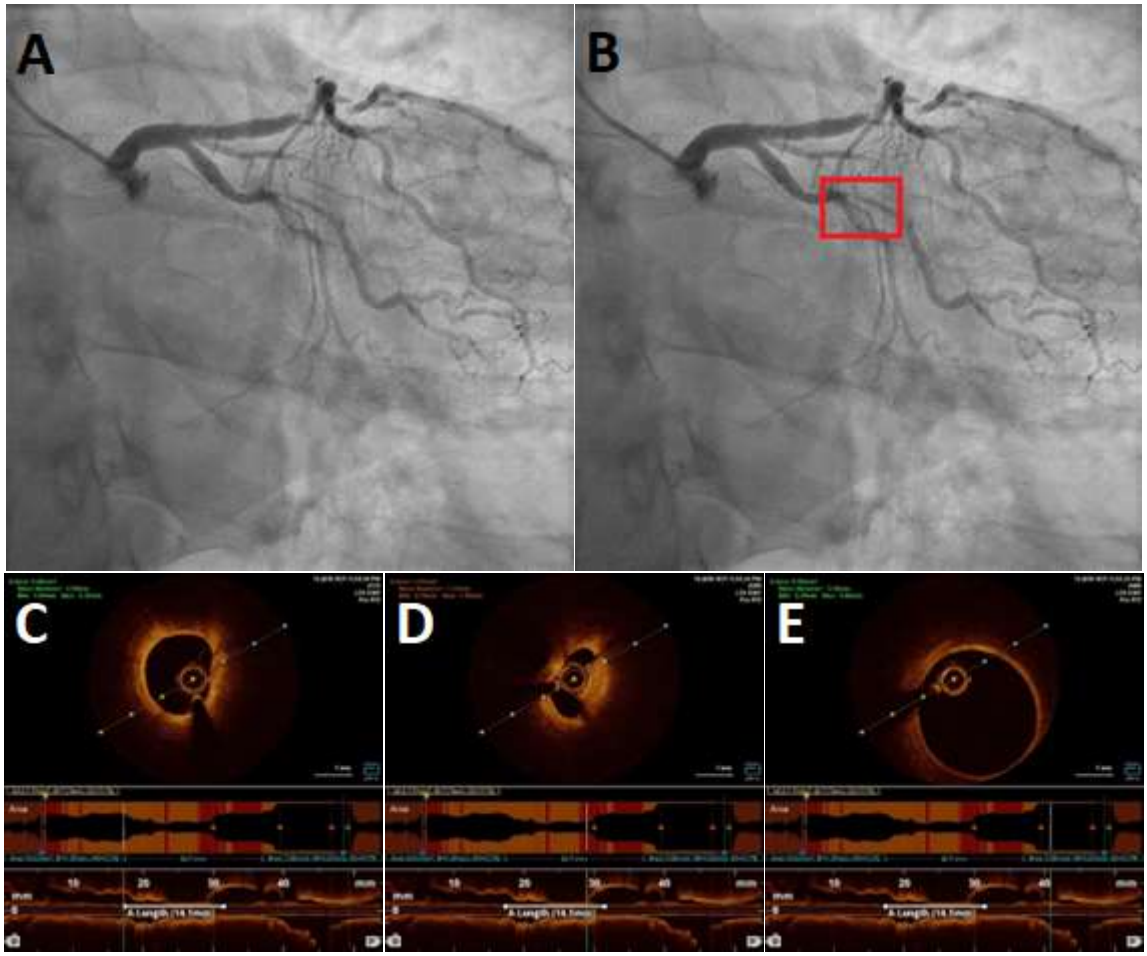

Figure 23 - N007 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 23 demonstrates a culprit lesion in the left circumflex coronary artery; there was good distal and proximal clearance of blood, and the culprit stenosis had small amounts of irregular white thrombus adherent – categorised as an intact fibrous plaque (plaque erosion).

3.2.3 N008 Angiogram and OCT images

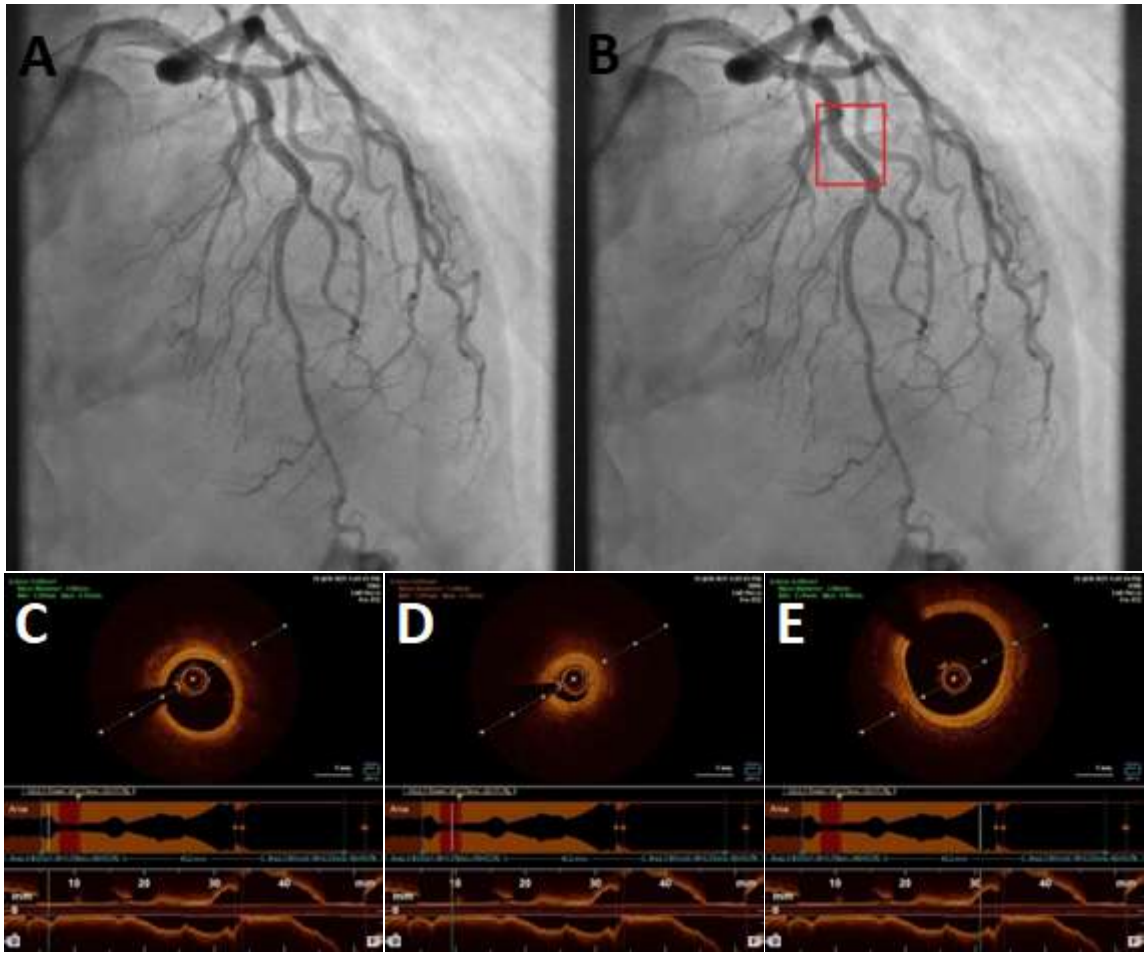

Figure 24 - N008 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 24 shows an LAD coronary artery, felt to be the culprit of the NSTEMI based on ECG changes. The OCT images show a smooth coronary artery without coronary thrombosis. This was classified as an undefined lesion. Following discharge, this patient subsequently had a cardiac MRI scan, which suggested the cause of his presentation to be myocarditis rather than an ischemic insult (NSTEMI) to his heart.

3.2.4 N010 Angiogram and OCT images

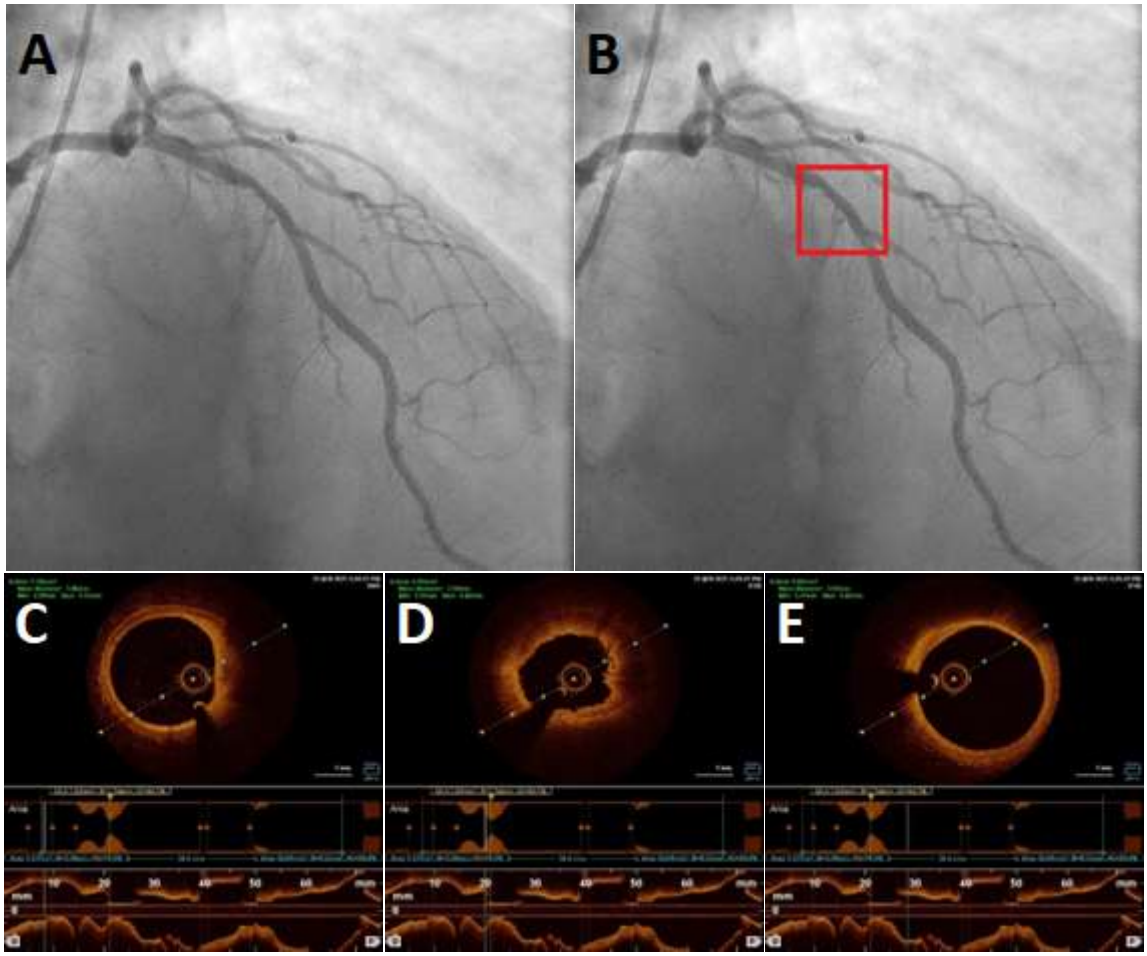

Figure 25 - N010 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 25 demonstrates a lesion within the mid-LAD artery. The OCT images showed an irregular surface to the endothelium with adherent white thrombus – characterised as an intact fibrous cap (plaque erosion).

3.2.5 N011 Angiogram and OCT images

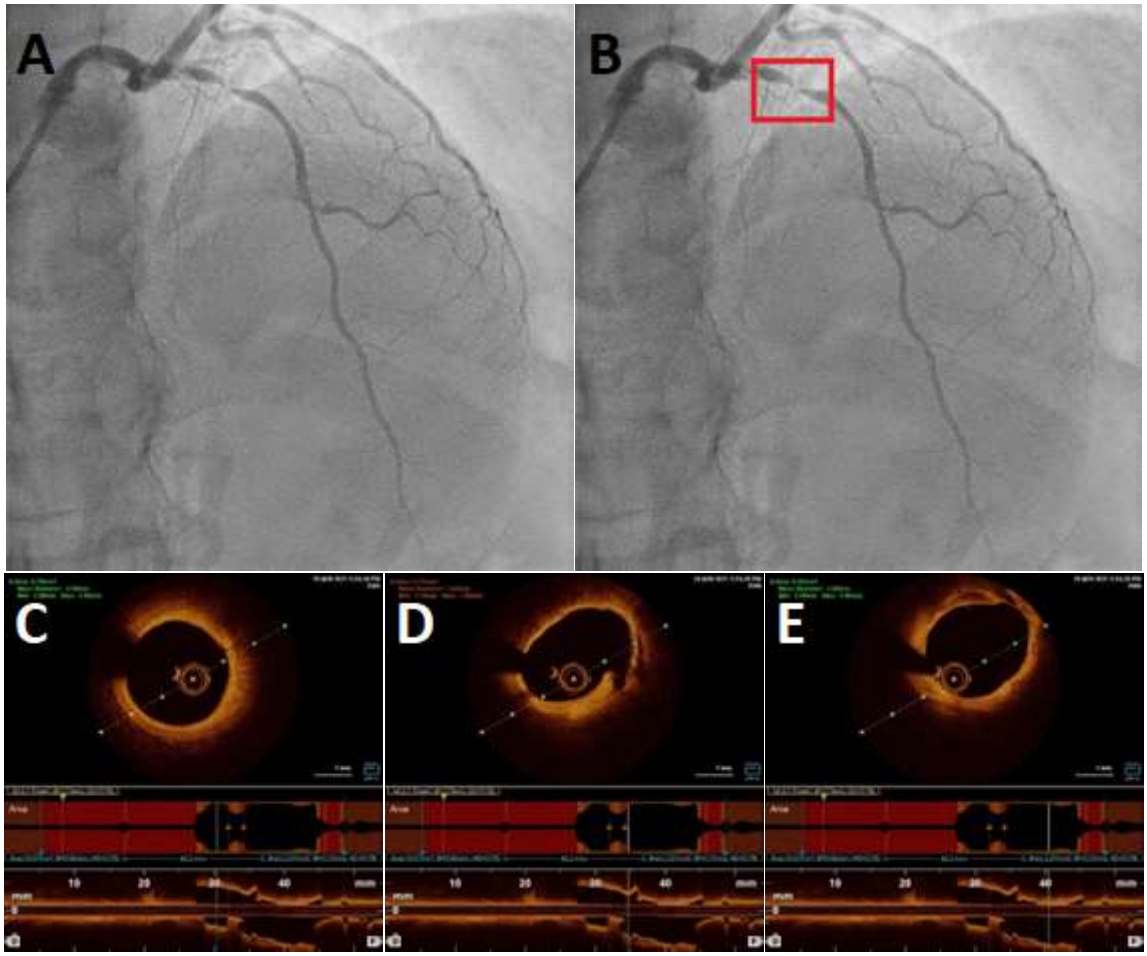

Figure 26 - N011 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 26 shows a culprit lesion within the mid-LAD artery; OCT analysis shows a red thrombus adherent to a disrupted fibrous cap with a cavity where a necrotic core would have been – characterised as ruptured fibrous plaque (plaque rupture).

3.2.6 N018 Angiogram and OCT images

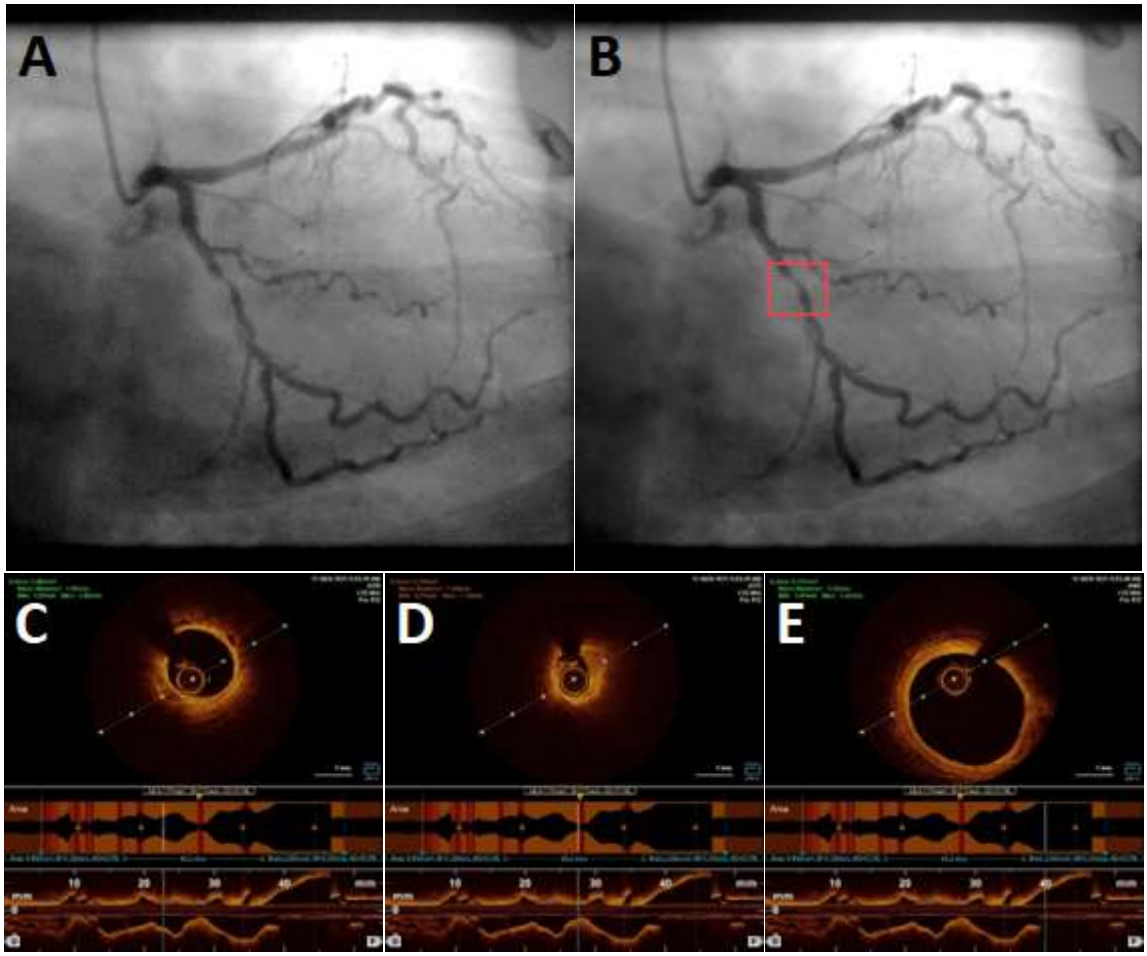

Figure 27 - N018 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 27 demonstrates a lesion within the mid-left circumflex artery. The OCT images showed an irregular surface to the endothelium with adherent white thrombus – characterised as an intact fibrous cap (plaque erosion).

3.2.7 N024 Angiogram and OCT images

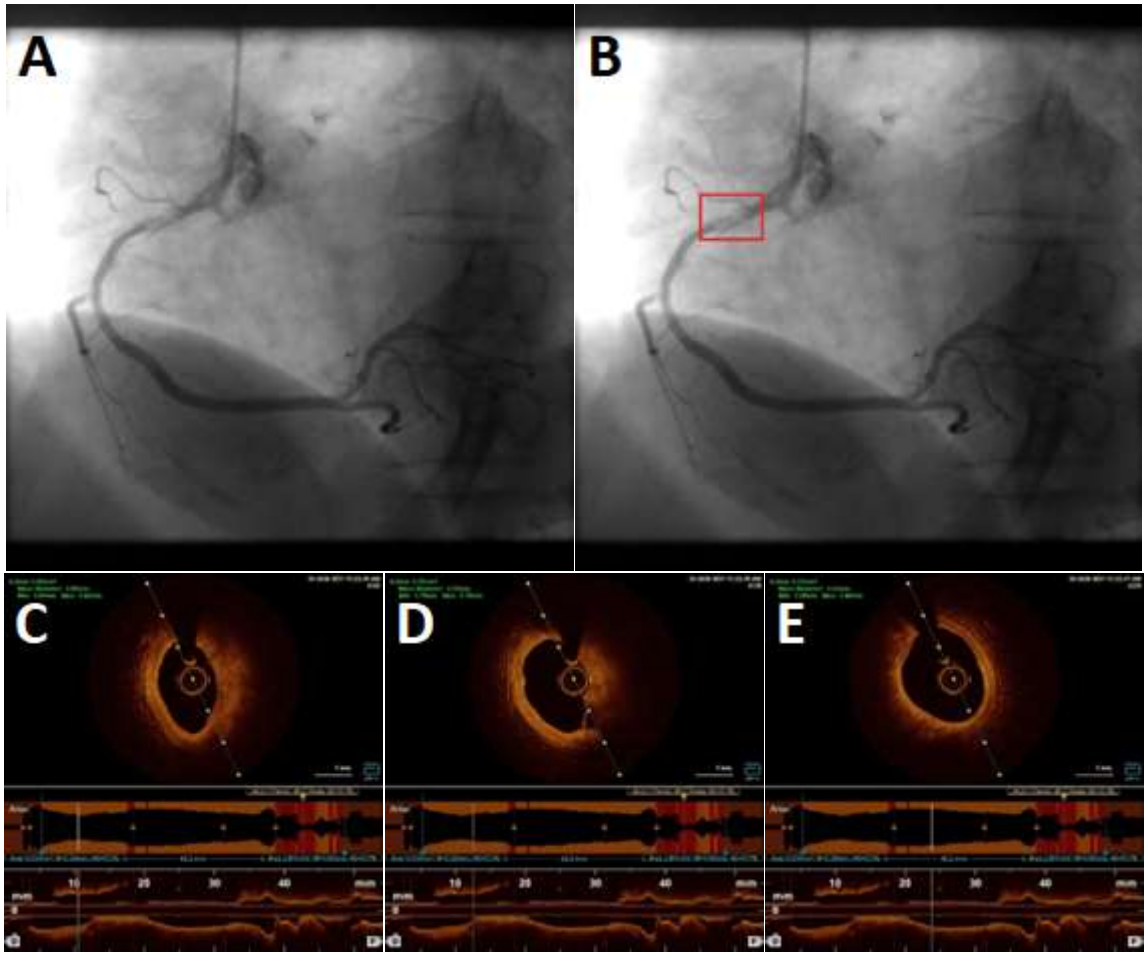

Figure 28 - N024 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 28 shows a culprit lesion within the proximal right artery; OCT analysis shows a red thrombus adherent to a disrupted fibrous cap with a cavity where a necrotic core would have been – characterised as ruptured fibrous plaque (plaque rupture).

3.2.8 N025 Angiogram and OCT images

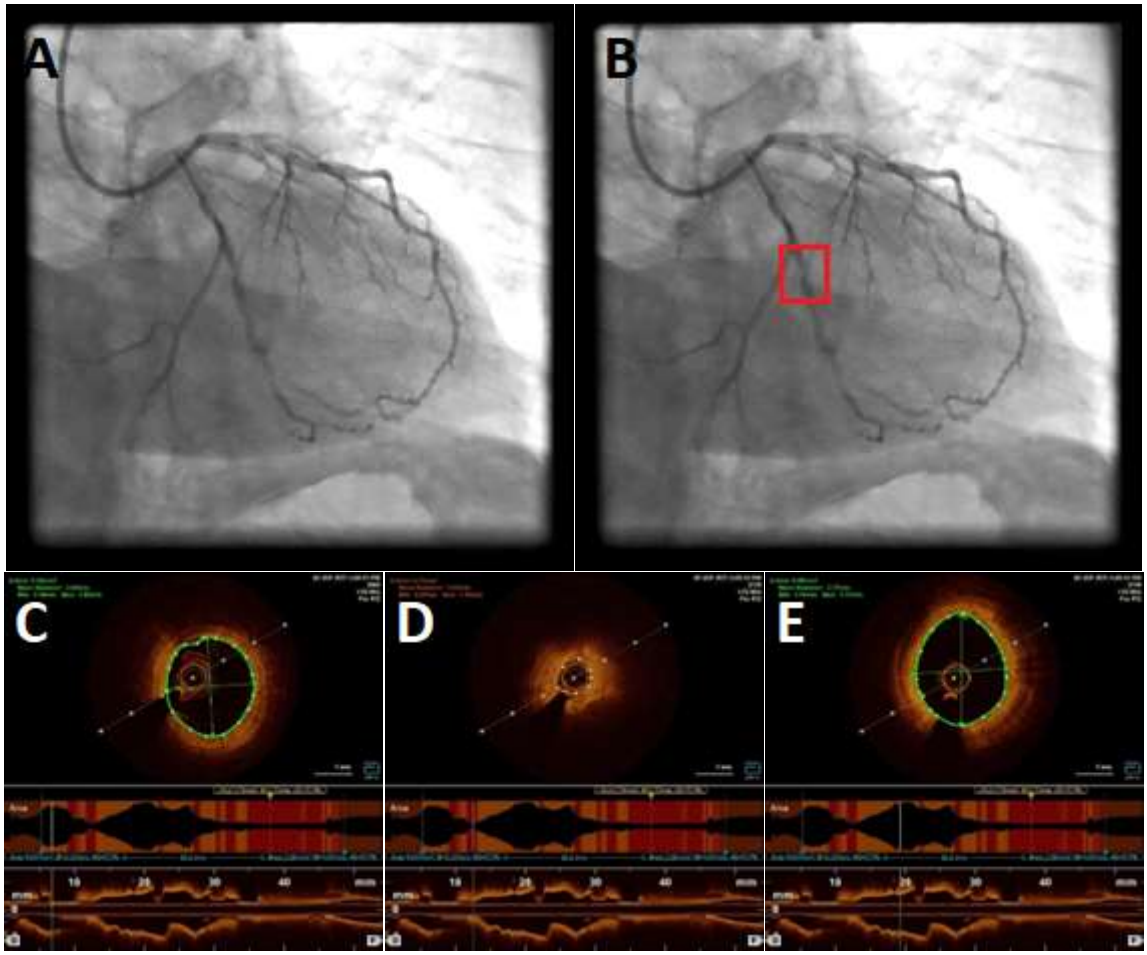

Figure 29 - N025 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 29 shows a culprit lesion within a mid-left circumflex coronary artery; unfortunately, it was impossible to classify this lesion due to poor blood pool washout (and the retention of the tracing tools when the images were transferred to the PACS system).

3.2.9 N026 Angiogram and OCT image

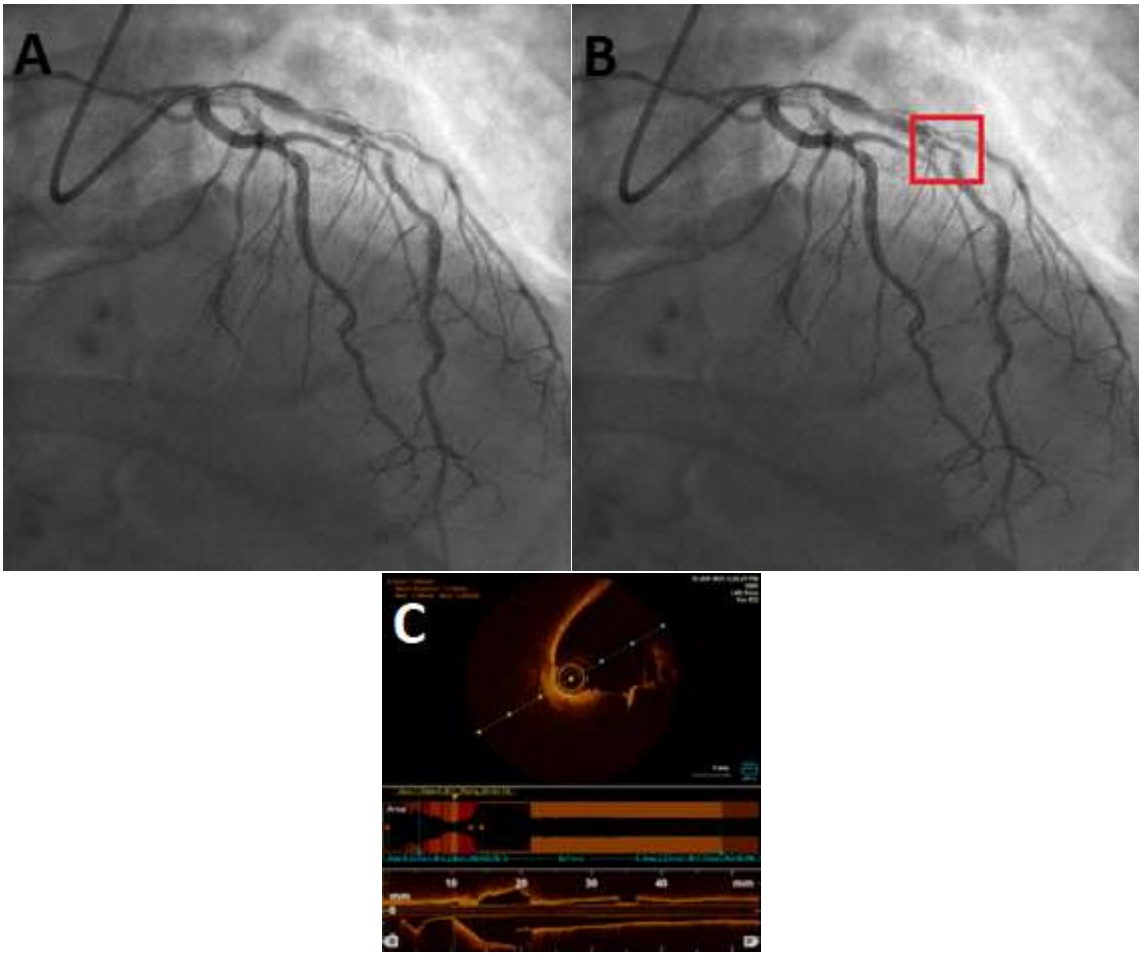

Figure 30 - N026 Angiogram and OCT image

(A) culprit artery (B) lesion of interest (C) distal vessel

Figure 30 shows a culprit plaque within a mid-LAD coronary artery. Unfortunately, only one frame of the OCT images had been transferred to the PACS system – classified as undefined.

3.2.10 N027 Angiogram and OCT images

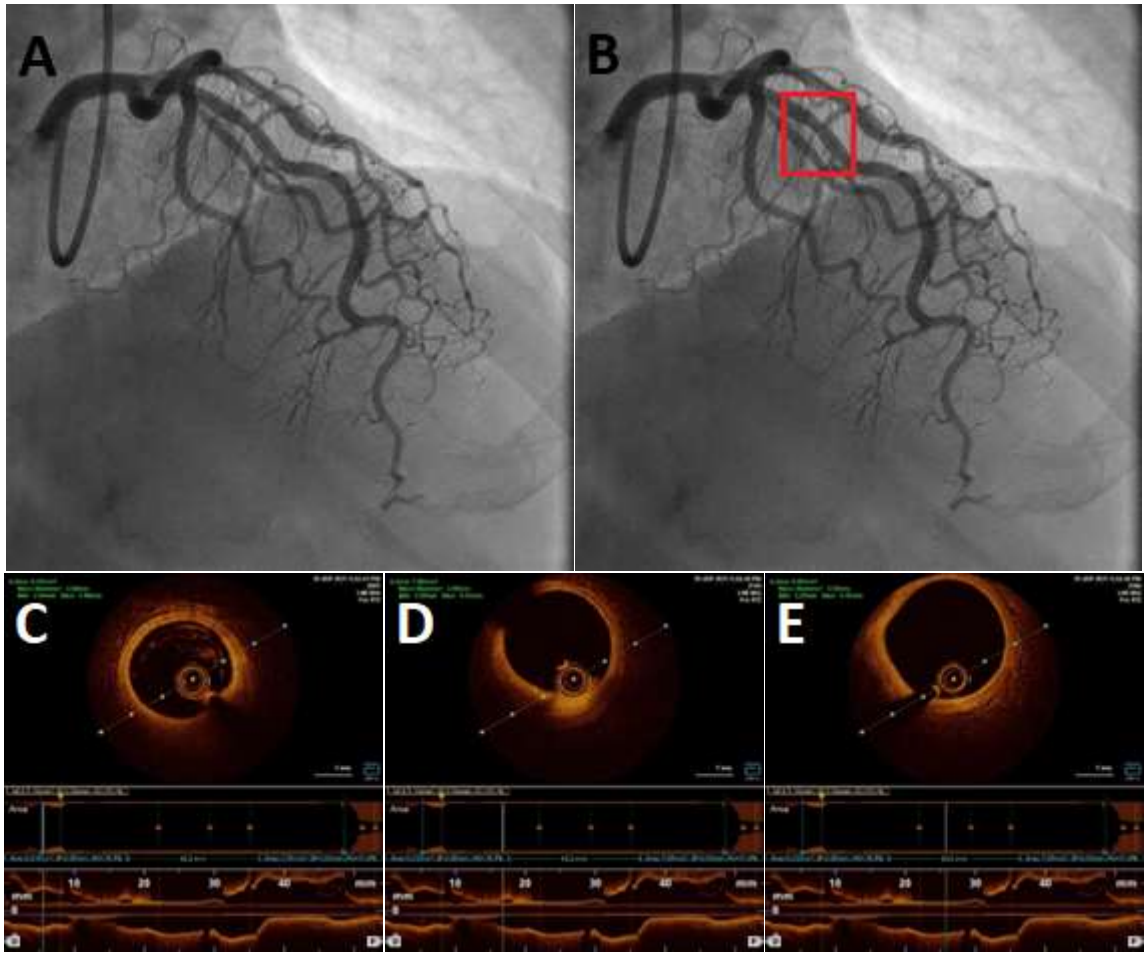

Figure 31 - N027 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 31 shows a culprit lesion and OCT run in an LAD. As the OCT showed smooth coronary arteries without any thrombus formation, this was classified as undefined.

3.2.11 N028 Angiogram and OCT images

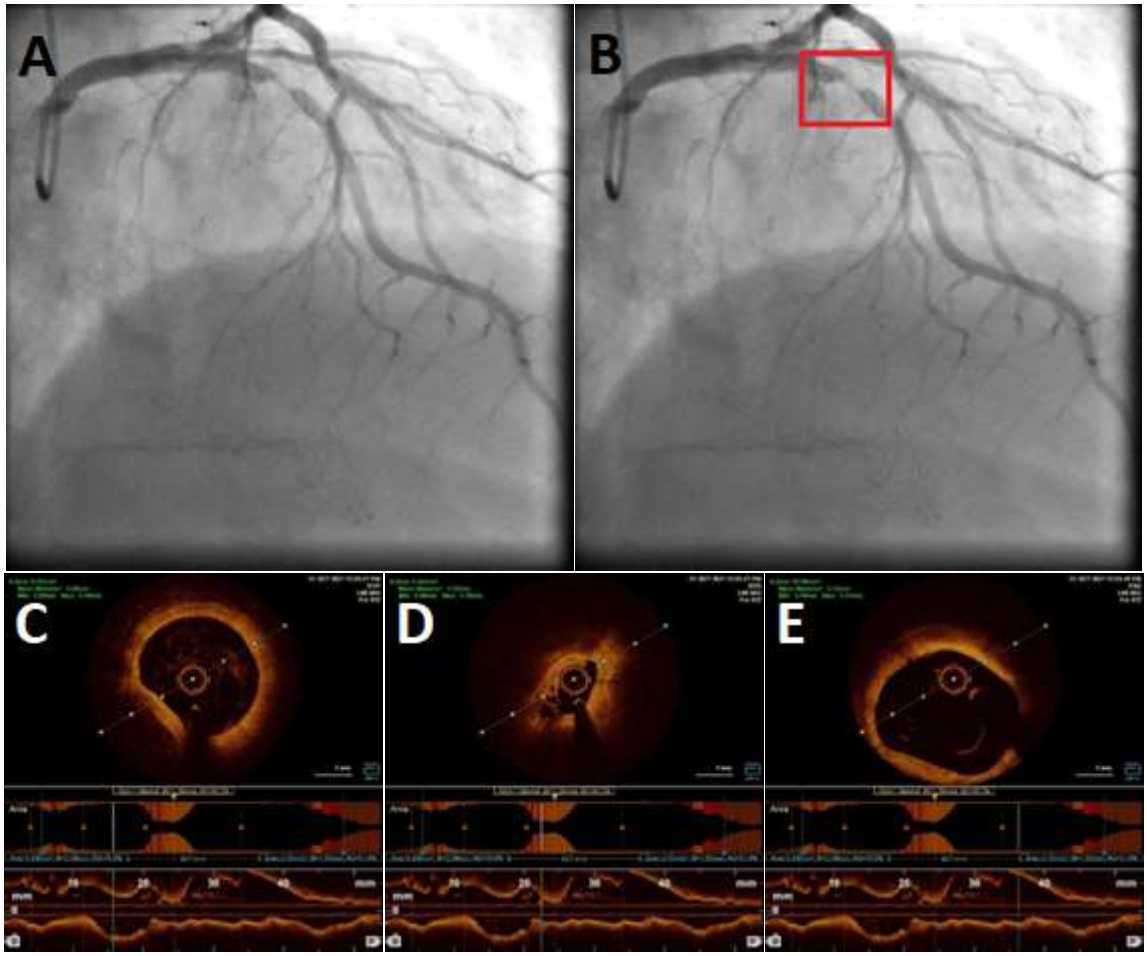

Figure 32 - N028 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 32 shows a culprit lesion within the mid-LAD. OCT images showed an irregular surface to the endothelium with adherent white thrombus – characterised as an intact fibrous cap (plaque erosion).

3.2.12 N029 Angiogram and OCT images

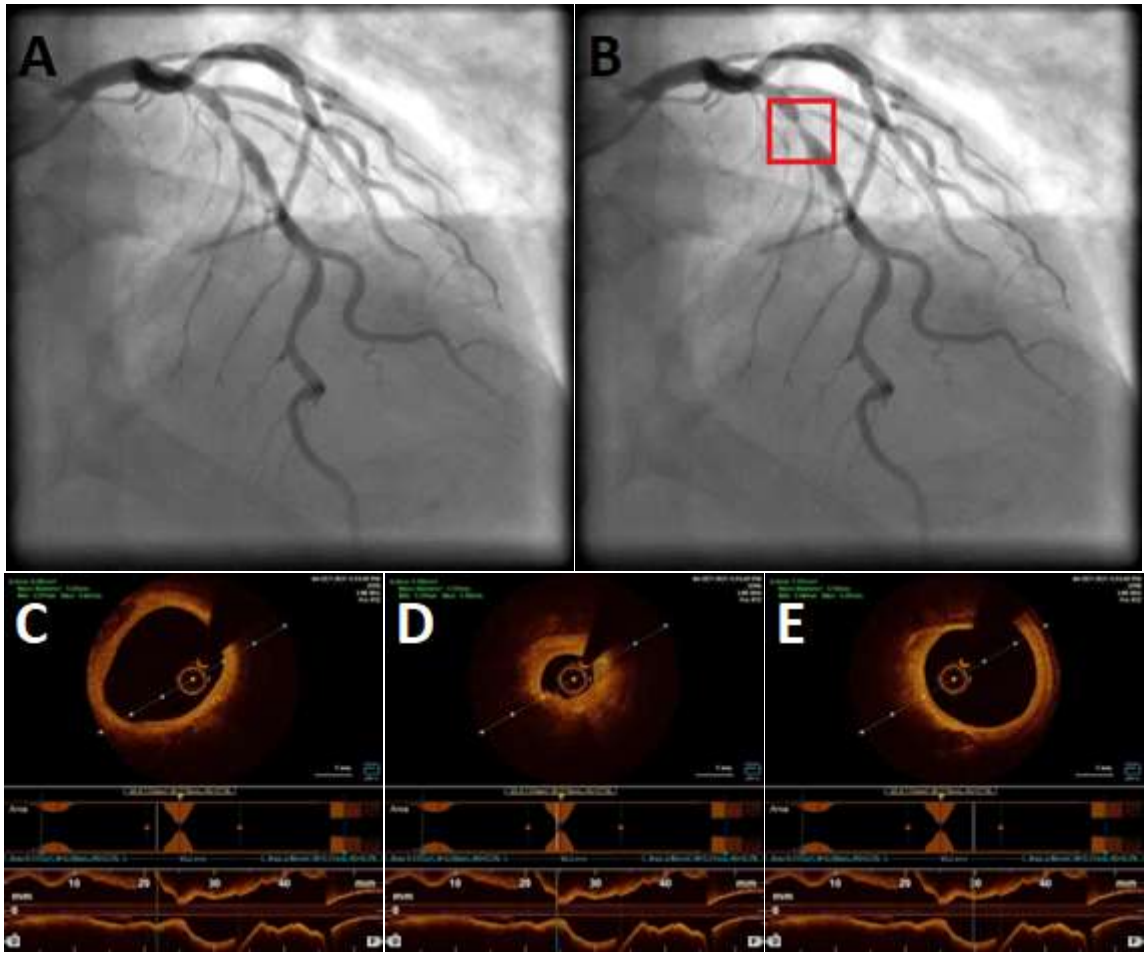

Figure 33 - N029 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 33 shows a culprit lesion within the mid-LAD. OCT images showed an irregular surface to the endothelium with adherent white thrombus – characterised as an intact fibrous cap (plaque erosion).

3.2.13 N030 Angiogram and OCT images

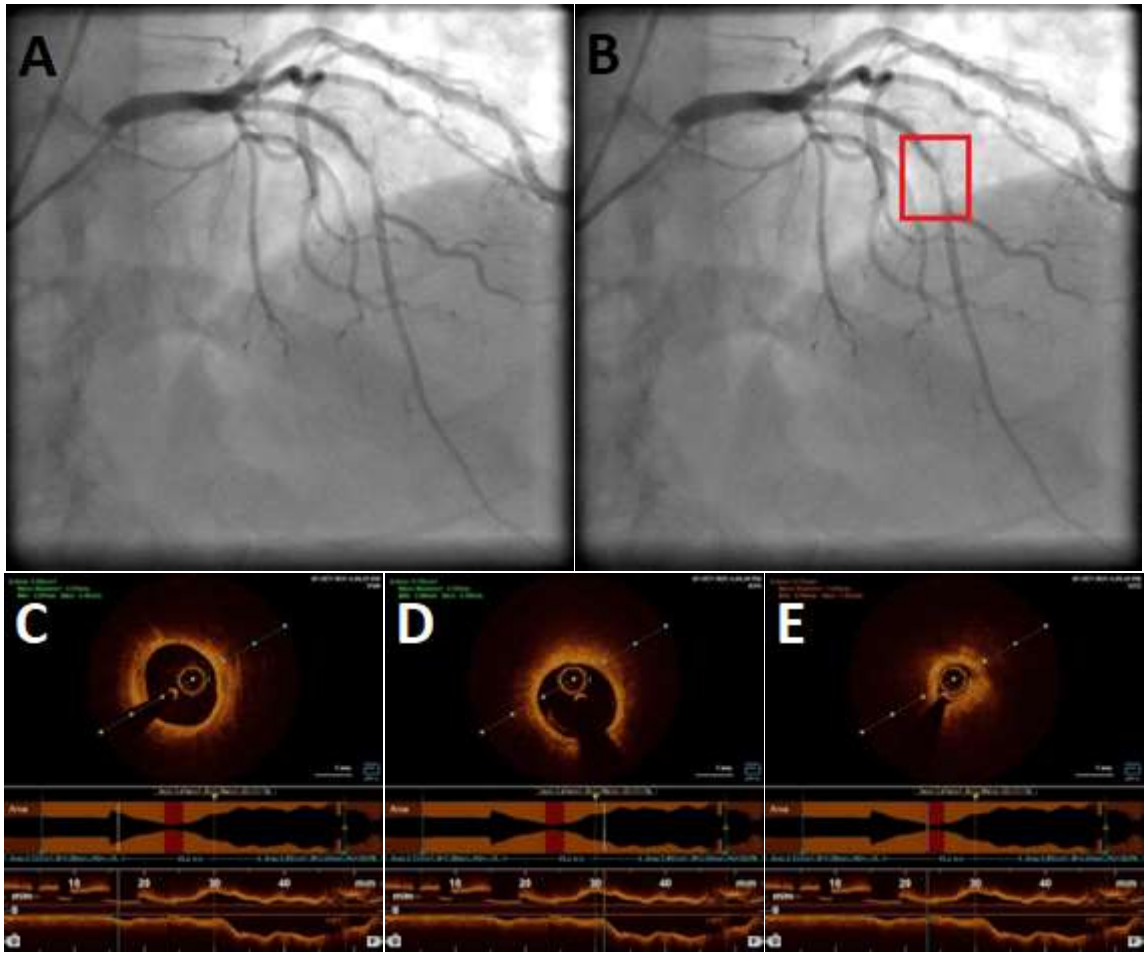

Figure 34 - N030 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 34 shows a culprit lesion within the mid-LAD; OCT analysis shows a red thrombus adherent to a disrupted fibrous cap with a cavity where a necrotic core would have been – characterised as ruptured fibrous plaque (plaque rupture).

3.2.14 N031 Angiogram and OCT images

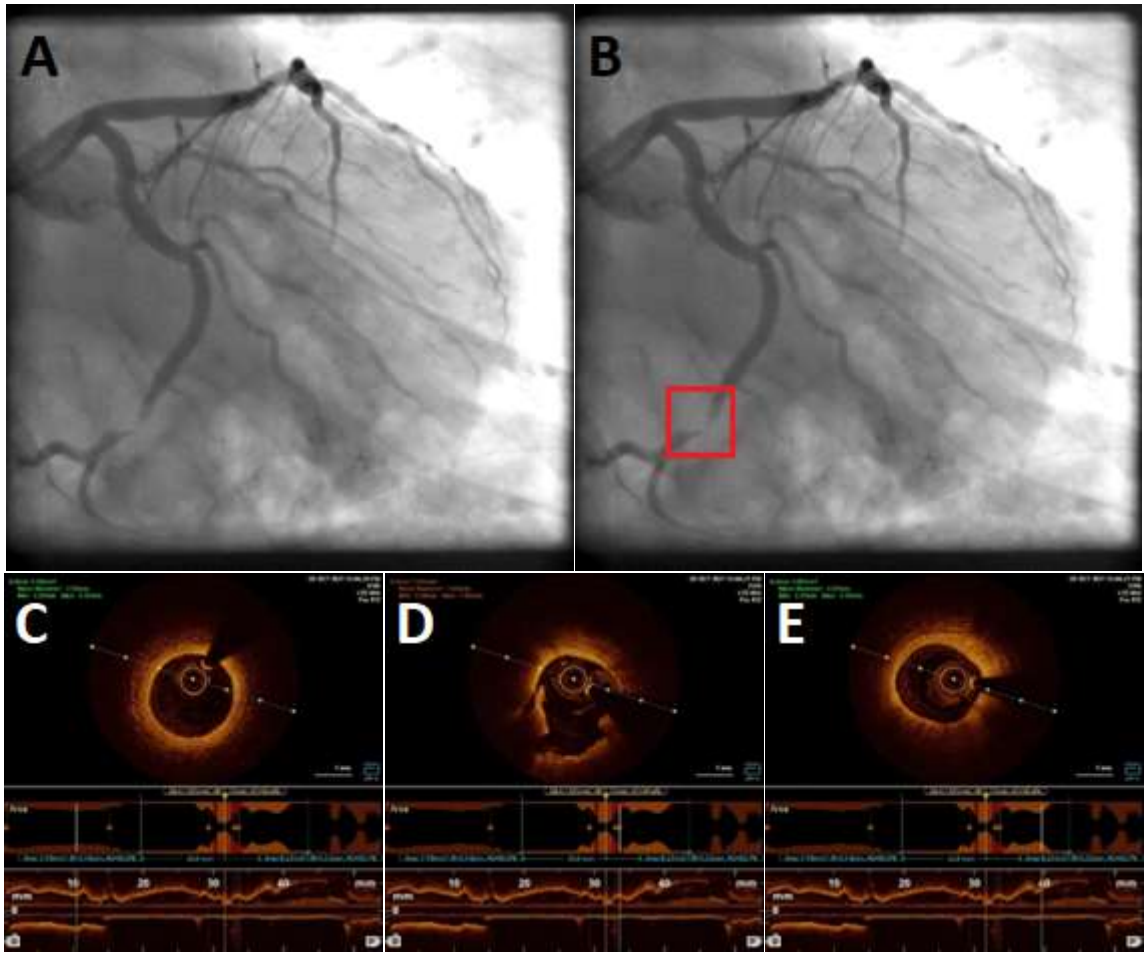

Figure 35 - N031 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 35 shows a culprit lesion within the distal AV circumflex artery; OCT analysis shows a red thrombus adherent to a disrupted fibrous cap with a cavity where a necrotic core would have been – characterised as ruptured fibrous plaque (plaque rupture).

3.2.15 N032 Angiogram and OCT images

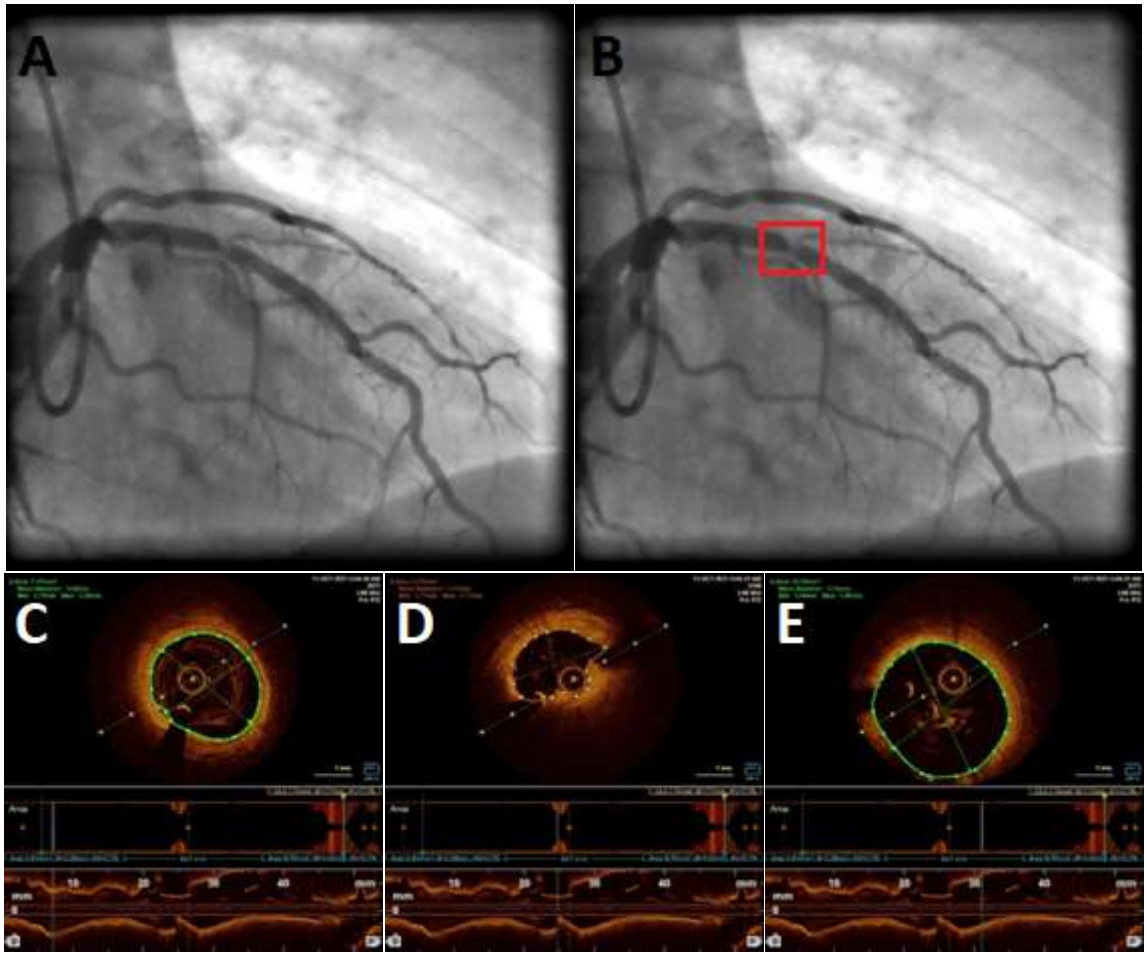

Figure 36 - N032 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 36 shows a culprit lesion within a mid-left circumflex coronary artery; unfortunately, it was impossible to classify this lesion due to poor blood pool washout (and the retention of the tracing tools when the images were transferred to the PACS system).

3.2.16 N033 Angiogram and OCT images

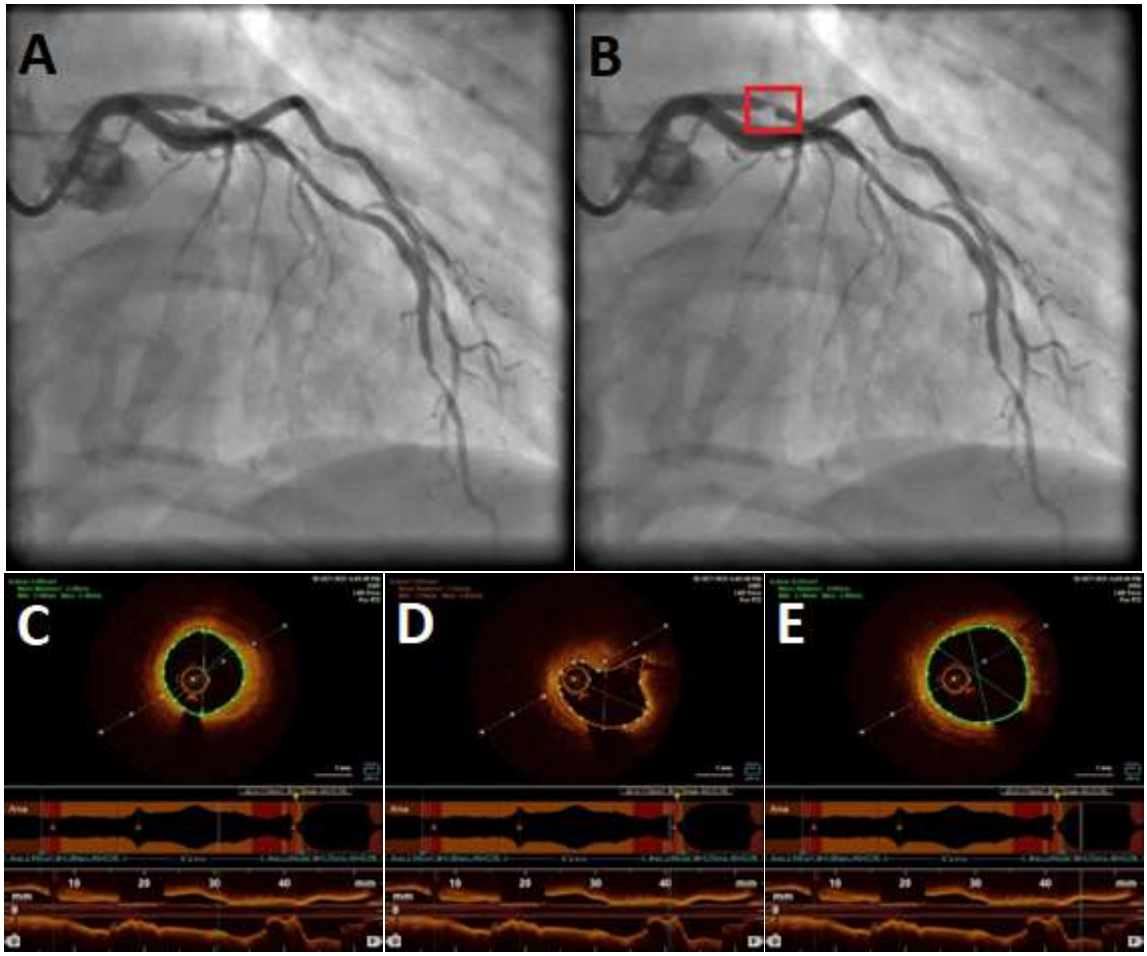

Figure 37 - N033 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 37 shows a culprit lesion within the proximal LAD artery; OCT analysis shows a red thrombus adherent to a disrupted fibrous cap with a cavity where a necrotic core would have been – characterised as ruptured fibrous plaque (plaque rupture).

3.2.17 N034 Angiogram and OCT images

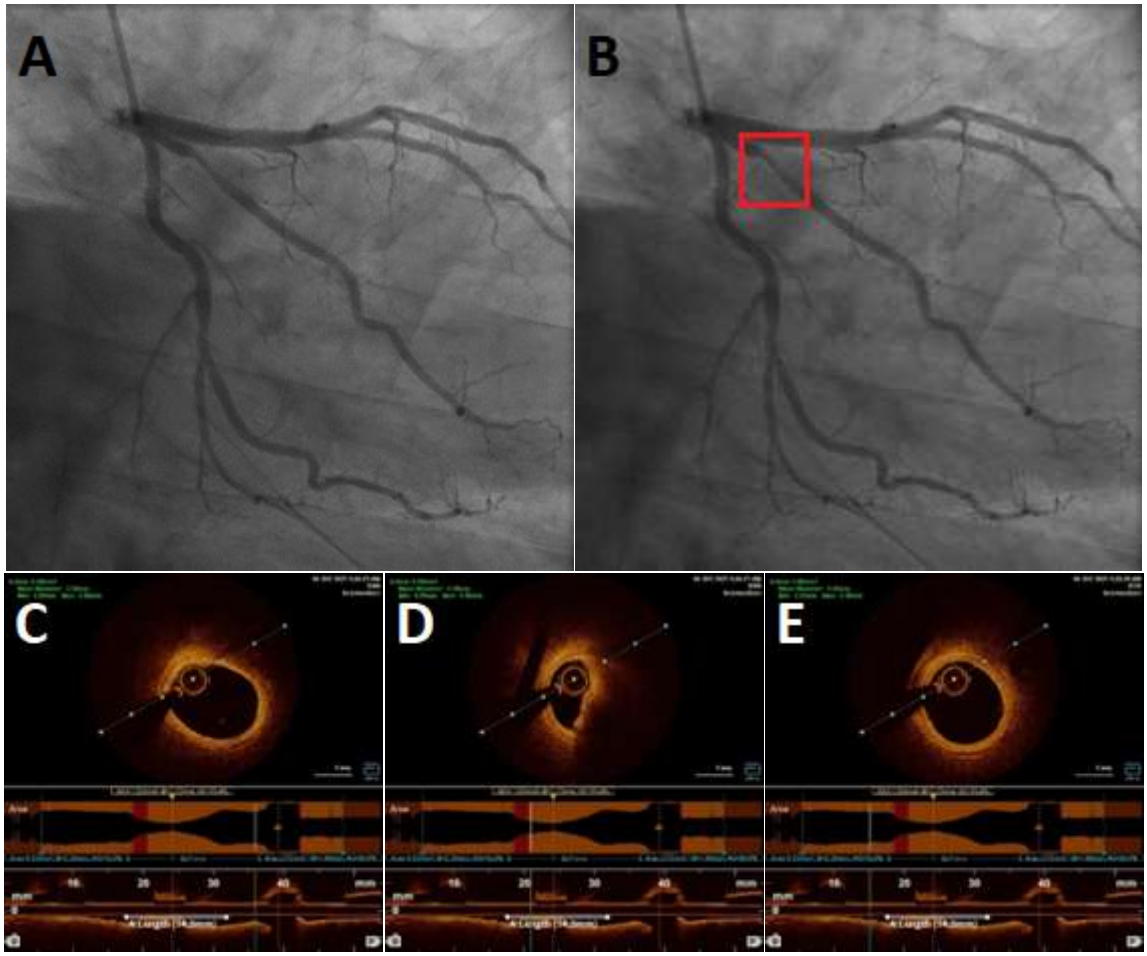

Figure 38 - N034 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 38 shows a culprit lesion within the Intermediate artery; OCT analysis shows a red thrombus adherent to a disrupted fibrous cap with a cavity where a necrotic core would have been – characterised as ruptured fibrous plaque (plaque rupture).

3.2.18 N035 Angiogram and OCT images

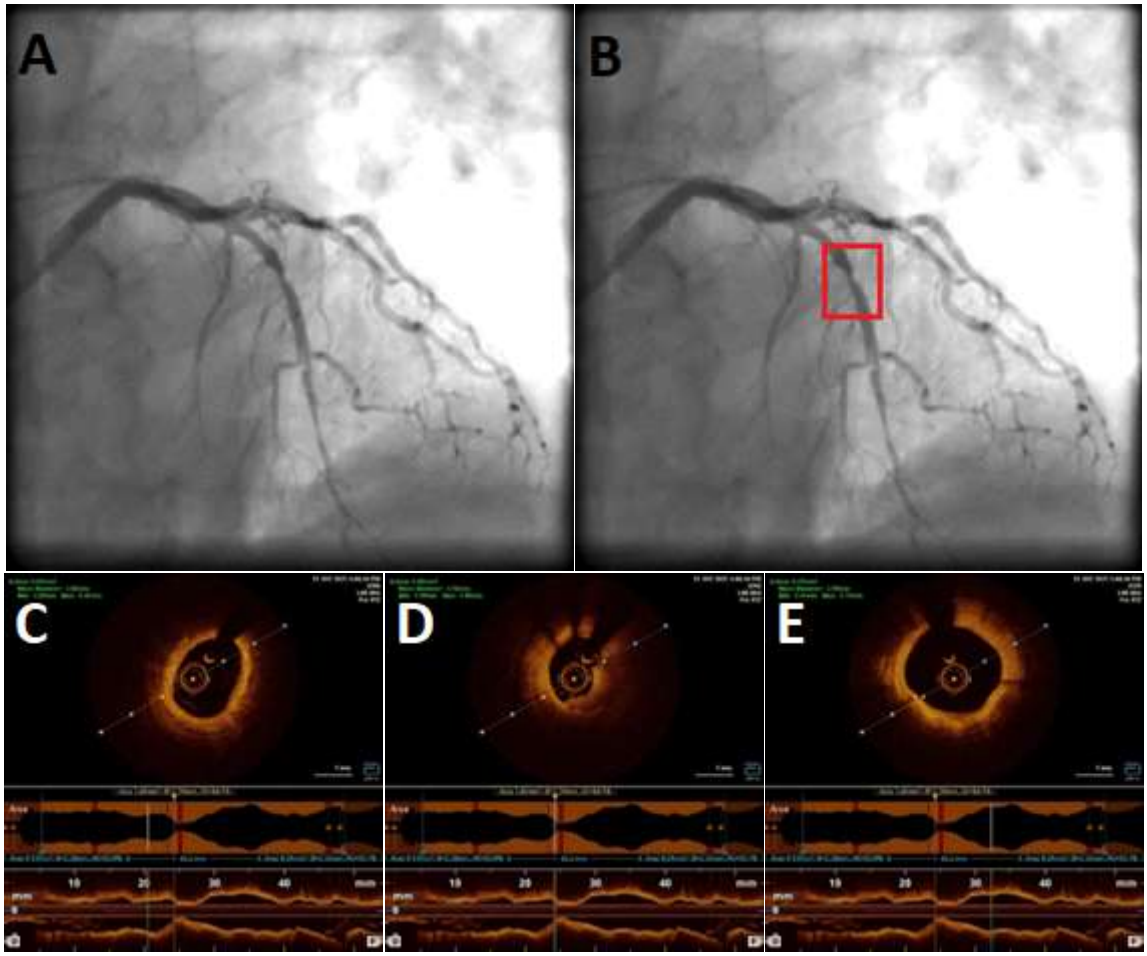

Figure 39 - N035 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 39 shows a culprit lesion within the mid-LAD. OCT images showed an irregular surface to the endothelium with adherent white thrombus – characterised as an intact fibrous cap (plaque erosion).

3.2.19 N036 Angiogram and OCT images

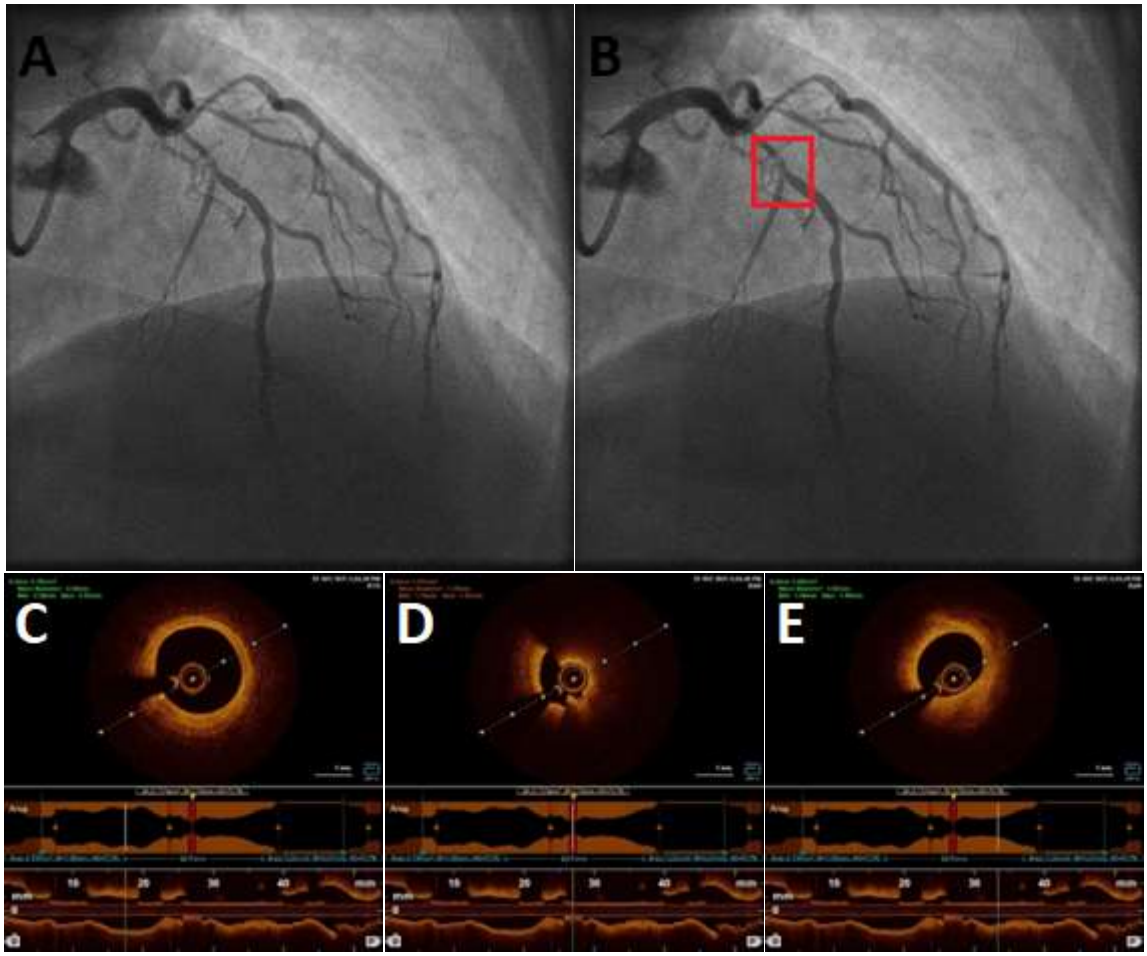

Figure 40 - N036 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 40 shows a culprit lesion within the Intermediate artery; OCT analysis shows a red thrombus adherent to a disrupted fibrous cap with a cavity where a necrotic core would have been – characterised as ruptured fibrous plaque (plaque rupture).

3.2.20 N037 Angiogram and OCT image

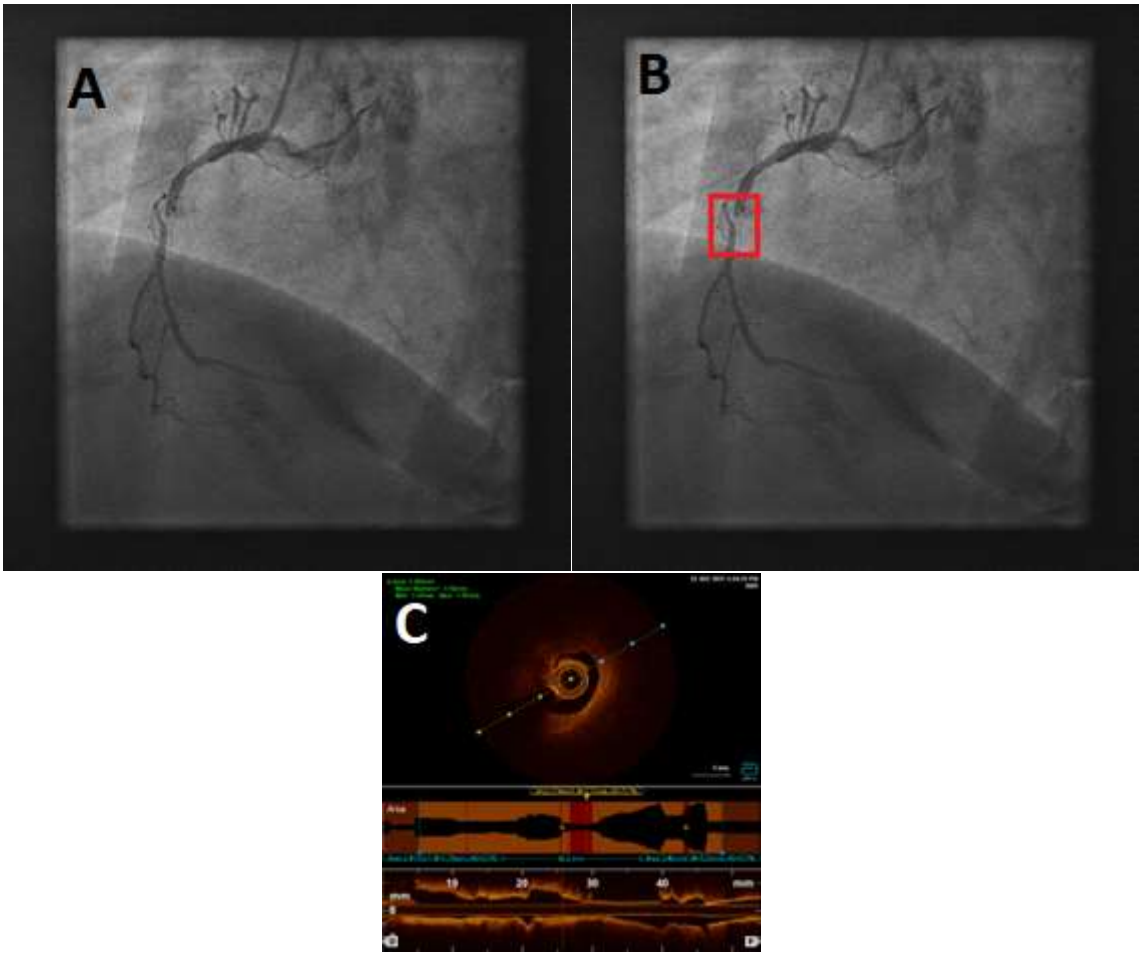

Figure 41 - N037 Angiogram and OCT image

(A) culprit artery (B) lesion of interest (C) distal vessel

Figure 41 shows a culprit plaque within a mid-right coronary artery. Unfortunately, only one frame of the OCT images had been transferred to the PACS system – classified as undefined.

3.2.21 N038 Angiogram and OCT images

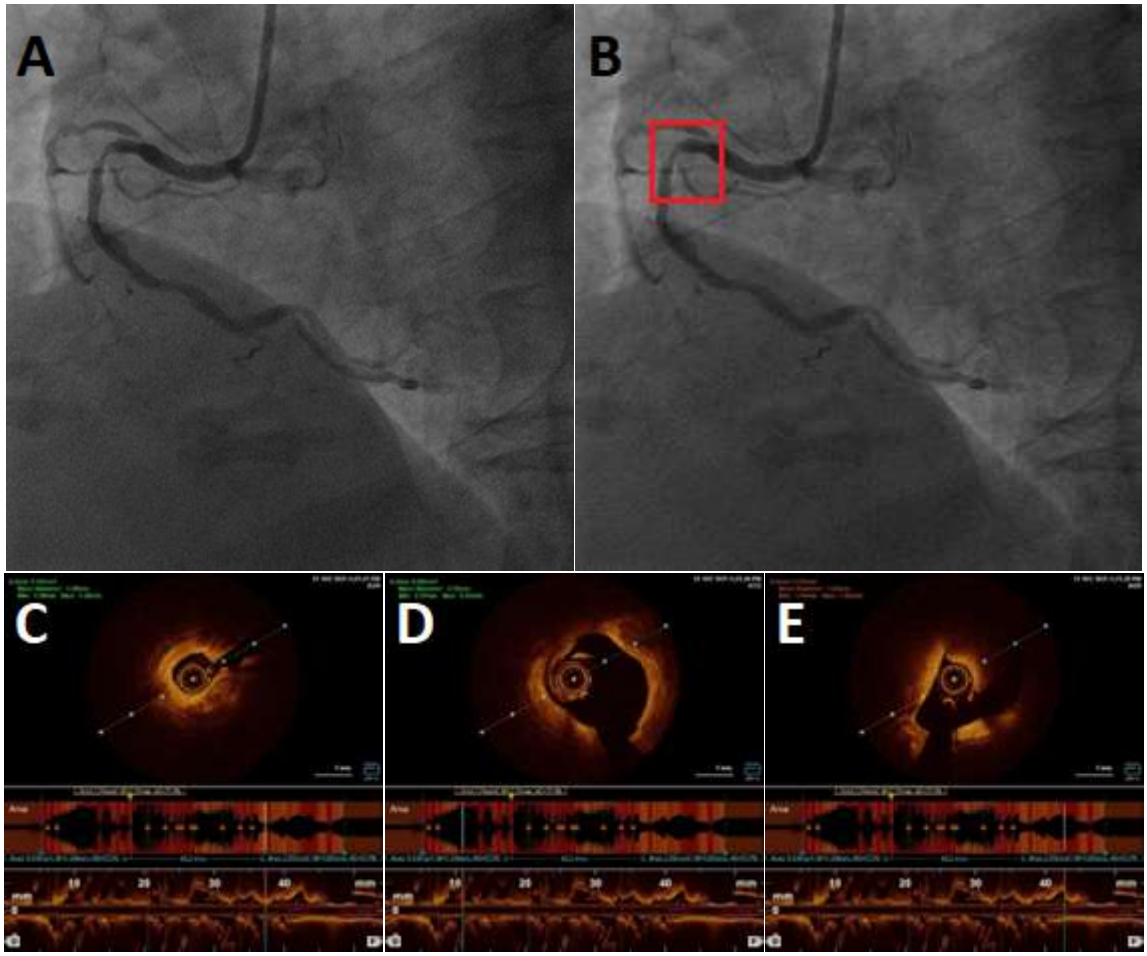

Figure 42 - N038 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 42 shows a culprit plaque within a mid-right coronary artery. Unfortunately, due to a high degree of artefact on the OCT images, it was impossible to interpret – classified as undefined

3.2.22 N039 Angiogram and OCT images

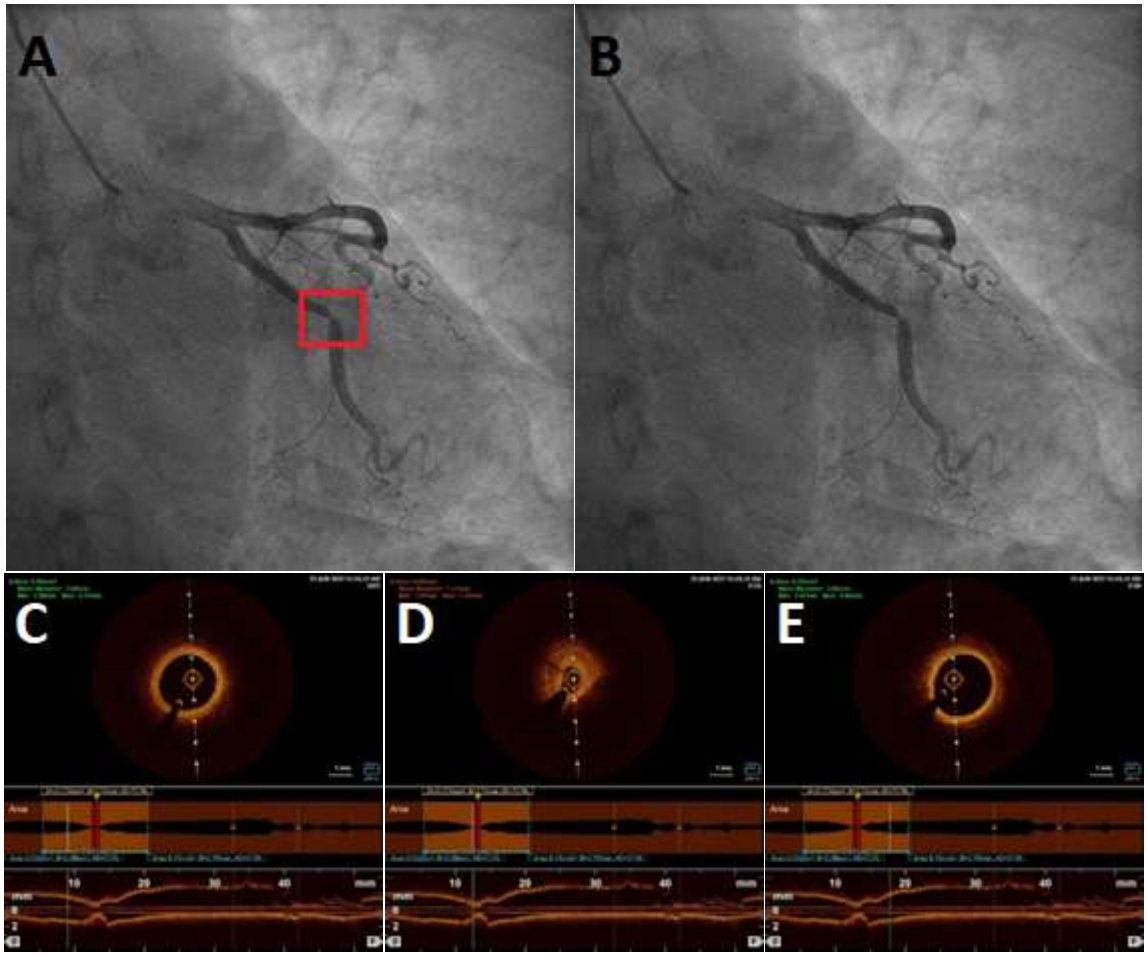

Figure 43 - N039 Angiogram and OCT images

(A) culprit artery (B) lesion of interest (C) distal vessel (D) culprit lesion (E) proximal vessel

Figure 43 shows a culprit lesion within the mid-left circumflex. OCT images showed an irregular surface to the endothelium with adherent white thrombus – characterised as an intact fibrous cap (plaque erosion).

3.3 Clinical Outcomes

One and 12-month clinical outcome data between NSTEMI and Feasibility (Table 3) and RFC/IFC (Table 4). 1-month follow-up reported no adverse events in the two groups recruited to the study. At the 12-month follow-up, one patient in the Feasibility arm had reinfarction, leading to hospital admission and subsequent target vessel revascularisation. At the 12-month follow-up, two patients in the NSTEMI arm had adverse events (both from the RFC group), with one patient being admitted with an NSTEMI (different vessel) and one patient requiring target vessel revascularisation for angina. There were no statistical differences between the two groups (even comparing the IFC to the RFC group).

	NSTEMI (22)	Feasibility (11)
One month		
MACE	0	0
Death	0	0
Cardiac Death	0	0
Non-Cardiac Death	0	0
Reinfarction	0	0
Stroke	0	0
Stent thrombosis	0	0
Target vessel revascularisation	0	0
12 months		
MACE	2	1
Death	0	0
Cardiac	0	0
Non-Cardiac	0	0
Reinfarction	1	1
Stroke	0	0
Stent thrombosis	0	0
Target vessel revascularisation	1	1

Table 5 - 1 and 12-month clinical outcome data between NSTEMI and Feasibility

**one patient in the IFC arm has only had nine months of follow-up*

	RFC (7)	IFC (8)
One month		
MACE	0	0
Death	0	0
Cardiac Death	0	0
Non-Cardiac Death	0	0
Reinfarction	0	0
Stroke	0	0
Stent thrombosis	0	0
Target vessel revascularisation	0	0
12 months		
MACE	2	0
Death	0	0
Cardiac	0	0
Non-Cardiac	0	0
Reinfarction	1	0
Stroke	0	0
Stent thrombosis	0	0
Target vessel revascularisation	1	0

Table 6 - 1 and 12-month clinical outcome data between RFC and IFC

**one patient in the IFC arm has only had nine months of follow-up*

3.4 Flow Cytometry Analysis

3.4.1 CEC Experiment

In the feasibility arm, there was a statistically significant increase in the percentage of circulating endothelial cells (CECs) 4 hours following elective angioplasty (Wilcoxon matched-pairs signed rank test P value 0.001). The mean percentage of CECs (CD31+ve, CD146+ve) out of CD45-ve peripheral blood mononuclear cells pre-angioplasty was 9.8%, with a standard deviation of 7.1%, and the mean percentage post-angioplasty was 16%, with a standard deviation of 6.9%. These results demonstrate that elective angioplasty is associated with an increase in the percentage of CECs in the peripheral blood (figure 44).

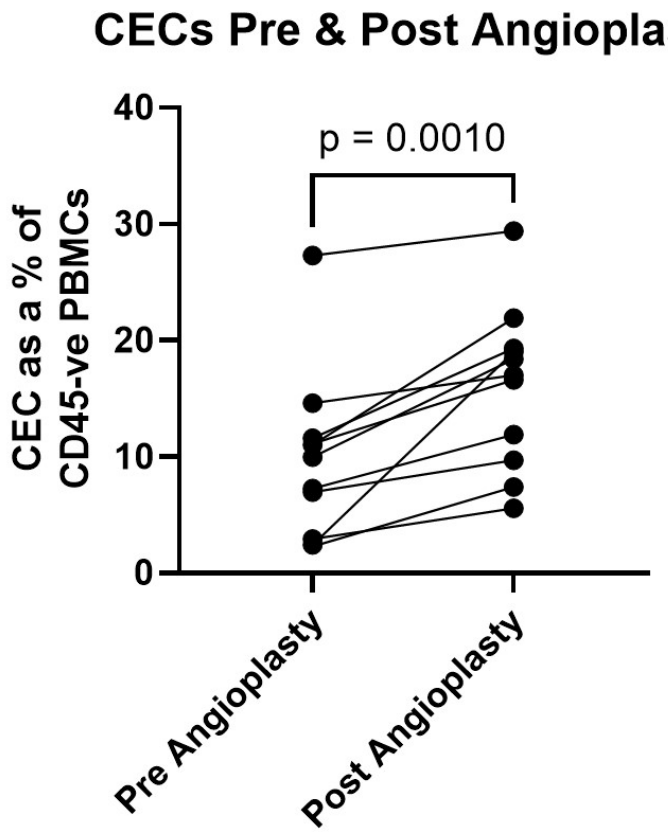

Figure 44 - Circulating Endothelial Cells (CECs) Before and After Coronary Angioplasty

The results indicate that the assay can identify circulating endothelial cells in peripheral blood, demonstrating its sensitivity by detecting changes as early as four hours. Furthermore, a positive correlation was observed; the percentage of circulating endothelial cells relative to CD45-negative peripheral blood mononuclear cells (PBMCs) was found to be significantly higher post-angioplasty (4.6 ± 2.1%) compared to pre-angioplasty (2.2 ± 0.9%) ($p < 0.01$). These findings suggest a link between coronary angioplasty and endothelial cell denudation, resulting in elevated circulating endothelial cells in the bloodstream.

PBMC = Peripheral blood mononuclear cells

Zombie staining was then assessed to detect the viability of the cells, with results expressed as a proportion of CECs positive for the dye. The mean percentage of Zombie-positive CECs pre-angioplasty was 1.2% (range 7.4), and post-angioplasty was 0.8% (range 5.1) (figure 45).

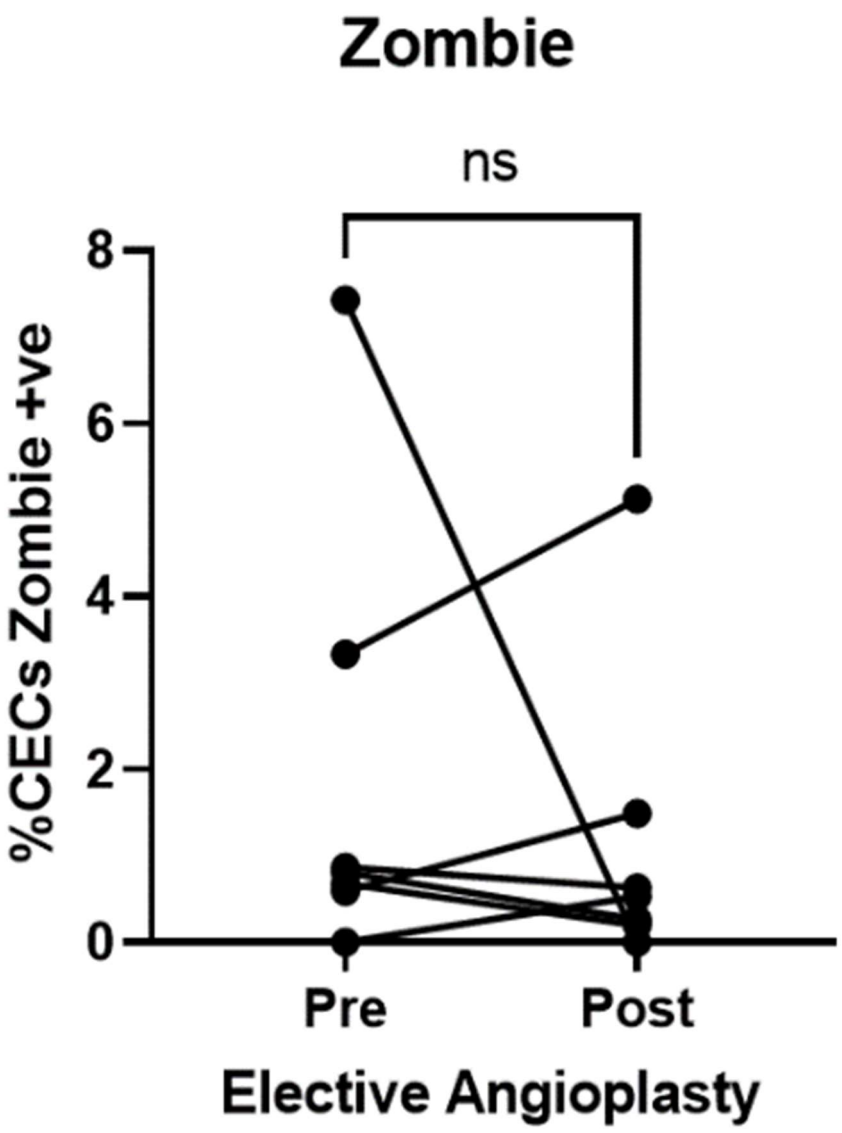

Figure 45 - Proportion of Feasibility group CECs Zombie +ve pre- and post-angioplasty

This figure shows that a tiny proportion of the isolated CECs tested positive for the Zombie stain, and there was a non-significant change pre-post elective angioplasty. Whilst this did not give us results for discussion, the experiment had been calibrated and gated using the Zombie stain within the panel. It was decided to continue to continue staining for Zombie throughout the experiment.

To determine whether the levels of circulating endothelial cells (CECs) in patients with NSTEMI were elevated compared to stable cardiovascular disease, we compared the mean percentage of CECs (CD31+ve, CD146+ve) out of peripheral blood mononuclear cells in the NSTEMI arm to that of the pre-angioplasty arm. The feasibility pre-angioplasty arm served as a control group since these patients had stable cardiovascular disease without recent acute myocardial infarction. We hypothesised that the percentage of CECs in the NSTEMI arm would be higher than in the pre-angioplasty arm, consistent with the notion that endothelial damage during NSTEMI would lead to increased levels of CECs in the peripheral blood.

The mean percentage of CECs (CD31+ve, CD146+ve) out of peripheral blood mononuclear cells in the NSTEMI arm was 13% (SD = 5.7, SEM = 1.2), with a range of 4.3% to 30.2% (n = 22). Compared to the pre-angioplasty arm, which had a mean percentage of CECs of 9.8% (SD = 7.1, SEM = 2.2) with a range of 2.3-27.3 (n=11), there was no significant difference between these two arms; a Mann-Whitney test p= 0.052 (Figure 46).

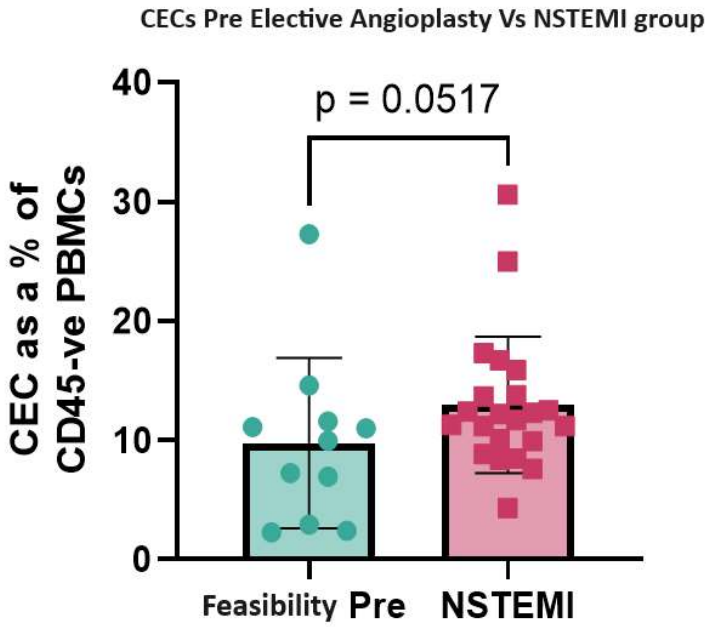

Figure 46 - CECs in MI patients compared to Feasibility

This figure illustrates the mean percentage of circulating endothelial cells (CECs), CD31+/CD146+/CD45-, within peripheral blood mononuclear cells from patients with angina and known coronary artery disease immediately prior to coronary angioplasty (blood from the feasibility group used as a control group) and the NSTEMI population immediately prior to coronary angioplasty. While both arms displayed outlier results that may introduce variability, the Mann-Whitney test revealed a p-value of 0.052, indicating a borderline significant difference between the two groups. These findings hint at CECs being a potential marker for NSTEMI events but do not reproduce positive findings seen in earlier work [51,145,146]

Next, I assessed the levels of CECs in patients with NSTEMI, categorised by the presence of either plaque rupture or erosion as determined by OCT analysis. The IFC (n=8, mean 17.4%, SD 6.8) group showed significantly higher levels of CECs compared to the RFC (n=7, mean 9%, SD 2.7) group (Mann-Whitney test p=0.012 (figure 47).

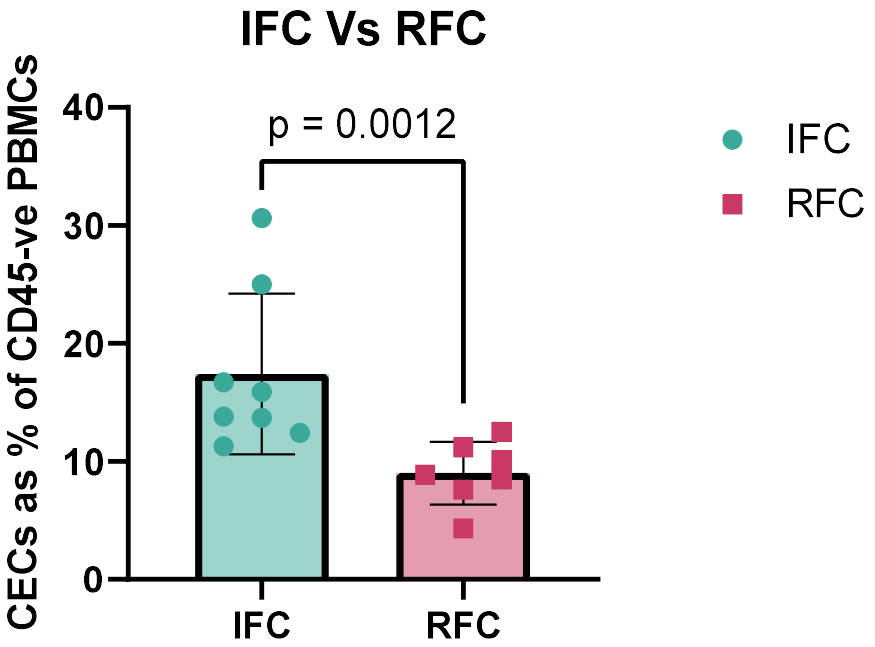

Figure 47 - CEC levels pre angioplasty NSTEMI arm stratified by IFC and RFC

This figure compares circulating endothelial cell (CEC) levels between the IFC (intact fibrous cap – plaque erosion) and RFC (ruptured fibrous cap – plaque rupture) groups within the NSTEMI arm of the study. Statistical analysis using the Mann-Whitney test revealed a significant difference between the two groups (p=0.012), with higher CEC levels observed in the IFC group

Next, we tried to ascertain the proportion of these isolated CECs or dead. Zombie staining of isolated CECs did not show any statistical differences between the IFC group (mean 2.5%, range 5.8%, n=8) compared to the RFC group (mean 4.1%, range 8.9%, n=7) Mann-Whitney test p=0.536 (figure 48).

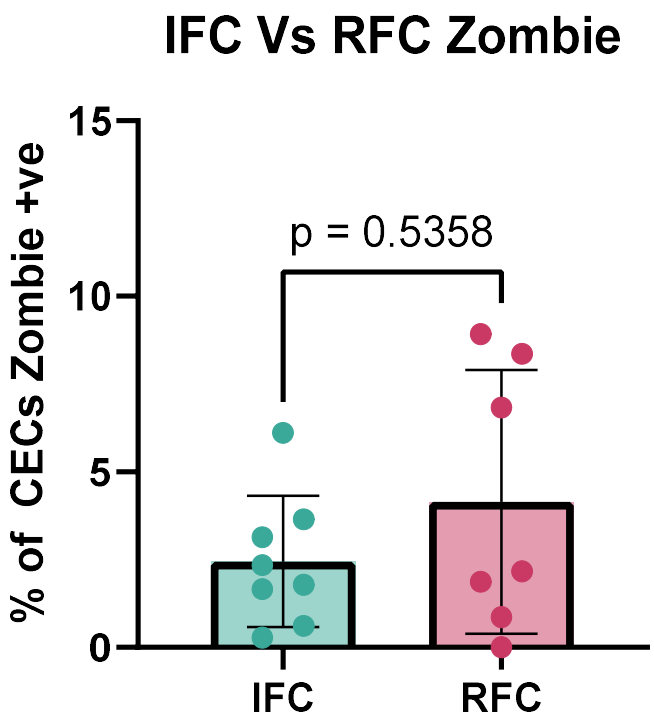

Figure 48 – Proportion of CECs dead/apoptotic between IFC and RFC groups

This figure illustrates that only a minimal fraction of the isolated circulating endothelial cells (CECs) tested positive for the Zombie stain. Additionally, a non-significant difference was observed between the RFC (ruptured fibrous cap – plaque rupture) and IFC (intact fibrous cap – plaque erosion) groups. It remains unclear whether these findings indicate the majority of these cells were viable (not dead/apoptotic) or if there was insufficient calibration of the dilution and staining protocol with the Zombie stain.

3.4.2 Platelet/Leucocyte Experiment

In the feasibility arm of the study, PBMCs were first isolated based on their forward and side scatter characteristics. This population of cells was then stained for CD41 to identify monocytes positive for platelets, and platelet-monocyte aggregates (PLAs) were quantified using flow cytometry. Two fewer patients were in the Feasibility group as we had been awaiting the CD41 antibody for delivery when the study began recruitment.

A Wilcoxon matched-pairs signed rank test showed no significant difference in PLAs between pre- and post-angioplasty samples in the Feasibility group (p = 0.164). In the pre-angioplasty group (n=9), the minimum PLA level was 1.0, the maximum was 33.5, the range was 32.5, the mean was 13.23, the standard deviation was 12.79, and the standard error of the mean was 4.3. In the post-angioplasty group (n=9), the minimum PLA level was 1.4, the maximum was 33.5, the range was 32.0, the mean was 14.7, the standard deviation was 13.9, and the standard error of the mean was 4.6(figure 49).

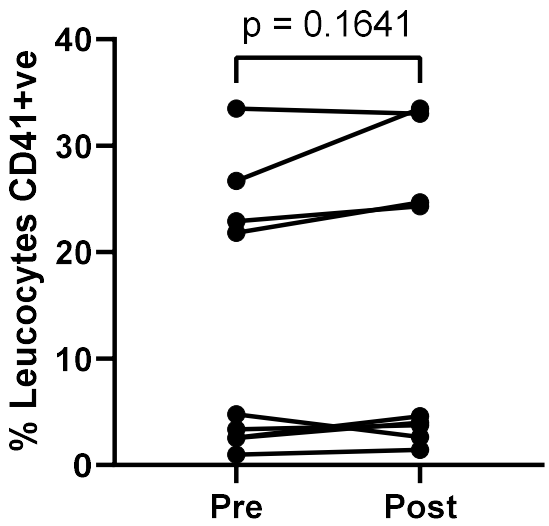

Figure 49 - Feasibility arm PLAs

This figure depicts a considerable variation in the levels of platelet/leucocyte aggregates isolated from peripheral blood taken pre-angioplasty in the feasibility group, with minimal differences observed in the matched pair taken 4 hours post-angioplasty. PLAs were measured by gating the CD45+ve population of PBMCs (Leukocytes) against the platelet marker CD41

Following on from the feasibility experiment, we analysed the levels of PLAs in the NSTEMI patient group (n=22) and compared them to the feasibility group (pre-angioplasty, n=9). A Mann-Whitney test showed a statistically significant difference in the levels of PLAs between the two groups, with a p-value of <0.0001. The mean level of PLAs in the NSTEMI group was 37.8% (SD=8.8), while the mean level in the Feasibility group was 13.2% (SD=12.8). The range of PMAs in the NSTEMI group was 32.3%,

with a minimum of 17.1% and a maximum of 49.9%. The range of PMAs in the feasibility group was 13.2%, with a minimum of 12.8% and a maximum of 33.5% (figure 50).

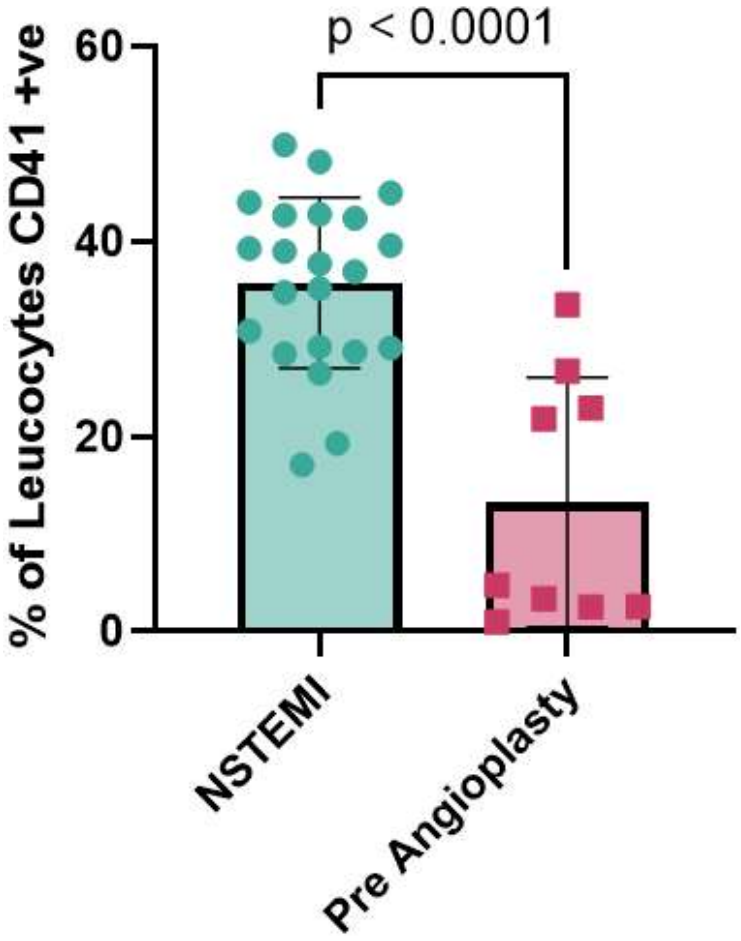

Figure 50 - NSTEMI PLAs compared to stable patients

This figure illustrates significantly elevated levels of platelet-leukocyte aggregates (PLAs) in the NSTEMI group when compared to the samples taken in the feasibility arm immediately pre-angioplasty, which served as a control (symptomatic, stable coronary artery disease patients immediately pre-coronary angioplasty)

Next, I took the levels of PLAs from the NSTEMI arm and separated them into IFC and RFC. There was no significant difference between the IFC or RFC arms (Mann-Whitney test p = 0.867).

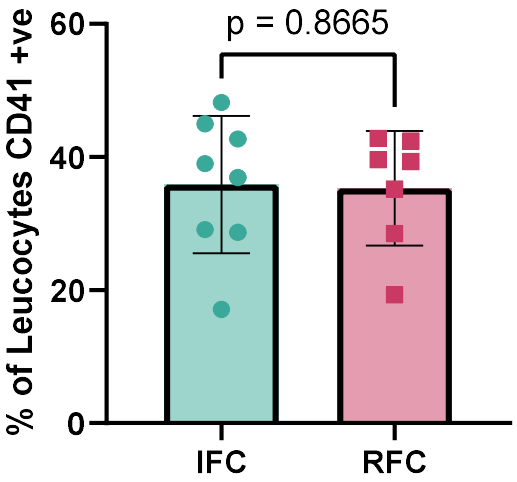

Figure 51 - NSTEMI PLAs in IFC compared to RFC

This figure demonstrates that, while significantly higher numbers of platelet-leukocyte aggregates (PLAs) were observed in the NSTEMI arm, there was no difference between the IFC (intact fibrous cap – plaque erosion) and RFC (ruptured fibrous cap – plaque rupture) populations.

4 Discussion

4.1 Principal findings

The key finding of this study was that patients admitted with NSTEMI, specifically those with an intact fibrous cap (IFC), my surrogate marker for plaque erosion, exhibited higher levels of circulating endothelial cells in peripheral venous blood compared to those with a ruptured fibrous cap (RFC), constituting the plaque rupture group. The feasibility group showed that the flow cytometry method effectively detected circulating endothelial cells in peripheral blood samples, demonstrating a significant increase four hours after elective angioplasty. Additionally, the study found increased circulating platelet/leucocyte interactions in the NSTEMI group compared to feasibility arm patients used as controls, consistent with previous research[147]. However, no differential levels were identified between IFC and RFC patients.

These NSTEMI group findings align with Quillard et al.'s (2015) hypothesis, suggesting that plaque erosions may be attributed to mass endothelial cell apoptosis and denudation from the culprit lesion site. Our primary hypothesis centres on this "two-hit" model Quillard et al. proposed, in which chronic endothelial damage first promotes endothelial cell loss through apoptotic stimuli, such as vessel wall shear stress[38] or hyaluronic acid (HA) in the extracellular matrix as the first "hit" in the cascade. The second "hit" is that the presence of neutrophils at the site of erosion amplifies the effects of TLR2 activation, leading to increased endothelial cell apoptosis and detachment, thereby making smooth muscle cell-rich plaques more susceptible to erosion[39]. We aimed to detect this mass denudation of cells with higher levels in the IFC group as a surrogate marker of this. As erosions can heal and form an overlying neointima, this will increase the shear stress at the site of this plaque; it may well be that the endpoint of a plaque erosion MI is a consequence of multiple, temporally isolated events at the site that culminate into a final athero-occlusive thrombus.

In the PEPSii study, which recruited 22 patients into the NSTEMI group and 11 into a Feasibility arm, baseline characteristics were similar between groups, and there was no increase in major adverse cardiovascular events (MACE) compared to similar published cohorts. Intracoronary optical coherence tomography (OCT) assessment revealed the presence of IFC in 37% of recruited NSTEMI patients (53% of interpretable OCTs) and RFC in 32% (47% of interpretable OCTs).

This study demonstrated increased circulating endothelial cells (CECs) in patients with stable cardiovascular disease following elective angioplasty. While higher levels of CECs were detected in the venous blood of the NSTEMI group compared to the feasibility arm (pre-angioplasty – stable angina patients with symptoms) used as a control group, this difference was not statistically significant.

However, a statistically significant difference in CEC levels was observed between the IFC and RFC groups, supporting the two-hit hypothesis of plaque erosion.

4.1.1 Recruitment

Recruitment for this study was challenging from several viewpoints, and I did not manage to make our target sample size of 50. Firstly, the COVID-19 pandemic meant that the NHS closed recruitment to all non-COVID projects, and the REC meetings were suspended through the first wave; I returned to clinical practice for these first four months whilst there was a pause. The time between the first and second waves was used to clear the project through the REC approval process. Again, the second wave of the pandemic resulted in a pause in potential recruitment, and I returned to clinical practice whilst I could not recruit patients. Once up and running, I was limited in the use of the laboratory time at the University (due to social distancing requirements, hoods were reduced and had to be pre-booked – this resulted in many patients being ineligible for recruitment as there was no capacity to process the blood samples). Once I was recruiting, I completed around two patients a week (with weekend lists most successful as the hoods at the university were not being used). The NHS flow cytometer had six weeks of prolonged maintenance (24/6/2021-13/8/2021); whilst I had fixed cells, some concerns holding fixed cells in formalin for such a long time would affect sample quality, so recruitment was again paused. Finally (as described in 4.3), the total loss of OCT data took the OCT machine out of action for a month (January 2021) whilst I attempted a data recovery solution.

4.1.2 Baseline characteristics

In our study, the incidence of IFC was 37%, while RFC was observed in 32% - this was likely skewed as 37% of our OCTs were uninterpretable due to data loss or poor blood pool washout when the operator performed the OCTs. Of the interpretable OCTs, the incidence of IFC was 54%, while RFC was observed in 46%. This is a higher incidence of erosion than other MI published series that have suggested erosion rates 20-36%[37,148.] However, a recent series suggested erosion rates may be higher (44%) than previously published[149] as described in the introduction.

I found no differences in sex between the two groups, although studies have previously suggested that plaque erosion occurs more frequently in women than men. However, our sample size is too small to draw any reliable conclusions. Similarly, I saw a non-statistically significant increase in current smoking status in the IFC group compared to RFC which had previously been noted in other series[7].

4.1.3 OCT and Angiographic Characteristics

There were no significant differences between the RFC and IFC groups in angiographic and procedural characteristics. In almost every case, the culprit artery was treated with PCI, with only one case in the IFC group that was managed conservatively. The LAD was the most treated artery in both groups.

I found no statistical difference in stent size or length between the two groups. Whilst our sample size is small, the operator was not blind to the OCT findings, and there was potentially less stent use in the IFC group than in the RFC group (1 case). This patient had an akinetic anterior wall, and the operator felt there would be no benefit to percutaneous intervention. Interestingly, at follow-up (one year), he remained well on optimum medical therapy with a normalisation of his left ventricular function and was angina-free. Whilst not a pre-determined part of the test group, this case would be consistent with the findings from the EROSION study, which suggested that for OCT-verified IFC, non-obstructive lesions might be managed without stenting. The EROSION study differs from our cohort as they recruited all acute coronary syndrome patients (not just NSTEMI), and any patients with an OCT-defined plaque erosion (IFC) and residual stenosis of <70% were treated with anti-thrombotic therapy without stenting and followed-up with OCT at one month. Four hundred five patients with analysable OCT images were screened, 103 plaque erosions were identified, and 60 patients were recruited to the study, with 55 completing the 1-month follow-up. Most (47 patients) cases had at least a 50% reduction in the thrombus burden, improving the minimal flow area within the artery. Twenty-two patients showed complete thrombus resolution after thrombus aspiration during angiography and peri-procedural Glycoprotein IIb/IIIa inhibitors. There was one death because of gastrointestinal bleeding, and one patient underwent coronary intervention to the culprit in the absence of symptoms[11]. Forty-nine patients who completed the study were followed up at one year with repeated OCT assessments, demonstrating a further decrease in the thrombus burden. However, even at one year, 27 patients appeared to have chronic, predominantly white thrombus burden[12]. Like our own, this work is limited by the resolution of OCT, and it is not always possible to see the neointimal layer forming over these thrombus plugs. Chronic laminated thrombus from this site, in keeping with the two-hit model, could increase shear stress by altering the flow dynamics of the coronary artery at this level, encouraging further apoptotic insults to the endothelial layer. Like our patient, their cohort had a year of dual antiplatelet therapy and remained symptom-free [12].

While a much smaller cohort, Souteyrand et al. screened ACS patients suitable for medical management of myocardial infarction for a prospective study of culprit healing using serial OCT assessments. They recruited ten patients who met their inclusion criteria: onset of symptoms <12 hours, achievement of “optimal reperfusion” with a Thrombolysis In Myocardial Infarction (TIMI) 3

flow with non-critical residual angiographic narrowing, and a small plaque/thrombus prolapse on OCT, after thrombus aspiration/thrombolysis. These patients had already had their coronary angiogram with the decision for medical therapy, and baseline OCT assessments were taken after 5.2±2.6 days with an RFC culprit lesion in five patients and an IFC culprit in the remaining five patients, with OCT aspects of thrombosis being revealed in all cases. Follow-up OCT assessments of these plaques varied but appeared to show continued communication with the core of the RFC lesions, with neointimal formation closing the communication in those follow-up studies taken later (4-6 months). The IFC lesions showed that the irregular white thrombus had become smoother with a two-layer appearance and a rim of circumferential transition[73], suggesting the incorporation of thrombus behind endothelial cells forming a neointima as seen in the histological work and classification schedule presented by Virmani[3].

A four-year follow-up of the EROSION study cohort was undertaken to try and ascertain the prognosis of such a conservative strategy and to see if there was a determinant of any requirement for target lesion revascularisation. They completed follow-ups in 52 patients (55 in the original EROSION study) and split them into two categories: those with target lesion revascularisation (the TLR group) and the non-TLR group. The 11 patients who had required revascularisation at the site of their index culprit tended to be those that had a lesser reduction in the thrombus volume with less of an improvement in the diameter stenosis or resolution of thrombus at the initial 1-month follow-up than the non-TLR group[126]. Potent glycoprotein inhibitor use at the index procedure was the strongest determinant of thrombus resolution[11]. PEPSii supports the endothelial apoptosis model of plaque erosion, with the neo-endothelialisation seen by Virmani [3] a crucial part in the healing of the plaque; in cases where the thrombus does not resolve, there is a higher risk of further events driving TLR.

It is worth noting that drug-coated balloons were used to treat the culprit plaque in 3 patients in the feasibility group and 2 in the NSTEMI group (not categorised into RFC or IFC). This is primarily attributable to the interventionist's strategy to avoid the potential early and late sequelae of stent deployment.

4.1.4 Clinical outcomes

One and 12 (Table 3,4) month outcome data reports only one reinfarction and one target lesion revascularisation (both 14%) occurring during follow-up in the NSTEMI cohort (both in the RFC group). There were no statistical differences in clinical outcomes between the two groups (Feasibility Vs NSTEMI nor RFC Vs IFC). Published data on target lesion revascularisation rates [150] show a 10-year risk of around 20%, most of which occurs within the first year [13], and the rate of recurrent MI in a non-culprit vessel from registry data is around 10%[151]. Although the small sample size in our study has the

potential to introduce bias in measuring event rates related to MACCE, our findings remain comparable to published data, indicating that our study provides valuable insights into this area despite its limitations.

4.1.5 OCT data

OCT analysis revealed residual thrombus in most (88%) of ACS cases, with no significant differences between the two groups. Predominantly white thrombus was observed in the IFC group and red thrombus in the RFC group. Due to the poor resolution of the DICOM compared to native OCT files, I could not comment on any small amounts of red or white thrombus mixed in with the main thrombus. However, our blinded analysis by separate operators showed consensus. This is in keeping with published work from Niccoli et al.[97], who demonstrated a difference in the presence of white thrombus (96.6% vs 29.2%) and red thrombus (3.4% vs 70.8%) between IFC and RFC ($p < 0.001$).

Virmani et al. have shown that eroded plaques do not have a large lipid core but a proteoglycan-rich matrix and that the prevalence of inflammation is lower than that in ruptured plaques[3]. Other autopsy case series have demonstrated that thrombus overlying a plaque erosion are more likely to be late stage with layers of smooth muscle cells, proteoglycan deposition and endothelial infiltration surrounded by acute fibrin and platelets compared to plaque rupture thrombi which superimposed a necrotic core and were composed of alternating layers of platelets mixed with fibrin and intact neutrophils[152]. The REC did not feel that three vessel OCT was a safe enough addition to this work. However, other groups have been able to demonstrate both culprit and non-culprit lesions showing a layered effect to suggest prior healing at a site of thrombosis associated with endothelial discontinuity; perhaps this is further evidence for the two hit hypothesis where there needs to be a critical mass of damage to the endothelial layer to result in a propagating thrombus[153]. This may explain the baseline levels of CECs seen in stable patients in the feasibility arm, though without confirmatory intracoronary imaging, it would be difficult to investigate this conjecture.

Unfortunately, with the loss of the raw OCT data, I could not export the files to an offline review station and obtain data on the proximal/distal lumen areas and the degree of luminal stenosis at the culprit plaque. Similarly, the DICOM files used to categorise into IFC and RFC were of a significantly lower resolution than native OCT cart/ORW, so I was not able to comment on all of the lesion characteristics, including fibrous cap thickness, lipid, cholesterol crystals, thrombus, dissections, macrophage accumulations, calcium, and intimal neo-vasculature mentioned in consensus standards from the International Working group[9].

While the work of Prati[125] and Jia[11] are good exploratory studies into a more personalised approach to plaque erosion, they were not powered for MACCE endpoints and have not yet made guideline

recommendations. This study was not designed to test options for personalised medicine; the single medically managed IFC patient responded well to optimum medical therapy and remains symptom-free at 1-year follow-up in keeping with their propositions.

4.1.6 Flow cytometry

4.1.6.1 CEC experiment

Circulating endothelial cells have previously been used as markers of endothelial damage[50–54,56–60]. Our study has used the flow cytometry method (only previously used to compare CEC levels in control patients to AMI patents[51,145,146]) to detect vascular injury to the coronary endothelium following percutaneous coronary angioplasty. I demonstrated a significant increase between the levels in the plaque erosion (IFC group) compared to the plaque rupture (RFC) group, even though the sample size was smaller than planned. This was done using an established flow cytometry method to isolate CECs in venous blood[51], and the proportion was compared as a percentage of CD45-ve peripheral blood mononuclear cells. Whilst the measurement of CECs has previously been shown to be a diagnostic biomarker of acute myocardial infarction [51,61–64], this method is both effort and time-consuming. It would make a poor discriminatory test between IFC and RFC in clinical practice.

As CECs are already known to increase as a result of endothelial damage, particularly post-myocardial infarction [47,49,50,52–54,56–60,145,146,154], and whilst they have been considered as a marker of acute myocardial infarction, a differential to the mechanism of injury has not previously been considered. Our data adds critical *in vivo* evidence to the proposed mechanism of plaque erosion by demonstrating an increased proportion of CECs measurable in blood compared to plaque rupture. This suggests that an active process at the endothelial layer results in this greater denudation of cells into the bloodstream than the traumatic denudation of cells when a thin-capped atheroma bursts. There will likely be an edge of endothelial cells surrounding a ruptured plaque, leading to aggressive activation of the clotting cascade from the necrotic core, resulting in the initial hit on endothelial cells. This then stimulates them to activate platelets to seal the breach, and the inflammatory reaction from atherothrombosis could be the second hit to form a penumbra of activated/apoptotic endothelial cells supporting atherothrombosis.

Unfortunately, I could not use the Annexin stain marker for apoptosis because this stain does not work with fixed cells, and our surrogate maker could only determine the cell's vitality (alive/dead). This fluorescent conjugated marker was poorly taken up by the cells isolated by my flow cytometry method, which is not what I would have expected considering multiple other studies either finding apoptotic CECs or endothelial microparticles as a result of CEC breakdown using an Annexin stain[61–64].

4.1.6.2 Platelet/leucocyte experiment

The PLA experiment did not produce differential results between IFC and RFC but is in line with previous work demonstrating utility as a biomarker following a recent myocardial infarction[147]. Whilst I was confident that I had isolated the PLAs from flow cytometry, there were widely variable levels in the proportion of these positive for platelets pre-angioplasty, with some of the samples post-angioplasty falling and some elevating. Whilst these patients had been loaded with potent antiplatelet therapy (in the form of Clopidogrel) pre-procedure, aspirin therapy has not previously been shown to lower levels of circulating PLAs in healthy controls[155]. The average levels for the PLAs in the feasibility arm pre-angioplasty were lower compared to the levels seen in the NSTEMI arm, though with the widely divergent results in the feasibility arm (where bloodletting was temporally controlled – unlike the NSTEMI arm where bloodletting was pre-angiography but could have been up to 48 hours from the NSTEMI event) make me question the validity of these results. This may have been due to our limited access to the flow cytometer and the need to fix our samples in 4% formaldehyde; on occasions, these samples could wait 2-3 weeks to access the machine. Previous work has shown that the fixation process can dramatically alter the results of PLA experiments[156] and that the fixation process does not necessarily prevent their formation over time[157].

4.2 Feasibility of the study

An important objective was to demonstrate the utility and feasibility of our methodology. I chose an elective cohort as a feasibility group to validate our method to identify CECs against previous work [57] before applying the methodology to our NSTEMI cohort using an OCT assessment method used previously at our centre[37]. I targeted patients presenting with short ischaemic times to reflect the inflammatory process around the onset of the myocardial infarction to minimise the impact of secondary inflammatory changes.

I was unable to recruit our target of 50 NSTEMI patients to the study due to a combination of factors, namely the impact of COVID delaying the ability to recruit at the NHS centre and limiting the available bench time in the UEA labs, equipment failures (the NHS flow cytometer was toward the end of its natural life needing prolonged periods of maintenance) and the total loss of OCT data from our imaging cart caused us to pause recruitment whilst I endeavoured to achieve a data recovery solution.

I successfully recruited 22 patients into the study without complications from performing OCT (or delay in treatment). I did not expect any major complications as, from the literature, OCT assessment is associated with a very low adverse event rate (0.16%)[158]. The REC panel was concerned about device-related coronary dissection risks, so it mandated only culprit vessel assessment (rather than the initially proposed three-vessel assessment to look for bystander plaque morphologies). One

series[158] had over a thousand OCT patients without a dissection[158], and another series suggested a dissection rate of 0.2% (although this occurred in a patient who used an older method of OCT acquisition where a low-pressure, short over-the-wire perfusion balloon occludes the artery and coronary flow is replaced by ringers lactate before acquisition)[72].

As a result of the patients being included in the PEPSii project, OCT was frequently used as a further adjunct for PCI to help size the vessel and, therefore, appropriately select the required balloon or stent with the utility of being able to use the OCT catheter after deployment of the treatment to assess the immediate result and allow for clinical optimisation. Intra-coronary imaging guided PCI (in the form of intravascular ultrasound) has been shown to reduce the risk of target vessel failure (occlusion, restenosis, or requirement for vessel revascularisation) for complex interventions with results now out to 3 years post-intervention[159] and hopefully the ILUMIEN IV: OPTIMAL PCI study currently active having completed recruitment will be able demonstrate this utility for all comer lesions requiring intervention later this year.

4.2.1 Study Limitations

The most obvious limitations of our study are the small sample size and the single-centre design; more extensive studies would be required to validate our findings.

In conducting this study, I deliberately chose to utilise peripheral blood sampling as the analysis method exclusively. This decision was rooted in several considerations, primarily assessing Circulating Endothelial Cells (CECs) detectability. The feasibility arm of our study successfully demonstrated the practicality of this approach, showcasing its non-invasive nature and the ability to discern alterations in CECs. Peripheral sampling prioritised patient comfort and minimising potential risks associated with more intrusive procedures. However, the selection of peripheral blood sampling is not without its limitations. This systemic approach may pose challenges in precisely localising changes related to specific coronary lesions or areas of interest. In contrast, localised sampling methods, such as those conducted in the coronary sinus or up/downstream of plaque using an aspiration catheter, offer a more targeted assessment. The systemic nature of peripheral blood sampling may inadvertently overlook localised changes, particularly in the context of NSTEMI, a condition known for its potential to have pan-coronary inflammation[160]. The predecessor study to this project compared peripheral blood samples to those taken from the coronary artery using a thrombus aspiration catheter and found a high concordance of cytokines between coronary and peripheral samples[10].

Plaque erosion is essentially a histological diagnosis, and although I classified our OCT findings into different morphologies, there was no pathological validation. True pathological validation is impossible because studying the samples post-mortem of patients who had died from myocardial

infarction is fundamentally very different from classifying *in vivo* those who have survived and are having definitive treatment for this. The absence of endothelial cells is a crucial pathological criterion for plaque erosion, but OCT cannot detect endothelial cell loss. The OCT definition of plaque erosion is based primarily on a diagnosis of exclusion requiring the absence of a fibrous cap rupture. I have used an *intact fibrous cap* (IFC) to reflect this.

Our study cohort did not represent the entire NSTEMI population. I had specific exclusion criteria to ensure I was only recruiting patients on whom it was safe to perform OCT while only slightly delaying the procedure time. Therefore, I could not include every NSTEMI patient; given the nature of the study, this was the only feasible, safe approach I could use. For example, I excluded patients with poor renal function, primarily for safety reasons, as I did not want to increase their risk of contrast-induced nephropathy from the contrast dose of the procedure, something with which the REC had concerns, and I strengthened our exclusion criteria in response to this.

I had to exclude patients for whom I was unable to perform OCT, leading to a reduction in recruitment adequately, in addition to the patients I was unable to classify due to data loss, poor (OCT technique) blood washout or excess residual thrombus, accounting for 36% of our recruited (OCT suitable) NSTEMI population. This meant I eliminated patients from our subsequent analysis that could have made our findings more generalisable; it is a recognised problem in other OCT studies with a similar loss of categorisation due to undefinable OCTs[161,162]. I used a blinded method to categorise the NSTEMI OCT cohort into IFC or RFC, and I feel that this was reliable; disagreements between the blinded reviews did not classify a single patient into the opposite cohort, and with the assistance of a third, blinded, external reviewer the unblinding lead to consensus for all lesions. In the future, using the OCT offline review workstation would make this process much more accurate as the resolution of the raw OCT data is much higher than the exported DICOM files.

COVID-19 and the OCT data loss were not our only issues with this project. I had limited access to a (clinical) flow cytometer to run our experiments towards the end of its working life, which required multiple periods of downtime and recalibration. Several times, I ran aliquots of the samples and found the returned flow data spurious (non-sensical) or the cytometer failed to give any results, so we had to save the fixed samples for longer than I had hoped whilst the cytometer was repaired and recalibrated; the impact of this on the viability of our results is unknown.

4.2.1.1 OCT data loss

OCT data for the PEPSii project was stored both in the raw form on the stand-alone cart and as DICOM (Digital Imaging and Communications in Medicine Standard) transfers (as video files) to the PACS (picture archiving and communication system). Transfers to the DICOM system are manual and

depend on the console operator selecting the files and uploading them with or without overlayed lumen tools. I performed most transfers from the OCT cart (including for non-study patients); several of the transfers were performed by clinical staff when I was either not around or immediately after the patient's coronary intervention (each blood sample took about 2 hours to process to reach the fixed state ready for flow cytometry). When this study was proposed (before the COVID-19 pandemic), I had access to a St Jude OCT offline review workstation, which was placed into the archives whilst there were significant transformations of clinical workspaces. The intention had been to transfer the images to this workstation as the backup and to review the studies post anonymisation (with separate, blinded copies sent to collaborators who are published experts in the intracoronary imaging field - Patrick Calvert and Tom Johnson - for review to ensure the robustness of the OCT review). As I was unable to do this, I left the images stored on the OCT cart (which had been in use for several years, with many hundreds of raw OCT files used for teaching and more than 80% left on the hard drive) to download the data en-bloc after finding a space for the offline review workstation after the pandemic. Unfortunately, one afternoon, when I was covering clinical duties, a non-study team member had difficulty uploading a non-study OCT to the DICOM system (as the PACS server allocated to storing such images was full). Seeing the "out of memory" error message on the OCT cart, they proceeded to format the hard drive on the machine, believing that all other files had already been transferred to DICOM and were, therefore, effectively backed up. This was apparent the next day when I was working with the OCT cart, and use of this machine was immediately suspended whilst I reviewed our options; Abbott (who had taken over as the vendor for the machine) reported that once formatted, this data was not able to be retrieved by them but were able to remove the hard drive from the system for us safely. I sent the hard drive to DataTrack (a specialist data recovery company that had previously worked with the NHS); as the Abbott security software is encrypted, they could not perform conventional data recovery and quoted a forensic solution with no guarantee of success (costing way over our budget at £24480), I had already lost the OCT cart for one month (losing potential recruits) so decided to cut our losses and reassembled the cart to finish recruitment. Interrogation of the DICOM data showed that 2/22 uploaded OCT files had not transferred properly (with only single frames from the whole run on file). The video files from the PACS system were downloaded into anonymised .avi files and shared with collaborators for blinded offline reporting. On reflection, it had not occurred to us that this data could be lost in this way (the cart gives several prompts advising against wiping stored data), and I should have individually burnt DVDs (straight from the cart) after each case as a hard backup. I should have been more proactive in downloading the raw files as I lost several cases and a month of potential patient recruits (during the study, despite limiting confounders, I completed recruitment at a rate of around 2/week).

4.3 Considerations for the NSTEMI Population

While the most significant limiting factor for patient enrolment was logistical, with 404 patients deemed ineligible for inclusion due to not meeting the inclusion criteria or the unavailability of a lab space to process blood samples, there was still a significant attrition rate of screen failures (65%). In these cases, the operator felt that lesions were unsuitable for OCT assessment, primarily due to the severity of stenosis and the complexity of the lesions.

This marked a notable difference from the predecessor study, PEPS[10], which successfully performed OCT on 90% of recruited patients. PEPS[10] dealt with a different population presenting occlusive thrombus from a STEMI, allowing for thrombectomy and balloon dilatation to facilitate OCT passage—an approach I intentionally avoided in this project to preserve the underlying architecture of the plaque.

Practically, conducting OCT before any coronary intervention might not make sense clinically. However, in cases where culprit lesions are unclear, but there is compelling evidence for an event in a coronary territory, this approach could be sensible to make a formal clinical diagnosis in MINOCA (myocardial infarction with nonobstructive coronary arteries) cases as suggested in other literature[163].

In other cases, when there is a need for balloon dilatation to facilitate the passage of the imaging catheter, it can be employed when the results aid in coronary intervention, such as assessing calcium and reference luminal areas.

While reliable and reproducible in isolating Circulating Endothelial Cells (CECs) and ensuring accurate identification, the employed sampling methods face challenges in terms of clinical practicality. The process, taking approximately 2 hours to complete, is time-consuming, posing limitations on its feasibility within routine clinical practices. Additionally, the subsequent flow cytometry analysis, a critical step for further characterisation of CECs, lacks automation, further contributing to the impracticality of widespread clinical application. Although flow cytometry is clinically valuable for assessing haematological malignancies, the vast number of myocardial infarction patients surpasses the demand for such assessments. There is a call for reliable biomarkers to address the need for differentiating between erosions and ruptures, especially outside the realm of intracoronary imaging. Ideally, these biomarkers should be measurable through a single high-throughput assay, facilitating a more efficient and accessible diagnostic approach for a larger population of patients.

4.4 Future directions for research and clinical care

Despite significant hurdles, this study has generated pilot data supporting the hypothesis that CEC levels would be higher in patients presenting with an NSTEMI from plaque erosion than in a plaque

rupture. I have learnt a lot about our methodology and the practicality of running such studies with the limitations of lab space (particularly exacerbated by COVID-19 social distancing), which will contribute to our department's shared experience and future research.

Our group has previously demonstrated differential intracoronary cytokine expression in RFC and IFC and that elevated thrombospondin 1 and epidermal growth factor may play an etiological role in erosion. The method of achieving this by cytokine array, followed by ELISA, was reproducible but onerous[37]. Other technologies are using large-scale proteomics that can, from small samples of serum, create relative and absolute quantifications in larger sample sizes that are reproducible[164]. The earlier study used a thrombus extraction catheter to sample blood from the coronary artery. However, these are no longer used routinely in practice due to the risk of systemic embolisation as the clot is removed from the coronary artery[165]. Now, devices can deploy within the coronary artery and use baffles to change the flow dynamics so that blood from before, at and distal to the culprit artery can be sampled and investigated by proteomics or even by interrogation of the cellular component[166]. Before the COVID pandemic, I was working with Plaque Tech to consider using this project as part of the CE accreditation for their updated device. Unfortunately, manufacturing issues prevented it from being available for this study. Another alternative would be to mandate coronary sinus blood sampling from enrolled patients to allow an upstream (from the cardiac guide catheter) and a downstream (from the venous return of the heart) comparison of biomarkers and cellular complexes across a culprit lesion.

Considering the white thrombus prevalent in plaque erosion, it would be beneficial to look in further detail at platelet activity[167] and PLAs between erosion and rupture in future. I would propose staining for CD14 (monocytes) and CD41 (platelets) with fluorophores spaced far apart in their excitation/emission spectra to reduce the risk of contamination. The below panel shows the excitation and emission profiles of BV421 and APC; these are fluorophores which can be conjugated to anti-CD14 and anti-CD14 antibodies, which have their excitation and emission spectra far apart to reduce any risks of crosstalk contamination in the flow acquisition (figure 54). The BCRE labs now have a flow cytometer for independent use, so samples would no longer need to be fixed and could be processed in real-time. A collaborative approach with other groups with more experience working with platelets would help us refine our blood processing methodology to reduce confounding factors.

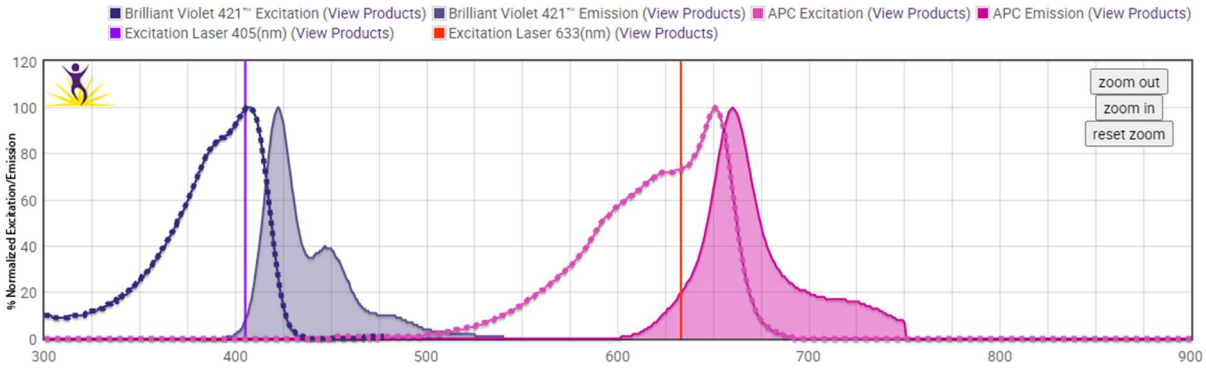

Figure 52 - Proposed flow cytometry panel for the measurement of PMAs

Biolegend Spectra Analyzer[140]

With the challenges and the learning curve for the processing of samples and interpretation of results, I would propose that any continuation of this project leads with a run-in period (as I tried to do with the feasibility arm) without taking OCTs and a pilot whereby ten control patients are compared to 10 NSTEMI to assess the reproducibility of the methods before commitment to full recruitment and OCT analysis. As the vendors are rolling out new and updated OCT consoles that integrate with the catheter laboratories, collating this co-registered data for collaboration with groups who have developed flow modelling methods to look at predictive factors behind plaque erosions would be sensible. Similarly, micro-OCT has been designed with 1 to 2 µm resolution, 10-fold better than incumbent technologies. This technology has not yet reached mainstream practice. It is currently being validated *ex vivo*, but it promises to better understand the endothelial layer around plaque erosion by allowing direct visualisation of the endothelium and inflammatory cells[168].

Co-registered OCT interrogation has already been helpful in mapping areas of endothelial sheer stress as a predictor for plaque erosion using flow dynamics[38]. CT-FFR has been developed and is used clinically to help predict the physiological effects of coronary plaque to predict whether lesions are flow-limiting. Future work could look at these flow dynamic effects to help develop risk models for the development of future coronary disease.

Our methodology is feasible and can be replicated with a small cohort of patients. However, the project has faced challenges due to various institutional circumstances such as delays in recruitment caused by COVID, limited access to lab workbenches due to social distancing, and data loss due to non-study team use of the OCT equipment. To overcome these challenges, it would be beneficial to have collaborations between multiple centers to better understand the causes of plaque erosion. By increasing the number of patients from different centers, we can obtain more reliable data and draw stronger conclusions. If we can analyze the serum using proteomic methods to explore differential cytokine expressions between RFC and IFC, it may help in identifying a clinically relevant biomarker of

plaque erosion. Although we stored serum from these patients to reduce the risk of contamination of the EDTA blood with endothelial cells from the veins, we did not recruit enough patients for meaningful proteomic analysis. O-Link, the company we had proposed to undertake this analysis, suggests a minimum sample size of 96 for meaningful statistical power from their proteomic arrays.

Finally, attention also needs to be paid to the clinical implications of this mechanistic work. Prati[125] and Jia[11] have demonstrated the potential benefits of a reduced intervention approach in the erosion cohort. Is there anything that can be added to upfront (powerful) GIIbIIIa receptor antagonists and prolonged antiplatelet medications for plaque erosion patients to reduce their ongoing risk of events from the culprit site, for example, with sustained anti-inflammatory medications such as colchicine or direct delivery of anti-inflammatory with a drug-coated balloon at the time of angiography? Prolonged dual antiplatelet therapy regimes have been demonstrated to reduce the risk of de novo MIs in patients who have previously presented and been treated for an MI at the expense of non-fatal major bleeding[169]. As healed erosions have been seen both on autopsy series[3] and in OCT assessment of non-culprit arteries to recurrently occur, this cohort of patients could receive the most benefit, thereby supporting a population approach to the reduction in cardiac risk.

5 Conclusion

I have demonstrated a larger proportion of circulating endothelial cells between IFC and RFC *in vivo*. These results add weight to the working hypothesis of the underlying pathology of plaque erosion being a primary endothelial insult resulting in cell death and denudation from the vessel wall. These results may help further understand plaque erosion's pathophysiology and suggest a target (endothelial cell apoptosis) for further preventative strategies. Our results indicate that our methodology is a safe and feasible approach for studying the potential triggers of plaque erosion despite having a high screen failure rate in this cohort.

I have achieved the primary objective of demonstrating the utility, safety, and feasibility of this combined methodology, correlating flow-cytometry assessment of circulating endothelial cells with plaque morphology in patients with MI. As a result, we have improved our understanding of the pathophysiology of plaque erosion in real-time and with actual patients.

Further work in more extensive studies is required to establish whether or not these observed differences result from endothelial cell apoptosis as the animal and cell-tank models suggest and to apply therapeutic strategies targeting this pathway to treat plaque erosion better.

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Appendix i. Invited BCIS Research Committee Review of Protocol

BRITISH CARDIOVASCULAR INTERVENTION SOCIETY

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Dr Alisdair Ryding

Via email

27 November 2019

Dear Dr Ryding

BCIS R&D group peer review
Study Title: Plaque Erosion Pilot Study II (PEPS-ii)
Study Sponsor: Norfolk and Norwich University Hospital
Principle Investigator: Dr Alisdair Ryding

Reviewer 1
This is an interesting and ambitious study of differing cellular and cytokine responses to distinct plaque processes in patients suffering a NSTEMI event i.e. erosion vs. rupture. I have a few specific comments:

1. What was the rationale for excluding patients over 75?

2. Why was the control group single vessel PCI only?

3. The whole study hinges on the ability of OCT to differentiate plaque rupture from plaque erosion. Therefore, there needs to be much more detail in the methods of the supportive evidence of OCT and its established sensitivity/specificity in defining erosion vs. rupture. This question is particularly important given the presence of thrombus obscuring the underlying tissue. If this cannot be robustly assessed then the whole study will be futile.

4. If thrombectomy is allowed, are the investigators confident that an aspiration catheter will not impact on the endothelial morphology and other study end-points?

5. I would rephrase the sentence "To the best of our knowledge this study is completely unique." as this is likely to raise some antibodies in grant review.

6. Just for clarification, will the two OCT experts analyse images together and reach a consensus or will they review images independently? In the latter case,

how will the investigators manage disagreement between the OCT experts in the assessment of the plaque morphology?

7. Given that there will be 4 OCT runs (and therefore perhaps up to 80mls of extra contrast) I wonder whether a more liberal exclusion of patients with low eGFR would be wise. Perhaps 50mls/min/1.73m2??

8. The centre performs ~500 PCIs a year for NSTEMI. However, given the exclusion criteria (especially age, low eGFR, and inability to predilate) along with the excess contrast which needs to be explicitly explained to the patients (resulting in some drop out), recruiting 1:5 patients and therefore completing the study in a year I suspect might be optimistic.

9. There is little discussion as to where this research might lead aside from academic interest i.e. what would be the potential clinical benefits should their findings being significant. This should be added.

10. Data on baseline and procedural drugs used should also be collected as well as data on the PCI procedure itself, particularly if there is longer term follow-up to avoid confounders in the potential association between the variables of interest and outcomes.

11. The definitions of adverse events should be included. As a minimum, this should include dissection, slow flow, contrast induced AKI.

12. Given the potential for a significant extra contrast load, should a post-PCI U&E not be included in the protocol (24-hrs at the earliest)?

13. For many PCIs, the amount of extra contrast will be a lot more than the proposed 5-10%. I would estimate it to be nearer 20-30% extra.

14. Should the additive risk of an OCT run x4, coronary sampling and contrast load not be added to the patient information sheets?

Reviewer 2

The research aim is laudable and the study should generate novel biomedical data re plaque erosion – rupture.

The PIS presently does not make clear to patients that 3-vessel OCT is not standard care and the procedure will be prolonged.

Consent – the angiogram findings will be unknown hence when will consent be obtained, during the procedure? In which case, a process for witnessed, verbal assent would be needed followed by written consent on the ward, or (2) take the patient off the table for consent and then back to the lab. This is not ideal since the standard care procedure would be interrupted without necessarily having the consent of the patient.

100 patients with 3-vessel OCT is not trivial – if enrolment is lower than anticipated then out-of-hours activity may be needed.

The hypothesis is implicit but it is neither explicitly stated or specific.

There is no power calculation. This should be linked to the main (active) hypothesis.

The power calculation should support the 80:20 distribution of cases vs. controls and/or be modified accordingly.

Will the care of the patient be influenced by the OCT findings in the non-culprit vessels? Eg PCI?

What is the purpose of the follow-up visit. The study is underpowered for clinical outcomes. Therefore, what will be the meaningful information that might be gathered at this time… a repeat of the biomarkers to assess for within subject change? Apologies if I missed this.

Reviewer 3

This is an interesting study proposal which builds on the work done by Chandran, Ryding and colleagues in STEMI, which demonstrated differential cytokine expression in ic samples from 15 patients with intact thick fibrous caps on OCT compared to 23 with ruptured thin fibrous caps. The planned study seeks to recruit 80 patients with non-STEACS as well as 20 patients undergoing elective PCI with similar analyses planned as in the STEMI study.

1. Given this is a NSTEAC group, it isn't always possible to reliably identify the culprit vessel/lesion cf STEMI. Often the most haemodynamically significant lesion is treated in clinical practice but this isn't reliable for the current study. Will ECG/echo/angiographic criteria needs used for this? Allowance needs to be made for drop out
2. Is the erosion/rupture ratio expected to be different in NSTEMI compared to STEMI, do you have any pilot data? The ability to distinguish erosion from rupture on OCT is key to the success of the study. In the 2017 JAHA paper, the consort diagram suggests a remarkably low rate of exclusion and inability to classify aetiology (presumably as discrepancies were resolved by consensus) but it would be worth allowing for greater loss of patients in the current proposal
3. The extra burden of OCT may have been underplayed in the PIS, there is no mention of increased contrast use
4. The hypothesis should be clearly stated and the power calculation built around this. The translational steps that would be needed to improve the care of patients should also be elaborated
5. It seems overambitious to recruit 100 patients (more if potential drop out is incorporated) - founders would like to see a more realistic timeline

Best wishes
Prof Divaka Perera
Peer Review Lead, BCIS R&D Committee

Appendix ii. PEPSii – Response to BCIS Review

The PEPSii protocol underwent changes directly related to the BCIS review process, the reviewers were given Protocol Draft 3.3. This document explains how we have responded with our protocol update 3.4.

Reviewer 1

1. What was the rationale for excluding patients over 75?
    a. These were more likely to have renal dysfunction, are a higher risk of contrast induced nephropathy and a higher likelihood of tortuosity/calcification making the OCT analysis more difficult. Plaque erosion is also more commonly seen in younger population.
2. Why was the control group single vessel PCI only?
    a. For ease of recruitment and to reduce confounding factors, most of our elective angioplasty is single vessel. Initially we had intended to take OCT assessments of the control group but it was thought this would not add any further detail to our study whilst increasing patient risk.
3. The whole study hinges on the ability of OCT to differentiate plaque rupture from plaque erosion. Therefore, there needs to be much more detail in the methods of the supportive evidence of OCT and its established sensitivity/specificity in defining erosion vs. rupture. This question is particularly important given the presence of thrombus obscuring the underlying tissue. If this cannot be robustly assessed then the whole study will be futile.
    a. OCT is the only in-vivo approach to assessing the difference between plaque erosion and plaque rupture; this has been validated and remains the gold standard. A review article suggests that the sensitivity / specificity of OCT to detect plaque erosion is about 80%. Our previous study PEPSi managed to differentiate with minimal uninterpretable scans. The NSTEMI population are likely to have a more readable OCT as there is time for the thrombus to dissipate on antiplatelet therapy (shown in registry studies). We have factored in 12.5% non-diagnostic OCT studies in our power calculation.
4. If thrombectomy is allowed, are the investigators confident that an aspiration catheter will not impact on the endothelial morphology and other study end-points?
    a. Thrombectomy is not allowed, it was used in the previous (STEMI) study as a way to get intracoronary blood samples

5. I would rephrase the sentence “To the best of our knowledge this study is completely unique.” as this is likely to raise some antibodies in grant review.
    a. Rephrased
6. Just for clarification, will the two OCT experts analyse images together and reach a consensus or will they review images independently? In the latter case, how will the investigators manage disagreement between the OCT experts in the assessment of the plaque morphology?
    a. Images will be reviewed independently by experts (Dr Johnson and Dr Calvert). In cases of discordance, consensus will be reached through discussion between the reviewers. We expect there to be a small rate of OCT which will be uninterpretable
7. Given that there will be 4 OCT runs (and therefore perhaps up to 80mls of extra contrast) I wonder whether a more liberal exclusion of patients with low eGFR would be wise. Perhaps 50mls/min/1.73m2??7
    a. There will now just be 1 OCT run with a with a more liberal exclusion of patients now to an eGFR of <45 (in keeping with most OCT based studies requiring multiple runs)
8. The centre performs ~500 PCIs a year for NSTEMI. However, given the exclusion criteria (especially age, low eGFR, and inability to predilate) along with the excess contrast which needs to be explicitly explained to the patients (resulting in some drop out), recruiting 1:5 patients and therefore completing the study in a year I suspect might be optimistic.
    a. PEPSi managed to recruit 40 patients in 6 months locally, NSTEMI outnumbers STEMI 3:1 so we think this will be possible, we have funding for an 18 month recruitment window if required
9. There is little discussion as to where this research might lead aside from academic interest i.e. what would be the potential clinical benefits should their findings being significant. This should be added.
    a. The importance of plaque erosion as a cause of myocardial infarction is increasingly recognised, as is the potential need for treatment to be tailored to the underlying pathology. Endothelial cell detachment and neutrophil activation appear to be critical to plaque erosion and we believe that research focused on these processes is fundamental to developing new approaches to diagnosis, treatment and prevention. The adoption of specific treatment strategies for plaque erosion currently requires OCT examination of the culprit lesion, and expert interpretation of the images. Identifying biomarkers that reliably differentiate plaque erosion or rupture non-invasively would revolutionise the way that MIs are managed in the future. For example, diagnosing plaque erosion from a blood test would avoid the need for OCT examination which could allow a significant

proportion of patients to avoid stenting. This would therefore save resources and potentially improve health outcomes. PEPSii is a collaboration with experts at other UK institutions, and we believe that our data will provide the basis for a larger multicentre study that may change clinical practice.

10. Data on baseline and procedural drugs used should also be collected as well as data on the PCI procedure itself, particularly if there is longer term follow-up to avoid confounders in the potential association between the variables of interest and outcomes.
    a. This will be added to the CRF
11. The definitions of adverse events should be included. As a minimum, this should include dissection, slow flow, contrast induced AKI.
    a. This was added
12. Given the potential for a significant extra contrast load, should a post-PCI U&E not be included in the protocol (24-hrs at the earliest)?
    a. Contrast load now significantly reduced, U&Es at 24Hrs post PCI is standard of care at our centre
13. For many PCIs, the amount of extra contrast will be a lot more than the proposed 5-10%. I would estimate it to be nearer 20-30% extra.
    a. This was based on 4 OCT runs, we are now only taking 1 run
14. Should the additive risk of an OCT run x4, coronary sampling and contrast load not be added to the patient information sheets?
    a. OCT is only 1 run now, but risks have been added to PIS (as per REC recommendation)

Reviewer 2

1. The PIS presently does not make clear to patients that 3-vessel OCT is not standard care and the procedure will be prolonged.
    a. No-longer taking 3 vessel OCT, but the prolongation of procedure now in PIS
2. Consent – the angiogram findings will be unknown hence when will consent be obtained, during the procedure? In which case, a process for witnessed, verbal assent would be needed followed by written consent on the ward, or (2) take the patient off the table for consent and then back to the lab. This is not ideal since the standard care procedure would be interrupted without necessarily having the consent of the patient.
    a. Written consent prior to study, if patient unsuitable for OCT then will proceed no further in the study and blood samples will be discarded.

3. 100 patients with 3-vessel OCT is not trivial – if enrolment is lower than anticipated then out-of-hours activity may be needed.
    a. 80 patients with 1 vessel OCT is now the requirement for the study
4. The hypothesis is implicit but it is neither explicitly stated or specific.
    a. Now stated
5. There is no power calculation. This should be linked to the main (active) hypothesis.
    a. Power calculation in Protocol 3.4 undertaken calculating the difference between two means
    b. Pilot data has been uncovered from a 1999 study looking at circulating endothelial cells. Full power calculation was performed as part of our BHF grant application "Our previous study of 40 patients showed a statistically significant difference in some cytokine levels between plaque erosion and plaque rupture. In PEPSii we are interested in differences between circulating endothelial cells post MI ; the only previous relevant work done in this field shows that CEC levels are raised post-MI15, although this was undertaken before plaque erosion and plaque rupture were able to be defined in-vivo. Review of this data shows that there are two clear groupings of CEC levels; a low count in around 75% of samples seen which appears to correlate with plaque rupture (mean 26 cells/ml, SD 17) and a group with a higher count potentially representing plaque erosion (mean 339 cells/ml, SD 170), and the overall population variance was 21000. If we assume CECs will be normally distributed around their means (although in reality the distributions are not normal), alpha 0.05, beta 0.2 gives us an overall sample size of about 70 to detect a minimum difference of 70 in CEC levels between erosion and rupture. We intend to recruit 80 patients as some of the OCT assessments will not be able to distinguish between erosion and rupture. Based on registry data of 405 consecutive OCT assessments of all-comer MI patients from the EROSION study and our own PEPSi study we would expect a final analysable sample of 21 patients with plaque erosion and 49 with plaque rupture."
6. What is the purpose of the follow-up visit. The study is underpowered for clinical outcomes. Therefore, what will be the meaningful information that might be gathered at this time… a repeat of the biomarkers to assess for within subject change? Apologies if I missed this.
    a. To determine assess for any complications as a result of the study, reassurance for the patients who are volunteering their time.

Reviewer 3

1. Given this is a NSTEAC group, it isn't always possible to reliably identify the culprit vessel/lesion cf STEMI. Often the most haemodynamically significant lesion is treated in clinical practice but this isn't reliable for the current study. Will ECG/echo/angiographic criteria needs used for this? Allowance needs to be made for drop out
    a. ECG/Echo/Angiographic data will be used to assess the culprit lesion, if this is not clear then the patient will go no further in the study
2. Is the erosion/rupture ratio expected to be different in NSTEMI compared to STEMI, do you have any pilot data? The ability to distinguish erosion from rupture on OCT is key to the success of the study. In the 2017 JAHA paper, the consort diagram suggests a remarkably low rate of exclusion and inability to classify aetiology (presumably as discrepancies were resolved by consensus) but it would be worth allowing for greater loss of patients in the current proposal
    a. One published study suggests that plaque erosion accounts for a larger proportion of NSTEMI compared to STEMI (60:40). We have the pilot data of our PEPSi study and the JAHA paper. We are allowing for a 12.5% loss of patients to uninterpretable OCT.
3. The extra burden of OCT may have been underplayed in the PIS, there is no mention of increased contrast use
    a. This has been corrected
4. The hypothesis should be clearly stated and the power calculation built around this.
    a. Please see above comments – Reviewer 2 4 & 5
5. The translational steps that would be needed to improve the care of patients should also be elaborated
    a. Please see above comments Reviewer 1 – 9a
6. It seems overambitious to recruit 100 patients (more if potential drop out is incorporated) - founders would like to see a more realistic timeline
    a. Now looking to recruit 80 patients, ideal timeline is 1 year but we have funding for 18 months of recruitment. PEPSi managed to recruit 40 patients in 6 months at our centre

Appendix iii. Research and Ethics committee letter of approval

Health Research Authority

East of England - Cambridgeshire and Hertfordshire Research Ethics Committee

The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0207 1048106

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

16 September 2020

Dr Alisdair Ryding
Consultant Interventional Cardiologist
Norfolk and Norwich University Hospital
Norfolk and Norwich University Hospital
Colney Lane, Norwich
NR4 7UY

Dear Dr Ryding

Study title:	**Plaque Erosion Pilot Study IIA single centre, prospective observational pilot study comparing molecular and cellular differences between plaque rupture and plaque erosion in patients with non-ST elevation myocardial infarction undergoing PCI.**
REC reference:	**20/EE/0094**
Protocol number:	**270706**
IRAS project ID:	**270706**

Thank you for your correspondence, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Confirmation of Capacity and Capability (in England, Northern Ireland and Wales) or NHS management permission (in Scotland) should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA and HCRW Approval (England and Wales)/ NHS permission for research is available in the Integrated Research Application System.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

It is a condition of the REC favourable opinion that **all clinical trials are registered** on a publicly accessible database. For this purpose, 'clinical trials' are defined as the first four project categories in IRAS project filter question 2. Registration is a legal requirement for clinical trials of investigational medicinal products (CTIMPs), except for phase I trials in healthy volunteers (these must still register as a condition of the REC favourable opinion).

Registration should take place as early as possible and within six weeks of recruiting the first research participant at the latest. Failure to register is a breach of these approval conditions, unless a deferral has been agreed by or on behalf of the Research Ethics Committee (see here for more information on requesting a deferral:
https://www.hra.nhs.uk/planning-and-improving-research/research-planning/research-registration-research-project-identifiers/

As set out in the UK Policy Framework, research sponsors are responsible for making information about research publicly available before it starts e.g. by registering the research project on a publicly accessible register. Further guidance on registration is available at:
https://www.hra.nhs.uk/planning-and-improving-research/research-planning/transparency-responsibilities/

You should notify the REC of the registration details. We will audit these as part of the annual progress reporting process.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

After ethical review: Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study, including early termination of the study
- Final report

The latest guidance on these topics can be found at
https://www.hra.nhs.uk/approvals-amendments/managing-your-approval/.

Ethical review of research sites

NHS/HSC sites

The favourable opinion applies to all NHS/HSC sites listed in the application subject to confirmation of Capacity and Capability (in England, Northern Ireland and Wales) or management permission (in Scotland) being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS/HSC sites

I am pleased to confirm that the favourable opinion applies to any non-NHS/HSC sites listed in the application, subject to site management permission being obtained prior to the start of the study at the site.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
GP/consultant information sheets or letters [GP Letter Control]	1.0	10 March 2020
GP/consultant information sheets or letters [GP letter NSTEMI]	1.0	10 March 2020
IRAS Application Form [IRAS_Form_04062020]		04 June 2020
Letter from sponsor [Letter from NNUH]	1	05 March 2020
Other [PEPSii BCIS Response for IRAS]	1.0	12 May 2020
Participant consent form [PEPSii Consent Form - Control Group]	3.0	12 August 2020
Participant consent form [PEPSii Consent Form - NSTEMI Group]	3.1	20 August 2020
Participant information sheet (PIS) [Patient Information Sheet - Control Group]	3.0	12 August 2020
Participant information sheet (PIS) [Patient Information Sheet - NSTEMI Group]	3.1	20 August 2020
Referee's report or other scientific critique report		
Research protocol or project proposal [PEPSii Protocal]	3.4.1	27 February 2020
Summary CV for Chief Investigator (CI) [ADR CV]		05 March 2020
Summary CV for student		03 January 2020
Summary, synopsis or diagram (flowchart) of protocol in non technical language		
Summary, synopsis or diagram (flowchart) of protocol in non technical language [Study Flow Diagram]	3.4.1	10 March 2020

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Learning

We are pleased to welcome researchers and research staff to our HRA Learning Events and online learning opportunities– see details at:
https://www.hra.nhs.uk/planning-and-improving-research/learning/

IRAS project ID: 270706	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

pp. V. Sutton

Professor Barry Hunt
Chair

Email: cambsandherts.rec@hra.nhs.uk

Copy to: Ms Julie Dawson

Appendix iv. HRA Approval Letter

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Dr Alisdair Ryding
Consultant Interventional Cardiologist
Norfolk and Norwich University Hospital
Norfolk and Norwich University Hospital
Colney Lane
Norwich
NR4 7UY

Email: approvals@hra.nhs.uk
HCRW.approvals@wales.nhs.uk

16 September 2020

Dear Dr. Ryding,

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**Plaque Erosion Pilot Study IIA single centre, prospective observational pilot study comparing molecular and cellular differences between plaque rupture and plaque erosion in patients with non-ST elevation myocardial infarction undergoing PCI.**
IRAS project ID:	**270706**
Protocol number:	**270706**
REC reference:	**20/EE/0094**
Sponsor	**Norfolk and Norwich University Hospital**

I am pleased to confirm that HRA and Health and Care Research Wales (HCRW) Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

Please now work with participating NHS organisations to confirm capacity and capability, in line with the instructions provided in the "Information to support study set up" section towards the end of this letter.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) have been sent to the coordinating centre of each participating nation. The relevant national coordinating function/s will contact you as appropriate.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?

The standard conditions document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:

- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

Who should I contact for further information?
Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **270706**. Please quote this on all correspondence.

Yours sincerely,
Laura Greenfield

Approvals Specialist

Email: approvals@hra.nhs.uk

Copy to: *Ms Julie Dawson*

Appendix v. Patient Information Sheets

Our Vision
To provide every patient with the care we want for those we love the most

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Plaque Erosion Pilot Study ii

Summary Patient Information Sheet

The study is being conducted in patients who have had a heart attacks to try and help establish the cause. You are due to undergo an elective coronary angioplasty and we would like to take some samples of your blood to use as controls for we analyse the blood of patients who have had attacks.

We know that heart attacks are caused by the sudden blockage of an artery supplying blood to the heart. In most cases the artery is furred up due to fatty material, known as atherosclerosis. In about two thirds of heart attacks the fatty material suddenly bursts (known as plaque rupture) and this triggers a blood clot which blocks the artery.

In the other third of cases there appears to be a more subtle trigger for clotting/blockage (known as plaque erosion). Very little is known about the causes of this, and the purpose of this study is to improve our understanding. Eventually this may help us to improve the treatment and prevention of heart attacks in the future.

If you participate in this study, we will take and analyse a blood sample before and after your coronary angioplasty. Apart from this, you will receive the same treatment as someone who has had similar angioplasty treatment.

In this research study we will use information from you, your medical records and your GP. We will only use information that we need for the research study. We will let very few people know your name or contact details, and only if they really need it for this study.

Everyone involved in this study will keep your data safe and secure. We will also follow all privacy rules. At the end of the study we will save some of the data to allow us to communicate the results to you.

We will make sure no-one can work out who you are from the reports we write.

The following information pack tells you more about this.

PEPSii Plaque Erosion Pilot Study ii

PEPSii: A single centre, prospective observational pilot study comparing the molecular biology of plaque rupture and plaque erosion in patients with non-ST Elevation Myocardial Infarction undergoing PCI.

Patient Information Pack

We are inviting you to take part in a research project called PEPSii

You do not have to take part if you do not want to.

Please read this information which will help you decide.

IRAS Reference Number: 270706

Why am I being asked to take part in this research?
You are about to have a procedure called coronary angioplasty with the aim of improving the blood supply to your heart. This is because you have a narrowing in your coronary artery that limits flow. In most cases there is furring up of this affected heart artery due to a build-up of fatty material, known as atherosclerosis.

We are undertaking a study into the causes of heart attacks, we are interested in comparing samples of blood from patients who have had a heart attack with healthy people who have not. We would like to take a small sample of your blood before and after your angioplasty to help us with this study.

You are being asked to give permission for us to take these samples so that these can be analysed to improve our understanding of the causes of heart attacks. The samples will be stored anonymously and at any time you can withdraw your consent for your involvement in this project. We will store the samples in the Norwich Bio-repository to allow us to make further tests to investigate as they become available. No further treatment will be necessary as a result of this study. Although you would not receive any extra benefit from taking part, research like this helps to continually improve the treatments and care provided to all patients now and in the future.

Do I have to take part?
No. It is entirely up to you to decide. If you do not want to take part that's OK. Your decision will not affect the quality of care you receive.

If you do decide to take part you are free to withdraw at any time, without giving a reason, by contacting any member of the Cardiology Team.

What will I need to do if I take part?
If you agree to take part in this study we will take a small sample of blood before and after you have the angioplasty. Angiogram tests are routinely used to investigate and treat heart attacks in the NHS. All the information needed for the research (but not anything that could identify you) will be collected from your medical records and shared with the researchers.

If you choose to take part in this study, it will last for your planned procedure. You will not have to make any extra visits to your doctor over and above those needed for your normal care.

What are the disadvantages/risks?
There are no increased risks from participation in this study to you, we will take the samples of blood along with your regular pre-assessment tests before your angiogram, and ask that samples are taken from the sheath at the end of your procedure.

A summary of the results of this research will be made available to all those taking part; the investigators will write to you with a summary of the information we have learnt once the study is completed.

What will happen to information collected about me during the study?
All information that is collected about you during the course of this study will be kept strictly confidential according to the Data Protection Act 2018. Information on paper will be kept in locked filing cabinets and where possible behind security coded, locked doors. Electronic information will be kept on computers that are protected by passwords.

The electronic data we store for this study will be kept on a database. You will be assigned a unique study code in place of your name. Only members of the research team will have access to your name and address from the study code, so that you can be contacted as part of the follow up.

Any information about you that leaves the hospital will be anonymous and anything that could identify you (name, date of birth, address, hospital number) will be removed and you will only be identified by a study code. When the study is reported to the funding body, published in medical journals or presented at conferences it will not be possible to identify you personally.

Representatives from regulatory authorities may need to look at your medical records and the data collected in the study to check that the study was carried out correctly. All will have a duty of confidentiality to you.

What will happen to the results of the research study?
Once the study is complete and analysed, the results will be submitted for publication in a scientific journal and presented at scientific conferences. Your confidentiality will be maintained and you will not be identified in any report or publication of this study. If you wish to see the results when they are published let the researcher who obtains consent from you know and a copy of the results can be sent to you.

Who is organising and funding the research?
The research is organised and sponsored by Norfolk and Norwich University Hospitals NHS Trust.

Who has reviewed the study?
The study was reviewed by independent experts from the British Cardiovascular Intervention Society and has been given a favourable ethical opinion by the Cambridgeshire and Hertfordshire Research Ethics Committee. It has been reviewed and gained Trust approval from Norfolk and Norwich University Hospital Research & Development Department.

What if there is a problem?
If you are concerned about any aspect of this study you should ask to speak to one of the researchers who will do their best to answer your questions.

If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the Patient Advice and Liaison Service (PALS) (01603 289036).

If something goes wrong and you are harmed during the study due to someone's negligence then you may have grounds for a legal action for compensation against the hospital involved, but you may have to pay your legal costs. The normal NHS complaints mechanisms will still be available to you.

NHS hospitals are unable to agree in advance to pay compensation for non-negligent harm (situations where no one can be blamed for what happened). However, NHS Trusts are able to consider offering an ex-gratia payment in the case of a claim.

Who is organising and funding the research?
This study is being carried out by Dr Alisdair Ryding and sponsored by the Norfolk and Norwich University Hospital.

The research is funded by The Norfolk and Norwich University Hospital and has been supported by a grant from The Norfolk Heart Trust.

How will we use information about you?
We will need to use information from you, your medical records and your GP for this research project.

This information will include your NHS number/name/contact details. People will use this information to do the research or to check your records to make sure that the research is being done properly.

People who do not need to know who you are will not be able to see your name or contact details. Your data will have a code number instead.

We will keep all information about you safe and secure.

Once we have finished the study, we will keep some of the data so we can check the results. We will write our reports in a way that no-one can work out that you took part in the study.

What are your choices about how your information is used?
You can stop being part of the study at any time, without giving a reason, but we will keep information about you that we already have.

If you choose to stop taking part in the study, we would like to continue collecting information about your health from central NHS records/ your hospital/your GP. If you do not want this to happen, tell us and we will stop.

We need to manage your records in specific ways for the research to be reliable. This means that we won't be able to let you see or change the data we hold about you.

If you agree to take part in this study, you will have the option to take part in future research using your data saved from this study. The bloods samples you have provided will be stored anonymously in the Norwich Biorepository to allow us to test for further molecules of interest that may become apparent in the future

Where can you find out more about how your information is used?

You can find out more about how we use your information

- www.hra.nhs.uk/information-about-patients/
- www.hra.nhs.uk/patientdataandresearch
- by asking one of the research team
    - sending an email to james.wardley@nnuh.nhs.uk
    - ringing us on 01603 287690
- Speaking to the Norfolk and Norwich Data Protection Officer <insert here>

If you have any questions about this study please contact

Co-investigator: Dr James Wardley, Cardiology Research Fellow
Principal Investigator: Dr Alisdair Ryding (01603 387930)

Thank you for considering taking part in this study.

If you decide to participate you will be asked to sign a consent form and will be given a copy of this information sheet and the consent form to keep.

Our Vision
To provide every patient with the care we want for those we love the most

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Plaque Erosion Pilot Study ii

Summary Patient Information Sheet

The study is being conducted in patients who have had a heart attack. We know that heart attacks are caused by the sudden blockage of an artery supplying blood to the heart. In most cases the artery is furred up due to fatty material, known as atherosclerosis. In about two thirds of heart attacks the fatty material suddenly bursts (known as plaque rupture) and this triggers a blood clot which blocks the artery.

In the other third of cases there appears to be a more subtle trigger for clotting/blockage (known as plaque erosion). Very little is known about the causes of this, and the purpose of this study is to improve our understanding. Eventually this may help us to improve the treatment and prevention of heart attacks in the future.

If you participate in this study, we will take and analyse a blood sample and ask our colleagues to take the extra pictures inside of your heart arteries during the angiogram you will shortly have. You will not have to do anything, but we will contact you over the telephone for updates on your health. Apart from this, you will receive the same treatment as someone who has had similar treatment for a heart attack but is not participating in the study.

In this research study we will use information from you, your medical records and your GP. We will only use information that we need for the research study. We will let very few people know your name or contact details, and only if they really need it for this study.

Everyone involved in this study will keep your data safe and secure. We will also follow all privacy rules. At the end of the study we will save some of the data to allow us to communicate the results to you.

We will make sure no-one can work out who you are from the reports we write.

The following information pack tells you more about this.

PEPSii Plaque Erosion Pilot Study ii

PEPSii: A single centre, prospective observational pilot study comparing the molecular biology of plaque rupture and plaque erosion in patients with non-ST Elevation Myocardial Infarction undergoing PCI.

Patient Information Pack

We are inviting you to take part in a research project called PEPSii

You do not have to take part if you do not want to.

Please read this information which will help you decide.

IRAS Reference Number: 270706

Why am I being asked to take part in this research?
You have presented to the hospital with a heart attack and we are trying to understand the causes of heart attacks. Heart attacks are caused by the formation of a blood clot in a coronary artery, which interrupts the blood supply to part of the heart. In most cases there is furring up of the affected heart artery due to a build-up of fatty material, known as atherosclerosis. In most cases the fatty material bursts which triggers the formation of a blood clot with in the artery. The second most common cause of this clotting is where the cells that line arteries detach from the vessel wall, this is known as plaque erosion. The mechanism of plaque erosion is less clear and we are undertaking studies to try to understand the causes of this.

It is planned for you to have a procedure called a coronary angiogram where your arteries are visualised using contrast agents under x-ray guidance, this will help in the assessment of the cause of your heart attack and on-going treatment. To help us understand the causes of heart attacks better we would like to take a small amount of blood to analyse these for cells and specific molecules that might be important in this process. In order to find out whether a plaque erosion or plaque rupture cause your heart attack we would like to take extra detailed pictures from the inside of your heart arteries.

You are being asked to give permission for us to take these samples and take the extra high definition pictures of the inside of your coronary arteries so that these can be analysed to improve our understanding of the causes of heart attacks. The samples and pictures will be stored anonymously and at any time you can withdraw your consent for your involvement in this project. We will store the samples in the Norwich Bio-repository to allow us to make further tests to investigate as they become available. No further treatment will be necessary as a result of this study, we will however contact you by telephone at 1 month after your heart attack to check how you are. Although you would not receive any extra benefit from taking part, research like this helps to continually improve the treatments and care provided to all patients now and in the future.

Do I have to take part?
No. It is entirely up to you to decide. If you do not want to take part that's OK. Your decision will not affect the quality of care you receive.

If you decide NOT to take part you and your Consultant will agree on which treatment you will receive. This may be the same investigations you would have received by taking part in this research project.

If you do decide to take part you are free to withdraw at any time, without giving a reason, by contacting any member of the Cardiology Team.

What will I need to do if I take part?
If you agree to take part in this study we will take a small sample of blood before you have the angiogram. Angiogram tests are routinely used to investigate and treat heart attacks in the NHS. After the angiogram we will take some extra high resolution scans of the inside of the artery that caused the heart attack to try and work out if this was a plaque erosion or plaque rupture, these scans are routinely used to help guide specific cases but are not used in all procedures. After these pictures have been taken then your procedure will carry on as it already would. You do not need to do anything more. All the information needed for the research (but not anything that could identify you) will be collected from your medical records and shared with the researchers.

If you choose to take part in this study, it will last for your hospital admission, the research team will review your records and telephone you a month after discharge to see how you are doing. The entire research project will last for 18 months. You will not have to make any extra visits to your doctor over and above those needed for your normal care.

What are the disadvantages/risks?
There are only minimal risks involved in this research study over and above those for a coronary angioplasty.

- There is a very small risk of trauma to the vessel when this imaging catheter is placed (1 in 1200), this compares to significant vessel wall disruption as part of the angioplasty procedure (1 in 13) and are easily treatable with the placement of a stent.
- We will be using around 15mls of X-ray contrast (dye) in addition to the ~150ml normally required, there is a risk of renal dysfunction when volumes of more than 300ml are used (you are being asked to participate as you are at low risk for contrast related complications).
- Your angiogram procedure will be extended by up to 15 minutes to allow the set-up of the imaging catheter and the collection of the images, this does not increase the risk of your angiogram.
- If you take part in this study you will have fluoroscopic angiography pictures. Some of these will be extra to those that you would have if you did not take part. These procedures use ionising radiation to form images of your heart arteries, provide treatment and provide your doctor with other clinical information. Ionising radiation can cause cell damage that may, after many years or decades, turn cancerous. If you choose to take part in this study the extra pictures will add an additional 2% to the total dose. The additional risk of cancer from the extra dose involved this study would be 1 in 300000.

What will happen to information collected about me during the study?
All information that is collected about you during the course of this study will be kept strictly confidential according to the Data Protection Act 2018. Information on paper will be kept in locked filing cabinets and where possible behind security coded, locked doors. Electronic information will be kept on computers that are protected by passwords.

The electronic data we store for this study will be kept on a database. You will be assigned a unique study code in place of your name. Only members of the research team will have access to your name and address from the study code, so that you can be contacted as part of the follow up.

Any information about you that leaves the hospital will be anonymous and anything that could identify you (name, date of birth, address, hospital number) will be removed and you will only be identified by a study code. When the study is reported to the funding body, published in medical journals or presented at conferences it will not be possible to identify you personally.

Representatives from regulatory authorities may need to look at your medical records and the data collected in the study to check that the study was carried out correctly. All will have a duty of confidentiality to you.

What will happen to the results of the research study?
Once the study is complete and analysed, the results will be submitted for publication in a scientific journal and presented at scientific conferences. Your confidentiality will be maintained and you will not be identified in any report or publication of this study. If you wish to see the results when they are published let the researcher who obtains consent from you know and a copy of the results can be sent to you.

Who is organising and funding the research?
The research is organised and sponsored by Norfolk and Norwich University Hospitals NHS Trust.

Who has reviewed the study?
The study was reviewed by independent experts from the British Cardiovascular Intervention Society and has been given a favourable ethical opinion by the Cambridgeshire and Hertfordshire Research Ethics Committee. It has been reviewed and gained Trust approval from Norfolk and Norwich University Hospital Research & Development Department.

What if there is a problem?
If you are concerned about any aspect of this study you should ask to speak to one of the researchers who will do their best to answer your questions.

If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the Patient Advice and Liaison Service (PALS) (01603 289036).

If something goes wrong and you are harmed during the study due to someone's negligence then you may have grounds for a legal action for compensation against the hospital involved, but you may have to pay your legal costs. The normal NHS complaints mechanisms will still be available to you.

NHS hospitals are unable to agree in advance to pay compensation for non-negligent harm (situations where no one can be blamed for what happened). However, NHS Trusts are able to consider offering an ex-gratia payment in the case of a claim.

Who is organising and funding the research?
This study is being carried out by Dr Alisdair Ryding and sponsored by the Norfolk and Norwich University Hospital.

The research is funded by The Norfolk and Norwich University Hospital and has been supported by a grant from The Norfolk Heart Trust.

How will we use information about you?
We will need to use information from you, your medical records and your GP for this research project.

This information will include your NHS number/name/contact details. People will use this information to do the research or to check your records to make sure that the research is being done properly.

People who do not need to know who you are will not be able to see your name or contact details. Your data will have a code number instead.

We will keep all information about you safe and secure.

Once we have finished the study, we will keep some of the data so we can check the results. We will write our reports in a way that no-one can work out that you took part in the study.

What are your choices about how your information is used?
You can stop being part of the study at any time, without giving a reason, but we will keep information about you that we already have.

If you choose to stop taking part in the study, we would like to continue collecting information about your health from central NHS records/ your hospital/your GP. If you do not want this to happen, tell us and we will stop.

We need to manage your records in specific ways for the research to be reliable. This means that we won't be able to let you see or change the data we hold about you.

If you agree to take part in this study, you will have the option to take part in future research using your data saved from this study. The bloods samples you have provided will be stored anonymously in the Norwich Biorepository to allow us to test for further molecules of interest that may become apparent in the future

Where can you find out more about how your information is used?

You can find out more about how we use your information

- www.hra.nhs.uk/information-about-patients/
- www.hra.nhs.uk/patientdataandresearch
- by asking one of the research team
    - sending an email to james.wardley@nnuh.nhs.uk
    - ringing us on 01603 287690
- Speaking to the Norfolk and Norwich Data Protection Officer <insert here>

If you have any questions about this study please contact
Co-investigator: Dr James Wardley, Cardiology Research Fellow
Principal Investigator: Dr Alisdair Ryding (01603 387930)

Thank you for considering taking part in this study.

If you decide to participate you will be asked to sign a consent form and will be given a copy of this information sheet and the consent form to keep.

Appendix vi. Consent Forms

Our Vision
To provide every patient with the care we want for those we love the most

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

PEPSii – Plaque Erosion Pilot Study II

PEPSii: A single centre, prospective observational pilot study comparing the molecular biology of plaque rupture and plaque erosion in patients with non-ST Elevation Myocardial Infarction undergoing PCI.

Thank you for taking the time to read the "Control" Summary Patient Information Sheet (V3 12.8.20) and the "Control" Patient Information Pack (V3 12.8.20)

If you decide to participate you will be asked to sign the enclosed consent form and will be given a copy of this sheet, the Control patient information pack (V3 12.8.20) *and the consent form to keep.*

If you have any further questions about this study please contact

Co-investigator: Dr James Wardley, Cardiology Research Fellow
Norfolk and Norwich University Hospital
Colney Lane
Norwich
Norfolk
NR4 7UY
United Kingdom
Principal Investigator: Dr Alisdair Ryding (01603 387930)

Consent Form

Study Title: Plaque Erosion Pilot Study II – PEPSii

Study Number: 270706

Principal Investigator: Dr Alisdair Ryding

Patient Name | CRF ID

	Please read the following statements and put your initials in the box to show that you have read and understood them and that you agree with them	Please initial each box
1	I confirm that I have read and understand the "Control" patient information sheet/pack V3 dated 12th August 2020 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2	I understand that my involvement is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected.	
3	I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from the Sponsor or authorised by the Sponsor, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records	
4	I understand that a member of the research team will contact my GP informing them of my participation the study.	
5	I understand that if I withdraw from the study early, or the study staff are unable to contact me, the study site (study doctor and/or staff) would like permission to make the following contacts. **PLEASE TICK THE BOXES IF YOU AGREE:** ☐ The study site has my permission to contact me to collect information or to review publicly available records (if available and allowed by local law) on how I am doing at what would have been the end of the study. ☐ The study site has my permission to contact my GP (General Practitioner) who will review my medical records and tell the study staff how I am doing at what would have been the end of the study.	
6	In the event that I withdraw from the study early, I understand that the information already collected about me will used in the study unless I inform the team this is against my wishes	

To be filled in by the patient I agree to take part in the above study		
Your name	Date (Day/Month/Year) (e.g. 14/July/2019)	Signature

To be filled in by the person obtaining consent (investigator) I confirm that I have explained the nature, purposes and possible effects of the research study to the person whose name is printed above. They agreed to take part by signing and dating above.		
Name of Investigator (or person obtaining consent if different from Investigator)	Date (Day/Month/Year) (e.g. 14/July/2019)	Signature

Impartial Witness *At least one impartial witness is mandatory when the patient, is unable to read or write. An impartial witness must be present during the entire informed consent discussion.* I confirm that the information in the consent form was accurately explained to, and apparently understood by, the patient, and that consent was freely given by the patient.		
Name of Investigator (or person obtaining consent if different from Investigator)	Date (Day/Month/Year) (e.g. 14/July/2019)	Signature

Instructions to Study Staff

- File one copy in the patients notes
- File one copy in the trial folder
- Give one copy to the patient

If the study doctor signing this form is not the Principal Investigator, they must be an authorised representative

PEPSii – Plaque Erosion Pilot Study II

PEPSii: A single centre, prospective observational pilot study comparing the molecular biology of plaque rupture and plaque erosion in patients with non-ST Elevation Myocardial Infarction undergoing PCI.

Thank you for taking the time to read the "NSTEMI" Summary Patient Information Sheet (V3.2 18.08.2021) and the "NSTEMI" Patient Information Pack (V3.2 18.08.2021)

If you decide to participate you will be asked to sign the enclosed consent form and will be given a copy of this sheet, the NSTEMI *patient information pack* (V3.2 18.08.2021) *and the consent form to keep.*

If you have any further questions about this study please contact

Co-investigator: Dr James Wardley, Cardiology Research Fellow
Norfolk and Norwich University Hospital
Colney Lane
Norwich
Norfolk
NR4 7UY
United Kingdom
Principal Investigator: Dr Alisdair Ryding (01603 387930)

Consent Form

Study Title: Plaque Erosion Pilot Study II – PEPSii

Study Number: 270706

Principal Investigator: Dr Alisdair Ryding

Patient Name | CRF ID

	Please read the following statements and put your initials in the box to show that you have read and understood them and that you agree with them	Please initial each box
1	I confirm that I have read and understand the "NSTEMI" patient information sheet/pack V3.2 dated 18th August 2021 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2	I understand that my involvement is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected.	
3	I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from the Sponsor or authorised by the Sponsor, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records	
4	I understand that a member of the research team will contact my GP informing them of my participation the study.	
5	I understand that if I withdraw from the study early, or the study staff are unable to contact me, the study site (study doctor and/or staff) would like permission to make the following contacts. **PLEASE TICK THE BOXES IF YOU AGREE:** ☐ The study site has my permission to contact me to collect information or to review publicly available records (if available and allowed by local law) on how I am doing at what would have been the end of the study. ☐ The study site has my permission to contact my GP (General Practitioner) who will review my medical records and tell the study staff how I am doing at what would have been the end of the study.	
6	In the event that I withdraw from the study early, I understand that the information already collected about me will used in the study unless I inform the team this is against my wishes	

To be filled in by the patient I agree to take part in the above study		
Your name	Today's Date (Day/Month/Year) (e.g. 14/July/2019)	Signature

To be filled in by the person obtaining consent (investigator) I confirm that I have explained the nature, purposes and possible effects of the research study to the person whose name is printed above. They agreed to take part by signing and dating above.		
Name of Investigator (or person obtaining consent if different from Investigator)	Date (Day/Month/Year) (e.g. 14/July/2019)	Signature

Impartial Witness *At least one impartial witness is mandatory when the patient, is unable to read or write.* *An impartial witness must be present during the entire informed consent discussion.* I confirm that the information in the consent form was accurately explained to, and apparently understood by, the patient, and that consent was freely given by the patient.		
Name of Impartial witness	Date (Day/Month/Year) (e.g. 14/July/2019)	Signature

Instructions to Study Staff

- File one copy in the patients notes
- File one copy in the trial folder
- Give one copy to the patient
- Post copy to GP

If the study doctor signing this form is not the Principal Investigator, they must be an authorised representative

Appendix vii. GP notification letters

Our Vision
To provide every patient with the care we want for those we love the most

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Plaque Erosion Pilot Study ii

Dr Alisdair Ryding
Department of Cardiology
Norfolk and Norwich University Hospital

<GP Name>
<GP Address 1>
<GP Address 2>
<GP Address 3>

Dear Doctor,

<Patient name> was enrolled into the control arm of the Plaque Erosion Pilot Study ii prior to <his/her> angioplasty procedure using consent form "Control <version>" dated <date>. A copy of the Patient Information Sheet <version> dated <date> was posted to him prior to recruitment. <He/she> had the opportunity to ask and have answered all questions pertaining to the above, provided informed consent to take part and a copy of the signed/dated Consent Form was given to <him/her> for <his/her> records.

Bloods were taken pre & post angioplasty and assessed using flow cytometry methods to look at differing cell populations with serum saved for further cytokine analysis. We are looking to compare these samples from patients with myocardial infarctions (plaque erosion and plaque rupture) with the stable plaque (control) disease arm.

<Patient name> will be called at 1 month to assess his vital status, and once enrolment is complete and data is analysed, we will be contacting patients (and yourselves) with the results.

Please do not hesitate to contact me should you have any queries about this study.

Yours sincerely,

Dr James Wardley

Research Fellow

Investigator: Dr A Ryding
Protocol: Plaque Erosion Pilot Study II (PEPSii)
GP Letter Control Group
Version 2.0, dated 18th August 2021

Our Vision
To provide every patient with the care we want for those we love the most

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Plaque Erosion Pilot Study ii

Dr Alisdair Ryding
Department of Cardiology
Norfolk and Norwich University Hospital

<GP Name>
<GP Address 1>
<GP Address 2>
<GP Address 3>

Dear Doctor,

Your patient has been admitted to the Norfolk and Norwich University Hospital with an NSTEMI and has been recruited into the Plaque Erosion Prospective Study ii on <date>. This study is looking for evidence to support animal models and hypothesised mechanisms of plaque erosion. <Patient name> was enrolled into the NSTEMI arm of the Plaque Erosion Pilot Study ii prior to his angioplasty procedure using consent form "NSTEMI <version number>" dated <date>. A copy of the Patient Information Sheet <version> dated <date> was given to him/her prior to recruitment. <He/she> had the opportunity to ask and have answered all questions pertaining to the above, provided informed consent to take part and a copy of the signed/dated Consent Form was given to <him/her> for <his/her> records.

Samples of blood were taken and flow cytometry used to isolate different cell populations of interest. Remaining serum is stored in the Norwich Bio-repository where it can be tested for cytokines of interest at a later date. At their diagnostic angiogram, they had extra images taken from the inside of their coronary arteries using Optical Coherence Tomography which provide detailed 3-dimensional maps and allow us to establish whether the coronary event was due to plaque erosion or plaque rupture. They then received normal standard of care for the rest of the admission.

One month after enrolment into this study patients will be contacted by telephone to determine vital status. Once enrolment is complete and data is analysed we will be contacting patients (and yourselves) with the results.

Please do not hesitate to contact me should you have any queries about this study.

Yours sincerely,

Dr James Wardley

Research Fellow

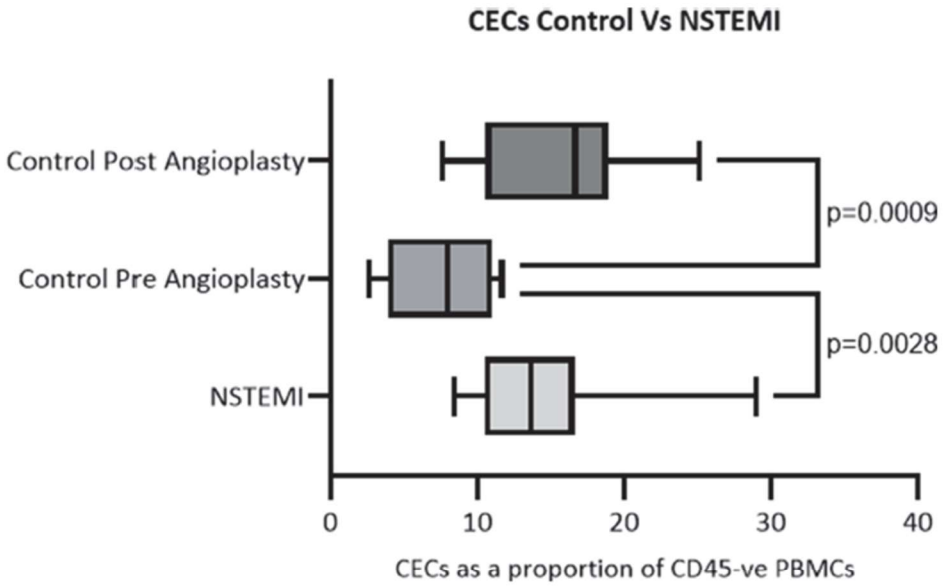
Appendix viii. Publications arising from work

35 **PLAQUE EROSION PILOT STUDY II (PEPSII): IS THERE AN ASSOCIATION BETWEEN CIRCULATING ENDOTHELIAL CELLS AND PLAQUE EROSION**

[1]James Wardley, [2]Stuart Rushworth, [3]Patrick Calvert, [4]Alisdair Ryding, [2]Marcus Flather, [5]Tom Johnson. [1]*Norfolk and Norwich University Hospital;* [2]*University of East Anglia;* [3]*Royal Papworth Hospital;* [4]*Norfolk and Norwich University Hospitals Foundation Trust;* [5]*University Hospitals Bristol*

10.1136/heartjnl-2023-BCS.35

Introduction Plaque erosion accounts for 30-40% of cases associated with a Type 1 MI and appears to have a different



Abstract 35 Figure 1 Differential CEC levels in controls, controls post-angioplasty and in NSTEMI population

Abstract 35 Table 1 Baseline characteristics

	NSTEMI (22)	Control (11)	p
Age	63.27 (43-78)	62 (52-70)	0.6653
Sex (males)	61%	73%	0.4585
Smoker	32% Current	9% Current	
	45% Ex	36% Ex	
	12% Never	55% Never	
BMI	28.9 (24.3-45.8)	32.7 (22.3-52.5)	0.2897
Hypertension	41%	55%	0.4740
Diabetes	14%	27%	0.3539
Hypercholesterolemia	41%	36%	0.8085
Creatinine	72.77 (55-105)	81 (53-105)	0.1145
Haemoglobin level	149.7 (118-186)	144.3 (124-169)	0.3197
Neutrophil count	7.335 (3.1-15.8)	4.758 (2.96-6.64)	0.0073

Abstract 35 Table 2 Baseline clinical characteristics RFC Vs IFC

	RFC (6)	IFC (8)	P value
Age	61.67 (50-72)	64.63 (55-78)	0.6836
Male	50%	63%	0.674
Smoking	17% Current	37.5% Current	
	66% Ex-smoker	25% Ex-smoker	
	17% Never smoked	37.5% Never smoked	
Hypertension	33%	25%	0.7563
Diabetes	17%	25%	0.7325
Cholesterol	4.833 (3-7)	5.913 (4.4-7.9)	0.1430
Creatinine	72.50 (58-91)	67.75 (57-89)	0.4406
Haemoglobin level	145.2 (127-163.0)	148.8 (137-162)	0.5634
Neutrophil count	5.782 (3.10-9.42)	7.301 (4.60-10.34)	0.2222

pathology compared to plaque rupture but the underlying mechanisms are uncertain. The Plaque Erosion Pilot Study carried out previously by our group demonstrated differential intracoronary cytokine expression in plaque rupture and erosion MI as defined by optical coherence tomography (OCT). We hypothesise that the mechanism of plaque erosion is due to propagation of apoptosis in the endothelial cell layer exposing the underlying extracellular matrix leading to thrombosis and release of circulating endothelial cells (CECs) with higher release in patients with erosion compared to rupture. Our aim was to test the apoptotic hypothesis of plaque erosion by investigating differences in CECs between plaque erosion and plaque rupture in patients with acute MI.

Methods

Methods 11 control patients had venous blood taken before and 4 hours after elective angioplasty. Flow cytometry was used to assess relative CEC levels by using antibodies against CD45, CD146 and CD31. Blood samples were taken from as close to presentation and processed from 22 NSTEMI patients presenting early to hospital who also had OCT carried out to group them into ruptured fibrous cap (RFC) or erosion with intact fibrous cap (IFC) by standard criteria during coronary angiography and validation by independent review.

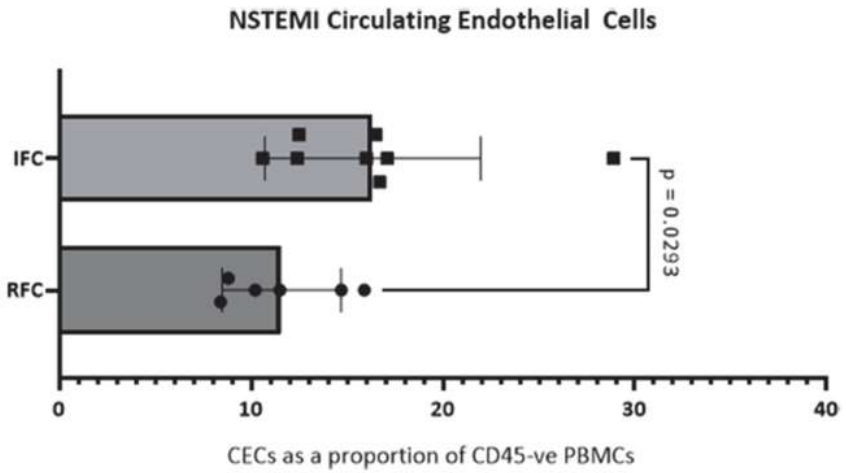
Results Table 1

We demonstrated an increase in circulating endothelial cells from baseline to post-angioplasty ($p=0.0293$) in control patients. In patients presenting with an NSTEMI we found higher levels of CECs compared to controls with known coronary artery disease ($p=0.0003$).

Figure 1

Following OCT assessment of the culprit coronary artery, six lesions were classified as RFC (27%), eight as IFC (36%), and eight were undefined (36%).

Table 2



Abstract 35 Figure 2 Differential CEC levels in the RFC Vs IFC group

There were higher levels of CECs in the IFC group compared to the RFC group (p=0.0293).

Figure 2

We attempted to differentiate between the cells isolating being alive (having burst free from endothelial layer at the time of plaque rupture) and dead (having undergone apoptosis and detaching off the endothelial layer at the time of plaque erosion) using Zombie Green™ dye that is non-permeant to live cells but permeant to the cells with compromised membranes. We saw numerically higher levels of cells positive for this stain in the IFC group that did not reach significance (p= 0.1812).

Conclusion We demonstrated higher CEC levels post-coronary angioplasty (iatrogenic endothelial injury) compared to baseline. We also detected higher levels of CECs in the IFC cohort (plaque erosion) compared to the RFC group (plaque rupture). This supports the hypothesis that plaque erosion occurs when endothelial cells denude off the vessel wall, exposing the underlying extracellular matrix and providing a nidus for thrombosis. These observations may help to target therapy for plaque erosion in the future.

Conflict of Interest None