A Flow Cytometric and Optical Coherence Analysis of the Role of Microparticles as Determinants of Plaque Instability in Acute Coronary Syndrome (FOAM Study)

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Dedication

I dedicate this work to my wife Anitha who provided constant support and to our boys Likhit & Nikhil. This work would not have been possible with out the support and encouragement given by my parents. My father always wished for my success as a doctor, researcher and to be a person with good humility. I am forever indebted to the constant support, encouragement and words of wisdom given by him. Unfortunately he passed away during the middle of this project and his thoughts remain forever with me.

Declaration

I, Dr Sudheer Koganti, hereby declare that this thesis is my own work. Other sources of information, when used, have been duly acknowledged.

I confirm that the practical procedures resulting in experimental data presented in this thesis were performed by me except for the following:

Dr Roby Rakhit, my primary supervisor, did majority of the interventional procedures and acquisition of OCT images. I operated the OCT console, did all the live measurements of coronary arteries and gave input into the interventional aspects.

Signed:

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Publications Related to this Thesis

- **Koganti S**, Karanasos A, Tu S, Rakhit RD, Regar E. *Visualization of extensive intraplaque neovascularization by optical coherence tomography.* **Hellenic J Cardiol**. 2017 Feb 2. pii: S1109-9666(17)30028-3. doi: 10.1016/j.hjc.2017.01.011.

- **Koganti S**, Eleftheriou D, Brogan PA, Kotecha T, Hong Y, Rakhit RD. *Microparticles and their role in coronary artery disease*. **Int J Cardiol**. 2017 Mar 1;230:339-345. doi10.1016/j.ijcard.2016.12.108. Epub 2016 Dec 22.

- **Koganti S**, Kotecha T, Rakhit RD. *Choice Of Intracoronary Imaging: When To Use Intravascular Ultrasound Or Optical Coherence Tomography.* Interventional Cardiology (London) 11(1):epub. March 2016. DOI10.15420/ICR.2016.11.01.11

Abstracts from this Thesis

- *Correlation between circulating microparticles and optical coherence tomography derived coronary atherosclerotic plaque characteristics*. Poster presentation, **Optics in Cardiology**, Rotterdam 2017.

- **S Koganti**, A Karanasos, CD Loder, T Lockie, **E Regar**, RD Rakhit **.** *OCT characterisation of coronary atherosclerosis in a cohort of patients with non-ST elevation acute coronary syndrome and stable angina: Analysis on patient and artery level*. Poster presentation, **Optics in Cardiology**, Rotterdam 2017.

- **Sudheer Koganti**, Despite Eleftheriou, Tushar Kotecha, Paul Brogan, Roby Rakhit: *Microparticle-mediated thrombin in generation coronary artery disease*. Poster presentation, **ESC**, Rome 08/2016. European Heart Journal 2016 abstracts supplement.

- **Sudheer Koganti**, Despite Eleftheriou, Tushar Kotecha, Paul Brogan, Roby Rakhit Persistent circulating platelet and endothelial derived microparticle signature during 6 month follow up of patients undergoing PCI for ACS versus stable angina. Poster presentation, **ESC**, Rome 08/2016. **European Heart Journal** 2016 abstracts supplement.

- **Sudheer Koganti**, Despite Eleftheriou, Tushar Kotecha, Paul Brogan, Roby Rakhit: *Enhanced microparticle-mediated thrombin generation in stable and unstable coronary artery disease*. EuroPCR, Paris; 05/2016. **Eurointervention** abstracts 29016.

- **S Koganti**, A Karanasos, T Kotecha, N Patel, CD Loder, T Lockie, **E Regar**, RD Rakhit: *34 Culprit coronary arteries in stable angina and unstable coronary artery disease have more vulnerable features when compared to non-culprit coronary arteries*. **Heart** (British Cardiac Society) 05/2016; 102(Suppl 4). DOI:10.1136/heartjnl-2016-309588.34

- Tushar Kotecha, **Sudheer Koganti**, Chrysostomos Mavroudis, Despina Eleftheriou, Paul Brogan, Roby Rakhit: *14 Differential microparticle signature in acute coronary syndromes and stable angina*. **Heart** (British Cardiac Society) 05/2016; 102(Suppl 4). DOI:10.1136/heartjnl-2016-309588.14

Abbreviations

AA	Arachidonic Acid
ACS	Acute Coronary Syndrome
AFM	Atomic force microscopy
AMI	Acute Myocardial Infarction
AnV	Annexin V
AnV+MP	Annexin V + Micoparticle
AO	Aorta
AUC	Area Under Curve
BNP	B type natriuretic peptide
CABG	Coronary Artery Bypass Grafting
CAC	Coronary Artery Calcification
CAD	Coronary Artery Disease
CD40 L	CD40 Ligand
CO	Coronary Artery
COX-1	Cyclooxygenase - 1
CT	Computed Tomography
CVD	Cardiovascular disease
CVVH	Continuous veno-venous hemofiltration
DAPT	Dual Antiplatelet Therapy
DSE	Dobutamine Stress Echocardiography
EDTA	Ethylenediaminetetra-acetic acid
eGFR	Estimated Glomerular Filtration rate
EMP	Endothelial Derived Microparticle
EPA	Eicosapentaenoic Acid
FC	Flow Cytometry
FCT	Fibrous Cap Thickness
FD	Frequency Domain
FH	Familial Hypercholesterolemia
FITC	Flourescein isothiocyanate
FFR	Fractional Flow Reserve
HDL	High Density Lipoprotein
HUVECs	Human Umbilical Vein Endothelial Cells
hs-CRP	High sensitivity C-Reactive Protein
ICAM-1	Intercellular Cell Adhesion Molecule - 1

INF - ϒ	Interferon - ϒ
IL	Interleukin
IMR	Index of Microvascular Resistance
IQR	Interquartile Rang
ISR	Instent Restensosi
IVUS	Intravascular Ultrasound
LAD	Left Anterior Descending Artery
LCX	Left Circumflex Artery
LDL	Low Density Lipoprotein
LMPs	Leucocyte Microparticles
LMWH	Low Molecular Weight Heparin
LPS	Lipopolyscharide
Lp-PLA2	Lipoprotein-associated phospholipase A2
LRP	Lipid Rich Plaque
LVEF	Left Ventricular Ejection Fraction
LVH	Left Ventricular Hypertrophy
MACE	Major Adverse Cardiovascular Events
MACCE	Major Adverse Cardiovascular & Cerebrovascular Events
Mac-1	Macrophage 1 Antigen
MAGE	Mean Amplitude Glycemic Excursion
mCRP	Monomeric C- Reactive Protein
MDA - LDL	Malonaldehyde Low Density Lipoprotein
MLA	Minimal Lumen Area
MLD	Minimal Lumen Diameter
MMP	Monocyte Microparticles
MPO	Myeloperoxidase
MVD	Microvascular Dysfunction
MVO	Microvascular Obstruction
mRNA	Messenger Ribonucleic Acid
miRNA	Micro Ribonucleic Acid
NSTEMI	Non ST Elevation Myocardial Infarction
NF-kB	Nuclear Factor Kappa light chain enhancer of activated B cells
NO	Nitrous Oxide
NMP	Neutrophil Microparticle
OCT	Optical Coherence Tomography
OFDI	Optical Frequency Domain Imaging

Ox - LDL	Oxidised Low Density Lipoprotein
PCI	Percutaneous Coronary Intervention
pCRP	Pentameric C- Reactive Protein
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PerCP	Peridinin – Chlorophyll-Protein Complex
PerCP-Cy5	Peridinin – Chlorophyll-Protein Complex with Cyanine 5.5
PMA	Platelet Monocyte Aggregate
pPCI	Primary Percutaneous Coronary Intervention
PPP	Platelet Poor Plasma
PS	P - Selectin
PSGL-1	P Selectin Glycoprotein Ligand 1
PTX-3	Pentraxin - 3
PUFA	Poly Unsaturated Fatty Acids
RA	Right atrium
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RCA	Right Coronary Artery
ROCK - I	Rho associated kinase - I
ROCK - II	Rho associated kinase - II
SA	Stable angina
SAA	Serum Amyloid Antigen
SD	Standard Deviation
SMCs	Smooth Muscle Cells
SMMPs	Smooth Muscle Microparticles
SSC	Side-Scatter
STEMI	ST Elevation Myocardial Infarction
TCFA	Thin Cap Fibroatheroma
TEM	Transmission electron microscopy
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibition
TGA	Thrombin Generation Assay
TGF- β	Transforming Growth Factor - β
TIMI	Thrombolysis in Myocardial Infarction
TNF - α	Tumour Necrosis Factor - α
TRAP	Thrombin Receptor Agonist Peptide
TnI & T	Troponin I & T

TXA2	Thromboxane – A2
UA	Unstable Angina
VCAM-1	Vascular Cell Adhesion Molecule - 1
VEGF	Vascular Endothelial Growth Factor
VP	Vulnerable Plaque
vWF	Von Willebrand Factor
WCC	White Cell Count

Abstract

Introduction

Microparticles (MPs) are implicated in the pathogenesis of coronary artery disease (CAD).

Aims

To determine whether circulating MPs correlate with high-risk coronary atherosclerotic plaque phenotype.

Methods

25 patients with CAD undergoing percutaneous coronary intervention (PCI) were recruited; 13 were diagnosed to have acute coronary syndrome (ACS) & 12 with stable angina (SA). We characterized and compared coronary atherosclerotic plaque burden and vulnerable plaque phenotype by three-vessel optical coherence tomography (OCT) between ACS and SA groups. Endothelial (EMPs), platelet (PMPs), Neutrophil (NMPs), tissue factor (TFMPs) and smooth muscle (SMMPs) were quantified by flow cytometry, compared between groups pre PCI, post PCI, at days 1,7, 30 and 180. Pre and post PCI MPs measured on day 1 were correlated with OCT parameters. The levels of total and individual phenotypes of MPs were also compared based on presence or absence of thin cap fibroatheroma (TCFA) – a feature of plaque vulnerability. Procoagulant potential of MPs was determined by measuring thrombin generation assay (TGA) at above mentioned time points and compared between ACS and SA groups and also with disease free controls.

Results

OCT analysis revealed that ACS patients had more vulnerable features. The total AnnexinV+ MP (ANV+MP) levels were similar in ACS and SA groups at baseline, peaked immediately after PCI and were at their lowest on day 1. At six months CD54+EMPs [Median 9.9 (IQR 3.8,18.7) Vs. 2.6 (0.7, 5.2); p=0.008] and CD62p+PMPs [3.6 (1.2, 6.7) vs. 0.7 (0.4, 2.7); p=0.03] were significantly elevated in the ACS cohort. Patients with CAD showed abnormal thrombograms when compared to controls. Furthermore, thrombin parameters remained abnormal in ACS & SA patients at 6 months as demonstrated on AUC. Patients with TCFA exhibited enhanced thrombogenecity.

Conclusion

MPs may have a role as novel biomarkers in identifying vulnerable patients. Patients with TCFA expressed enhanced thrombogenecity irrespective of clinical presentation suggesting vulnerability.

IMPACT STATEMENT

Through FOAM Study I was able to demonstrate culprit and non-culprit coronary arteries in patients with acute coronary syndrome (ACS) harbour more vulnerable plaque features. The vulnerable features include higher lipid plaque burden and also presence of thin cap fibroatheroma (TCFA). To the best of my knowledge for the first time a study associating microparticles (MPs) with Optical Coherence Tomography (OCT) derived plaque characteristics was carried out. I have successfully demonstrated that total and individual MPs were elevated in acute coronary syndrome (ACS) patients at the time of index event when compared to stable angina (SA) cohort and thrombin generation assay (TGA) was abnormal in both ACS and stable angina (SA) cohorts when compared to controls. A significant correlation was noted between various MPs and plaque characteristics. Platelet derived and subtypes of endothelial-derived microparticles were elevated in ACS patients when compared to SA patients at six months despite being on guideline directed antiplatelet therapy. These subtypes of MPs need to be evaluated further in larger studies to establish their role in coronary artery disease.

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Chapter 1. Introduction

1.1 Acute coronary syndrome, vulnerable plaque and microparticles

Acute coronary syndrome (ACS) is caused by disruption of vulnerable atherosclerotic plaque (VP). Early identification of VP was proposed as a key to improving risk stratification and prevention of recurrent events in patients with coronary artery disease (CAD). However, VP approach to risk stratification has limitations, as it's poor in terms of personalised risk assessment and treatment effects. Furthermore, VP approach is limited as only structural factors such as plaque phenotype at a time point are considered. Providing Regional Observations to Study Predictors of Events in the Coronary Tree (PROSPECT) study demonstrated increased rate of nonculprit lesion related adverse events, driven mainly by symptoms secondary to recurrent coronary ischemia. Therefore the concepts of "vulnerable patient" holds promise when compared to "vulnerable plaque" to better risk stratify individuals at risk of adverse cardiovascular events. One of the key components of vulnerable patient is vulnerable blood due to hyperocoagulable state and resultant atherothrmobosis leading to acute coronary events. Furthermore, clinical and angiographic characteristics have limitations in detecting changes in coronary plaque phenotype. Imaging with advanced tools such as Optical Coherence Tomography (OCT) is required for accurate plaque characterisation and adequate risk stratification. Microparticles (MPs) are membrane vesicles released from activated or apoptotic cells either systemically or within the atheromatous plaque and may contribute to both the formation, progression of atherosclerosis and plaque rupture. Several in vitro studies implicate MPs of different cellular origins in the recruitment of inflammatory cells into endothelium, endothelial dysfunction, prothrombosis and progression of atherosclerosis. However, in vivo the role of circulating MPs in the progression of atherosclerosis before an acute event and their clearance afterwards is unclear.

1.2 Microparticles in Coronary Artery Disease

1.1.1 Background

Despite significant advances in the medical and interventional management of coronary artery disease (CAD) mortality and morbidity remains high with ischemic heart disease being the leading cause of death worldwide in the last 5 years (1). Atherosclerotic disease, the hallmark of CAD, is now considered a chronic inflammatory process (2). Over the last twenty years, data have emerged showing that immune cells are involved in the pathogenesis, formation and evolution of atherosclerotic plaques causing either stable angina (SA) or acute coronary syndromes (ACS) (3). Early identification of features that define possible atherosclerotic plaque instability is vital to improve cardiovascular risk stratification and prognosis. As our understanding of CAD pathophysiology has evolved from not just a focal but ultimately a systemic disease, approaches to identify these high-risk patients may need to combine identification of local vulnerable plaques or myocardial damage but also novel plasma biomarkers relating to cumulative atherosclerosis burden.

Microparticles (MPs) are now considered key mediators of inflammation(4,5) and therefore may play a role in both the formation and progression of atherosclerosis and subsequent plaque rupture leading to ACS. MPs were referred to as “platelet dust” when first reported in 1967 (6). The perception that MPs were merely “innocent debris” rapidly changed due to an increasing body of evidence suggesting that they have potent pro-coagulant and pro-inflammatory properties (7). MPs are sub cellular particles (measuring <1 μm) derived from the plasma membrane of any eukaryotic cell. Although MPs are formed in response to various biological processes such as cellular activation and apoptosis, evidence for their role in pathological states comes from their presence in excess numbers in disease states such as ACS, sepsis, systemic inflammation (including vasculitis), and malignancy (8). Over the last decade an increasing number of studies have explored the mechanisms of formation of MP, their content, and contribution to pathological states through a number of mechanisms such as angiogenesis, inflammation and coagulation. Pertinent to

the pathology of ACS are MPs derived from platelets, representing 70% of total MP, but also those of endothelial cells, erythrocytes and leukocytes (9).

1.2.1 MP formation and composition

The normal plasma membrane consists of a phospholipid bilayer (10). The distribution of phospholipids in this bi-layer is asymmetric with the outer layer consisting of phosphatidylcholine/sphingomyelin and inner layer consisting of phosphatidylserine (PS) (11). This pattern of distribution of phospholipids is under the control of three proteins; Flippase, Floppase and Scramblase (12). Increased intracellular calcium following cell activation alters the function of these three proteins and results in movement of phospholipids towards the outer layer exposing intensely pro-coagulant PS. This reorganisation of plasma membrane lipid bi-layer is associated with loss of asymmetry of cytoskeleton thus leading to vesicle formation; these vesicles are then cleaved by Caspases into MP (10) (Figure 1). Caspases were further shown to play a role in the release of MPs by the cleavage of a Rho associated kinase (ROCK I) protein during apoptosis (13). Thrombin induced endothelial cell vesiculation has also been shown to involve nuclear factor (NF)-κβ signaling and ROCK II activation (14). The exposed PS is a potent pro-coagulant as it provides an excellent substrate for the pro-thrombinase complex (15).

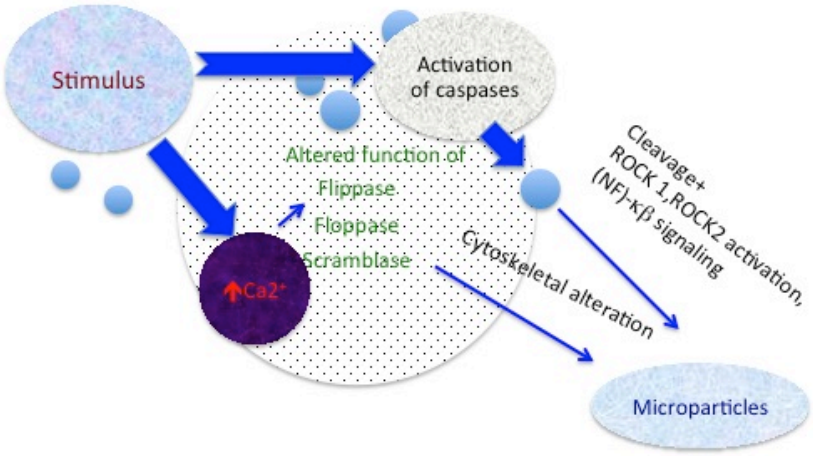


Figure 1. Schematic diagram showing cellular activation and apoptosis leading to microparticle formation (adapted from **Koganti S**, et al; **Int J Cardiol**. 2017 Mar 1; 230:339-345).

1.2.2 MP function

The biological function of MPs depends upon the parent cell they are derived from (16) (Table-1). MP lipid and protein composition also varies according to the parent cell and the stimulus that triggered their formation (17). Broadly they have a role in inflammation, coagulation, endothelial dysfunction, and angiogenesis. Evidence is also emerging of their role in microvascular dysfunction (MvD; see below). The pro-coagulant potential of MPs is largely secondary to PS exposure that acts as a platform for the assembly of several pro-coagulant factors along with the tissue factor expression (TF)(15). MPs also act as communicators or messengers carrying cytokines, mRNA and viruses (18). In addition MPs also act as transporters of specific micro RNAs (miRNAs), of particular relevance to cardiovascular diseases (19). Importantly, not all functions of MP appear to be detrimental as anticoagulant and fibrinolytic functions have also been reported (15) (20). In addition they also contain increased concentrations of oxidized phospholipids and caspases when compared to parent cell thus playing a role in cellular waste processes (21).

Table 1. Biologic function of various MPs and indicative surface markers based on cellular origin

Cell type	Surface marker	Biological function
Platelet (PMP)	CD31, CD42a, CD42b, CD61	Inflammation, Thrombogenesis, Angiogenesis*
Endothelial cell (EMP)	CD105, CD31, CD146, CD51, CD54, CD62E, CD144, CD34, CD18	Inflammation, Thrombogenesis, Angiogenesis†, Endothelial dysfunction
Monocytes (MMP)	CD105, CD14, CD11a, TF+, CD40 L	Inflammation, Thrombogenesis, Angiogenesis
Leukocyte (LMP)(22)	CD45	Inflammation, Thrombogenesis, Endothelial dysfunction
Erythrocyte (23)	CD235a	Thrombogenesis
Neutrophil (NMP)	CD15, CD64, CD66b, CD66e, CD11b, MPO(24)	Inflammation, Thrombogenesis, Anti-inflammatory effect on Macrophages

Note there may be overlap on expressed surface markers: CD146 has been found on activated T-cells; CD54 (Inter-Cellular Adhesion Molecule-1; ICAM-1) is also expressed by leukocytes; and CD51 is present on monocytes/macrophages and platelets. EMP – Endothelial derived MP, LMP – Leukocyte derived MP, MMP – Monocyte derived MP, NMP – Neutrophil derived MP, PMP – Platelet derived MP. † Low levels of EMP shown to promote angiogenesis where as high levels abolish angiogenesis (25), ∗in vitro only (26)

1.2.3 Quantification and phenotyping of MPs

Although there are various methods available to quantify MPs (Table-2), flow cytometry (FC) remains the most commonly used method (27). Staining with flurochrome-conjugated Annexin V (AnV), which binds to PS, is commonly used to identify MP of mixed cellular origin with FC (28). However some MPs don't bind with AnV. Whether this is a reflection of low PS content and/or a limitation of the technique using AnV staining to identify all MPs; or whether truly these AnV-negative MPs exist and have other functions remains to be established (29). As MPs carry parent cell proteins and receptors, fluorochrome-conjugated antibodies directed at these components allow us to quantify the specific type of MP using FC (Table-1). The procoagulant function of MPs can be confirmed in vitro by thrombin generation assay (TGA) (30) (Table -2).

Table 2. Methods to determine quantity of MPs and functional aspects

Method	Technical Aspects	Advantages	Disadvantages
Flow Cytometry	Detects surface antigens specific to the cellular origin. An-V can be used as a general marker for PS+ MPs	Widely used Can simultaneously detect two or more MP antigens	Inability to quantify PMPs sized approximately 400–500 nm (31)
Microscopy 1. TEM 2. AFM (31)	TEM Visualizes isolated MPs and determines their structure AFM is a type of scanning probe microscopy, allows high-resolution topographic imaging of PMPs	AFM can detect 1000-fold more PMPs than FC, mostly those of a small size	The time needed for sample handling and preparation renders current AFM procedures unsuitable for the screening of many samples
Dynamic Light Scattering (32)	Analyzes PMPs exposed to monochromatic light from a laser	Small sample volume (<0.4 ml) suffices	MPs can be significantly smaller post-filtration. Monodisperse system required

Method	Technical Aspects	Advantages	Disadvantages
Nanoparticle tracking analysis (33)	Analyzes particle movements by Brownian motion under a laser beam, with counting in real time	Small sample volume suffices	Studies of large vesicles (>500 nm) alone may be better carried out by flow cytometry Inability to accurately resolve heterogeneous mixtures of vesicles
Tunable resistive pulse sensing (34)	A high-resolution technique used to monitor individual and aggregated particles, of 50–1000 nm or more, as they move through tunable nanopores	Small sample volume (<0.1 ml) required	Limited data
Immunological & Procoagulant based assays (29,35)	These assays determine the procoagulant activity, in relation to the presence of circulating phospholipid or exposure to PS, tissue factor, or other PMP surface markers	Large number of samples can be screened	Limited to the capture system used soluble antigens
Proteomics (36)	Exploration of the nuclear material in PMP by processing and by various analytical methods such as 2D-electrophoresis and tandem mass spectrometry, spectral count analysis	Potential prognostic biomarker role in future in various cardiovascular disorders	Requires sophisticated equipment with high maintenance
Cellular assays or cell culture (24)	MP function evaluated by looking at the impact on the tissue	Can be used to elucidate therapeutic role	Requires time

The detailed methodologies of various assays mentioned in the table are beyond the scope of this review. References are provided as a guide to readers. AFM – Atomic force microscopy, TEM – Transmission electron microscopy, PMPs – Platelet microparticles, FC – flow cytometry.

1.2.4 Biologic function of MPs pertinent to CAD

MPs may contribute to formation and progression of atherosclerosis through a number of mechanisms such as angiogenesis, inflammation, coagulation, endothelial dysfunction, and MvD (Figure-2). These are considered in more detail below.

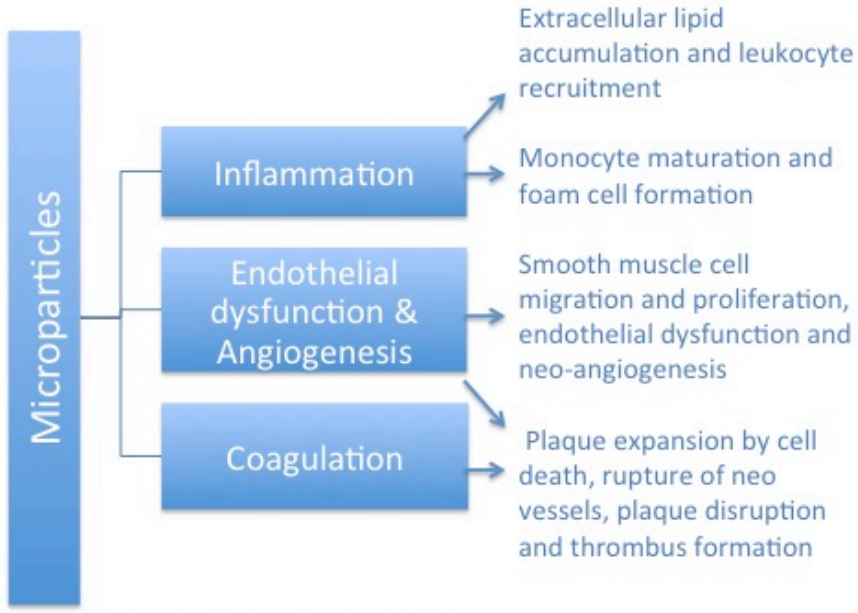


Figure 2. Flow chart depicting the interplay between the biologic function of MP and the atherosclerotic process. Key steps in the formation and progression of the atherosclerosis (right panel). Potential role played by MP through their biologic function in the atherosclerotic process (middle panel). (adapted from Koganti S, et al; Int J Cardiol. 2017 Mar 1;230:339-345.)

1.2.4.1 Angiogenesis

Vulnerable atherosclerotic plaques (VP) have characteristic features such as increased necrotic core, increased apoptotic macrophages and vasa vasorum (37). Atherosclerotic plaques develop their own microcirculation as they grow and this process is driven by angiogenic factors such as vascular endothelial growth factor (VEGF) (38). These micro vessels provide an avenue so that leukocytes and erythrocytes can enter and exit the atheromatous plaque, supplying oxygen and nutrients thus promoting the growth of the plaque. These micro-vessels are not stable and can rupture easily leading to intra-plaque haemorrhage (39) (40). MPs may play role in this angiogenesis-related plaque instability as demonstrated in the in vitro and in vivo studies described below.

1.2.4.1(a) In Vitro studies

Kim et al. demonstrated how PMPs could promote the proliferation and survival, migration, and tube formation in human umbilical vein endothelial cells (HUVECs) (25). When PMPs were treated with activated charcoal, a procedure

known to remove the lipid growth factors, the MP angiogenic activity was significantly reduced. These results suggest that the lipid components of the PMP may be major activating factors of protein components. In pathological states such as a growing tumour, PMPs shed from the circulating platelets may reach adequate concentrations contributing to florid neoangiogenesis (26). In another elegant study Leroyer et al demonstrated the potential role played by MPs in intra-plaque angiogenesis and thus plaque vulnerability. MPs were isolated from carotid endarterectomy specimens surgically obtained from 26 patients. The MPs thus isolated were further characterized by PS exposure and by identification of cellular origin. Plaque MPs (93% macrophage in origin) increased both endothelial proliferation and stimulated in vivo angiogenesis in matrigel assays performed in mice, whereas circulating MPs had no effect. MPs from symptomatic patients expressed more CD40 ligand (CD40L) and were more potent in inducing endothelial proliferation, when compared with asymptomatic plaque MPs. MP-induced endothelial proliferation was impaired by CD40L or CD40-neutralizing antibodies and abolished after endothelial CD40-ribonucleic acid silencing. In addition, the proangiogenic effect of plaque MPs was abolished in matrigel assays performed in the presence of CD40L-neutralizing antibodies or in CD40-deficient mice (41).

1.2.4.1(b) In Vivo study

In a clinical study LMP levels were found to be elevated in patients with high – grade carotid stenosis with underlying unstable plaque. Forty two asymptomatic patients with > 70% stenosis due for carotid endarterectomy had LMP levels quantified by high sensitive FC before surgery and plaque analysis post surgery. Based on the morphology, plaques were classified into stable and unstable as per American Heart Association criteria (42). Plaques with disrupted endothelium and intra-plaque hemorrhage were classified as unstable. Neurologic symptoms and the level of CD11bCD66b+ MPs independently predicted plaque instability thus underpinning the role of LMPs as a promising biomarker in predicting neurologic events (43). The discussed studies were not carried out in coronary specimens thus the relevance of these needs to be established before extrapolating to CAD.

1.2.4.2 Inflammation and Coagulation:

There are various mechanisms by which MPs can act as inflammatory mediators. In vitro studies have shown that binding of MPs to endothelial cells induces the expression of pro inflammatory molecules. PMPs and EMPs induce the expression of proinflammatory interstitial cell adhesion molecule -1 (ICAM-1), whereas MMP induce expression of ICAM-1 and Interleukin (IL)-8 (4). Inflammation and coagulation are linked processes in many diseases, and MMP, owing to their TF content, are intensely procoagulant.

1.2.4.2(a) In Vitro studies

Although animal studies have indicated a direct pathogenic role of C- reactive protein (CRP), the underlying mechanism remains elusive. Dissociation of pentameric CRP (pCRP) into pro-inflammatory monomers (mCRP) may directly link CRP to inflammation. Habersberger et al. investigated whether cellular MPs can convert pCRP to mCRP and transport mCRP following myocardial infarction (MI). In vitro experiments demonstrated that MPs were capable of converting pCRP to mCRP, which could be inhibited by the anti-CRP compound 1,6 bis-phosphocholine. Significantly more mCRP was detected on MPs from patients following MI compared with control groups. They further demonstrated that MPs containing mCRP were able to bind to the surface of endothelial cells and generate pro-inflammatory signals in vitro, suggesting a possible role of MPs in transport and delivery of pro-inflammatory mCRP in vascular disease (44).

One of the key features of MPs is their procoagulant potential. Aliman et al. have sought to establish the mechanism by which MPs promote thrombin generation and modulate fibrin density and stability. They isolated platelets and monocytes from healthy donors, which were then stimulated with calcium ionophore, thrombin receptor agonist peptide (TRAP) and lipopolysaccharide. MPs were isolated, washed by high-speed centrifugation and assessed using the following: transmission electron microscopy, nanoparticle tracking analysis, FC, TF activity, prothrombinase activity, thrombin generation, and clot formation, density and stability. MMPs had TF activity, supported prothrombinase activity, and triggered shorter thrombin generation lag times

than controls. Compared with controls, MMPs supported faster fibrin formation, 38% higher fibrin network density and higher clot stability. In contrast, PMPs did not have TF activity and supported 2.8-fold lower prothrombinase activity than MMPs. PMPs supported contact-dependent thrombin generation, but did not independently increase fibrin network density or stability (45) (46).

1.2.4.2(b) In Vivo studies

High levels of CRP seen in patients presenting with ACS suggest that atherosclerosis is an inflammatory process (47). Ueba et al. demonstrated a positive correlation between PMP and IL6 in 464 healthy volunteers (47). Biasucci et al. showed a correlation between EMPs and PMPs with high sensitive C-reactive protein (hs-CRP) in patients undergoing percutaneous coronary intervention (PCI) following presentation with ACS (48). Cui et al. showed that EMP, PMP, TF+MPs were significantly elevated in myocardial infarction (MI) and unstable angina when compared to a stable angina (SA) group. Good correlation with IL6, CRP (49) and higher CRP concentrations in patients with complex vulnerable plaques was shown(50).

More recently our group have demonstrated a positive correlation between MP expression and markers of inflammation and myocardial necrosis in patients with ACS or SA undergoing PCI. AnV+MP were quantified using FC from blood samples taken from the right atrium (RA) and culprit coronary artery (CO) and correlated with hs-CRP, IL-6, serum amyloid antigen (SAA) and Troponin T. Total and cell specific AnV+MP expression was higher in the ACS than SA group in both the CO and RA sites with CO MP levels being higher than the RA site in both groups. In the CO and RA sites of the ACS group SSA, IL-6, Troponin – T correlated positively with AnV+MP (51).

Above studies confirmed that MP levels are elevated in ACS with a gradient in different vascular compartments and good correlation with CRP and Troponin – T. However, these studies were associative in nature with no clear direction on the utility of MPs – could the correlation with inflammatory markers be used to further increase the sensitivity and specifity?

1.2.4.3 Endothelial dysfunction

Vascular endothelium plays a very important role in regulation of vascular tone and maintenance of vascular homeostasis. Endothelial dysfunction is one of the early steps in the development of atherosclerosis. It is also implicated in plaque progression and atherothrombotic events (52).

1.2.4.3(a) In Vitro studies

Rautou et al have shown that MP isolated from human atherosclerotic plaques transfer ICAM-1 to endothelial cells to recruit inflammatory cells thus promoting atherosclerotic plaque progression (5). Boulanger et al investigated whether or not MPs would affect endothelium-dependent responses. Rat aortic rings with endothelium were exposed for 24 hours to circulating MPs isolated from peripheral blood of 7 patients with non ischemic (NI) syndromes and 19 patients with MI. Endothelium-dependent relaxations to acetylcholine were not affected by high concentrations of MPs from NI patients. However, significant impairment was observed in preparations exposed to MPs from patients with MI at low and high concentrations (53).

1.2.4.3(b) In Vivo study

In another study endothelial-dependent vasodilatation was invasively assessed in 51 patients with CAD by quantitative coronary angiography during intracoronary acetylcholine infusion. Circulating CD31+/AnV+ apoptotic MPs analyzed by FC positively correlated with impairment of coronary endothelial function. Multivariate analysis revealed that increased apoptotic MP counts predicted severe endothelial dysfunction independent of traditional risk factors of CAD (54).

While CD31 EMP levels show good correlation with endothelial function it is not certain at what stage and spectrum of CAD it has a role as biomarker. Prospective population based studies in high risk cohorts are required prior to incorporating CD31 EMP into risk stratification models with confidence.

1.2.4.4 Microvascular dysfunction

Up to 30% of patients undergoing primary PCI following MI do not achieve adequate reperfusion. This phenomenon is called “no-reflow” and may be due to microvascular obstruction (MVO) (55). Porto et al correlated PMP, EMP with indices of MVO or MvD such as thrombolysis in myocardial infarction (TIMI) flow, thrombus score, corrected TIMI frame count, myocardial blush grade, quantitative blush evaluator score, and 90 minute ST segment resolution from sequential aortic and culprit coronary artery blood in 78 ST elevation myocardial infarction (STEMI) patients undergoing successful primary PCI. This is the first study to suggest a role of MP in the pathogenesis of MVO. However; correlation with absolute indices of MvD such as Index of Microvascular Resistance (IMR) are not yet available.

1.3 MP in patients considered to be at high risk of CAD or with stable CAD

Abnormal lipid levels, smoking, hypertension and diabetes are the major risk factors for CAD (56,57). In a large community based study of 844 individuals from a Framingham offspring cohort, various phenotypes of EMPs were shown to be associated with traditional risk factors in subjects without CAD. Multivariable analyses showed hypertension and metabolic syndrome to be associated with CD144+ EMPs, and hypertriglyceridemia to be associated with CD144+, CD31+/CD41- EMPs (58). Demonstrating acute release of MPs with nuclear material inside showed the deleterious impact of cigarette smoking on the vasculature as assessed in healthy volunteers who were exposed to smoking (59). Saudes et al. have shown in their cross-sectional study MP concentration and phenotype in familial hypercholesterolemia differed markedly from controls. Levels of AnV+-total, CD45+-pan-leukocyte and CD45+/CD3+-lymphocyte-derived circulating MPs were significantly higher in FH-patients with subclinical lipid-rich atherosclerotic plaques than fibrous plaques. This suggests that patients with life-long high low density lipoprotein exposure have higher endothelial activation and higher pro inflammatory profile, even under current state-of-the-art lipid lowering therapy suggesting long term effects of vascular damage (60). Augustine et al carried out a panel of MP assays on a cohort of

patients referred for Dobutamine stress echocardiography (DSE) looking for inducible ischaemia. They found that procoagulant, platelet, erythrocyte, and endothelial but not leukocyte, granulocyte, or monocyte-derived MPs were elevated immediately after a standardized DSE and decreased after 1 hour. Interestingly in twenty-five patients who had positive DSE test the MP levels did not change during stress. Similar findings were also noticed in patients with negative DSE test but with previous vascular disease. In those who subsequently underwent coronary angiography MP rise during DSE had occurred only in those with normal coronary arteries suggesting blunted response of MP in patients with or at risk of vascular disease (61). A coronary CT scan based study on post-menopausal healthy women considered to be at low risk of CAD by Framingham score, showed that EMPs, PMPs, and their TGA were greatest in women with high coronary artery calcification (CAC) scores, thus emphasizing their value in identifying women with premature CAD (62). EMPs expressing CD62E were shown to predict cardiovascular outcomes in patients with stroke history. Three hundred patients with a history of stroke in the preceding three months were recruited and their EMPs were assayed by FC. Of the 298 subjects who completed the study according to protocol for 36 months major cardiovascular events occurred in 29 patients (9.7%) correlating with patients who were noted to have higher expression of CD62E+MPs (63). In an only outcome based study to date Sinning et al correlated CD31+/AnV+ MPs in 200 patients with stable CAD undergoing angiography with cardiovascular outcomes. At median follow-up of 6.1 years major adverse cardiovascular and cerebral events (MACCE) occurred in 72 patients (37%). MP levels were significantly higher in the group of patients that experienced MACCE when compared the group of patients that did not experience any MACCE. In multivariate analyses high MP levels were associated with a higher risk for cardiovascular death, the need for revascularization, and the occurrence of a first MACCE. Inclusion of the MP level into a classical risk factor model substantially increased c-statistics from 0.637 to 0.702. This study gives robust evidence that the level of circulating CD31+/Annexin V+ MPs as an independent predictor of cardiovascular events in stable CAD patients and may be used for risk stratification by incorporating MPs into existing risk scoring models (64).

1.4 MP in patients with ACS

Circulating PMPs have been shown to be associated with the risk of future atherothrombotic events. Namba et al found PMPs to be significantly higher in patients with ACS than those in the group with SA. A positive correlation was observed between arc of calcification in the culprit coronary artery noted on IVUS with levels of circulating PMPs. Furthermore, the cohort of subjects with high load of circulating PMPs had low event free survival suggesting a potential role for circulating high PMPs in risk stratification (65). Crea et al noted higher levels of EMPs and PMPs in intra-coronary blood when compared to systemic blood in AMI patients undergoing primary PCI (48). Min et al. not only reproduced similar results in their cohort but went on to demonstrate significant reduction in MP levels post PCI thus implicating their role in thrombus formation (66).

One of the problems of PCI is a subsequent clinical event due to in-stent restenosis (ISR). MPs may have a role in predicting which cohorts of patients are at risk of developing ISR. Inoue et al in their study analysed circulating PMP, hs-CRP and activated Mac-1 on surface of neutrophils in 61 patients undergoing PCI. All three markers have increased in time dependent manner with maximum response at 48 hours in coronary sinus blood. Inflammation as well as platelet activation at the site of local vessel-wall injury plays an essential role in the mechanism of restenosis after PCI. Multiple regression analysis showed that each of PMPs, hs-CRP and Mac-1 as independent predictors of the late lumen loss (67).

1.5 Treatment directed towards MP

A number of different studies have explored the effect of various therapies on modulating circulating levels of MPs in cardiovascular disease. These treatment modalities range from dietary substitutes, prognostic medications used in CAD and mechanistic procedures such as haemofiltration. However most of these studies were carried out in small number of participants with no large scale randomized trial data. This is not surprising given the lack of standardization in quantifying MPs and time and effort required for their assay. For instance, treatment with n-3 fatty acids after MI appears to exert favorable effects on

levels of PMP, MMPs and their procoagulant activity (68). Total MP TF-procoagulant activity was also reduced in the n-3 fatty acid group compared to that in the placebo group. Similar results were also noted with dietary flavanol (69) and a diet rich in oats (70). Reducing oxidative stress by administering vitamin C appears to decrease the levels of MPs (71). Aspirin at a dose of 100 mg/day over 8 weeks was shown to reduce the number of PMPs and EMPs by 62.7% and 28.4% respectively; but no effect was noted on flow mediated dilatation used to assess endothelial function in patients with CAD(72). Studies involving lipid-lowering therapy did not show much promise in attenuating the levels of MPs. In a cohort of patients with CAD simvastatin/ezetimibe combination did not change platelet aggregation or the amount of circulating EMPs and PMPs. (73). However Huang et al noted reduction of levels of EMPs with high dose atorvastatin (40 mg) when compared to lower dose atorvastatin (20 mg) group although this did not translate into minimizing events in the higher statin group at the end of one year (74). In STEMI patients undergoing primary PCI significant reduction was noticed in peripheral procoagulant MP levels in the group who were given abciximab (potent platelet group IIb/IIIa inhibitor) prior to PCI when compared to the group which did not receive abciximab (75). In an in vitro study Abdelhafeez et al have shown that a standard continuous veno-venous hemofiltration (CVVH) model can decrease EMP levels. MPs generated from HUVECs were circulated through a standard CVVH filter (pore size 200 mum, flow rate 250 mL/hr.) for a period of 70 minutes. A 50% reduction in EMPs was noted within the first 30 minutes (76). In an earlier study Hong et al demonstrated filtration by standard therapeutic size filter not only removed NMPs but also abolished the pro-inflammatory effects in a group of children with vasculitis (24). However, large-scale clinical studies are required before these results can be extrapolated to patients with CAD. More recently Van Craenenbroeck showed inverse relation between baseline EMPs and the magnitude of increase in peak oxygen consumption following aerobic exercise training suggesting EMPs may predict increments in aerobic capacity and endothelial function (77).

1.6 Procoagulant potential of MPs

As mentioned above MPs are submicron vesicles and can be released from any eukaryotic cell following activation or apoptosis (78) and have procoagulant potential (78). The coagulation cascade in humans is regulated by fine balance between procoagulant and anticoagulant components. MPs by virtue of their procoagulant potential due to higher thrombin generation have the potential to tip this balance towards coagulation (79). The phospholipid bilayer externalised by flip-flop mechanism forms a good substrate for assembly of prothrombinase complexes and thus promotes thrombus formation(80). It is now well established that atheroscelrosis is an inflammatory process. The seminal event that leads to occlusion of a coronary artery in myocardial infarction is formation of thrombus over ruptured or eroded plaque (40). Patients with previous AMI were shown to have abnormal thrombograms with earlier lag phase, faster and higher thrombin generation when compared to a group of patients with stable angina (81). Significant correlation was also noted between the number of circulating PMPs and parameters of in vitro thrombin generation assay (82).

1.7 Future research directions

There is a need for standardization in each and every aspect of quantifying MPs, from collection of blood to centrifugation and storage to analysis. The possibility of incorporating absolute levels of MPs in traditional CAD risk scoring systems to enhance the predictability of an individual suffering a cardiac event need to be explored. Correlating the global atherosclerotic plaque burden by using adjunctive imaging such as optical coherence tomography with MPs may throw some insight into the utility of MPs as a potential biomarker. Correlating pro-coagulant function of MPs with IMR would further clarify their role, if any, in MvD and help in developing specific well-directed therapies. Furthermore confirmation of the role of MPs by demonstrating the changes related to atherosclerosis such as genesis of atherosclerosis itself will help in settle the debate whether presence of MPs in CAD is just an epiphenomenon or not. Exploration of nuclear material of MPs in various presentations of CAD by proteomics based research may help in establishing MPs as a prognostic biomarker. Once a pathophysiological role of MPs in CAD is established,

methods to quantify and functional assays are standardized the attention can then be turned to the development of a definitive therapeutic target. Further research is required beyond associative data to directly implicate MPs in disease pathogenesis whilst developing novel inhibitor molecules targeted against MP and testing these in the clinical setting.

1.8 Summary of the role of MPs in CAD

Even though strong evidence suggests that MPs are important biological mediators in the development and progression of CAD the lack of standardized methods for enumeration and identification restricts their current use in routine clinical practice. What is evident is that MPs are not only implicated in the pathogenesis of disease but they may have a role as biomarkers predicting acute events and perhaps represent a systemic signal, which may help in identifying the patients with vulnerable atherosclerotic plaques. However, it remains to be shown whether specific targeting of MPs is an effective means for reducing cardiovascular morbidly and mortality.

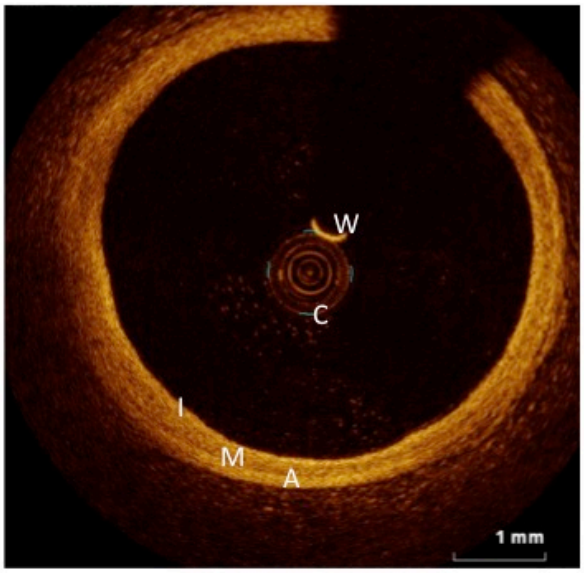
1.9 Optical Coherence Tomography

Introduction

Intracoronary imaging has the capability of accurately measuring vessel and stenosis dimensions, assessing vessel integrity, characterising lesion morphology and in guiding optimal percutaneous coronary intervention (PCI). Coronary angiography used to detect coronary stenosis severity and assess plaque characteristics has limitations as the 2 dimensional nature of fluoroscopic imaging provides lumen profile only. Performing PCI based on coronary angiography alone is inadequate for determining key metrics of the vessel such as dimension, extent of disease, plaque distribution and composition. The advent of intracoronary imaging has offset the limitations of angiography and has shifted the paradigm to allow a detailed objective appreciation of disease extent and morphology. Optical Coherence Tomography (OCT) is one of the several modalities available to carry out intracoronary imaging but is superior due to high resolution of images.

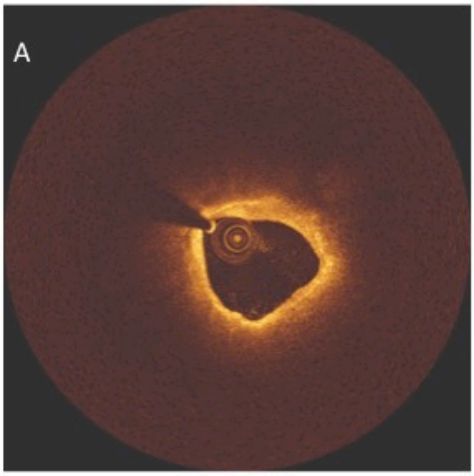
1.10 Technology of OCT

Intracoronary imaging by OCT is obtained using near-infrared light. The first generation of OCT imaging was based on occlusive balloon technology called time domain (TD) imaging. Frequency Domain (FD) imaging, also referred to as Fourier Domain spectral imaging or OFDI, has now surpassed TD imaging (83). FD imaging does not require occlusion of the proximal artery with a balloon as high viscosity liquids such as contrast media can be used to purge blood from the vessel while imaging is completed rapidly. Current commercially available OCT catheters consist of a single-mode optical fibre in a hollow metal wire torque that rotates at a speed of 100rps. The axial and lateral resolutions of OCT are 10-20 μm and 20 μm respectively (Figure 3). However, better resolution comes at a drawback of limited penetration - a maximum of 2mm (84). With acquisition speeds of up to 25 mm/s, rapid imaging of coronary artery can be achieved within a few seconds. Commercially available OCT catheters can be inserted into coronary artery on a 0.014" guide wire and are compatible with 5Fr or larger guiding catheters. For optimal imaging quality, a bloodless field is required which can be achieved by injection of 12-15 ml of highly viscous medium such as contrast either by hand or by using a power injector. Blood clearance can be challenging through a 5F catheter, therefore 6F or larger is generally recommended. Caution needs to be exercised in people with renal impairment where multiple pullbacks are contemplated due to the risk of contrast nephropathy. Current commercially available OCT software automatically detects the lumen, allows marking of every frame and gives user defined proximal and distal reference frames with dimensions. Furthermore, every pullback of the coronary artery can be viewed in cross-sectional frames (Figure 4), longitudinal view and lumen profile view. Cross sectional views are helpful in detailed study of plaque where as longitudinal and lumen profile views can be used for longitudinal measurements such as stent length. 3D reconstruction is possible and may assist in detailed assessment of lesions (Figure 5).



OCT showing a relatively normal segment of coronary artery. All three layers of artery are depicted.
I – intima
M – Media
A – Adventitia
C – OCT catheter
W - Guide wire

Figure 3. OCT showing a relatively normal segment of coronary artery



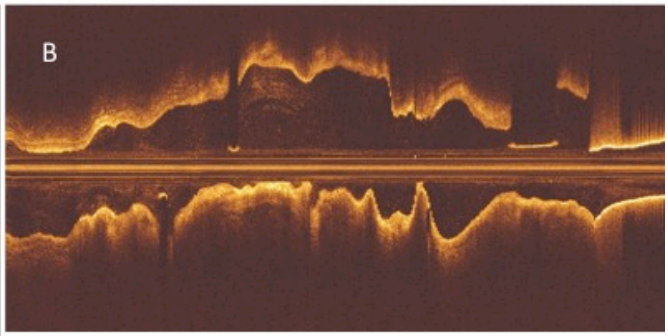


Figure:
A – Circumferential lipid rich plaque with no normal intima identified.
B – Longitudinal image of the same arterial wall

Figure 4. Cross sectional and longitudinal images of a lipid rich plaque in coronary artery

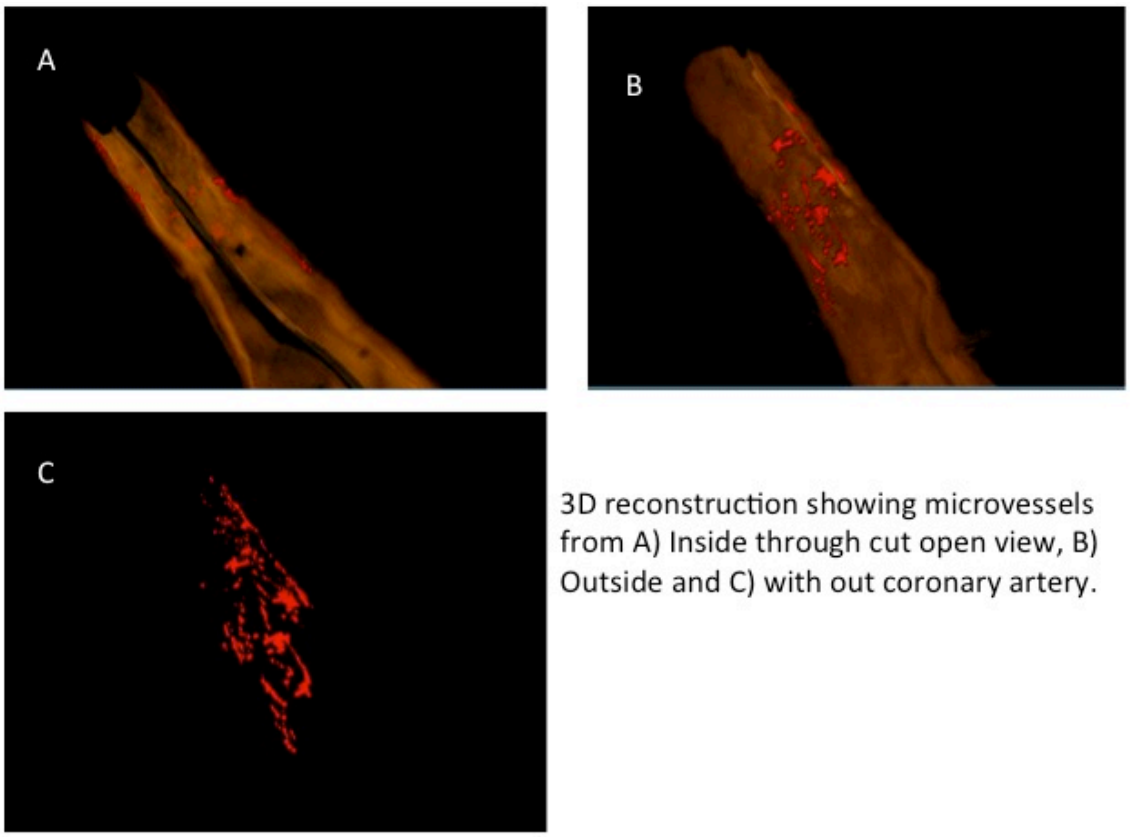


Figure 5. 3D reconstruction of a coronary artery (adapted from Koganti etal; Hellenic J Cardiol. 2017 Feb 2. pii: S1109-9666(17)30028-3).

1.11 Safety & Feasibility of OCT

One of the limitations of earlier TD – OCT was the requirement to have a bloodless field for adequate imaging. This was achieved by proximal occlusion of the coronary artery with a semi-compliant balloon followed by flushing of the artery with ringer's lactate solution. Whilst this method was not associated with any serious complications, minor complications such as transient ST elevation with associated chest pain were common (85). Furthermore, the acquisition time was longer with a pullback speed of only 0.5-3 mm/s. In comparison, current FD-OCT does not require balloon occlusion of coronary artery and, with higher pullback speeds; a bloodless field can be achieved by contrast injection.

Current generation FD- OCT has been shown to have an excellent safety profile. Integrated biomarker and imaging sub-study (IBIS-4) assessed the feasibility, and the procedural and long-term safety of OCT and Intravascular Ultrasound (IVUS) in patients with ST-elevation myocardial infarction (STEMI)

undergoing primary PCI. In this prospective cohort study, 103 STEMI patients who underwent serial three-vessel coronary imaging during primary PCI and at 13 months were compared with 485 STEMI patients undergoing primary PCI without additional imaging. Feasibility, defined as number of pullbacks suitable for analysis, and safety, defined as frequency of peri-procedural complications and major adverse cardiac events (MACE, a composite of cardiac death, myocardial infarction (MI) and any clinically indicated revascularization at 2 years) were recorded. Successful imaging was achieved in over 90% of patents at baseline and follow up using OCT. Although OCT had slightly more peri-procedural complications (<2.0% with OCT vs. 0% with IVUS), long-term safety was excellent with both modalities with no significant difference in MACE rates at 2 years (86). Of note, the majority of OCT-related complications were transient ST elevation due to coronary spasm, which in clinical practice can be mitigated by administering intra coronary nitrates prior to imaging.

In another registry-based study, OCT was shown to have comparable safety and feasibility profile. Analysis of 3,045 OCT pullbacks from 1142 patients revealed 7 complications related to OCT. Transient ST-elevation requiring withdrawal of the imaging catheter was noted with OCT (87).

1.12 Utility of OCT

OCT, with even better resolution when compared to IVUS, can be used in assessing plaque characteristics (88), constituents(89) and in optimizing PCI(90). However, with limited penetration, assessment of plaques with thickness greater than 1-0-1.5 mm is not possible with OCT(83,91). Up to 25% of ACS events are secondary to thrombus present on a non-ruptured plaque, also called as erosion(40,89). OCT helps in accurately identifying eroded plaques where if no lumen narrowing is present stenting may not be needed. Presence of thin cap fibroatheroma (TCFA), defined as lipid plaque thickness <65 microns, is predictive of future adverse cardiac events(40). However, interpretation of TCFA on OCT requires caution as artifacts due to tangential drop out can lead to misinterpretation(92). The superior resolution of OCT means it can detect TCFA(93) with adequate sensitivity/specificity, and also other features such as presence of macrophages(94), lipid volume and

neovessels– the features of so called vulnerable plaque (Figure 4-5). Furthermore, OCT not only identifies the presence of thrombus but can also distinguish between red and white thrombus often seen in STEMI(93) (Figure 6). Injury to the vessel wall post-PCI reflected by the presence of intimal tears, edge dissections, tissue prolapse, presence of thrombus and incomplete stent apposition can be readily assessed by OCT which gives an option to optimize as required(90). Two further areas where imaging with OCT is useful are capability of detecting thin neo-intima in follow up imaging after drug eluting stent implantation(95) and in delineating tissue characteristics of in stent restenosis(96).

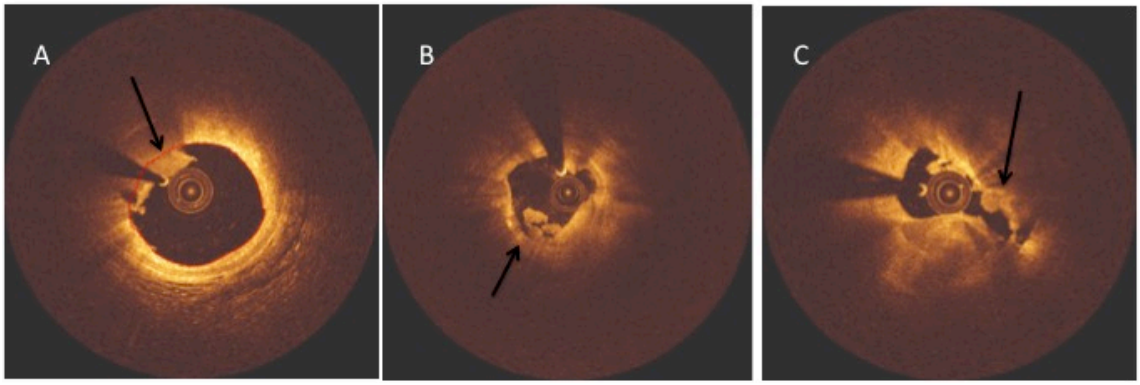


Figure 6. Classic appearance of intraluminal thrombus. A - Black arrow points to red thrombus (give light attenuation behind the thrombus) in a Segment of artery with preserved lumen suggesting the source to be elsewhere in the artery. B - Black arrow points to thrombus in a stenosed segment of artery. C - Thrombus inside a critically stenosed segment of artery.

1.13 Evidence base behind OCT

Currently trial data looking at OCT utility and clinical outcomes is limited. Angiography alone versus angiography plus OCT to guide decision – making during PCI (CLI-OPCI) study compared outcomes between patients undergoing PCI under angiography guidance alone vs. angiography & OCT guidance. The group that underwent PCI with angiography & OCT guidance had overall significantly less cardiac death, MI and repeat revascularization (97). Furthermore, OCT revealed adverse features following PCI in almost 35% patients that needed further intervention.

The ILUMIEN 1 study assessed how the clinical decision-making process is influenced when OCT is added to angiography and fractional flow reserve (FFR). The study enrolled 418 patients scheduled for PCI from 35 international centers, including patients with stable and unstable coronary syndromes prospectively in a non – randomized fashion. Once recruited, the majority of patients underwent pre-PCI FFR and OCT imaging. OCT imaging influenced physician decision-making process pre-PCI in 57% and post-PCI in 27% of cases. Additional in-stent post-dilatation was carried out in 81% and additional stent placement in 12% of the cases. Device-oriented MACE (cardiac death, MI and target lesion revascularization) and patient-oriented MACE (all cause death, MI and any repeat revascularization) were rare in hospital and at 30 days. Other events such as stent thrombosis were also extremely low(98). ILUMIEN 2 study showed the degree of stent expansion achieved after OCT vs IVUS guidance to be comparable. In this retrospective study propensity matched analysis of 354 patients who underwent OCT in the ILUMIEN I trial and 586 patients from the IVUS substudy of the ADAPT-DES trial based on reference vessel diameter, lesion length, calcification, and reference segment availability was comparable between OCT and IVUS guidance, as were the rates of major stent malapposition, tissue protrusion, and stent edge dissection (99).

1.14 Association of systematic inflammatory biomarkers with morphological characteristics of the coronary atherosclerotic plaque by intravascular optical coherence tomography.

Introduction

Ischemic heart disease remains the leading cause of death worldwide, despite advances in medical and interventional therapies (1). It is now widely accepted that atherosclerosis is an inflammatory process (100). All the key steps from initiation and progression of atherosclerosis to eventual plaque rupture or erosion and thrombus formation involve inflammatory pathways (2). Studies from cellular and molecular biology have shown how inflammatory pathways differ in each step of atherosclerosis (100). It is now well established that molecules such as cytokines and adhesion molecules alter the endothelial

integrity, whereby cells such as monocytes gain entry and alter the structural integrity of the arterial wall. Ensuing lipid accumulation and plaque progression involves degradation of interstitial collagen by matrix metalloproteinase (101). Both local and systemic inflammation contributes to plaque rupture with a subsequent role of activated platelets in thrombus formation and propagation by binding with monocytes. Yet again, it is the dynamic interplay between cells such as platelets and monocytes with molecules such as tissue factor and P-selectin glycoprotein ligand 1 that mediates thrombus formation and propagation. A greater knowledge of the role-played by various inflammatory cells and molecules involved in atherogenesis may translate into the development of biomarkers that can be used to screen individuals at risk and for monitoring interventions aimed at ameliorating atherosclerosis.

The majority of knowledge regarding the pathophysiology of atherosclerosis is based on histology. A vulnerable plaque is defined as a coronary atherosclerotic plaque that is prone to rupture, and that has morphological resemblance to ruptured plaques (102). The TCFA, a plaque with thin fibrous cap (< 65 μm), macrophage infiltration and large necrotic core, is currently considered the main phenotype of vulnerable plaque (103). Autopsy studies from patients who have suffered sudden cardiac death revealed the substrate for athero-thrombosis to be plaque rupture in 60-70% of the cases, plaque erosion in 20-30% of the cases and calcified nodules in the rest (40). However, autopsy studies have several limitations in that they represent the far-end of the clinical spectrum and ex vivo specimens need heavy processing and fixation with formalin thus leading to some degradation (104). Intracoronary imaging modalities such as optical coherence tomography (OCT) can be used for the in vivo imaging of coronary arteries and studying the atherosclerotic plaque characteristics due to high-resolution images (15 μm) obtained by near-infrared light (89,105,106). In their seminal study, Jang et al visualized the atherosclerotic plaque characteristics of culprit lesions by OCT in a cohort of 57 patients presenting with ST segment elevation myocardial infarction (STEMI, n=20), non STEMI (NSTEMI, n=20) and stable angina (SA, n=17) (107). OCT-identified TCFA and disrupted plaques were more prevalent in acute coronary syndromes (ACS), in contrast to plaques from stable coronary artery disease patients that had a higher incidence of calcifications. These findings were concordant with ex vivo

study conducted by same group (88) and previously reported autopsy studies (108)

As new data showing associations between biomarkers and cardiovascular events emerge, the finding of an association between the circulating levels of biomarkers and plaque morphology by OCT can provide new insights into the role of these biomarkers and the implicated mechanisms, thus providing more effective risk stratification.

1.14.1 Association with White blood cells (WBC)

Raffel et al conducted one of the first studies correlating inflammatory markers with plaque characteristics by OCT (109). The relationship between the peripheral WBC count, local macrophage density over the fibrous cap, other morphological features and presence of TCFA was evaluated in 43 patients undergoing angiography for ACS or SA. Patients with lipid rich plaques had higher WBC count in comparison to those with non–lipid-rich plaques, while there was a significant linear relationship between WBC count and plaque fibrous cap macrophage density irrespective of the presenting syndrome. Moreover, there was a strong linear relationship between WBC count and macrophage density in culprit plaque when compared to remote plaque, and an inverse linear relationship between WBC count, plaque macrophage density with fibrous cap thickness. Patients with culprit plaque with TCFA morphology had a higher median WBC count compared with those with culprit plaque without TCFA. **Although this study revealed a strong association between WBC and the presence of vulnerable plaque, it did not provide answers with respect to a mechanistic role of WBC in plaque destabilization.**

1.14.2 Association with C- reactive protein (CRP), high sensitivity (hs) CRP & Interleukins

Bouki et al. evaluated OCT-derived plaque characteristics between ACS and SA and correlated with hs-CRP and Interleukin (IL18) in 46 patients (32 ACS and 14 SA) (110). They noted more ruptured plaques, TCFA and lipid rich plaques in patients presenting with ACS and more calcific plaques in patients presenting with SA. IL18 and hs-CRP were significantly elevated in patients

presenting with ACS when compared to SA and correlated with presence of TCFA. Furthermore, on multivariate analysis hs-CRP was found to independently predict the presence of plaque rupture and detect one with a high degree of sensitivity and specificity. Niccoli et al. sought to evaluate the relationship between hs-CRP, Matrix metalloproteinase (MMP)-9, MMP-2, Myeloperoxidase (MPO) and Cystatin–C with the presence of plaque rupture, plaque erosion and significant stenosis with no thrombus (111). Their cohort of 84 patients consisted of 50 NSTE-ACS and 34 SA patients. On culprit artery only OCT there was no difference in plaque characteristics between ACS and SA. However, hs-CRP and MMP9 was associated with the presence of plaque rupture, whereas MPO with the presence of plaque erosion, and cystatin–c with the presence of significant stenosis without any superimposed thrombosis. Kashiwagi et al. evaluated the relationship between coronary arterial remodeling, fibrous cap thickness (FCT) and hs-CRP levels in 47 consecutive patients presenting with ACS. In this culprit plaque only study, arterial remodeling was assessed by intravascular ultrasound (IVUS), and FCT was measured by OCT (112). In total, positive remodeling (PR) was noted in 17 out of 47 patients and negative or intermittent remodeling in the remainder. Lipid-rich plaques and TCFA were more frequent in the PR group. Furthermore, high levels of hs-CRP were noted in the group with PR, as well as in those with documented TCFA. In a 3-vessel OCT study, involving 35 AMI and 20 SA patients, Fujii et al. showed hs-CRP levels to be associated with the presence of multiple TCFAs in ACS patients (113).

1.14.3 Pentraxin 3

Pentraxin 3 (PTX3) is an acute phase reactant and member of the pentraxin superfamily along with CRP. High levels of PTX3 are locally expressed in vascular endothelial and smooth muscle cells in human atherosclerotic lesions. Previously PTX-3 has been shown to be an early indicator of acute myocardial infarction (114). Koga et al. evaluated the association of PTX-3 with presence of TCFA and arterial remodeling index in 75 patients with CAD, of which 28 were diagnosed to have ACS and the remaining SA. Intravascular imaging was carried out with OCT and IVUS. Levels of PTX3 were significantly higher in patients with TCFA and correlated inversely with FCT and positively with the

remodeling index. Multivariate logistic regression analysis showed that a higher PTX3 level was the most powerful predictor of TCFA with receiver operating curve analysis showing PTX-3 levels of >3.24 ng/ml could predict presence of a TCFA with 84% sensitivity and 86% specificity.

1.14.4 Neopterin

Neopterin, a pteridine derivative is an intermediate metabolite in guanosine triphosphate metabolism and in tetrahydrobiopterin biosynthesis. Neopterin which is secreted by activated macrophages has previously been shown to be elevated in patients with ACS than SA (115). Furthermore, Kaski et al. showed that elevated neopterin levels at baseline in a cohort of patients with NSTE-ACS was independently associated with cardiac death, acute myocardial infarction and unstable angina after six months (116). More recently Sun et al. evaluated the levels of neopterin from peripheral venous blood in patients presenting with NSTE-ACS and SA, and evaluated a possible association with vulnerable plaque features from non–culprit plaques (117). Higher levels of neopterin were detected in non-culprit plaques with vulnerable features such as presence of TCFA, low FCT and high macrophage infiltration. However, a possible association of neopterin levels with culprit lesion morphology remains elusive, as it was not evaluated in the context of this study.

1.14.5 Myeloperoxidase

MPO, a hemoprotein released upon neutrophil activation has been shown to predict the outcome of patients with ACS (118,119) and is considered a marker of plaque vulnerability (119). A study evaluating the association of MPO with plaque erosion or rupture, in patients presenting with ACS has shown significantly higher MPO blood levels in eroded plaques. Furthermore, overlying thrombus in eroded plaques had significantly higher MPO levels when compared to thrombus from ruptured plaques in post mortem coronary samples of sudden death. Moreover, MPO levels were not elevated in the fibrous cap near rupture or the interface between thrombus and artery in eroded plaques but were significantly elevated in the thrombus overlying eroded plaque suggesting a role in thrombus formation. This could be probably explained by a mechanisms including hyaluronan-mediated loss of endothelial layer followed

by platelet adhesion and subsequent thrombus formation (120). Also, in line with previous studies MPO levels were elevated in smokers when compared to non-smokers (121), although this could be a confounder given the high percentage of smokers in the group with plaque erosion. In the same study, investigators showed no such discriminatory role for CRP

1.14.6 Impact of glucose fluctuation, monocyte subsets & P-selectin glycoprotein ligand 1 (PSGL-1)

Elevated blood glucose levels are commonly seen in patients presenting with AMI secondary to excessive sympathetic drive (122). This pattern is seen in both diabetics and non-diabetics. However, little is known if there is any correlation between glucose levels at the time of presentation with AMI or during recovery with atherosclerotic plaque characteristics. In a small study involving 37 consecutive patients with AMI that underwent OCT, Teraguchi et al. showed that glycemic variability, expressed as the mean amplitude of glycemic excursion (MAGE), was significantly higher in patients with plaque rupture than non-rupture patients (123). It is important to note that variability in glucose measurements was carried out prospectively up to 7 days, not allowing the evaluation of a possible prospective association of fluctuation in glucose levels with a future AMI. Furthermore, MAGE correlated positively and significantly with levels of CD14-bright, CD16fl- monocytes, which in turn were higher in patients with plaque rupture than in non-rupture patients. Whilst this suggests that glucose fluctuation is pro-inflammatory, no other correlation with traditional inflammatory markers such as WCC, CRP or cytokines was shown. Thus this study at best hypothesizes that dynamic glucose fluctuation in diabetic and non-diabetic patients is potentially associated with plaque rupture.

The role of monocytes in atherosclerosis is well established (124). Monocytes play a crucial role in the formation and propagation of thrombus in ACS patients. Activated platelets in ACS express P–selectin, an adhesion molecule to which monocytes bind through PSGL-1 resulting in platelet-rich arterial thrombi (125). In another monocyte-based study, expression of PSGL-1 on circulating peripheral CD14++CD16+ monocytes was significantly elevated in AMI patients with plaque rupture and intracoronary thrombus by OCT.

Interestingly, similar levels of PSGL-1 were not seen in plaque rupture associated with SA. Taking into account that thrombus formation secondary to plaque rupture or erosion is pathognomonic of ACS, whereas the culprit lesion in SA is usually obstructive in nature, plaque rupture in SA seen on OCT is usually an incidental finding that may have happened in the past with no clinical sequelae. Therefore, at the time of identification plaque rupture is probably no longer acute and as a result there is no thrombus, thus explaining the normal PSGL-1 levels (126).

1.14.7 Oxidized low density lipoproteins (Ox-LDL)

Malondialdehyde-modified LDL (MDA-LDL) is an oxLDL that was shown to be elevated in patients with ACS (127). Matsuo et al. evaluated the relationship between coronary plaque vulnerability assessed by OCT and circulating MDA-LDL in stable and unstable CAD. The circulating levels of MDA-LDL were significantly higher in patients with ACS, in SAP patients with TCFA and in ACS patients with ruptured TCFA (128). Similarly, experimental studies in mice have shown a transient increase in plasma Ox-LDL during the progression of atherosclerosis, not allowing however to discriminate whether of the observed elevated MDA-LDL levels in patients with vulnerable plaque are due to association or causation (129).

1.14.8 N3 and n6 polyunsaturated fatty acids (PUFA)

Eicosapentaenoic acid (EPA), a member of n3 PUFA family is derived from fish oil and has a role in preventing cardiovascular events (130). Arachidonic acid (AA), a member of n6 PUFA is known to promote CAD. Chronic imbalance of EPA & AA is known to promote CAD (131). A low EPA/AA ratio has been shown to be associated with thin cap fibroatheroma and wide lipid arc as seen on OCT in patients with stable angina (132). Similarly in ACS patients EPA/AA ratio was noted to be significantly lower in patients with TCFA than in those without TCFA (133). Addressing this imbalance and higher consumption of oily fish or fish oil supplements may impart stability to atherosclerotic plaque but that has to be studied in larger studies.

1.14.9 Troponin I

High sensitivity troponins at low levels were noted not only to be present in great majority of patients with stable coronary artery disease but also associated with the incidence of cardiovascular death and heart failure (134). Lee et al compared clinical and atherosclerotic plaque characteristics in patients undergoing PCI for stable CAD by dividing them into two groups; one with cTnI ≥0.03 ng/ml and one with cTnI <0.03 ng/ml (135). The group in which cTnI was elevated were noted to have frequent TCFAs, a greater lipid arc and longer lipid length. Furthermore, periprocedural myocardial injury occurred more frequently in the group with elevated cTnI, with OCT-identified TCFA being an independent predictor. This study suggests the presence of small levels of high sensitive troponins in some patients with CAD, which are associated with vulnerable plaque and an adverse outcome. However, large-scale studies have to be carried out to assess the feasibility of incorporating high sensitive troponin levels into traditional risk scoring systems to see if it has a good discriminator value.

1.14.10 Lipoprotein-associated phospholipase A2 (Lp-PLA2)

Lp-PLA2 is an enzyme known to hydrolyze ox-LDL particles leading to the production of byproducts that have been shown through in vitro experiments to cause plaque instability (136). Prior studies have shown association between higher levels of Lp-PLA2 mass and coronary events (137). Gu et al studied FCT and plaque volume in non-culprit lipid rich plaques at baseline and after 12 months by using OCT and IVUS in 24 patients presenting with ACS. Significant positive correlation was noted between Lp-PLA2 activity, FCT and plaque volume. Despite the potential role of Lp-PLA2 measurement as a biomarker for progression of CAD, the lack of any therapeutic benefit of direct inhibition of Lp-PLA2 activity in the SOLID-TIMI52 study suggests that this is not a suitable target for intervention (138).

1.15 Summary

In summary several studies (Table -3), though limited by relatively small number of participants, have shown good correlation between inflammatory markers

and OCT derived atherosclerotic plaque characteristics. However, the causation or association debate still remains, as predicting the levels of inflammatory markers and their association with vulnerable plaque in the run-up to acute event is a long shot. Furthermore, with the exception of traditional markers such as CRP, hs-CRP, WBC and Troponin, measuring novel inflammatory markers is cumbersome and is still at experimental level. These observations highlight the fact that research in this filed is still in its infancy and any conclusions are preliminary.

1.16 Conclusion

Prospective long-term and larger studies with a simplified way of assaying biomarkers are required before they can be adopted into risk stratification models. Until then the debate and pursuit on how best to predict the next acute coronary event goes on.

Table 3. Association of inflammatory biomarkers and plaque morphology in patients with ACS

Study	Population	Inflammatory marker	Association with plaque morphology
Inflammatory biomarkers			
Raffel et al (109)	32 ACS and 11 SA patients	WBC count	• WBC count correlated with cap thickness, macrophage density of the plaque • CRP levels higher in TCFA versus non-TCFA lesions
Kashiwagi et al (112)	47 ACS patients	CRP	• CRP levels higher in TCFA versus non-TCFA lesions
Fujii et al (113)	35 AMI and 20 SA patients	CRP	• CRP independent predictor of multiple TCFA in the coronary tree in AMI
Bouki et al (110)	32 ACS and 14 SA patients	CRP, IL-18	• CRP and IL-18 levels higher in TCFA versus non-TCFA lesions • CRP and IL-18 levels higher in ruptured plaques versus non-ruptured plaques • IL-18 levels lower in plaques with calcification • CRP levels independent predictor of ruptured plaque

Study	Population	Inflammatory marker	Association with plaque morphology
Inflammatory biomarkers			
Li et al	12 AMI, 23 UA and 11 SA patients	WBC count, CRP, IL-18, TNFα	• Cap thickness inversely correlated with WBC count and CRP, IL-18, and TNFα levels • WBC count and CRP, IL-18, and TNFα levels higher in TCFA versus non-TCFA lesions • No association for any factor with plaque rupture • CRP levels independent predictor of TCFA
Nicolli et al (111)	50 non-ST elevation ACS patients	CRP, MPO, MMP-9, MMP-2, Cystatin-C	• CRP and MMP-9 levels higher in plaque rupture compared to erosion and severe stenosis without thrombus • MPO levels higher in plaque erosion compared to rupture and severe stenosis without thrombus • Cystatin-C levels higher in severe stenosis without thrombus compared to plaque rupture or erosion • No association of MMP-2 with plaque morphology
Ferrante et al (139)	25 AMI patients	CRP, MPO	• No difference in CRP levels for plaque rupture versus plaque erosion • Elevated MPO levels in plaque erosion versus plaque rupture
Koga et al (114)	28 ACS and 47 SA patients	CRP, pentraxin-3	• Levels of pentraxin-3, but not CRP higher in TCFA versus non-TCFA lesions, both in ACS and SA lesions
Ozaki et al (126)	25 AMI and 20 UA patients	PSGL-1 expression in circulating monocytes	• Increased PSGL-1 expression in circulating monocytes in lesions with plaque rupture
Matsuo et al (128)	53 ACS and 49 SA patients	MDA-LDL, CRP	• Higher CRP levels but no difference in MDA-LDL levels in TCFA versus non-TCFA lesions in ACS • Significantly higher MDA-LDL levels in ruptured TCFA versus non-ruptured TCFA in ACS
Teraguchi et al (123)	37 AMI patients	MAGE, circulating monocytes	• Increased MAGE in plaque rupture compared to non-ruptured plaque • Increased circulating monocytes in plaque rupture
Sun et al (117)	81 CAD patients	Neopterin	• Higher levels of Neopterin in non-culprit plaque that have TCFA, smaller FCT and more macrophages

Study	Population	Inflammatory marker	Association with plaque morphology
Inflammatory biomarkers			
Wakabayashi et al (133)	59 ACS patients	Eicosapenatenoic acid/Arachidonic acid	• Low EPA/AA ratio in ACS patients with TCFA
Lee et al (135)	206 SA patients	Troponin	• Elevated cTnI group have frequent TCFAs, a greater lipid arc and longer lipid length
Gu et al (138)	24 ACS patients	Lp-PLA2	• Positive correlation between Lp-PLA2 activity, FCT and plaque volume

Abbreviations: ACS = Acute coronary syndrome, SA= Stable angina, AMI = Acute myocardial infarction, CAD = Coronary artery disease, UA = unstable angina, WBC=white blood cells, TCFA = Thin cap fibroatheroma, CRP=C-reactive protein, IL=interleukin, TNFα=tumor necrosis factor alpha, MPO=myeloperoxidase, MMP= Matrix metalloproteinase, PSGL-1=P-selectin glycoprotein ligand-1, MDA-LDL=malondialdehyde-modified low-density lipoprotein , MAGE= mean amplitude of glycemic excursion, FCT – Fibrous cap thickness, Lp-PLA2=Lipoprotein-associated phospholipase A2 .

Chapter 2. Patients & Methods

2.1 Pilot Data

In our last study we have shown a positive correlation between microparticle (MP) expression and markers of inflammation and myocardial necrosis in patients presenting with acute coronary syndrome (ACS) or stable angina (SA) (140). We have also examined the differences between the levels of MPs in right atrium (RA) and culprit coronary arteries (CO) in affected patients (140). 48 patients were recruited into the study (37 patients presented with ACS and 11 with SA). Blood samples aspirated sequentially from the right atrium (RA) and the coronary artery distal to the culprit lesion (CO) was used to assay AnnexinV+ Microparticle (AnV+MP) by flow cytometry. Markers of inflammation (hs-CRP, IL-6, TNF-α, serum amyloid antigen (SAA) and platelet activation (soluble p-selectin) and Platelet Monocyte Aggregates (PMA) were correlated with total and subtypes of MPs. Similarly indices of microvascualr dysfunction (MvD) ; index of microvascular resistance (IMR) and coronary wedge pressure (Pw) were correlated with MPs. We found that total AnV+MP and individual phenotypes of MPs were higher in the CO and RA compartments of ACS cohort vs. SA (p=0.04). AnV+MP were higher in the STEMI vs. NSTEMI and SA groups. Total and cell specific AnV+MP were higher in the CO vs. RA in the three groups. CD62P+PMA were also higher in ACS than the SA group in both CO (p=0.0003) and RA (p=0.04). Pw, but not IMR, was higher in the ACS vs. the SA group (p= 0.01). Pw and IMR correlated positively with total PMA (r^2=0.3; p=0.009) and (r^2=0.4; p=0.01) for Pw and IMR respectively in ACS; (r^2=0.2; p=0.03 and r^2=0.3; p=0.01) for Pw and IMR respectively in SA. In the ACS group only markers of inflammation and platelet activation correlated with total AnV+MP. In the CO, SAA correlated with total AnV+MP (r=0.5; p=0.01), PMP (r=0.5; p=0.02) and EMP (r=0.6; p=0.005). IL-6 also correlated with total AnV+ MP (r=0.6; p=0.004), EMP (r=0.4, p=0.04) and NMP (r=0.5; p=0.008). In the RA, IL-6 correlated with EMP (r=0.5; p=0.03) and TF+MMP (r=0.6, p=0.01). Troponin T levels correlated with PMP in both CO and RA of the ACS group (r=0.4 for both; p=0.04 and p=0.03 respectively). In the CO s-P-selectin correlated with total AnV+MP (r=0.61; p=0.001), PMP (r=0.6; p=0.002), EMP, (r=0.62; p=0.001) and NMP (r=0.54; p=0.006). CD62P+PMA also correlated

with PMP and EMP (r=0.5; p=0.04 for both). In the RA P-selectin correlated positively with total AnV+MP (r=0.81; p=0.001), PMP (r=0.77; p=0.003), EMP (r=0.78; p=0.002) and TF+MMP (r=0.72; p=0.007). High levels of AnV+MP were noted in the coronary artery of patients with ACS. Levels of AnV+MP correlated with severity of the ischaemic lesion as evidenced by higher MPs detected in the STEMI versus the NSTEMI and SA groups. Markers of platelet activation and inflammation in ACS patients, both at the site of the culprit lesion and in the systemic circulation, strongly correlated with total AnV+, platelet, endothelial, and neutrophil MP (51) (140). These observations warranted a further study to establish if a direct pathogenic link exists between inflammatory and prothrombotic pathways in the pathogenesis of ACS and myocardial necrosis.

The summary of pilot data can be found in Table 4.

Table 4. Summary of pilot data from previous study. Microparticles from right atrial and coronary compartments in acute coronary syndrome and stable angina patients

Clinical Presentation		STEMI (n=22)		NSTEMI (n=14)		Stable Angina (SA) (n=11)	
		Coronary	Right Atrium	Coronary	Right Atrium	Coronary	Right Atrium
Type of Microparticle (MP)	AnV+MP	5.35 X 10^6	1.72 X 10^6	2.85 X 10^6	0.29 X 10^6	2.51 X 10^6	1.1 X 10^6
	PMP	41%(2.19)	49%(0.84)	27%(0.8)	38%(0.11)	28%(0.7)	25%(0.27)
	EMP	13%(0.69)	8%(0.14)	8%(0.22)	10%(0.03)	9%(0.22)	4%(0.04)
	NMP	3%(0.16)	5%(0.09)	3%(0.09)	4%(0.01)	2%(0.05)	1%(0.01)
	TF+MMP	3%(0.16)	5%(0.09)	5%(0.14)	17%(0.05)	3%(0.07)	3%(0.03)
	Other AnV+MP	40%(2.14)	33%(0.6)	37%(1.04)	31%(0.09)	58%(1.4)	67%(0.7)

AnV+ MPs in the CO and RA sites were expressed as medians (10^6) with interquartile range. The absolute number of the total AnV+ MP in the STEMI, NSTEMI and SA groups in the CO and RA sites were 5.3 (3.0-12.0) and 1.7 (0.6-5.7) (p=0.01); 2.8 (1.9-4.5) and 0.3 (0.1-1.2) (p=0.004); 2.5 (0.9-5.7) and 1.1 (0.1-1.5) (p=0.03).*

ACS – Acute Coronary Syndrome, SA – Stable Angina, STEMI – ST segment elevation myocardial infarction, NSTEMI – Non ST segment elevation myocardial infarction, AnV+MP – Annexin V positive MP, PMP – Platelet derived MP, EMP – Endothelial derived MP, TF +MMP – Tissue Factor positive Monocyte derived MP, NMP – Neutrophil derived MP.

2.2 Aims of FOAM study

Several studies using in vitro-generated MPs of different cellular origins support the concept that MPs may modify endothelial function and promote the recruitment of inflammatory cells in the vascular wall, exacerbating the impaired endothelial function and favouring the progression of atherosclerosis. Little is however known about the in vivo role of circulating MPs in the progression of atherosclerosis before an acute event has occurred and their long term dynamics in patients with CAD. To clarify this, we phenotypically characterised MPs, defined their procoagulant activity and explored their association with morphological characteristics of atherosclerotic plaque characteristics detected by OCT in a cohort of subjects presenting with ACS and SA. Ultimately this combined approach may allow us to risk stratify an individual at risk of index and recurrent coronary events.

2.3 Original Hypothesis

MPs act as mediators of plaque instability thus contributing to ACS and have a role as biomarker.

MP expression correlates with features of vulnerable atherosclerotic plaque seen on OCT.

2.4 Experimental details and study design (Figure 7)

"FOAM" is an observational prospective cohort study of patients with CAD presented to the Royal Free Hospital cardiology team. Patients were recruited in 2 groups:

- Group 1 - Patients with ACS
- Group 2 - Patients with SA (control group)

In addition there was a separate control group included for thrombin generation analysis (TGA) only. This group consisted of subjects with no documented CAD.

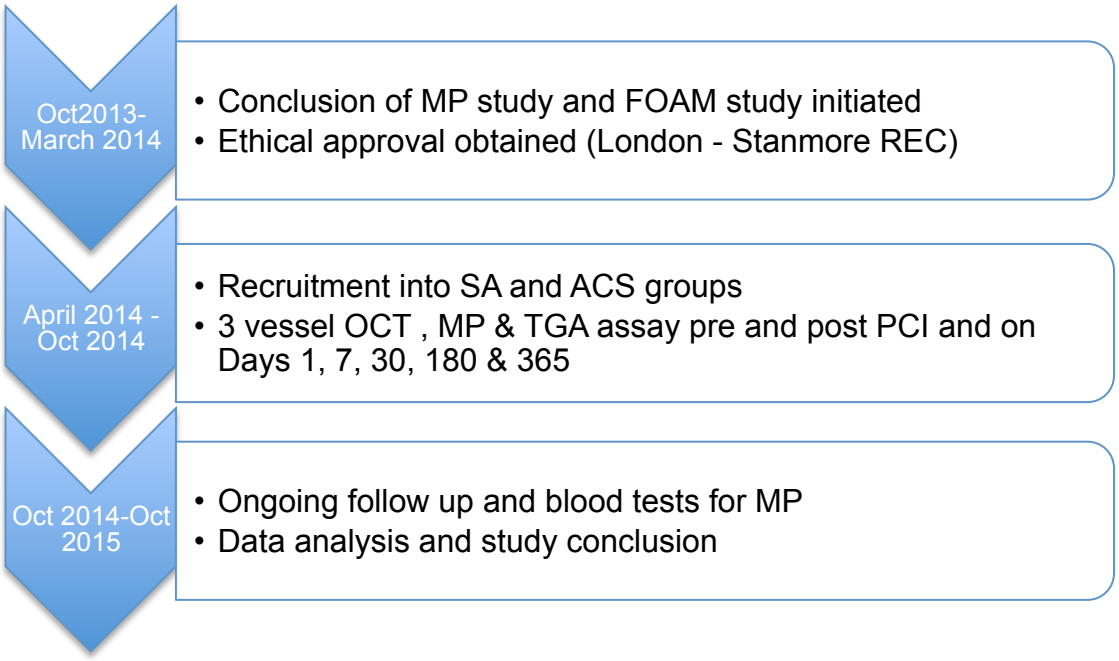


Figure 7. Study flow chart

REC – Research & Ethics Committee

ACS – Acute Coronary Syndrome

SA – Stable Angina

MP – Microparticle

OCT – Optical Coherence Tomography

TGA – Thrombin Generation Assay

PCI – Percutaneous Coronary Intervention

MP panel:

PMP – Platelet derived microparticles

MMP – Monocyte derived microparticles

EMP – Endothelial derived microparticles

TGA – Thrombin generation assay

The referral sources of patient groups were different but rest of the study pathway was similar (Figure 7). Patients in-group 1 were identified following admission to The Royal Free Hospital with ACS. The patients recruited into the ACS group would have been admitted directly to heart attack centre through high risk ACS pathway, referred via medical team by emergency department physicians or admitted through inter hospital transfer pathway. A full clinical examination was carried out, risk factor profile was recorded and global registry of acute coronary events (GRACE) score was calculated(141). On-going medical problems and medications were also recorded. This group of patients underwent baseline investigations with electrocardiogram (ECG) (142), chest X-ray (CXR), blood tests including renal profile, full blood count, cardiac troponin (143) and C-reactive protein (CRP) measurement. All ACS patients underwent coronary angiogram with a view to perform percutaneous coronary intervention (PCI) in a timely fashion (as soon as possible in high risk group and with in 72 hours in intermediate risk group) (144) in addition to medical therapy. Medical therapy included administration of dual antiplatelet therapy (DAPT) (145), high dose HMG co-A reductase inhibitor or statin(146), angiotensin converting enzyme inhibitor and a beta-blocker (147). DAPT included cyclo oxygenase inhibitor Aspirin at loading does of 300 mg orally (PO) followed by 75 mg daily and thienopyrimidine based antiplatelet drugs such as Clopidogrel at a loading dose of 600mg orally followed by 75 mg daily or non-thienopyrimidine drugs such as Ticagrelor at a loading dose of 180 mg orally followed by 90 mg twice daily PO (145,148). In addition low molecular weight heparin (LMWH - Enoxaparin) at a dose of 1mg/kg body weight twice daily– adjusted for renal

function in case of renal impairment or direct thrombin inhibitor such as Fondaparinux at a dose of 2.5 mg OD were also administered as subcutaneous injections till PCI or for 72 hours which ever happened first (149,150).

Patients in-group 2 were identified from out patient clinics diagnosed with stable angina and listed for invasive coronary angiogram with a view to perform PCI. Patients in this group may have undergone non-invasive ischemia testing by stress echo, myocardial perfusion scan, cardiac magnetic resonance imaging or anatomical imaging with CT coronary angiogram prior to being listed for PCI.

The control group for TGA analysis was randomly selected subjects in the hospital. These were age matched but there was no documented history of CAD.

Informed consent was obtained from all participants prior to participation in the study. Blood was collected for MP analysis and TGA prior to PCI. At the time of PCI OCT of all three coronary arteries was performed where feasible. Following PCI patients from both groups were discharged home with subsequent appointments for follow up. Further blood tests for MP and their TGA were done immediately post PCI and on days 1,7,30 and180 post PCI. This was similar in both the groups and the day 180 visit was defined as end of study for that particular subject.

In total 65 patients were screened and 25 recruited across both groups. 40 patients were excluded as either they had normal coronary arteries, had CAD with minimal luminal obstruction thus not requiring any intervention or required surgical revascuarisation.

2.5 Inclusion Criteria

- Male or Female, aged 20 years or above and below 80 years of age.
- Diagnosed with ACS or SA and requiring invasive coronary angiogram and PCI.
- Patients with denovo CAD.

2.6 Exclusion Criteria

- Unable to consent
- Patients who underwent CABG in the past
- Those with renal failure (eGFR<50)

2.7 Power calculations

Assuming an 80% power, and a 5% Type I error, with a Bonferroni correction to allow for three primary analyses (i.e. using a cut-off of 0.017) it was recommended to recruit 20 participants in the Stable angina group (group 2) and 50 participants to the ACS group (group 1). Using data from the referenced study (151) to provide assumed effect sizes for the control (angina) group; this means that the study has sufficient power to be able to detect:

- An increase in Lipid arc of 35 degrees or greater (assuming 120 degrees (SD 40 degrees) in group 1)
- An increase of 1.75mm in lipid length or greater (assuming 3.7 mm (SD 2 mm) in group 1)
- An increase in lipid volume index of 295 or greater (assuming 500 (SD 400) in group 1)

Differences of these magnitudes were thought to reflect clinically important effect sizes. These sample size estimates were calculated using the 'sampsi' command in Stata v13.1.

For the three primary MP (PMP, MMP, and EMP) markers, it is anticipated that they are normally distributed, as has been found previously. Based on previous study the above sample size would be sufficient to detect differences in the levels of MPs between both groups. However, plotting the distribution of these markers and assessing their distribution visually will check this assumption. Assuming the assumption holds, these three markers will be compared between groups using an unpaired t-test (Mann Whitney U test)

Secondary outcomes of neovascularisation, macrophages, thin cap fibroatheroma (TCFA), thrombus, minimal luminal area and minimal lumen

diameter (MLA, MLD) and plaque rupture were compared between the groups similarly.

2.8 Data analysis

2.8.1 MP assay

Data were analyzed using FlowJo (Treestar Inc, Ashland, OR). Further details are elaborated in detailed methodology.

2.8.2 OCT analysis

All OCT pullbacks were analyzed frame by frame (every 1mm) by using QCU CMS software**.** Plaque tagging was carried out by new, innovative and automated software (QCU CMS developed by Jouke Dijkstra at University of Leiden, Netherlands) with abilities for manual correction. As such only minor adjustments are required regarding cap thickness and borders. Two independent clinicians carried out the analysis and then any differences were agreed by consensus. No separate intra and inter observer variability was looked for. All measurements were as per consensus report published by International Working Group for Intravascular Optical Coherence Tomography Standardization and Validation. Two independent experienced observers analyzed the OCT images using validated criteria for plaque characterization (Drs Sudheer Koganti & Antonis Karanasos under the supervision of Dr Evelyn Regar) according to international intra vascular OCT working group consensus document (152) (Figures 8-17). Discordance between observers was resolved by taking a consensus reading. Signal-poor lesions with poorly delineated borders on OCT images were identified as lipid core and signal-rich homogeneous lesions overlying a lipid core as a fibrous cap. The thinnest part of a fibrous cap was measured 2 times, and the average was defined as fibrous cap thickness. The arc of a lipid core on cross-sectional OCT images was measured and semiquantified according to the number of involved quadrants. When a lipid core comprised > 2 quadrants, it was deemed to be lipid-rich plaque. We defined thin cap fibroatheroma (TCFA) as a lipid rich plaque with the thinnest part of the fibrous cap measuring < 65 **µ**m. Plaque rupture was defined as a presence of fibrous-cap discontinuity (fig 12) and a cavity formation

in the plaque. In order not to confuse with any edge or other dissections cavity extending more than 5 individual frames (5mm) was classed as rupture. Calcific plaques were defined as heterogeneous areas that are signal-poor with a sharply delineated border. Calcific nodule was defined as regions of calcium that protrude into the lumen. Intracoronary thrombus was identified as a mass protruding into the vessel lumen from the surface of the vessel wall. Cholesterol crystals were identified as thin & linear areas of high intensity with in a fibrous cap or necrotic core. Macrophages were identified as signal-rich, distinct, or confluent punctate regions in a fibroatheroma. Macrophages should only be evaluated in the context of a fibroatheroma, as no macrophage validation studies have been reported to date on normal vessel wall or pathological intimal thickening (PIT). Therefore Macrophages were only evaluated in the context of a fibroatheroma either with thick or thin cap. Neovessels were identified as signal poor tubuloluminal regions present in the adventitia and either in the thickened intima or plaque. Qualitative measurements such as lipid and calcific plaque volumes were calculated as product of average arc and length. The plaque volumes were further adjusted for analyzed arterial length to obtain relative volumes. Full list of OCT parameters analyzed are enlisted in Table 5. OCT images showing various plaque characteristics from study participants and segmenting models are shown in Figures 8-17.

Table 5. Summary of OCT parameters recorded

Lipid maximum arc	**(0-360$^{0)}$**
Lipid average arc	**(0-360$^{0)}$**
Lipid Length	**mm**
Lipid volume	**Average arc X lipid length**
Relative lipid volume	**Lipid volume/analyzed arterial length**
Neovascularisation	**% frames**
Macrophages	**%frames**
Cholesterol crystals	**% frames**
Calcium max arc	**(0-360$^{0)}$**
Calcium average arc	**(0-360$^{0)}$**
Calcium Length	**mm**
Calcium volume	**Average arc X calcium length**
Calcium relative volume	**Calcium volume/analyzed arterial length**
TCFA -	**<_ 65 (in µm)**
Calcific nodule	**% frames**
Thrombus –	**% frames**
Plaque rupture –	**present or absent**
Fibrous cap thickness	**in µm**

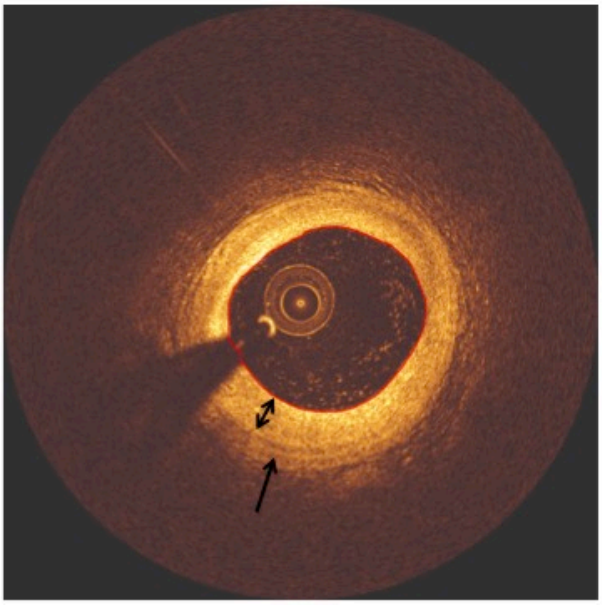


Figure 8. Typical example of pathological intimal thickening. Single headed arrow shows dark band of media. Double headed arrow shows layer of intima that is several hundred microns in thickness.

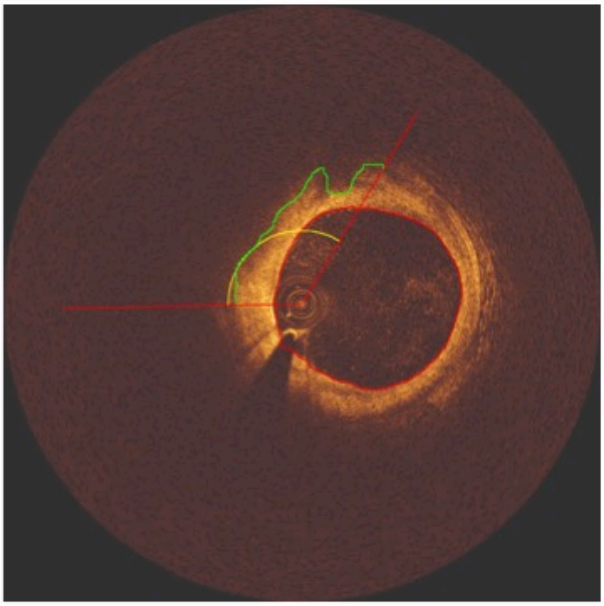


Figure 9. Image depicts segmentation of lipid rich Plaque. The plaque extends between red Lines which is also the arc. The green line indicates the fibrous cap thickness with the smallest measurement near 12 o'clock.

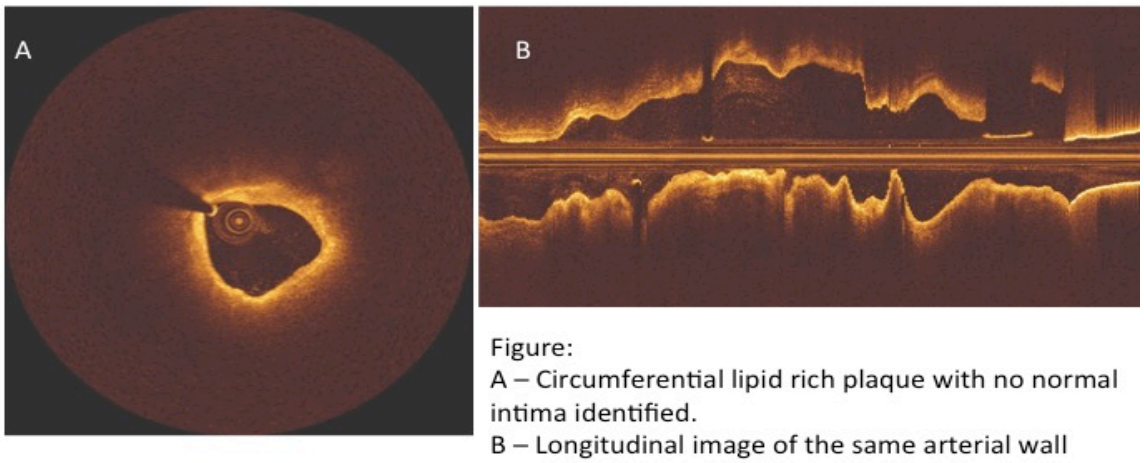


Figure 10. Lipid rich plaque

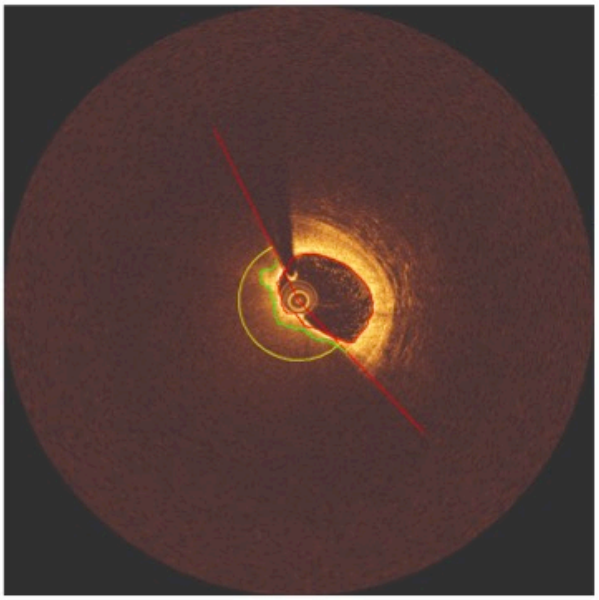


Figure 11. A classic example of thin cap fibroatheroma. Here the cap thickness was 29 microns. Red line is the arterial lumen and green line is the cap thickness. The minimal distance between two lines was calculated as fibrous cap thickness.

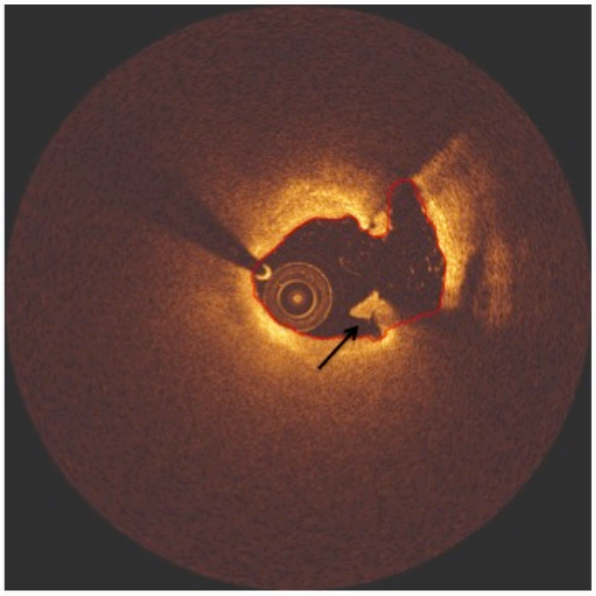


Figure 12. A classic example of plaque rupture in a lipid rich plaque. False lumen following rupture extends from 1 o'clock to 4 o'clock position. Superimposed thrombus is also seen (black arrow).

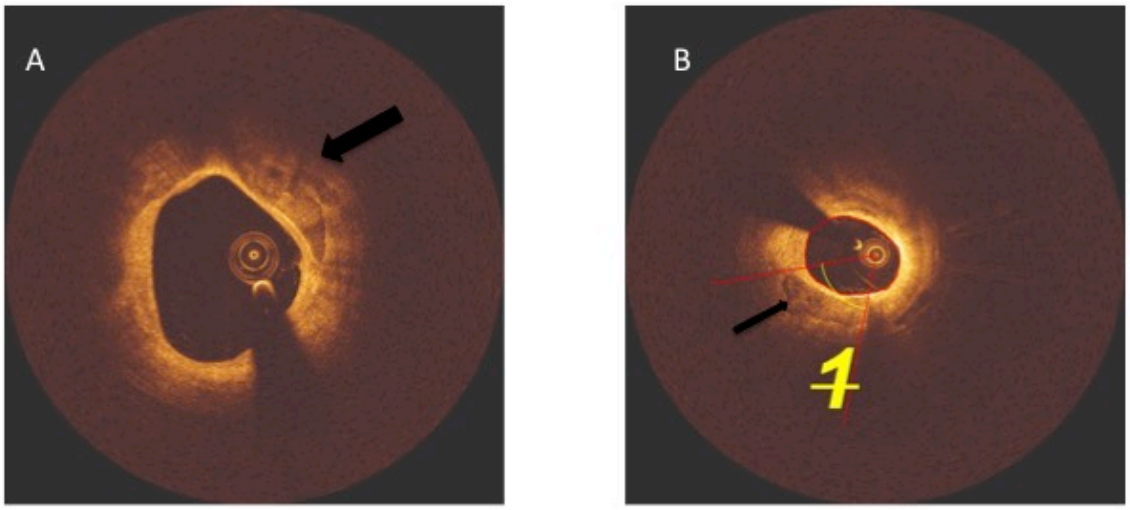


Figure 13. Figure depicting calcific plaque. A - Fibrocalcific plaque extending from 8 o'clock till 4 o'clock position. Black arrow points to dense calcium. B - Circumferential fibrocalcific plaque with dense calcium indicated by black arrow. Red lines indicate the arc of calcium and the method of segmenting.

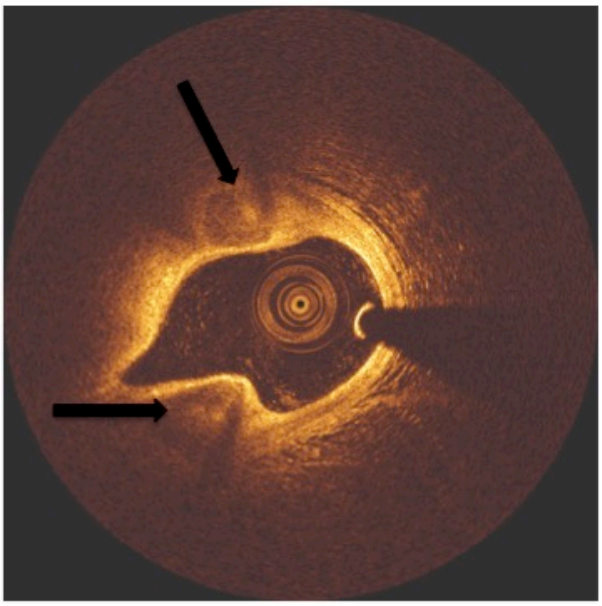


Figure 14. A good example of calcific nodule (black arrows) which accounts for culprit lesion in about 5-10% of acute coronary syndromes

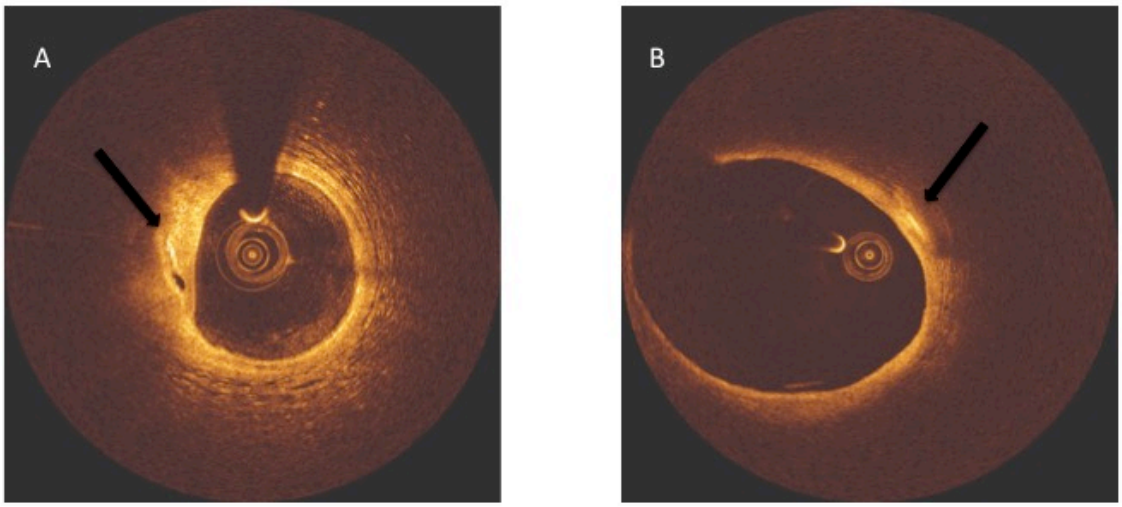


Figure 15. Figure depicting bright spots in the intima of coronary artery. A - Black arrow points to bright shadow with in lipid rich plaque - this is likely to be either dense macrophages or cholesterol crystals and almost certainly cholesterol crystal in image B (black arrow).

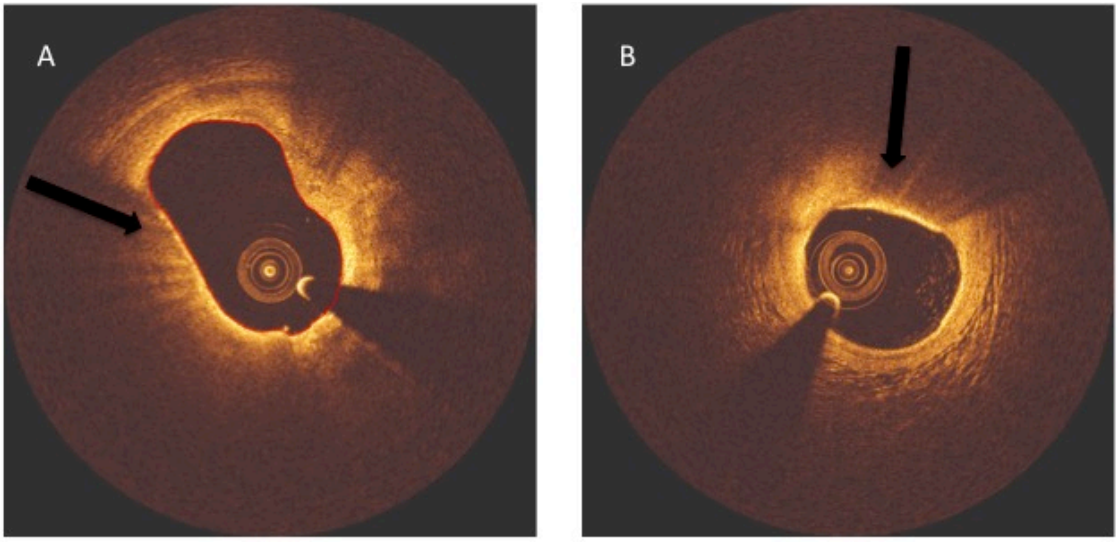


Figure 16. Black arrows in images A & B are highlighting macrophages. This appearance is classic similar to that described in literature.

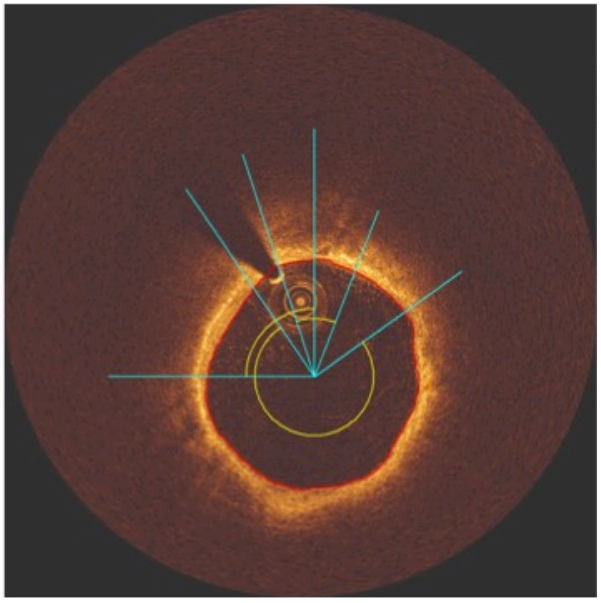


Figure 17. Typical example of how the plaque was segmented. This particular example once again shows thick layer of macrophages in a lipid rich plaque

2.9 Clinical benefits for patients

This study will provide an additional insight into the pathophysiology of coronary atherosclerotic plaque rupture and help us explore the role of MPs in plaque instability. This additional insight may be helpful eventually in identifying the vulnerable patient so that therapeutic avenues aimed to combat the deleterious biological effects of MP can be found. Whilst this is still early we anticipate the

therapeutic avenues may be in the form of intensifying secondary prevention therapy, close follow up and the possibility of targeted therapy directed at MPs.

2.10 Methodology in detail

2.10.1 MP assessment

Immediately prior to the PCI procedure whole blood was collected from a large antecubital peripheral vein using an 18 G needle and stored in 3.2% buffered citrate bottles. Blood was also collected from the aorta at the time of coronary angiography and post PCI. For the purpose of consistency and to minimise shear stress related platelet activation catheters with similar inner diameter were used (5 Fr Judkin's right 4 catheter by Cordis™ or optitorque catheter by Terumo corporation™ – both have an inner diameter of 0.11cm). Administering anticoagulants such as Heparin is mandatory prior to any PCI procedure where as administering glycoprotein IIb/IIIa inhibitors is decided on case-by-case approach (153,154). Our experience showed that anticoagulants interfere with the TGA. To prevent this pre PCI blood samples were collected prior to administration of any systemic anticoagulants. In ACS group all efforts were made where clinically possible to maintain a 12-hour delay from the last dose of low molecular weight heparin administration to aspiration of blood so that there are no errors in interpreting thrombogenic assay. PCI was performed according to standard procedures following collection of samples. DAPT with Aspirin and Clopidogrel or Ticagrelor was administered according to the hospital protocol. In addition ACS patients received LMWH as per the hospital protocol (see above). LMWH was not given to patients with stable angina. TGA was not interpretable on immediately post PCI blood samples for obvious reasons and hence not included in the analysis.

The whole blood (2 X 5mls) collected into 3.2% buffered citrate (Becton Dickinson) bottles was centrifuged to obtain platelet poor plasma (PPP). This process was completed with in 2 hours of collection of blood to prevent haemolysis. Centrifugation was carried out twice at 5000g for 5 minutes each. PPP was then stored at –80 ^{0} C for batch analysis. On the day of analysis PPP was thawed rapidly in a 37 ^{0}C water bath. 250µl of PPP was then centrifuged at 17000 g for 60 minutes and the supernatant decanted to obtain the

microparticle pellet. The MP were then reconstituted in AnV binding buffer (BD PharMingen, Oxford, United Kingdom), divided into 35 µl aliquots and plated onto the wells of a 96 well U-bottomed plate prior to staining with AnV and other monoclonal antibodies.

2.10.2 Labelling of microparticles with annexin V and monoclonal antibodies

The labelling and quantification of MP was achieved as follows: 5 µl of a 1 in 5 dilution Annexin V conjugated with fluorescein isothiocyanate (FITC; BD Pharmingen) or -phycoerythrin (PE; BD Pharmingen) or PECy5·5 (BD, Pharmingen) were added to the wells in a 96 well U bottomed plate as per plate plan (Plate plan is shown in Figure 24). In addition, antibodies against platelet, endothelial, neutrophil and monocyte surface markers conjugated to red (PE) or far-red (PerCP-Cy5) fluorochromes were used to identify MP of different cell origin. Platelet markers examined were the constitutively expressed platelet marker CD42a (mouse IgG1 anti-human CD42a-PerCP-Cy5, BD Pharmingen), Endothelial surface markers examined were E-selectin (mouse IgG1 anti-human CD62E-PE BD Pharmingen); CD105 (“Endoglin”; mouse anti-human IgG1 CD105-PE, BD Pharmingen); ICAM-1 (mouse IgG1 anti-human CD54, BD Pharmingen); and PECAM-1 (mouse IgG1 anti-human CD31-PE, BD Pharmingen). Neutrophil surface marker examined was CD66b (mouse IgG1 anti-human CD66b-PE, BD Pharmingen). Monocyte surface markers examined were lipopolysaccharide receptor (LPS-R) (mouse IgG1anti-human CD14-PerCP- Cy5, AbD Serotec) and to assess tissue factor (TF) expression on monocyte MPs, samples stained with mouse PE labeled anti-human CD142 (clone ME52, BD Pharmingen) were additionally incubated with mouse (FITC–labeled anti-human TF (clone VD8, American Diagnostica, USA). All antibodies were used at final dilutions listed in table 2-1. MPs were incubated with the labeled antibodies and annexin V for 20 minutes at room temperature in the dark with gentle shaking. The incubation was then terminated by adding 200 µl of annexin V buffer to each well, with the plate immediately transferred for flow cytometry. The plate plans used for MP detection are shown in Table 6. All samples were additionally stained with the constitutively expressed platelet marker CD42a (mouse IgG1 anti-human CD42a-PERCP, BD Pharmingen) to

exclude a platelet origin for these MPs as well as the relevant isotype controls (as per manufacturer recommendation).

2.10.3 Flow cytometric analysis of microparticles

To establish the limits of detection & optimize the gating strategy standard latex beads size of 1.1 µm & 3 µm (Sigma) were used. All analysis was performed on a FACS calibur flow cytometer (Becton Dickinson). To obtain optimal forward and side scatter instrument settings for MP 1.1 µm latex beads (Sigma) were run and logarithmic forward and side scatter plots obtained. Gates were then set to include particles less than approximately 1.1µm, but to exclude the first forward scatter channel containing maximal noise. Optimal compensation was set for green, red and far-red fluorescence. Specific binding for each antibody was determined using isotype control antibodies with equal protein: fluorochrome ratios, and at the same final dilution as per manufacturer recommendation. Since annexin V is a protein and not an antibody (and hence no isotype control antibody exists), the threshold for annexin V binding was determined by using the fluorescence threshold established for MP in the absence of labeled annexin V. Particles less than 1.1 µm in size and binding annexin V were then selected. Platelet derived MPs were defined as particles < 1.1 µm in size, staining for CD42a+/AnV+. Endothelial derived MPs were defined as CD62E+/AnV+/CD42a-, CD54+/AnV+/CD42a-, CD106+/AnV+/CD42a- or CD31+/AnV+/CD42a- MPs. Neutrophil MPs were defined as CD66b+/AnV+/CD42a- MPs. Smooth muscle MPs were defined as NG2+/AnV+/CD42a- and lastly monocyte derived MPs baring TF were identified as TF+/AnV+/CD14+ MPs. MP samples were run at medium flow rate with a cut-off time of 30 seconds, which resulted in capture of approximately 200,000 gated bead events. A representative set of flow cytometric plots, and the gating protocol are shown in figure 18-19 below. Data were analysed using FlowJo (Treestar Inc, Ashland, OR).

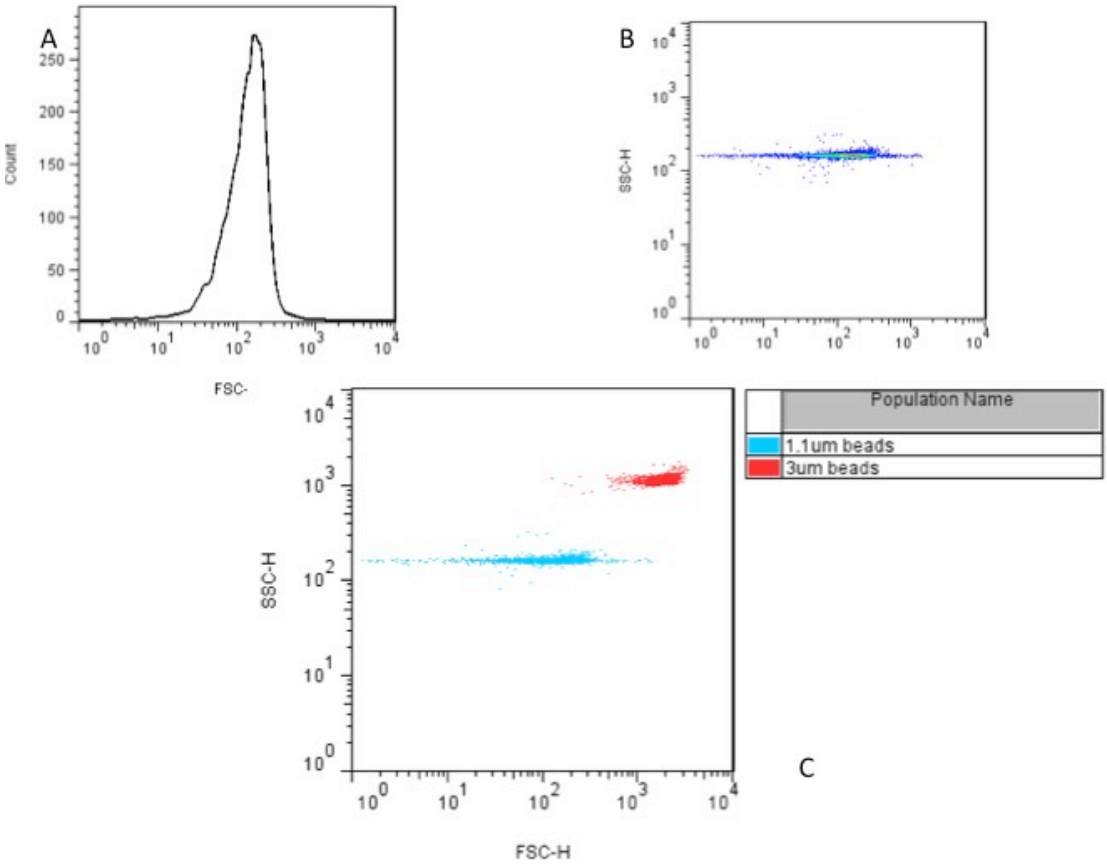


Figure 18. MP gating strategy using latex beads.

A - Histogram of 1.1µm beads on the forward scatter scale.

B - 1.1µm beads on flow cytometry. Data is presented as side scatter (particle granularity) vs. forward scatter (particle size).

C - 1.1µm and 3 µm beads on flow cytometry. Data is presented as side scatter (particle granularity) vs. forward scatter (particle size) and nicely shows two different bead populations. 1.1µm beads are used for gating, 3 µm beads are also concurrently run at the time of flow cytometry to determine absolute number of MPs/ml of plasma.

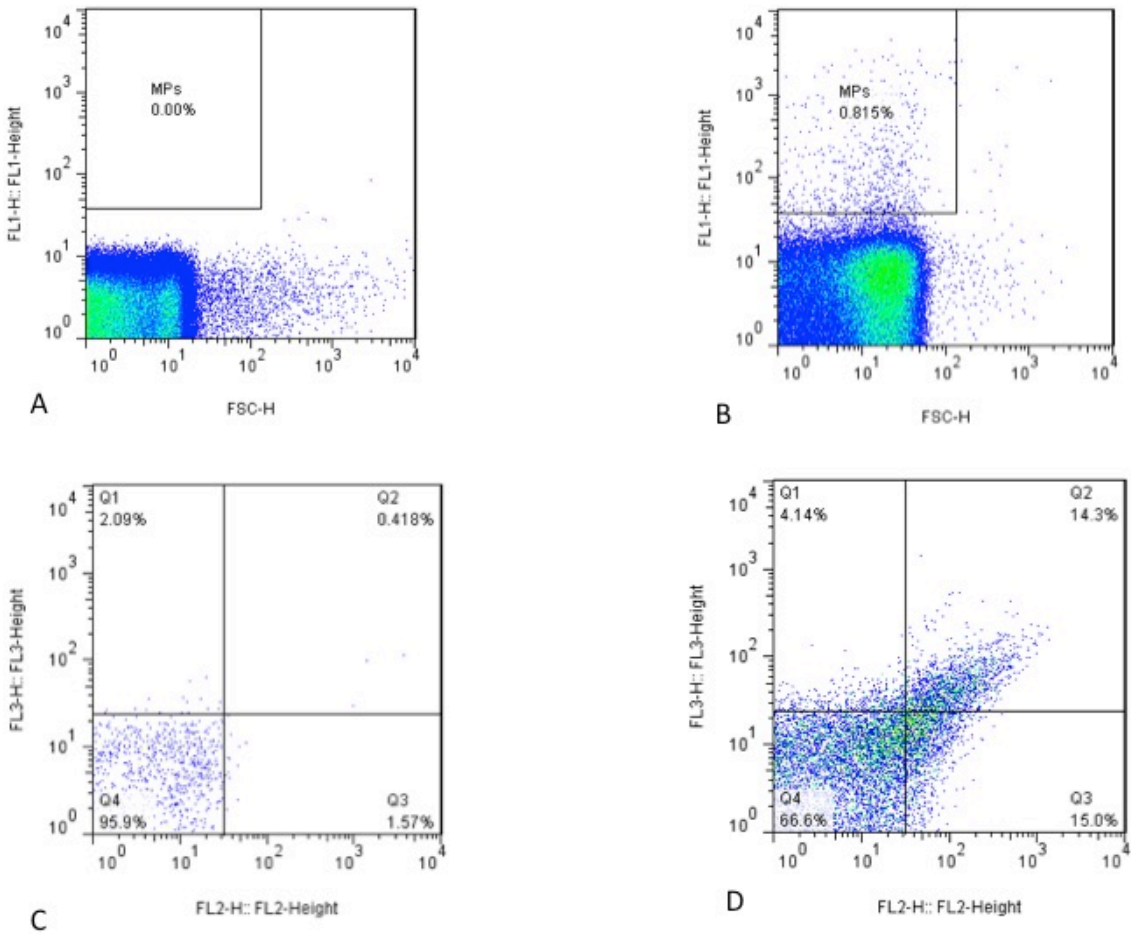


Figure 19. Flow cytometric analysis of annexin V+ MPs from platelet poor plasma (PPP) of a patient with Non ST elevation myocardial infarction (NSTEMI). These are post intervention samples from a peripheral vein.

A - Events shown here are not stained with annexin V and serve as control. This, in combination with 1.1µm latex beads is used to determine the gating of annexin V+ MPs.

B - Annexin V+ MPs acquired from PPP of a patient with NSTEMI.

C - IgG1k isotype control.

D - Figure depicting CD31+MPs.

2.10.4 Determination of absolute microparticle number per ml of plasma

To convert flow cytometer counts to an estimate of the number of MP per ml of plasma, a predetermined number (always 200000, calculated as per manufacturer recommendations) of 3 µm latex beads (Sigma) were run concurrently with the microparticle samples. The absolute number of annexin V binding microparticles per ml of plasma was then determined by using the proportion of beads counted and the exact volume of plasma from which the

microparticles were analysed, as described by Brogan et al.(155) (Figure 20). The following equation was thus derived to convert flow:-

Total number of MP/ ml of plasma =

(200 000 / no. of beads counted) X (no. of MP counted per well) X n / Number of mls of plasma (usually 200 µl***)***

n=The number of wells into which the sample was divided

no of beads counted = Usually 7000-9000 beads

Number of beads added per well = 200000

Figure 20. Brogan formula of calculating MP number from flow cytometer counts

2.10.5 Microparticle mediated thrombin generation assay (TGA) (30)

Blood was collected, centrifuged and stored as described above for MP. On the day of analysis PPP was thawed rapidly at room temperature and the centrifuged at 17,000 G for 60 minutes. The obtained MP pellet was resuspended in 200 µL normal pooled MPFP containing 30µg mL^{-1} corn trypsin inhibitor (CTI) to inhibit contact activation. Pooled MPFP was prepared as a batch from approximately 50 mL freshly drawn blood from healthy volunteers. Subsequently 40 µL of MPs suspended in control MPFP was added to the plate well, followed by 50 µL of calcium-fluorogenic substrate (0.5 mM/L of Z-G-G-R-AMC and 7.5 mM/L of calcium final reagent concentrations, Pathway Diagnostics). No exogenous TF or phospholipids were added at this stage to ensure that the thrombin generated was solely dependent on MP related coagulant activity. The thrombin generated was measured by fluorogenic excitation/ emission at 360/460 nm at 1minute time intervals for 60 minutes against a standard thrombin calibrator (Pathway Diagnostics) in an Optima fluorescence plate reader (BMG). Measures recorded were: (i) height of peak thrombin (nM); (ii) lag time (time to onset of thrombin generation); (iii) velocity index = rate of thrombin generation; and (iv) endogenous thrombin potential (ETP) = the area under the curve (Figure 21).

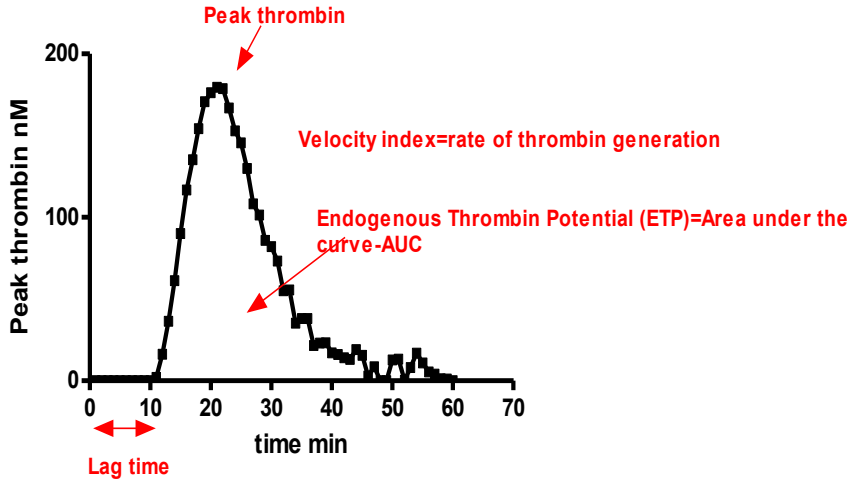


Figure 21. Typical TGA curves

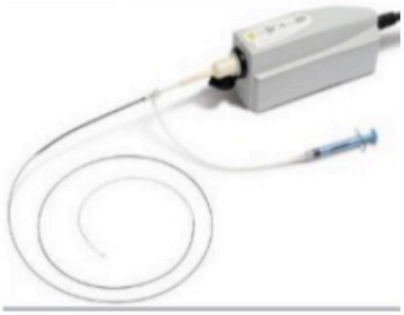
Thrombin generation assay curves.

Measures recorded are (i) peak height, in nM thrombin, (ii) lag time (time to onset of thrombin generation), (iii) velocity index = rate of thrombin generation, and (iv) endogenous thrombin potential (ETP) referring to the area under the curve.

2.10.6 2. OCT Assessment

Pre-procedure preparation is similar to the MP aspect of the study. Coronary angiography followed by PCI is performed via trans-radial or trans-femoral route. Upon identifying the culprit lesion a guiding catheter was used to engage the coronary ostia. Inadequate guide catheter support and poor engagement can result in poor OCT pullbacks due to flush back of contrast into aorta. To prevent this a guide catheter that provided adequate support and selective engagement of coronary ostia with minimal flush back was used. Heparin at a dose of 70 international units/kg was administered prior to introduction of guide wire into the coronary artery. Once the guide wire was introduced a prepared OCT catheter (Dragon Fly ™, St Jude Medical) (Figure 22) mounted on the guide wire and passed beyond the culprit lesion. Once in position OCT catheter was gently purged with 3mls of contrast to clear the blood. OCT catheter was positioned and manoeuvred under X-ray screening. Following this OCT catheter was withdrawn using automatic or manual pullback with intermittent flushing recording high quality infrared images of the artery. We always carried out 75mm pullbacks with a view to capture as much information as possible in single pullback rather than conventional 54 mm pullbacks.

Following this PCI was carried out as deemed appropriate by the operator. Once the PCI procedure was completed OCT pullbacks of rest of the coronary tree was completed. OCT was not performed in non-dominant coronary trees. Balloon predilatation of the culprit lesion was performed where the preprocedural thrombolysis in myocardial infarction (TIMI) grade flow was less than 3.



A: Dragonfly OCT catheter and docking station

B: OCT console

Figure 22. OCT equipment. Dragonfly catheter is attached to docking station after purging with normal saline. The docking station is wired to OCT console and is enabled to acquire rapid images.

Table 6. 96 – Well U-bottom plate plan used for MP analysis

	Unstained	CD105 42a	CD62P 42a	CD62E 42a	CD66b 42a	CD54 42a	CD31 42a	TF 14	NG2 42a	Total FITC	Total PE	PEcy5
V1												
A1												
A2												
V2												
V3										IgG1PE		
V4										IgG1KPE		
V5										IgG1FITC		3µ beads
V6										IgG1PECy5		1.1µbeads

Table 7. Cell specific flourochrome conjugated antibodies and other reagents used for MP analysis

Specificity	Isotype	Conjugate	Company	Clone	Dilution
CD42a	Mouse IgG1k	PERCP-Cy5	BD PharMingen	Beb1	1/50
CD62E	Mouse IgG1k	PE	BD PharMingen	68-5H11	1/50
CD54	Mouse IgG1k	PE	BD PharMingen	HA58	1/50
CD105	Mouse IgG1k	PE	BD PharMingen	266	1/50
CD31	Mouse IgG1k	PE	BD PharMingen	WM59	1/50
CD62P	Mouse IgG1k	PE	BD PharMingen	AK-4	1/50
CD66b	Mouse IgG1k	PE	BD PharMingen	G10F5	1/50
CD14	Mouse IgG1k	PE-Cy5	AbD Serotec	61D3	1/50
TF/CD142	IgG1	FITC	Sekisui Diagnostics	V1C7	1/50
NG2	IgG1	PE	R&DSystems	11711	1/50
Isotype controls					
	IgG1	PE	BD PharMingen	MOPC21	1/50
	IgG1	PECy5	BD PharMingen	MOPC21	1/50
	IgG1	FITC	BD PharMingen	MOPC21	1/50
	IgG1k	PE	BD PharMingen	MOPC21	1/50
Annexin V		FITC	BD PharMingen		

Other reagents:

Annexin – V binding buffer - BD Pharmingen

Latex beads (Sigma 3μ - LB 30 & 1μ - LB 11 beads)

Other equipment:

96 – well U-bottom plate (Greniner, polypropylene, cat 650201)

Chapter 3. Optical Coherence Tomographic Characterisation of Coronary Atherosclerosis in a Cohort of Patients with Non-ST Elevation Acute Coronary Syndrome and Stable Angina: Analysis on Patient and Artery Level

3.1 Summary

Introduction and aims

Previous studies have reported presence of multiple complex coronary plaques in the context of acute myocardial infarction. The aim of this study was to characterise morphological differences in atherosclerosis of the entire coronary tree by intravascular optical coherence tomography (OCT) in patients with non-ST elevation acute coronary syndrome (NSTEACS) and stable angina (SA) in contemporary clinical practice.

Methods

25 patients (13 NSTEACS & 12 SA) with coronary artery disease requiring percutaneous coronary intervention were prospectively enrolled. OCT of the whole coronary tree was carried out where feasible.

Results

OCT pullbacks were performed in 66 out of 75 coronary arteries. Patient-level analysis revealed higher lipid burden in NSTEACS cohort when compared to SA cohort as evidenced by statistically significant longer lipid length, wider lipid arc and higher lipid volume. Artery-level analysis revealed culprit coronary arteries in NSTEACS cohort to have lower cap thickness (Median 77 μm IQR (51,141) versus138 μm (91,126); p=0.01)) and non-culprit coronary (NC) arteries in NSTEACS to have higher lipid burden when compared to non-culprit arteries in SA cohort. Ruptured plaques in NC arteries were noted in 50% of NSTEACS patients and 23% SA patients (p=0.67). Thin cap fibroatheromas not

related to culprit lesion were present in 33% of NSTEACS and 23.3 % of patients with SA (p=0.45).

Conclusions

In this contemporary study NSTEACS patients had more widespread lipid-rich plaque disease both in culprit and non-culprit arteries. These findings warrant aggressive individualised secondary prevention approach.

3.2 Introduction and aims

Coronary artery disease (CAD) is an inflammatory process affecting the whole coronary artery tree (156). Previous intracoronary imaging studies revealed culprit plaques in acute coronary syndrome (ACS) to be distinct from non-culprit plaques with more unstable features, and non culprit (NC) plaques in ACS patients to have more vulnerable features when compared to non-culprit plaques in stable angina (SA) (157,158). Other imaging studies revealed detectable ruptured plaques and intracoronary thrombus in stable angina patients(159). This suggests that coronary events do occur silently in a proportion of patients with no apparent clinical manifestation. However, most of these comparisons have focused in focal lesions, not examining the entirety of the coronary tree and the morphology of angiographically unapparent lesions, thus not capturing the true extent of atherosclerosis in combination with its complexity (160). I sought to investigate potential differences in the extent and morphology of atherosclerosis by optical coherence tomography OCT in culprit and non-culprit segments of the coronary arteries between patients with NSTEACS and SA.

3.3 Patients and methods

3.3.1 Study Population

A total of 65 patients were screened and 25 were prospectively enrolled for a three-vessel OCT study. Patients with NSTEACS or SA and requiring invasive treatment were included in the study. Patients with prior coronary artery bypass graft surgery (CABG), renal failure with estimated glomerular filtration rate < 50

and those that were not able to consent were excluded. The NSTEACS group included patients with non-ST elevation–myocardial infarction (NSTEMI) and unstable angina (UA). NSTEMI was defined as ischemic symptoms with elevated cardiac Troponin T (TnT) and with or without ischaemic changes on the ECG. UA was defined as angina at rest without any elevation in TnT. SA was defined as chest pain on exertion with or without positive functional or anatomical testing. Out of 12 patients with SA, 4 patients underwent prior CT coronary angiography before proceeding to angiography and 8 patients proceeded directly to angiography based on history. Ethical approval was given by London – Stanmore research ethics committee.

3.3.2 Acquisition of OCT Images

Coronary angiography followed by percutaneous coronary intervention (PCI) was performed via trans-radial or trans-femoral route. Patients who did not have to proceed to PCI following coronary angiography were screened out. In those who required PCI, OCT of the culprit artery was carried out first followed by PCI of the culprit artery, and then by OCT of the non-culprit arteries. All OCT studies were carried out on ILUMIEN™ OPTIS™ system using Dragon Fly™ catheter (St Jude Medical, St Paul, Minnesota, USA). Culprit lesion was determined based on ECG localisation and angiographic appearances. Attempts were made to carry out 75 mm pullbacks in all arteries to capture as much information as possible in single pullback, with the exclusion of non-dominant small calibre coronary arteries. In cases, where the imaged length did not capture the entirety of the information, repeat pullbacks were performed. All images were anonymised and digitally stored for batch analysis.

3.3.3 OCT Data Analysis

All OCT pullbacks were analyzed frame by frame (every 1mm) by using QCU CMS software. Two independent experienced observers analyzed the OCT images using validated criteria for plaque characterization according to international intravascular OCT working group consensus document(83). Segments of OCT data that were non-interpretable due to artifacts or poor image quality were excluded from analysis. Discordance between observers was resolved by taking a consensus reading. Care was taken to analyze left

main stem (LMS) only once if it was recorded in both left anterior descending (LAD) and left circumflex (LCX) pullbacks. In principle the LAD pullback was used to analyze the morphology of LMS. Lipid core and calcium were recorded and their corresponding arc (in º) was measured. Measurements included mean and maximum lipid core and calcium arc, and also relative volumetric indexes of necrotic core and calcium content. When a lipid core comprised ≥90°, it was deemed to be lipid-rich plaque.

In cases of lipid core, the overlying cap was measured over its entire surface by a semi-automated algorithm, with subsequent manual corrections if necessary. The minimum and average cap thicknesses were then calculated. We defined thin cap fibroatheroma (TCFA) as a lipid rich plaque with the thinnest part of the fibrous cap measuring < 65 µm. Presence of macrophages, plaque rupture, and thrombus was also recorded. Ruptured plaque was defined as fibrous cap discontinuity and cavity formation in the plaque. Calcific plaques were defined as heterogeneous areas that are signal-poor with a sharply delineated border. Calcific nodule was defined as regions of calcium that protrude into the lumen. Intracoronary thrombus was identified as a mass protruding into the vessel lumen from the surface of the vessel wall. Cholesterol crystals were identified as thin & linear areas of high intensity with in a fibrous cap or necrotic core. Macrophages were identified as signal-rich, distinct, or confluent punctate regions in a fibroatheroma. Neovessels were identified as signal-poor tubuloluminal regions present in the adventitia and either in the thickened intima or plaque. Qualitative measurements such as lipid and calcific plaque volumes were calculated as product of average arc and length. The plaque volumes were further adjusted for analyzed arterial length to obtain relative volumes. Full list of OCT parameters analyzed are enlisted in appendix.

3.3.4 Statistical Analysis

An independent statistician performed all statistical analysis. Normal data is presented as mean ± SD.Non-parametric data are reported as median and interquartile range (IQR). Categorical variables were compared with chi-square or Fisher's exact test, as appropriate.

The OCT variables were measured in each of the three arteries for each patient. These values were compared between the two patient groups. Due to the skewed distribution of most OCT variables the comparisons were performed using the Mann-Whitney test. Non-culprit arteries between ACS and SA were also compared using Mann-Whitney test. Additionally, Wilcoxon matched-pairs test was used to compare between culprit and non-culprit values.

3.4 Results

3.4.1 Patient characteristics

Majority of the patients across both groups were of male sex. Patients in NSTEACS group were more often on statins. Both groups were evenly matched on other variables (Table-8). All 25 patients underwent angiography with 23 undergoing PCI. One patient in SA group did not undergo PCI as fractional flow reserve (FFR) in the LAD was 0.84. One patient from ACS group was referred for CABG based on positive FFR data in LAD with a culprit lesion in the LCX. Two other patients underwent FFR guided PCI in the SA group.

Table 8. Baseline characteristics

	ACS (n=13)	SA (n=12)	p value
Age (mean ± SD)	60.1 ± 8.14	56.6±8.9	0.32
Male (n, %)	12 (92.3)	11 (91.7)	0.79
Diabetes (n, %)	5 (38.4)	2 (16.7)	0.67
Hypertension (n, %)	5 (38.5)	4 (33.3)	1
Hyperlipidaemia (n, %)	12 (92.3)	8 (67)	0.15
Smoking (n, %)	3 (23.1)	4 (33.3)	0.4
Family History of CAD (n, %)	1 (7.7)	3 (25)	0.65
Previous PCI (n, %)	2 (15.3)	0	0.15
Creatinine (Median, IQR)	82.5 (76.25 – 88.50)	84 (75.2-87.5)	0.6
Troponin (Median, IQR)	0.24 (0.2 - 3.8)		
CRP (Median, IQR)	4 (1 - 6.25)		
LVEF (mean ± SD)	58.3±5.3	57.8±8.5	0.6
Medications:			
ACE- I / ARB (n, %)	3 (23.1)	4 (33.3)	0.82
Beta-blockers (n, %)	4 (31)	4 (33.3)	1
Aspirin (n, %)	7 (54)	4 (33.3)	0.69
Clopidogrel (n, %)	11(85)	12 (100)	0.8
Ticagrelor (n, %)	2 (15)	0 (0)	0.1
Statin (n, %)	10 (77)	4 (33.3)	0.08
Distribution of CAD (n, %):			
1 vessel disease	5 (38.5)	7 (58.3)	0.2
2 vessel disease	4 (31)	2 (17)	0.3
3 vessel disease	4 (31)	3 (25)	0.8
Culprit Artery (n,%)			0.4
LAD	8(61.5)	6(50)	
LCX	4(30.7)	3(25)	
RCA	1(7.6)	3(25)	

SD standard deviation, ACS – acute coronary syndrome, SA – stable angina, CAD – coronary artery disease, PCI – percutaneous coronary intervention, CRP – C- reactive protein, LVEF – left ventricular ejection fraction, ACE-I – angiotensin converting enzyme inhibitor, ARB – angiotensin receptor blocker, LAD – Left anterior descending artery, LCX – Left circumflex artery, RCA – Right coronary artery.

3.4.2 OCT data acquisition

In total OCT pullbacks were carried out in 66 coronary arteries (32 in ACS and 34 in SA) out of possible 75 excluding 9 non-dominant arteries of small calibre (7 RCA and 2 LCX).

3.4.3 Distribution of CAD

In both groups, LAD was the commonly diseased artery with plaque distribution predominantly in the proximal and mid segments. In contrast plaques were more evenly distributed in the LCX and RCA (Table – 9).

Table 9. Plaque distribution in relation in the segment of artery

		ACS (n=32)	SA (n=34)
LAD	Ostial	1 (3%)	2(5.8%)
	Proximal	7 (21.8%)	6 (17.6)
	Mid	9 (28.1%)	6 (17.6)
	Distal	1(3%)	0 (0)
LCX	Ostial	0(0%)	0(0)
	Proximal	6(18.75)	1 (2.9)
	Mid	2(6.25)	2 (5.8)
	Distal	2(6.25)	0(0)
RCA	Ostial	(0)	0(0)
	Proximal	3 (9.38)	4(11.6)
	Mid	2(6.25)	4(11.6)
	Distal	4(12.5)	1(2.9)

LAD - Left anterior descending artery, LCX – Left circumflex artery, RCA – Right coronary artery

3.4.4 Analysed pullback length

The adjusted length of pullbacks and consequent number of frames (1mm = 5 frames) used for analysis are elaborated in table 10.

Table 10. Table showing analysed length and frames per pullback

		ACS	SA	p
LAD	Analysed length of pullback (mm)	73.4 (56-74.9)	70.6(50.9-74.8)	0.6
	Analysed frames in pullback	366 (279-373)	330 (236-372.3)	0.2
	Segmented as normal on OCT (mm)	19(1.5-23)	10(2-17.8)	0.12
	Segmented as PIT on OCT (mm)	12.5(4-20)	21.4(5.7-30)	0.17
LCX	Analysed length of pullback (mm)	60.2(48.2-74.8)	67.9(53.2-73.8)	0.56
	Analysed frames in pullback	254(185-351)	324(247-360)	0.52
	Segmented as normal on OCT (mm)	7(1-19)	20(8.7-33)	0.07
	Segmented as PIT on OCT (mm)	14(6-29)	19.6(12-41)	0.4
RCA	Analysed length of pullback (mm)	74.8(65.2-74.8)	69(49.8-74.85)	0.53
	Analysed frames in pullback	373(325-373)	344(248-373)	0.54
	Segmented as normal on OCT (mm)	14(1-18)	16.5(5.2-20.2)	0.5
	Segmented as PIT on OCT (mm)	19(9-40)	19.2(7.2-30.5)	0.6

All values are in median and inter quartile range. mm – millimetres, NSTEACS – non-ST elevation acute coronary syndrome, SA – Stable angina LAD – Left anterior descending artery, LCX – Left circumflex artery, RCA – right coronary artery. OCT – Optical coherence tomography, PIT – pathological intimal thickening.

3.4.5 Comparison of plaque characteristics at patient level

ACS patients had more lipid burden when compared to SA cohort. This was evident by longer lipid length, wider maximum lipid arc, higher lipid and relative lipid volumes. No difference was noted in other parameters (Table -11).

Table 11. Comparison of OCT variables between ACS and SA

OCT variable	ACS Median (IQR)	SA Median (IQR)	p-value
Lipid length (mm)	20 (11, 35)	13 (5.25, 20.75)	**0.014**
Lipid max arc (°)	165.1(109.4,253.3)	125.3 (78.5, 161.7)	**0.024**
Lipid average arc **(°)**	81.85 (63.5,110.4)	69.10(51.70,88.10)	0.115
Lipid Volume	1819.5 (694.5, 3638.8)	860.3 (314.4,1620.6)	**0.014**
Lipid Relative Volume	8 (2.2, 14)	3.4 (1.9, 7.2)	**0.019**
Lipid cap thickness - minimum **(**µm)	77.5 (36, 114.5)	85.1 (51, 123)	0.62
Calcium length (mm)	6.5 (0,19.25)	6.0(0.5-11)	0.9
Calcium max arc **(°)**	89 (0,138)	73 (14.5, 144.6)	0.7
Calcium average arc **(°)**	60.8 (0,93.6)	48 (13.5,76.8)	0.5
Calcium Volume	398.3 (0, 1452)	210 (0, 910)	0.64
Calcium Relative Volume	1.8 (0, 4.9)	1.3 (0, 5.2)	0.82
Ruptured plaques	7 (2,12)	5(3,10)	0.09
Macrophages in % frames	2.7 (0, 7.7)	2.7 (0, 5.5)	0.49
Neovessels in % frames	1.9 (0, 8.2)	5.3 (1.5, 14.7)	0.1
Cholesterol crystals in % frames	0 (0.0, 1.3)	0.0 (0.0, 0.0)	0.6
Thrombus in % frames	0 (0, 0)	0 (0, 0)	0.76
Calcific nodules % frames	0(0, 0)	0(0, 0)	0.6

IQR - inter quartile range, mm - millimetres

3.4.6 Comparison of plaque characteristics at artery level

Comparison between culprit and non-culprit coronary arteries revealed thinner lipid cap in culprit arteries (77 (µm) vs. 138(µm); p=0.01) (Table -11). Non-culprit arteries in ACS patients had longer lipid length and higher lipid volume when compared to NC arteries in SA cohort (Table -12). Comparison of OCT variables between non-culprit arteries in ACS and SA are shown in Table 13. Comparison of OCT variables between culprit arteries in ACS and SA are shown in 14.

Table 12. Comparison of OCT variables between culprit and non-culprit arteries across both groups

OCT variable	Culprit arteries Median (IQR)	NC arteries Median (IQR)	p-value
Lipid length (mm)	18(13, 25)	24 (10, 49)	0.17
Lipid max arc **(°)**	183 (135, 257)	174(123, 282)	0.78
Lipid average arc **(°)**	91(67,112)	100(59,180)	0.55
Lipid Volume	1601 (751, 2201)	1631 (743, 4190)	0.46
Lipid Relative Volume	7.5 (2.8, 11.2)	6.3 (2.3,16.6)	0.65
Lipid cap thickness – minimum (µm)	77 (51, 141)	138 (91, 216)	**0.01**
Calcium length (mm)	9 (1,17)	3 (1, 23)	0.52
Calcium max arc **(°)**	110 (34,189)	76(42, 212)	0.79
Calcium average arc **(°)**	63 (27,100)	48 (30,115)	0.95
Calcium Volume	664 (14, 1495)	239 (54, 2022)	0.51
Calcium Relative Volume	2.7 (0.1, 5.6)	1.4 (0.1, 8.1)	0.25
Ruptured plaques	4(2,12)	3(3,10)	0.16
Macrophages in % frames	3.3 (0, 6.5)	2.4 (0.3, 13.0)	0.59
Neovessels in % frames	2.8 (1.4,14.3)	8.1 (0, 20.8)	0.25
Cholesterol crystals in % frames	0 (0, 1)	0.0 (0.0, 0.0)	0.23
Thrombus in % frames	0 (0, 0)	0 (0, 0)	0.66
Calcific nodules % frames	0(0, 0)	0(0, 0)	0.56

IQR - inter quartile range, NC – non culprit, mm - millimetres

Table 13. Comparison of OCT variables between non-culprit arteries in ACS and SA

OCT variable	Non-culprit (ACS) Median (IQR)	Non-culprit (SA) Median (IQR)	P-value
Lipid length (mm)	25(4, 36)	11 (2.5, 16.5)	**0.03**
Lipid max arc **(°)**	154.9 (90, 173.9)	112.35(61.2, 141.5)	0.09
Lipid average arc **(°)**	76.2(56,103)	66.25(45.93,82.2)	0.23
Lipid Volume	1772.5(307, 4035.6)	701 (106, 1307)	**0.035**
Lipid Relative Volume	7.45 (1.06, 15.5)	2.9(1.08,4.6)	0.062
Lipid cap thickness - minimum (μm)	92 (34, 116)	102 (57.5, 128)	0.7
Calcium length (mm)	3 (0,20)	4 (0, 10.25)	0.79
Calcium max arc **(°)**	76 (0,139.4)	64.17(0, 93.8)	0.63
Calcium average arc **(°)**	58 (0,76)	43.6 (0,50.2)	0.41
Calcium Volume	204 (0, 1510)	144.5 (0, 612)	0.87
Calcium Relative Volume	1.3 (0.1, 4.9)	0.9 (0.1, 4.2)	0.86
Ruptured plaques	2(1,5)	3(1,5)	0.7
Macrophages in % frames	3.3 (0.2, 8.2)	1.5 (0.3, 4.3)	0.14
Neovessels in % frames	0.28 (0,7.4)	4 (1.1, 9.6)	0.19
Cholesterol crystals in % frames	0 (0, 1)	0.0 (0.0, 0.0)	0.8
Thrombus in % frames	0 (0, 0)	0 (0, 0)	0.9
Calcific nodules % frames	0(0, 0)	0(0, 0)	0.2

IQR - inter quartile range, NC – non culprit.

Table 14. Comparison of OCT variables between culprit arteries in ACS and SA

OCT variable	Culprit arteries ACS Median (IQR)	Culprit arteries SA Median (IQR)	p-value
Lipid length (mm)	18 (13.25, 37.25)	20 (13, 24.5)	0.76
Lipid max arc (°)	230.86 (147.24,268.32)	164 (125.35, 248.96)	0.29
Lipid average arc (°)	92.90 (67.25,113.45)	85.80(58.9,122.3)	0.977
Lipid Volume	1751.41 (1345, 3416.8)	1616 (787.4,2542.6)	0.611
Lipid Relative Volume	8.8 (5.86, 13.3)	7.18 (3.2, 11)	0.58
Lipid cap thickness - minimum (υm)	55 (26.25, 126.5)	70.5 (49.5, 96.3)	0.47
Calcium length (mm)	8 (0.25,15)	10(1.25-15.50)	0.41
Calcium max arc (°)	99.79 (19.71,99.79)	174 (36.5, 273.6)	0.18
Calcium average arc (°)	60.8 (10.50,91.63)	82.7 (37.1,105.7)	0.22
Calcium Volume	449.4 (15, 1216)	885 (29.2, 1543)	0.57
Calcium Relative Volume	2 (0.6, 4.8)	3.90 (0.11, 6.09)	0.46
Ruptured plaques	3(1,5)	3(1,05	0.9
Macrophages in % frames	1.8 (0, 11.5)	4 (1.33, 9.28)	0.44
Neo Vessels in % frames	4.25 (1.3, 11.6)	11.9 (1.5, 16)	0.53
Cholesterol crystals in % frames	0.05 (0.0, 2.86)	0.06 (0.02, 0.66)	0.34
Thrombus in % frames	0 (0, 0)	0 (0, 0)	1
Calcific nodules % frames	0(0, 0)	0(0, 0)	1

3.4.7 Culprit lesion, ruptured plaques & TCFA

The culprit lesion was predominantly lipid rich plaque (LRP) in ACS cohort (n=10, 77%); it was fibroatheroma in 1 patient and unable to interpret in 2 patients. Plaque rupture was evident in 7 (53%) culprit lesions of ACS. In SA cohort culprit lesion was LRP in 10 (83%) patients and fibroatheroma in 2 patients.

Ruptured plaques in non-culprit segments were present in 50% of ACS patients and 23% of SA patients (p=0.67).

TCFA not related to ruptured plaque were present in 33.3% of ACS and 23 % of patients with SA (p=0.45). TCFA were predominantly located in the proximal to mid segments of LAD but more widely distributed in LCX and RCA.

3.5 Discussion

My study examined differences in plaque characteristics in patients with NSTEACS versus SA, focusing on the entire coronary tree rather than isolated plaques.The main findings of our study were: 1) In ACS there is high incidence of ruptured plaques and TCFA not related to the culprit lesion, i.e. 50% and 33% respectively; 2). In ACS, in the entirety of the coronary tree of lipid burden is higher than in SA both after including or excluding culprit arteries, implying a more extensive advanced coronary artery disease. Collectively, these findings support the notion that ACS is a panvascular process, rather than a focal manifestation.

The findings of my study are in line with the findings of Kato et al who demonstrated that non-culprit arteries in ACS patients had more vulnerable features when compared to non-culprit arteries in non-ACS patients (158). We also know from PROSPECT study that angiographically mild non-culprit lesions but with vulnerable features were associated with adverse cardiovascular events (161). We also noted incidental ruptured plaques in 30% and TCFA in 23% of SA patients. This is despite of the fact that ACS patients on the whole and NC arteries in ACS had more lipid burden. Previous studies have reported that atherothrombotic events are often silent and contribute to plaque progression (162). Thieme et al reported 11% of stable angina patients to have ruptured plaques and associated thrombi detected on angioscopy. Their figure is less than my findings perhaps explained by the limitations of angioscopy (163).Thus I may have just picked up these findings at an early stage of atherosclerotic process. Coronary atherosclerosis is an inflammatory process that progresses over years (2). Inflammation probably plays a role in regulating plaque biology that triggers the thrombotic complications silently in the so-called stable patients. Evidence for coronary inflammatory process on OCT comes in the way of segmenting the LRP cross-sections with macrophages (164). In my study both cohorts of patients had similar percentage of frames of macrophages

whereas NC arteries in ACS cohort had numerically more but statistically non-significant frames with macrophages. Macrophages play a dual role as markers of on-going inflammation in the coronary bed and as the causative agent for plaque rupture by collagen breakdown in the fibrous cap through matrix metalloproteinases (165). In contrast to previous studies, the macrophage content in our study was similar between ACS and SA cohort of patients. The role of neovessels in progression of atherosclerosis has been reported before (166). Ensuing hypoxia with thickening of intima results in proliferation of neovessels, which become major source nutrients to the vessel wall (167,168). In addition to aiding in plaque progression due to lack of structural integrity these neovessels can rupture easily and become a source of intraplaque haemorrhage (39). In my study we have found long segments of coronary artery with pathological intimal thickening – similar between ACS and SA. Likewise the percentage of frames with neovessels was also similar between ACS & SA and between non-culprit ACS and non-culprit SA arteries. The possible explanation is that a higher number of ACS patients in my cohort was on statins. Raber et al recently reported regression of atherosclerosis in non-infarct related artery with high dose statin regime at follow up (169). It is possible that statin treatment could have stabilised the fibrous cap and prevented rupture in my cohort and other inflammatory features. The beneficial effects of statins in reducing cardiac events by lowering LDL and their anti-inflammatory effects are well known (170,171). Despite of this the ACS cohort in my study had higher lipid burden along with evidence of on-going inflammation across both groups. Despite adequate preventive therapy recurrence of coronary events is common in patients with ACS (172). Aggressive secondary prevention with and beyond statins is probably required to decrease lipid plaque burden.

3.6 Limitations

Limitations associated with a prospective observational study involving small sample size at a single centre are worthy of note. The small sample size is underpowered to associate lipid burden with any outcome data. More patients in ACS cohort were on statins – this may have reduced the vulnerable features of plaque. However, the small sample size meant I was able to carry out detailed frame be frame analysis thus obtaining robust data.

3.7 Conclusions

In contemporary clinical practice patients with ACS and NC ACS arteries had more lipid burden despite of current evidences based therapy.

Chapter 4. Persistent Circulating Platelet and Endothelial Derived Microparticle Signature May Explain On-Going Pro-Thrombogenicity After Acute Coronary Syndrome

4.1 Summary

Background

Microparticles (MPs) are submicron vesicles, released from activated, and apoptotic cells. MPs are shown to be elevated in the circulation of patients with coronary artery disease (CAD) and have pro-thrombotic potential. However, limited data exists on MP signature over time following an acute coronary event.

Methods

Circulating total annexin v+ (Anv+) MPs of endothelial (EMP), platelet (PMP), monocyte (MMP), neutrophil (NMP) and smooth muscle cell (SMMP) origin were quantified by flow cytometry. 13 patients with acute coronary syndrome (ACS) were prospectively enrolled and 12 patients with stable angina (SA) were included as a comparator group. A panel of MP was measured at baseline, after percutaneous coronary intervention (PCI) and at days 1, 7, 30 and 6 months. Intra & inter group comparison was made between various time points. MP mediated thrombin generation was measured by recording lag phase, velocity index, peak thrombin and endogenous thrombin potential at these time points and compared with healthy controls.

Results

The total AnV+ MP levels were similar in ACS and SA groups at baseline, peaked immediately after PCI and were at their lowest on day 1. PMP & EMP levels remained significantly elevated in ACS patients at 6 months when compared to SA. No such difference was noted with NMP, MMP and SMMP. Patients with CAD showed abnormal thrombograms when compared to

controls. Peak thrombin (nano moles) was significantly higher in CAD when compared to controls (254 IQR [226, 239] in ACS, 255 IQR [219, 328] in SA and 132 IQR [57, 252] in controls; p = 0.006). Differences in thrombin generation between ACS and SA were not significant (p=1). Furthermore, thrombin parameters remained abnormal in ACS & SA patients at 6 months as demonstrated on AUC (3467 IQR [2691, 4243] at baseline & 3572 IQR [2827, 4317] at six months for ACS vs. 3411 IQR [2726, 4096] at baseline & 3726 IQR [2975, 4477] at six months for SA; p<0.004)) respectively when compared to baseline.

Conclusions

Total MP and individual MP phenotypes were significantly elevated after PCI reflecting endothelial injury. Elevated PMP and EMP levels at 6 months in ACS patients is suggestive of on-going inflammation, endothelial injury and may explain on-going pro-thrombogenicity seen up to 6 months after ACS despite dual antiplatelet therapy.

4.2 Introduction and Aims

Atherosclerotic coronary artery disease (CAD) is a pro-inflammatory and pro-coagulant condition (2,37). CAD remains a major cause of global mortality and morbidity (173). Furthermore, recurrence of coronary events due to progression of de novo disease in patients with a history of CAD remains high (161,172). Better risk stratification and aggressive secondary prevention tailored to the individual is required to combat this problem as we now know significant individual variation exists in disease phenotype. Development of novel biomarkers may yield patient specific information reflecting the underlying pathophysiologic process of CAD. The concept of “vulnerable patient” has been proposed in place of “vulnerable plaque” to better risk stratify individuals at risk of adverse cardiovascular events. The vulnerable patient can be characterized by a triad of vulnerable plaque, vulnerable blood and vulnerable myocardium; the vulnerability in blood is derived from a hypercoagulable state (174). Over the last two decades, emerging data has demonstrated the role of sub-micron vesicles called microparticles (MPs) in mediating inflammation (4) in atherosclerotic CAD (5). MPs are cell membrane-derived particles released

from activated or apoptotic cells (18) and have been shown to be elevated in the circulation of patients with CAD (175) but no information exists regarding their long-term clearance following an acute event. The biological function of MPs depends upon the parent cell they originate from (16). Broadly they have a role in inflammation & coagulation (44,47), endothelial dysfunction, angiogenesis(41) and microvascular dysfunction (176). I hypothesized that the levels of circulating endothelial derived (EMP), platelet derived (PMP), neutrophil derived (NMP), monocyte derived (MMP) and smooth muscle cell derived (SMMP) MPs in acute coronary syndrome (ACS) are dynamic. Furthermore I sought to assess MP mediated thrombin generation as a quantifiable measure of their pro-thrombotic potential. The evaluation of MP expression over time and their pro-thrombotic potential may have a role as a biomarker aiding in better patient risk stratification and represent a future therapeutic target.

4.3 Patients and Methods

13 patients with ACS scheduled for invasive angiography and percutaneous coronary intervention (PCI) were included in the study. 12 patients with SA were recruited as a comparator group. In addition 10 health controls were recruited just for baseline TGA analysis. The healthy controls consisted of 10 patients (5 male and 5 female subjects) and were age matched with ACS and SA groups but did not have any documented CAD, risk factors for CAD nor on any medications. Comparison with healthy controls was only carried out at baseline and not at any other time points. Ethical approval was obtained from London – Stanmore research ethics committee. Informed consent was obtained from every patient prior to participation in the study. Blood was collected for MP analysis and thrombin generation assay (TGA) immediately pre and post PCI, on days 1,7,30 and 180. For comparative purposes the TGA was also analysed from an age-matched control group with no CAD. Inclusion and exclusion criteria were as mentioned before in chapter 3.

4.3.1 MP assessment

Dual antiplatelet therapy with Aspirin and Clopidogrel or Ticagrelor was given according to global risk for cardiovascular events (GRACE) score (177). Pre-

PCI samples were collected before the administration of unfractionated heparin or GP IIb/IIIa antagonists. In addition, ACS patients received treatment with Low molecular weight heparin (LMWH) up until PCI. However, where clinically possible we ensured that there was a delay of 12 hours between the last dose of LMWH and blood collection to minimise any potential effect on TGA levels.

Venous blood from antecubital vein was collected pre (V1) and post PCI (V2) and on days 1 (V3), 7 (V4), 30 (V5) and 180 (V6) in citrate bottles. The collected blood was centrifuged to obtain platelet poor plasma (PPP) and then stored at -80^{o}C until analysed by flow cytometry using the BD FACS Calibur^{TM} Cell Analyzer. Particles <1.1**µl** in size and binding annexin V+ were selected for analysis. Double and triple staining was used to define MP subpopulations to identify their cellular origin. PMPs (activated) were defined as AnV+/CD42a+/CD62P+, activated EMPs were defined as AnV+/CD42a-/CD105+ or CD31+ or CD54+ or CD62E+ or CD31+, activated NMPs were defined as AnV+/CD42a-/CD66b+, MMP with tissue factor (TF) expression were defined as AnV+/TF+/CD14+ and SMMPs as AnV+/CD42a-/NG2+. Data collected was analysed with FlowJo software (version 8.8.3; Tree Star, Inc., OR, USA). Gating strategy was elaborated in chapter 2.

4.3.2 Microparticle mediated thrombin generation assay

The procedure for blood collection, storage and preparation for assay was similar to MP assessment. MPs were sedimented from PPP and resuspended in 200 **µl** of control microparticle-free plasma (MPFP) containing 30 mg/mL of corn trypsin inhibitor (Sigma) to inhibit contact activation. The MPFP was prepared from approximately 50 **µl** plasma obtained from healthy volunteers. Subsequently, 40 **µl** of MPs resuspended in control MPFP was added to the plate well, followed by 50 **µl** of calcium-fluorogenic substrate (0.5 mmol/L of Z-G-G-R-AMC and 7.5 mmol/L of calcium final concentrations (Pathway Diagnostics). No exogenous TF or phospholipids were added. The thrombin generated was measured by fluorogenic excitation/emission at 360/460 nm at 1-minute time intervals for 60 minutes in an Optima fluorescence plate reader (BMG). Measures of peak thrombin, lag time, velocity index, and endogenous thrombin potential were recorded. As patients with ACS were treated with

LMWH a time delay of at least 12 hours was maintained after the last dose of LMWH before aspirating blood so that there are no errors in interpreting thrombin generation assay (TGA). TGA was not interpretable on immediate post PCI samples due to administration of systemic heparin and hence excluded from analysis.

4.3.3 Statistics

Normally distributed data were presented as mean ± SD. Non-parametric data were reported as median and interquartile range (IQR). Categorical variables were compared with chi-square or Fisher's exact test, as appropriate. All numeric parameters of interest were measured on continuous scales. As the same patients were assessed at the different time points, the analysis was performed using multilevel linear regression. Two-level models were used with individual measurements contained within patients. Variables with positively skewed distributions were log transformed before analysis. Analyses were performed for all patients combined, and then separately for ACS and SA patients. Additional analyses compared between the thrombin parameters at the first time point between the patient groups and a control group. The analyses were performed using Analysis of Variance (ANOVA). Variables with skewed distributions were analysed on a log scale. In addition to an overall comparison of the three groups, post-hoc tests were used to compare between each pair of groups. The p-values from these post-hoc comparisons were given a Bonferroni adjustment to allow for an increased risk of finding a statistically significant result due to multiple comparisons. The strength of association between the microparticle measurements and the thrombin variables was performed using Spearman's rank correlation (due to the skewed distribution of the microparticle measurements (and some of the thrombin variables). Analyses were performed for the values at each time point.

4.4 Results

4.4.1 Patient characteristics

The majority of the patients across both groups were of male sex. More numbers of patients in the ACS group were on statins. Both groups were evenly matched for other variables (Table-15).

Table 15. Baseline characteristics

	ACS (n=13)	SA (n=12)	p value
Age (mean ± SD)	60.1 ± 8.14	56.6±8.9	0.32
Male (n, %)	12 (92.3)	11 (91.7)	0.79
Diabetes (n, %)	5 (38.4)	2 (16.7)	0.67
Hypertension (n, %)	5 (38.5)	4 (33.3)	1.0
Hyperlipidaemia (n, %)	12 (92.3)	8 (67)	0.15
Smoking (n, %)	3 (23.1)	4 (33.3)	0.4
Family History of CAD (n, %)	1 (7.7)	3 (25)	0.65
Previous PCI (n, %)	2 (15.3)	0	0.15
Creatinine (Median, IQR)	82.5 (76.25 – 88.50)	84 (75.2-87.5)	0.6
Troponin (Median, IQR)	0.24 (0.2 - 3.8)		
CRP (Median, IQR)	4 (1 - 6.25)		
LVEF (mean ± SD)	58.3±5.3	57.8±8.5	0.6
Medications:			
ACE- I / ARB (n, %)	3 (23.1)	4 (33.3)	0.82
Beta-blockers (n, %)	4 (31)	4 (33.3)	1
Aspirin (n, %)	7 (54)	4 (33.3)	0.69
Clopidogrel (n, %)	11(85)	12 (100)	0.8
Ticagrelor (n, %)	2 (15)	0 (0)	0.1
Statin (n, %)	10 (77)	4 (33.3)	0.08
Distribution of CAD (n, %):			
1 vessel disease	5 (38.5)	7 (58.3)	0.2
2 vessel disease	4 (31)	2 (17)	0.3
3 vessel disease	4 (31)	3 (25)	0.8

Male, CAD – Coronary artery disease, PCI – Percutaneous coronary intervention, CRP - C-reactive protein, LVEF – left ventricular ejection fraction, ACE – I – Angiotensin converting enzyme inhibitors, ARB – Angiotensin receptor blockers, LAD – Left anterior descending, LCX – Left circumflex and RCA – Right coronary artery.

4.4.2 Total Microparticles

In all patients, total MPs increased considerably following PCI and rapidly decreased at day 1, though these changes were not significant (Figure 23). At all time points except immediately post PCI total MPs were numerically higher in the ACS group, though not statistically significant (Figure 24). The time points at which MPs were at their peak and nadir were immediately post PCI and on day 1 post PCI respectively. The results are as follows with values of microparticles in 100,000s and presented in median and interquartile range (IQR). In the ACS group, the levels of total AnV+MPs at baseline, post PCI, days 1, 7, 30 & 180 post PCI were 306 (270, 360), 728 (226, 1043), 114 (55, 264), 343 (192, 609), 117 (60, 206) and 245 (109, 354) respectively; p =0.007. In SA group the levels of total AnV+MPs at baseline, post PCI, days 1, 7, 30 & 180 post PCI were 202 (63, 502), 962 (422, 1323), 88 (40, 251), 294 (172, 951), 92 (44, 157) and 165 (77, 490) respectively; p<0.001. The following tables and box plots illustrate the levels of total and individual phenotypes of MPs (Tables 16-25).

Table 16. Total AnV+ MPs (in 100,000s) across all time points and comparing between ACS and SA cohorts

	All Median (IQR)	ACS Median (IQR)	SA Median (IQR)	Group P-value
V1	284 (102, 360)	306 (270, 360)	202 (63, 502)	0.89 (+)
A1	265 (87, 396)	254 (87, 400)	275 (86, 392)	
A2	517 (209, 733)	479 (208, 727)	527 (210, 738)	
V2	802 (365, 1139)	728 (226, 1043)	962 (422, 1323)	
V3	112 (54, 263)	114 (55, 264)	88 (40, 251)	
V4	294 (192, 730)	343 (192, 609)	294 (172, 951)	
V5	102 (44, 198)	117 (60, 206)	92 (44, 157)	
V6	198 (93, 401)	245 (109, 354)	165 (77, 490)	
Time p-value	**<0.001**			

V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

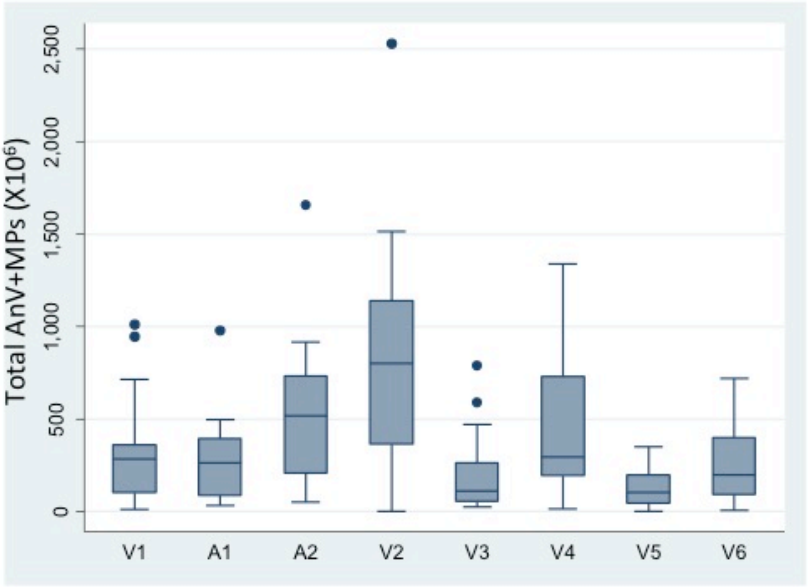


Figure 23. Total AnV+ MPs across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples

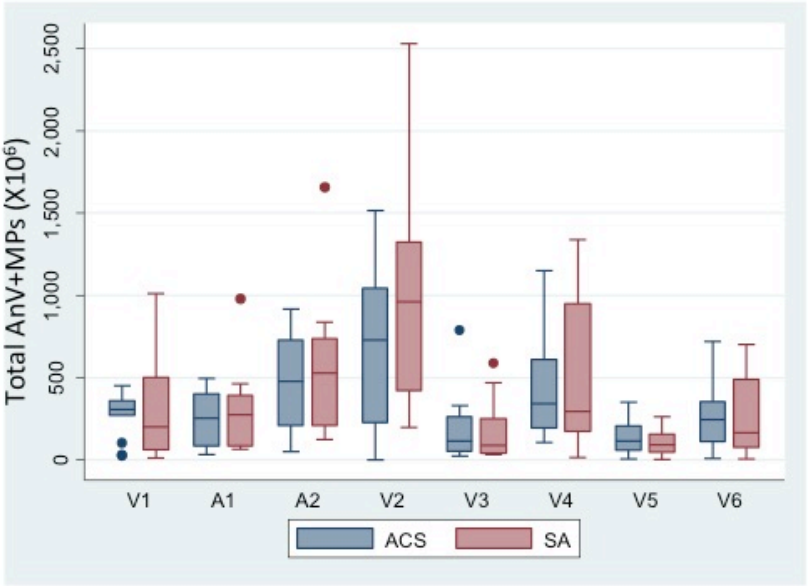


Figure 24. Box plots comparing total ANV+ MPs between ACS and SA across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

4.4.3 Anv+ CD42- CD105+EMP

CD105 EMP levels are enumerated below. Numerically higher levels were noted pre PCI in the ACS cohort with no statistical significance but broadly similar levels by 6 months (Figures 25-26).

Table 17. CD105 EMPs (in 100,000s) across all time points and comparing between ACS and SA cohorts

Time point	All Median (IQR)	ACS Median (IQR)	SA Median (IQR)	Group P-value
V1	13.8 (2.9, 35.9)	17.7 (4.7, 39.3)	11.0 (2.3, 28.6)	0.69 (+)
A1	13.2 (2.6, 29.0)	12.4 (2.3, 33.2)	14.8 (2.9, 28.5)	
A2	19.5 (12.7, 33.6)	19.5 (9.5, 25.8)	22.7 (13.2, 42.4)	
V2	25.4 (14.7, 63.0)	19.2 (2.4, 37.6)	28.5 (15.3, 86.1)	
V3	8.7 (2.9, 27.1)	8.7 (3.6, 37.8)	7.0 (2.2, 21.0)	
V4	6.2 (2.2, 19.6)	8.1 (8.1, 28.4)	4.2 (2.2, 6.2)	
V5	5.9 (1.9, 9.0)	4.7 (1.8, 9.3)	6.3 (1.9, 8.2)	
V6	3.6 (2.0, 10.7)	3.4 (1.1, 15.0)	3.7 (2.3, 7.6)	
Time p-value	**<0.001**			

V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

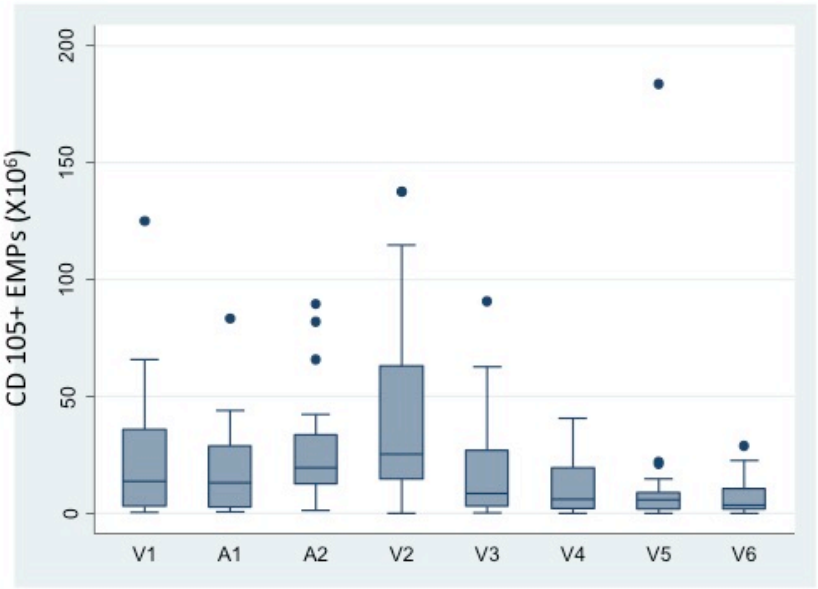


Figure 25. Anv+ CD42- CD105+EMP across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

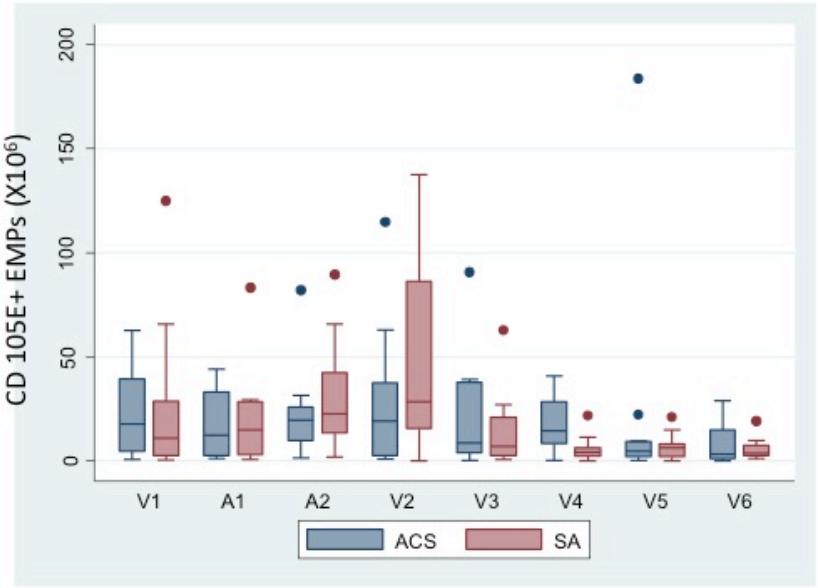


Figure 26. Box plots comparing ANV+CD42-CD105+EMPs between ACS and SA across all time points V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

4.4.4 Anv+ CD42- 62P+PMPs

A trend similar to total AnV+ MPs was also noted with PMPs (Figure 27-28). In the ACS group the levels of activated PMPs at six months were higher than the SA group; 3.6 (1.2, 6.7) vs. 0.7 (0.4, 2.7); p=0.03.

Table 18. CD62P+ PMPs (in 100,000s) across all time points and comparing between ACS and SA cohorts

Time point	All Median (IQR)	ACS Median (IQR)	SA Median (IQR)	Group P-value
V1	4.5 (2.1, 24.9)	5.9 (3.3, 24.4)	4.0 (1.8, 27.9)	0.42
A1	4.6 (1.7, 21.5)	3.5 (1.4, 19.2)	5.7 (1.8, 31.7)	0.60
A2	15.6 (8.3, 26.1)	12.3 (6.0, 20.4)	18.6 (10.0, 40.7)	0.18
V2	11.6 (3.5, 38.2)	9.0 (2.5, 30.2)	16.0 (10.2, 63.7)	0.18
V3	2.4 (0.9, 7.2)	3.6 (0.9, 7.2)	1.7 (0.9, 3.7)	0.14
V4	1.9 (0.5, 4.4)	3.4 (1.4, 10.3)	1.6 (0.4, 2.7)	**0.04**
V5	2.1 (0.6, 5.3)	2.8 (0.8, 8.0)	1.5 (0.6, 4.1)	0.43
V6	1.7 (0.9, 5.4)	3.6 (1.2, 6.7)	0.7 (0.4, 2.7)	**0.03**
Time p-value	**<0.001**	**0.01**	**<0.001**	

V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

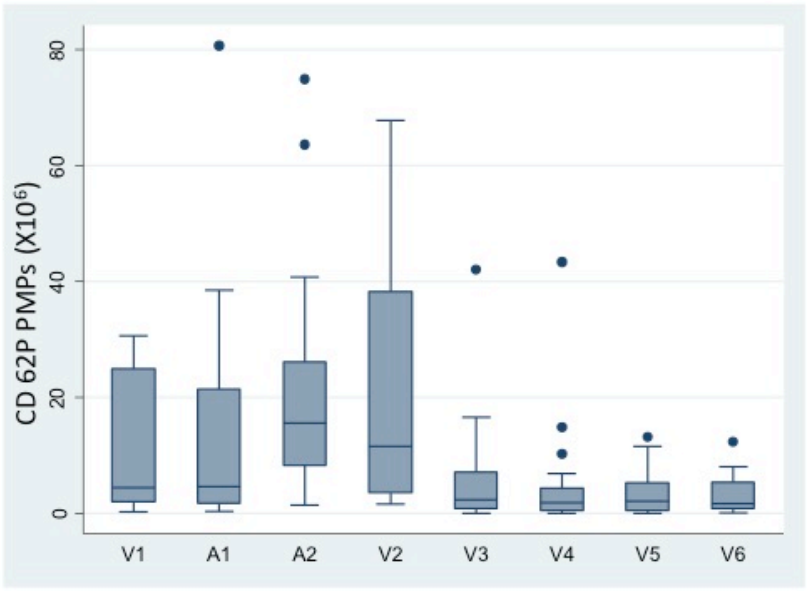


Figure 27. Anv+ CD42- CD62P+PMPs across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

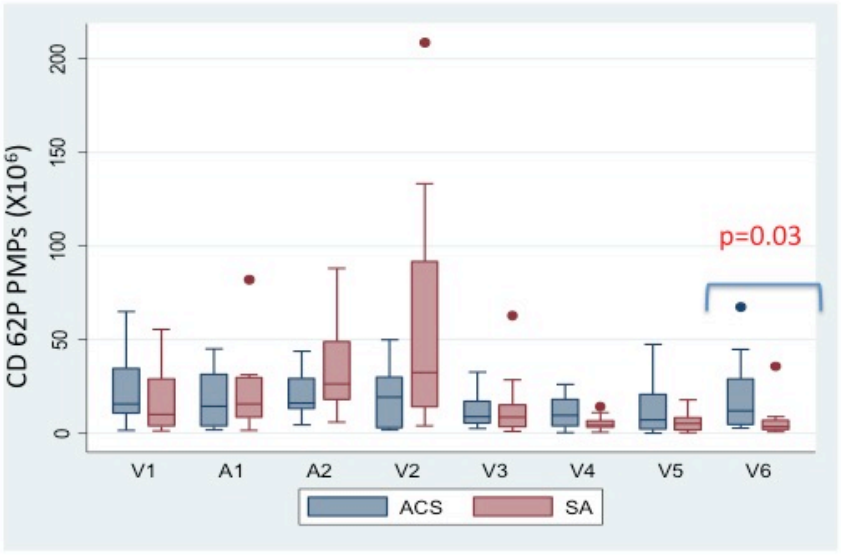


Figure 28. Box plots comparing ANV+CD42-CD62P+PMPs between ACS and SA across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

4.4.5 Anv+ CD42- 62E+EMP

Levels of CD62E+ EMPs were higher in the ACS group when compared to the SA group at six months – 11.9 (4.7, 29.0) vs. 3.4 (1.8, 6.7); p=0.02. (Figures 29-30).

Table 19. CD62E+ EMPs (in 100,000s) across all time points and comparing between ACS and SA cohorts

Time point	All Median (IQR)	ACS Median (IQR)	SA Median (IQR)	Group P-value
V1	15.2 (5.2, 29.2)	15.6 (10.8, 34.5)	10.0 (3.8, 28.9)	0.37
A1	14.9 (4.4, 30.5)	14.2 (3.6, 31.4)	15.5 (8.4, 29.7)	0.65
A2	21.7 (14.2, 34.9)	16.1 (13.2, 29.1)	26.3 (18.0, 48.9)	0.26
V2	23.5 (13.1, 34.3)	19.2 (2.8, 29.8)	32.4 (13.4, 91.6)	0.05
V3	8.8 (3.2, 17.0)	8.8 (5.4, 17.0)	8.6 (3.2, 15.1)	0.44
V4	5.8 (3.1, 11.1)	9.5 (3.7, 18.0)	4.3 (2.9, 6.5)	0.73
V5	6.3 (1.4, 9.0)	7.2 (2.0, 20.7)	5.2 (1.4, 8.4)	0.43
V6	5.3 (3.2, 28.0)	11.9 (4.7, 29.0)	3.4 (1.8, 6.7)	**0.02**
Time p-value	**<0.001**	0.05	**<0.001**	

V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

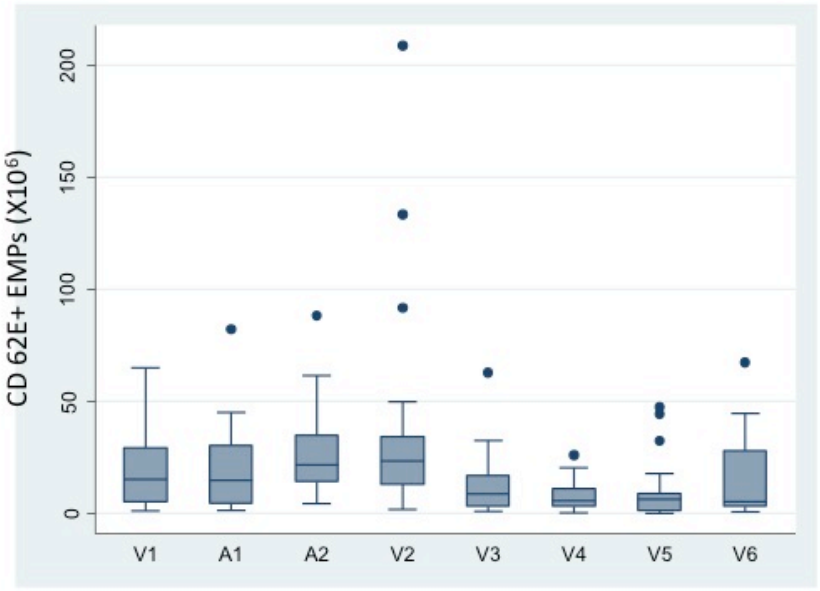


Figure 29. Anv+ CD42- CD62E+EMPs across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

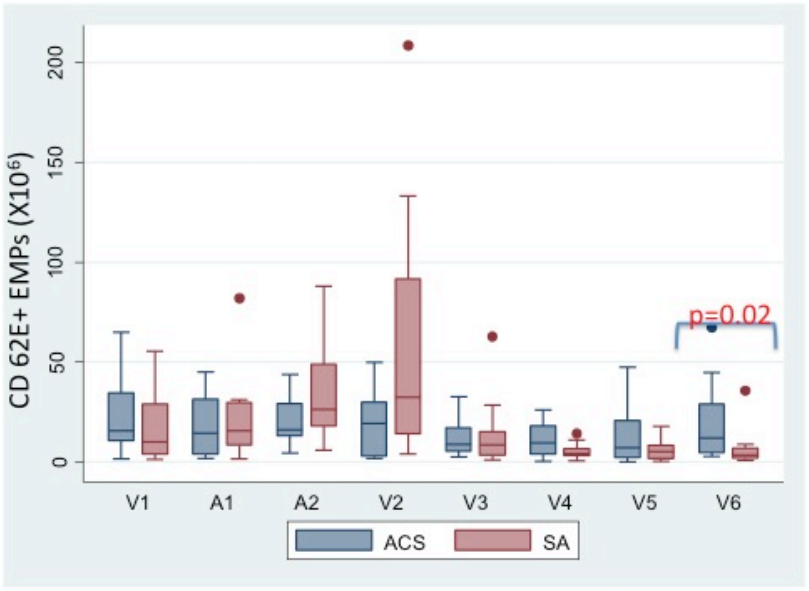


Figure 30. Box plots comparing ANV+CD42-CD62E+EMPs between ACS and SA across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

4.4.6 Micro Particle Measurement 4 – Anv+ CD42- 66B+

CD66b NMP levels are enumerated below. Numerically higher levels were noted pre PCI and at six months in the ACS cohort with no statistical significance. (Figures 31-32).

Table 20. CD66b+ NMPs (in 100,000s) across all time points and comparing between ACS and SA cohorts

Time point	All Median (IQR)	ACS Median (IQR)	SA Median (IQR)	Group P-value
V1	4.6 (1.5, 18.5)	8.2 (3.1, 18.5)	2.6 (0.7, 19.3)	0.59 (+)
A1	8.8 (1.8, 25.7)	10.2 (1.9, 15.8)	5.4 (1.3, 33.8)	
A2	18.2 (9.2, 32.9)	14.2 (3.9, 27.5)	24.8 (13.2, 50.6)	
V2	12.8 (3.9, 42.5)	9.9 (3.3, 34.5)	16.2 (9.1, 73.0)	
V3	4.0 (2.0, 7.5)	4.0 (2.0, 7.5)	3.6 (1.7, 4.8)	
V4	2.1 (0.4, 4.2)	2.2 (0.4, 6.4)	1.8 (0.3, 4.2)	
V5	3.3 (0.8, 4.3)	3.3 (0.6, 5.7)	2.6 (1.1, 4.3)	
V6	3.5 (1.4, 5.9)	4.2 (2.5, 9.8)	1.9 (0.7, 3.9)	
Time p-value	**<0.001**			

V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

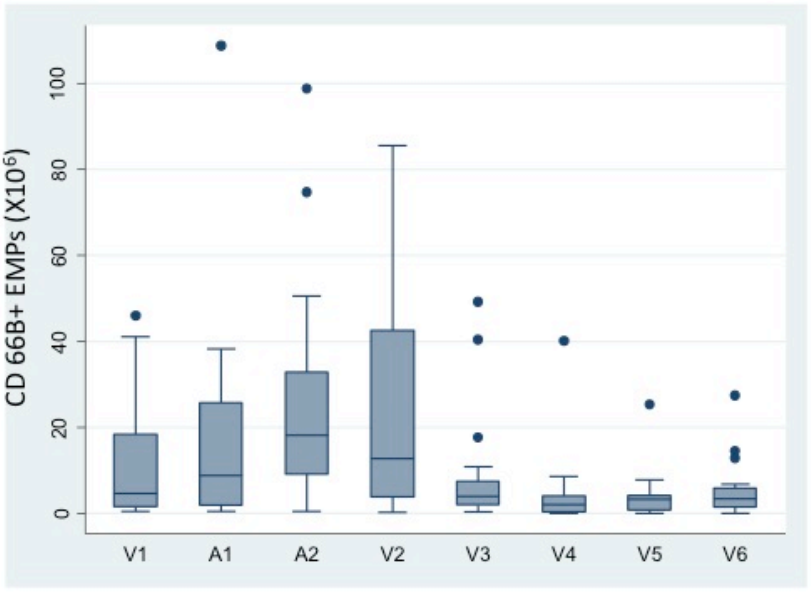


Figure 31. Anv+ CD42- CD66B+NMPs across all time points V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

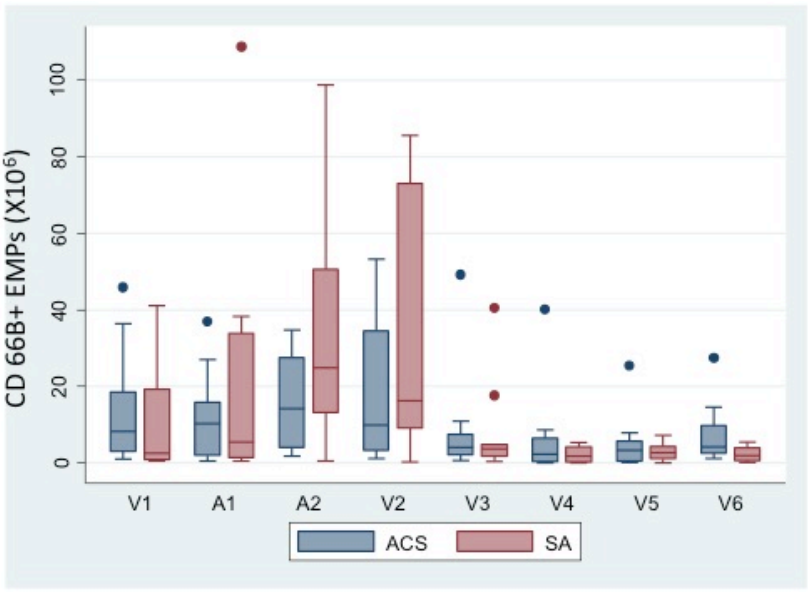


Figure 32. Box plots comparing ANV+CD42-CD66B+NMPs between ACS and SA across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

4.4.7 Micro Particle Measurement 5 – Anv+ CD42- CD54+EMPs

Levels of CD54+ EMPs were mostly higher in the ACS group when compared to the SA group particularly so at six months - 9.9 (3.8, 18.7) vs. 2.6 (0.7, 5.2); p=0.008. (Figures 33-34).

Table 21. CD54+ EMPs (in 100,000s) across all time points and comparing between ACS and SA cohorts

Time point	All Median (IQR)	ACS Median (IQR)	SA Median (IQR)	Group P-value
V1	10.2 (2.7, 30.3)	13.6 (9.8, 22.6)	6.0 (1.4, 30.9)	0.12
A1	10.1 (4.6, 26.8)	7.2 (4.5, 26.3)	10.4 (8.8, 27.2)	0.54
A2	17.3 (10.3, 36.1)	13.1 (5.5, 30.2)	30.0 (14.4, 41.9)	0.11
V2	20.5 (11.4, 38.9)	17.2 (5.7, 28.6)	22.8 (11.4, 55.9)	0.28
V3	4.7 (1.8, 14.5)	4.7 (2.0, 14.5)	5.2 (1.8, 12.2)	0.68
V4	4.2 (1.7, 8.3)	7.5 (4.5, 12.8)	2.7 (1.1, 4.4)	0.15
V5	3.5 (0.4, 9.5)	3.0 (1.4, 14.8)	4.2 (0.3, 9.5)	0.38
V6	4.0 (2.6, 13.5)	9.9 (3.8, 18.7)	2.6 (0.7, 5.2)	**0.008**
Time p-value	**<0.001**	**0.007**	**<0.001**	

V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

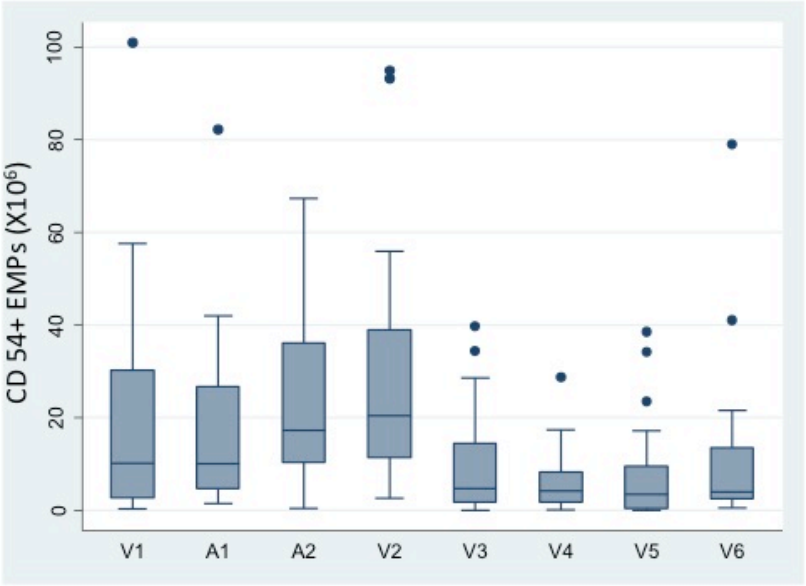


Figure 33. Anv+ CD42- CD54+EMPs across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

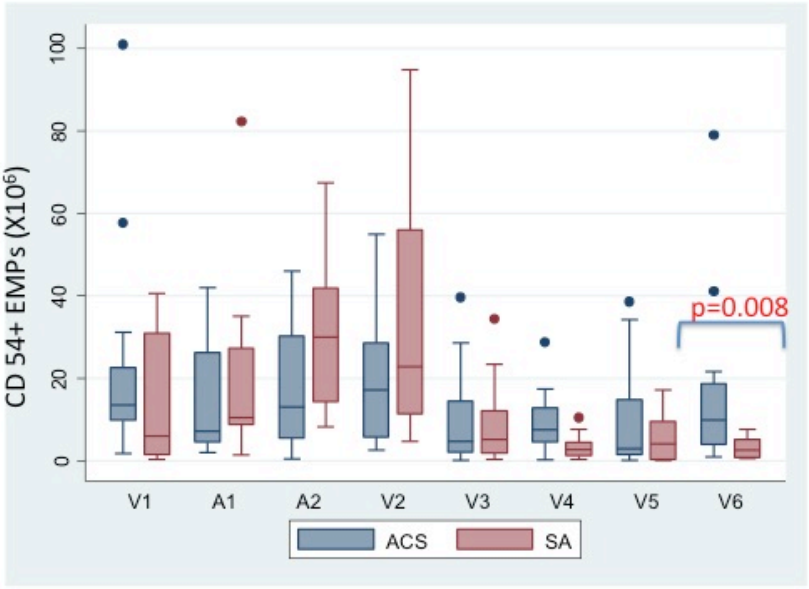


Figure 34. Box plots comparing ANV+CD42-CD54+EMPs between ACS and SA across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

4.4.8 Micro Particle Measurement 6 – Anv+ CD42- 31+

CD31+ EMP levels are enumerated below. Numerically higher levels were noted pre PCI. There was a steady trend of higher levels in the ACS cohort from one month onwards reaching borderline statistical significance at 6 months. (Figures 35-36).

Table 22. CD31+ EMPs (in 100,000s) across all time points and comparing between ACS and SA cohorts

Timepoint	All Median (IQR)	ACS Median (IQR)	SA Median (IQR)	Group P-value
V1	3.7 (1.0, 6.5)	5.5 (1.5, 6.9)	1.5 (0.7, 4.9)	0.31
A1	3.6 (1.7, 10.6)	3.0 (1.5, 11.8)	4.2 (2.2, 9.3)	0.98
A2	7.7 (3.2, 15.6)	5.4 (2.5, 13.7)	8.4 (4.2, 17.6)	0.40
V2	4.7 (2.6, 9.1)	4.2 (0.9, 9.1)	5.3 (3.1, 17.2)	0.24
V3	1.9 (0.4, 6.2)	4.5 (0.8, 6.2)	1.3 (0.4, 3.8)	0.16
V4	0.9 (0.6, 1.7)	1.6 (1.0, 4.9)	0.8 (0.6, 1.2)	0.17
V5	1.3 (0.5, 2.0)	1.8 (0.8, 3.7)	0.9 (0.3, 1.6)	0.12
V6	1.0 (0.4, 2.3)	1.8 (0.5, 3.1)	0.7 (0.3, 1.1)	0.05
Time p-value	**<0.001**	**0.01**	**<0.001**	

V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

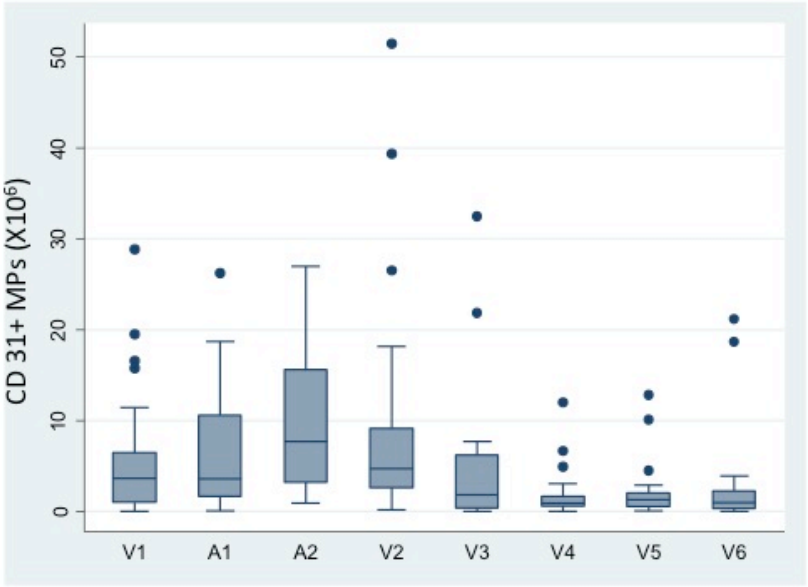


Figure 35. Anv+ CD42- CD31+EMPs across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

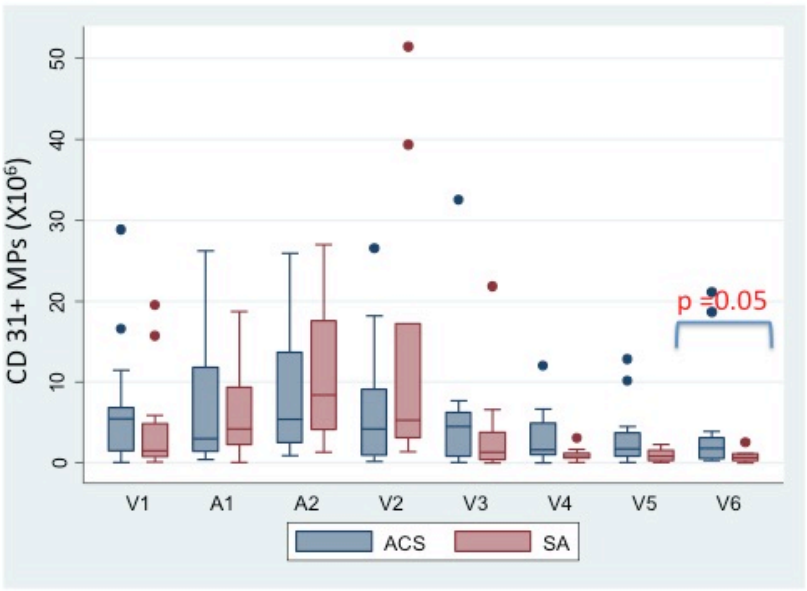


Figure 36. Box plots comparing ANV+CD42-CD31+EMPs between ACS and SA across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

4.4.9 Anv+ CD14+ TF+

CD14+TF+ MP levels are enumerated below. Broadly similar levels of MPs were noted between groups with no specific trend. (Figures 37-38)

Table 23. Anv+ CD14+ TF+ MPs (in 100,000s) across all time points and comparing between ACS and SA cohorts

Time point	All Median (IQR)	ACS Median (IQR)	SA Median (IQR)	Group P-value
V1	3.3 (1.0, 9.9)	3.4 (1.0, 13.4)	2.4 (0.8, 8.8)	0.72 (+)
A1	4.6 (2.4, 12.6)	4.9 (0.8, 22.0)	3.4 (2.5, 7.5)	
A2	6.5 (3.5, 10.9)	7.3 (5.0, 16.9)	4.9 (3.3, 8.5)	
V2	7.4 (2.6, 15.9)	6.6 (1.1, 15.9)	8.2 (3.4, 25.9)	
V3	2.4 (0.4, 21.3)	2.9 (0.5, 12.4)	1.3 (0.1, 21.3)	
V4	2.3 (0.2, 9.3)	3.1 (0.0, 6.0)	1.4 (0.4, 11.8)	
V5	3.1 (0.1, 9.9)	3.4 (0.4, 19.4)	1.7 (0.1, 5.8)	
V6	2.0 (0.3, 9.1)	1.9 (0.8, 14.8)	2.7 (0.2, 8.9)	
Time p-value	**<0.001**			

V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

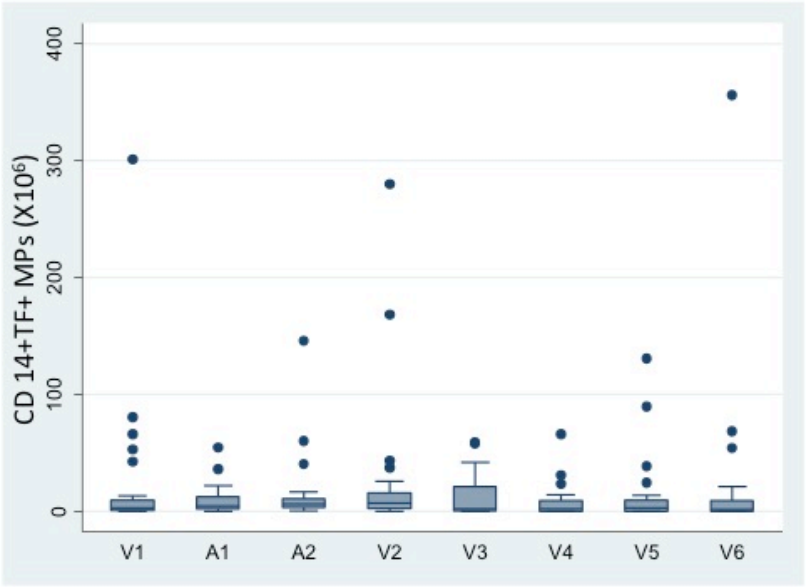


Figure 37. Anv+ CD14+ TF+ MPs across all time points V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

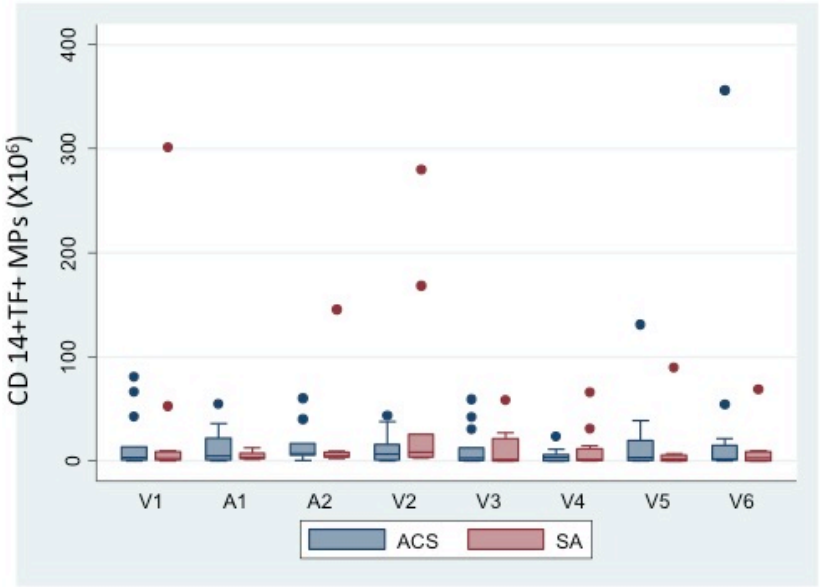


Figure 38. Anv+ CD14+ TF+ MPs between ACS and SA across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

4.4.10 Micro Particle Measurement 8 – Anv+ CD14- TF+

CD14+TF+ MP levels are enumerated below. Broadly similar levels of MPs were noted between groups with no specific trend. (Figures 39-40).

Table 24. Anv+ CD14- TF+ MPs (in 100,000s) across all time points and comparing between ACS and SA cohorts

Time point	All Median (IQR)	ACS Median (IQR)	SA Median (IQR)	Group P-value
V1	21.4 (6.5, 64.0)	20.8 (6.5, 64.0)	30.2 (7.0, 78.8)	0.85 (+)
A1	25.3 (7.2, 86.9)	10.2 (6.7, 108.3)	53.5 (29.9, 65.5)	
A2	40.1 (10.7, 66.1)	21.9 (7.7, 41.0)	62.3 (34.5, 79.6)	
V2	34.8 (13.5, 77.7)	14.6 (9.0, 26.9)	69.1 (42.6, 128.1)	
V3	23.6 (5.3, 50.4)	12.5 (5.3, 46.0)	26.1 (6.1, 50.4)	
V4	11.1 (1.4, 52.8)	6.4 (1.4, 44.8)	22.6 (1.9, 134.6)	
V5	10.7 (1.7, 38.3)	14.8 (1.9, 40.2)	8.4 (1.0, 35.3)	
V6	22.0 (2.4, 75.3)	21.2 (2.4, 65.4)	25.3 (3.0, 120.4)	
Time p-value	**0.001**			

V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

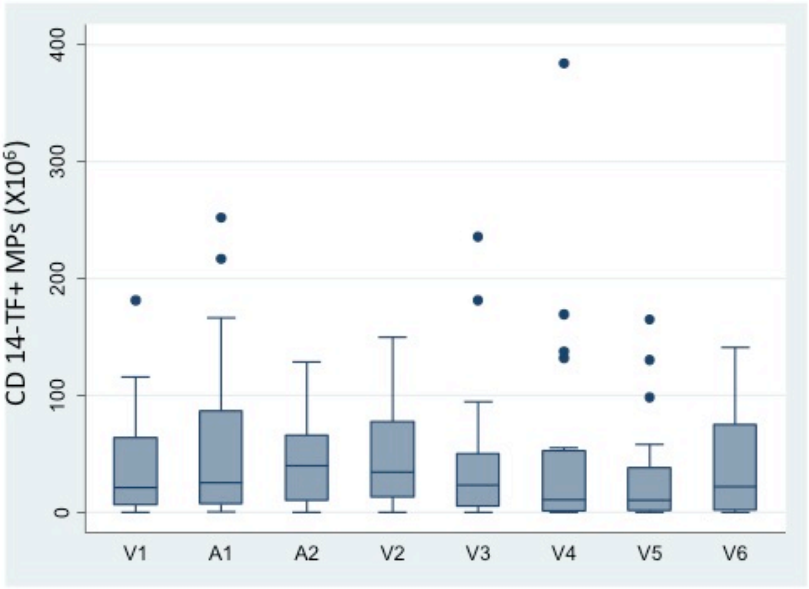


Figure 39. Anv+ CD14- TF+ MPs across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI A1-A2: Pre and post PCI aortic samples.

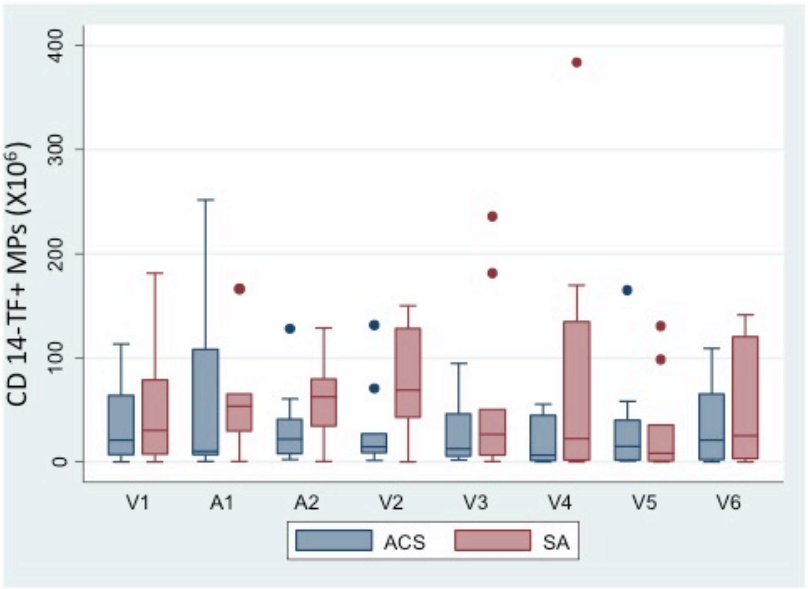


Figure 40. Anv+ CD14- TF+ MPs between ACS and SA across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

4.4.11 Anv+ CD42- NG2+

NG+ SMMP levels are enumerated below. ACS cohort clearly had higher levels though not statistically significant. (Figures 41-42).

Table 25. Anv+ CD42- NG2+MPs (in 100,000s) across all time points and comparing between ACS and SA cohorts

Time point	All Median (IQR)	ACS Median (IQR)	SA Median (IQR)	Group P-value
V1	17.8 (7.3, 29.5)	20.0 (7.3, 31.9)	10.6 (4.9, 27.9)	0.64 (+)
A1	9.4 (3.4, 25.9)	4.4 (3.3, 27.3)	11.6 (7.0, 24.4)	
A2	21.6 (11.7, 29.5)	18.3 (1.4, 27.7)	24.1 (13.5, 50.9)	
V2	28.5 (10.6, 44.3)	26.8 (8.1, 36.5)	31.4 (11.6, 74.9)	
V3	5.3 (2.2, 11.8)	5.3 (2.3, 11.8)	4.4 (2.2, 8.3)	
V4	4.6 (2.8, 15.1)	13.3 (3.5, 19.7)	3.9 (2.4, 6.1)	
V5	6.1 (1.6, 9.6)	7.3 (2.6, 15.0)	5.1 (1.0, 8.6)	
V6	4.7 (2.4, 13.8)	5.9 (2.4, 21.3)	3.1 (1.7, 8.1)	
Time p-value	**<0.001**			

V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

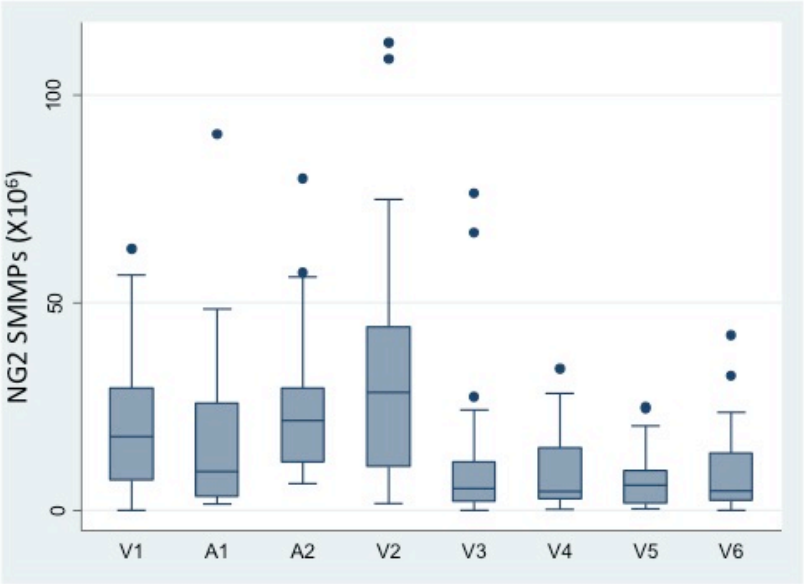


Figure 41. Anv+ CD42- NG2+ MPs across all time points. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

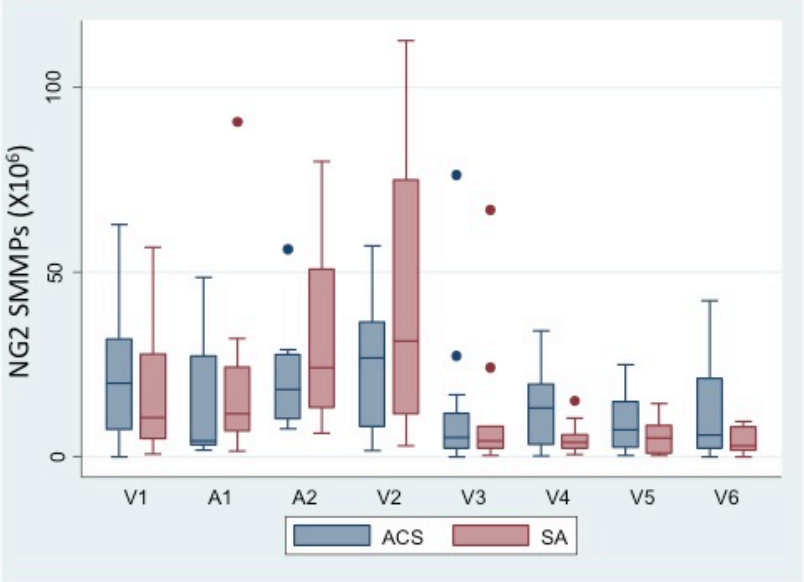


Figure 42. Anv+ CD42- NG2+ MPs between ACS and SA across all time points V1-6: venous samples pre, post and on days 1,7,30,180 post PCI. A1-A2: Pre and post PCI aortic samples.

Box plots of individual MP Phenotypes significantly elevated at 6 months in the ACS cohort (excluding aortic compartment) (Figures 43-45):

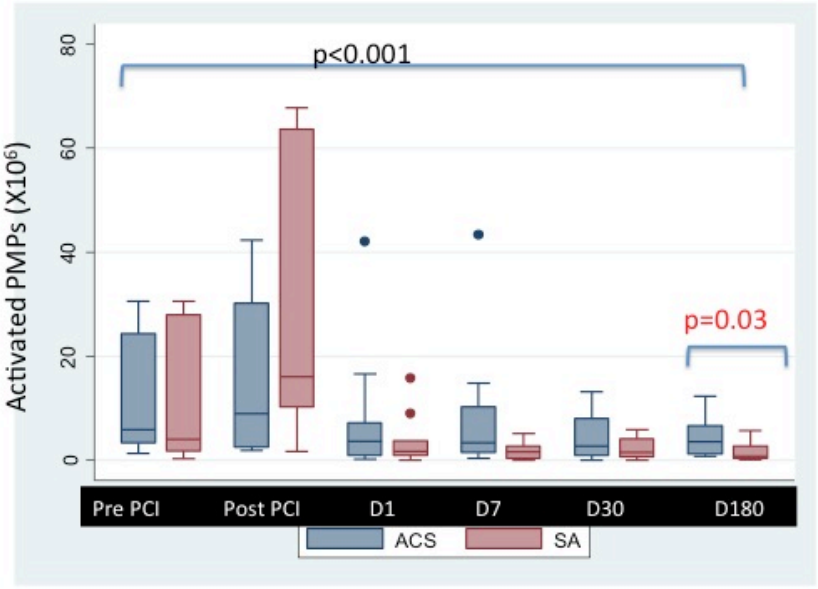


Figure 43. AnV+CD42-62P+PMP. As shown in the figure there was significant difference in the levels of MPs with in groups. PMP levels were high in ACS patients at 6 months when compared to SA. The difference between levels of PMPs between ACS and SA at other time points was not significant. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI.

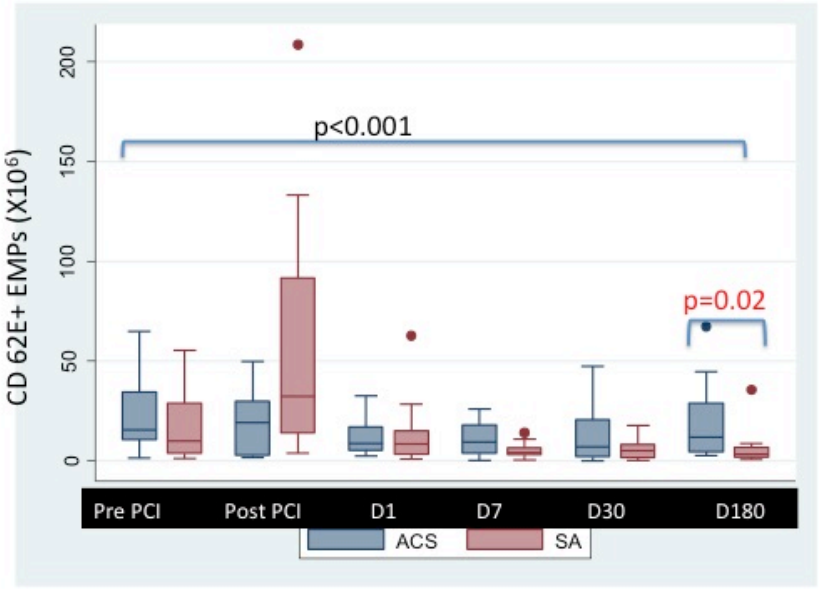


Figure 44. AnV+CD42-62E+EMP. As shown in the figure there was a significant difference in the levels of MPs within groups. EMP levels were high in ACS patients at 6 months when compared to SA. The difference between levels of EMPs between ACS and SA at other time points was not significant. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI.

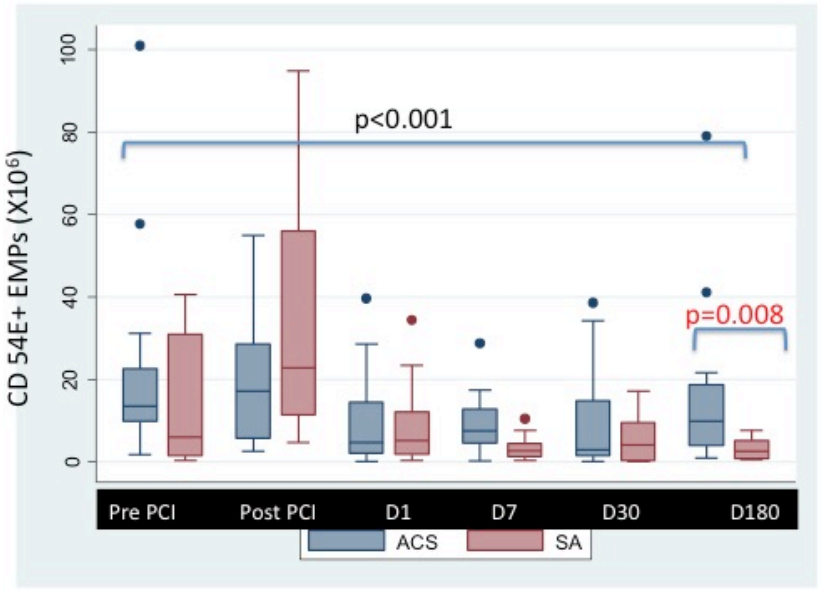


Figure 45. AnV+CD42-54+EMP. As shown in the figure there was significant difference in the levels of MPs with in groups. EMP levels were high in ACS patients at 6 months when compared to SA. The difference between levels of EMPs between ACS and SA at other time points was not significant. V1-6: venous samples pre, post and on days 1,7,30,180 post PCI.

4.4.12 MP mediated TGA

Further analyses were performed to examine how the thrombin parameters change over time. Firstly, the analysis was performed for all patients combined (Table 26). Parameters found to be normally distributed, and analysed without a transformation, were summarised by the mean and standard deviation at each time point. Parameters found to have a skewed distribution, and analysed on the log scale, were summarised by the median and inter-quartile range at each time point.

Table 26. Thrombin parameters across the whole cohort

Variable	Time point	Mean (SD) or Median [IQR]	P-value
Lag time	Time 1	16.3 (6.7)	**<0.001**
	Time 2	16.9 (7.0)	
	Time 3	16.6 (7.7)	
	Time 4	15.0 (5.2)	
	Time 5	8.4 (6.0)	
	Time 6	16.7 (3.9)	
Thrombin	Time 1	254 [225, 339]	0.12
	Time 2	281 [140, 357]	
	Time 3	271 [216, 331]	
	Time 4	287 [242, 304]	
	Time 5	303 [291, 304]	
	Time 6	316 [258, 536]	
Velocity index	Time 1	25.1 [16.1, 38.5]	0.82
	Time 2	28.7 [13.9, 51.0]	
	Time 3	28.0 [20.7, 37.8]	
	Time 4	32.4 [13.5, 46.6]	
	Time 5	28.3 [25.1, 33.4]	
	Time 6	28.7 [18.4, 38.8]	
AUC	Time 1	3562 (721)	**<0.001**
	Time 2	3325 (993)	
	Time 3	3885 (714)	
	Time 4	3636 (798)	
	Time 5	4376 (451)	
	Time 6	3509 (746)	

The analyses suggested significant differences between time points for lag time

& AUC. No significant differences were observed for thrombin and velocity index.

The results for lag time suggested a relatively constant average lag time at the different time points, with the exception of time point 5 (one month post PCI) (Figure 46) where the values were much lower than at other time points. There was no clear trend in values for AUC (Figure 47).

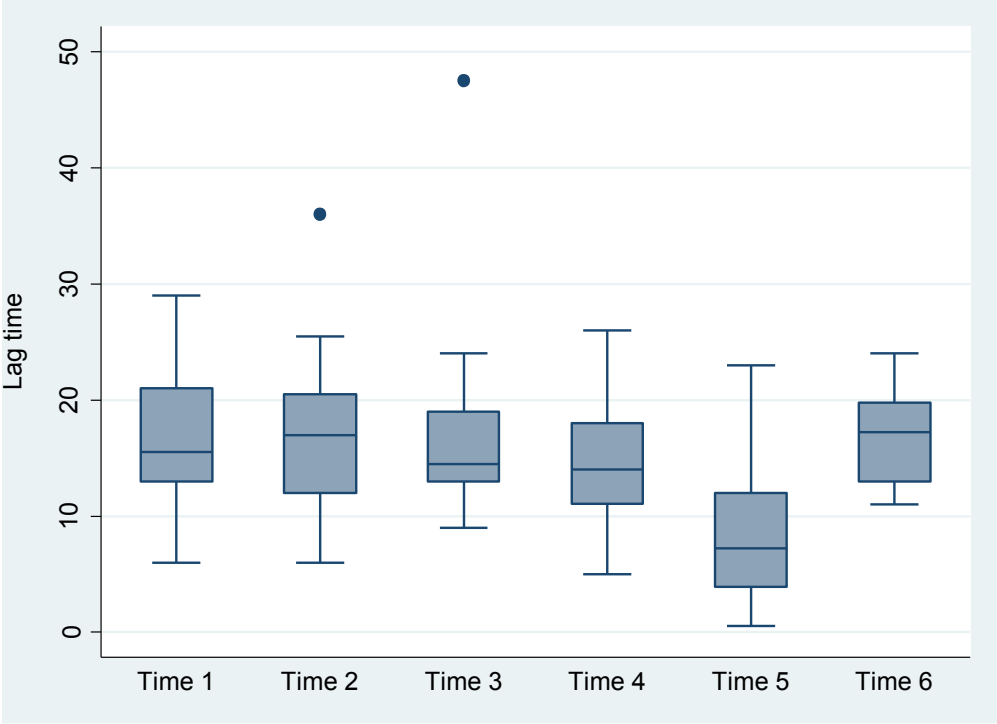


Figure 46. Lag time across the whole cohort. Time 1 – Pre PCI, Time 2 – post PCI, Time 3 – day 1post PCI, Time 4 – day 7 post PCI, Time 5 – day 30 post PCI and Time 6 – day 180 post PCI.

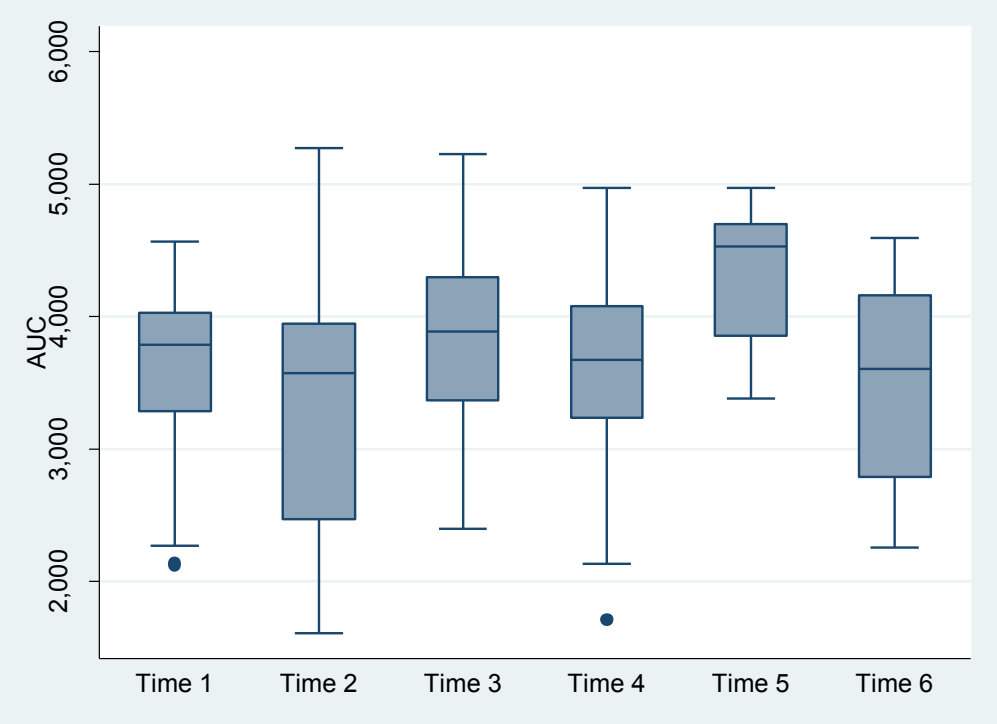


Figure 47. AUC across the whole cohort. Time 1 – Pre PCI, Time 2 – post PCI, Time 3 – day 1post PCI, Time 4 – day 7 post PCI, Time 5 – day 30 post PCI and Time 6 – day 180 post PCI.

When compared between ACS an SA groups MP mediated TGA at various time points revealed abnormal lag phase, peak thrombin, velocity index and endogenous thrombin potential as reflected by area under curve (AUC) in both ACS and SA groups compared to healthy controls (Table 27).

Table 27. Thrombin parameters comparing ACS and SA cohorts

Variable	Time point	ACS subgroup		SA subgroup	
		Mean (SD) or Median [IQR]	P-value	Mean (SD) or Median [IQR]	P-value
Lag time	Time 1	16.8 (7.1)	**0.04**	15.7 (6.5)	**<0.001**
	Time 2	15.7 (4.7)		18.6 (9.2)	
	Time 3	18.0 (10.2)		15.3 (4.6)	
	Time 4	16.1 (6.4)		13.8 (3.4)	
	Time 5	9.8 (7.0)		6.8 (4.5)	
	Time 6	15.8 (4.0)		18.0 (3.8)	
Thrombin	Time 1	254 [226, 339]	0.13	255 [219, 328]	0.46
	Time 2	276 [137, 346]		318 [223, 401]	
	Time 3	261 [229, 308]		275 [193, 445]	
	Time 4	288 [207, 338]		287 [242, 291]	
	Time 5	304 [296, 306]		304 [291, 305]	
	Time 6	316 [282, 583]		295 [249, 469]	
Vel index	Time 1	20.9 [12.3, 26.2]	0.31	35.4 [21.7, 45.6]	0.99
	Time 2	28.3 [13.3, 60.0]		28.7 [17.1, 45.4]	
	Time 3	27.1 [20.7, 38.3]		28.3 [19.9, 36.8]	
	Time 4	33.4 [11.1, 48.1]		30.6 [26.1, 46.6]	
	Time 5	28.6 [25.4, 36.0]		28.3 [24.4, 30.8]	
	Time 6	28.9 [18.4, 38.8]		28.0 [18.1, 37.6]	
AUC	Time 1	3411 (685)	**0.003**	3726 (751)	**0.004**
	Time 2	3275 (901)		3393 (1157)	
	Time 3	3979 (550)		3799 (853)	
	Time 4	3491 (693)		3795 (909)	
	Time 5	4198 (494)		4569 (315)	
	Time 6	3467 (776)		3572 (745)	

The results suggested significant differences in lag time between time points for both subgroups. The results for each subgroup mirrored those of the analysis of all patients, with values at one-month post PCI lower than at all other time points.

Both subgroups showed a significant difference in AUC values between time points, with the highest values again found one month after PCI. Illustrations of some of the significant results by subgroup are shown in subsequent graphs.

4.4.13 Individual TGA parameters (Figures 48-49)

4.4.13.1(a) Lag phase

Lag phase at baseline for ACS, SA and controls were; median16.8 (range 7.1), 15.7 (6.5) and 20.5 (7.1) seconds respectively (p=0.31). Lag phase was shortest at 1 month across both groups (9.8 (7.0) for ACS vs. 6.8 (4.5) seconds for SA; (p=0.45) before reaching baseline levels by 6 months (15.8 (4.0) for ACS vs. 18.0 (3.8) seconds for SA, p= 0.31).

4.4.13.1(b) Thrombin

The amount of thrombin generated was also similar between ACS, SA and controls at baseline was (254 (226, 339 vs. 255 (219, 328) vs. 132 (57, 252); p=0.006). However, at six months the ACS cohort generated more thrombin, although this result was not statistically significant (316 (282, 583) vs. 295 (249, 469); p=0.09).

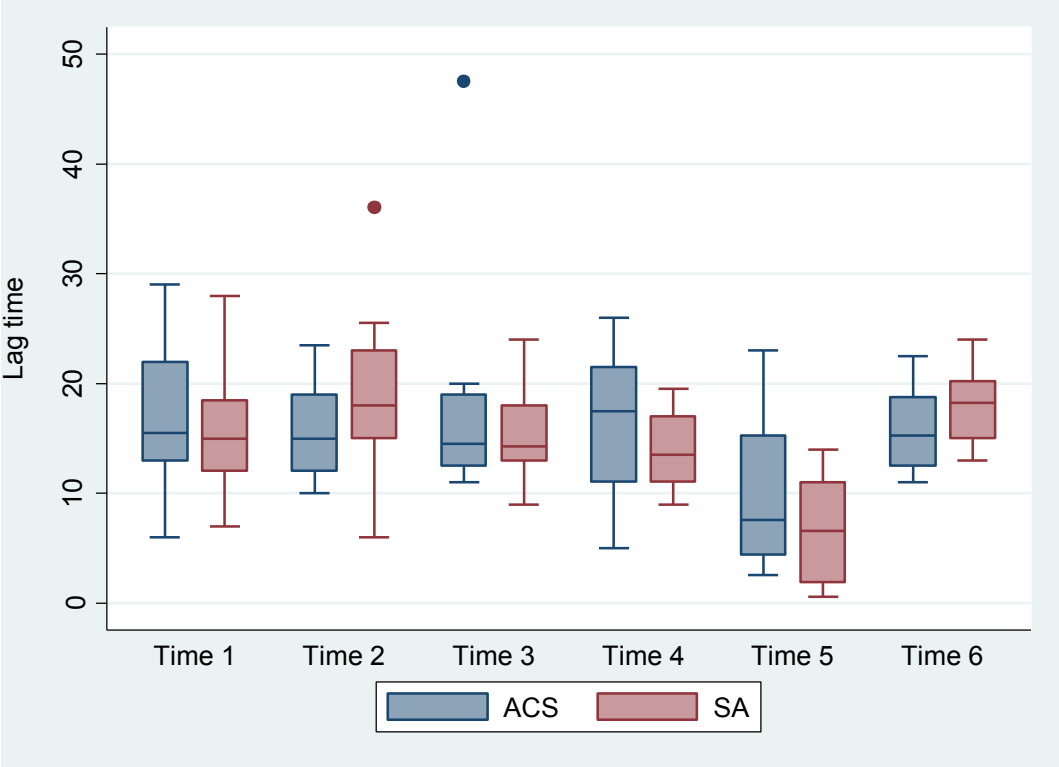


Figure 48. Lag time comparing ACS and SA cohorts. Time 1 – Pre PCI, Time 2 – post PCI, Time 3 – day 1post PCI, Time 4 – day 7 post PCI, Time 5 – day 30 post PCI and Time 6 – day 180 post PCI.

4.4.13.1(c) Area under curve (AUC)

AUC is a measure of endogenous thrombin generation potential and is a factor of peak thrombin and velocity index (rate of thrombin generation). At baseline the AUC for ACS, SA and control groups was 3411 (685), 3726 (751) and 2334 (1319) respectively (p=0.006). At six months these values were 3467 (776) and 3572 (745) for ACS and SA groups respectively (p=0.45).

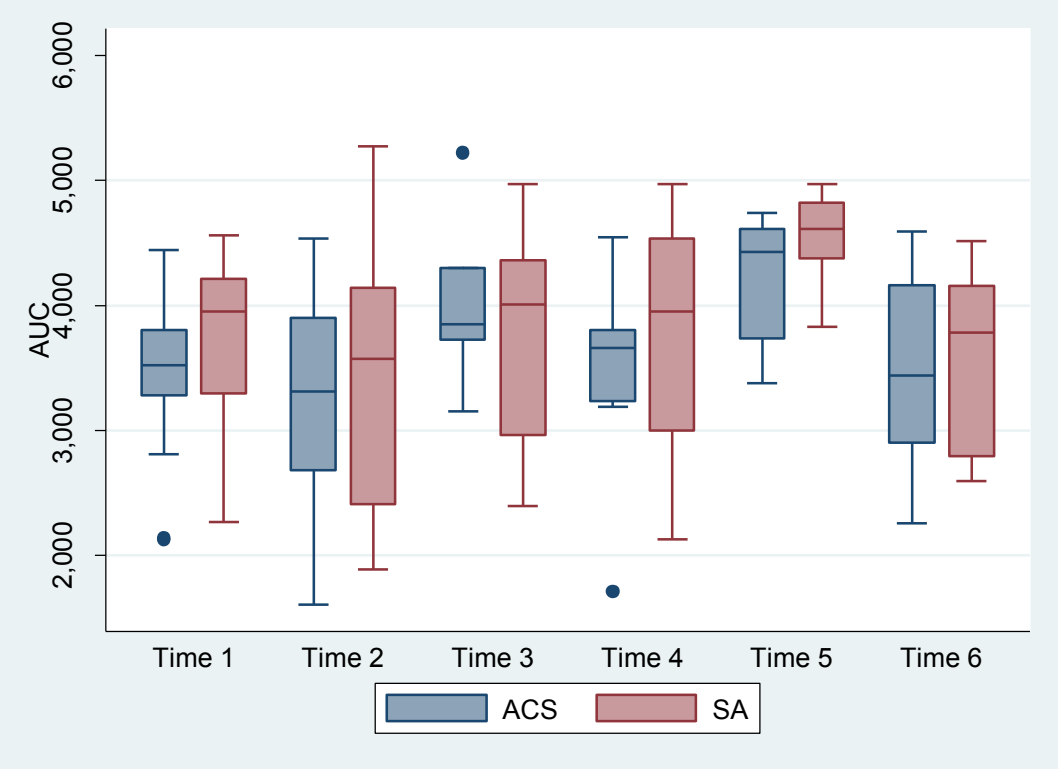


Figure 49. Lag time comparing ACS and SA cohorts. Time 1 – Pre PCI, Time 2 – post PCI, Time 3 – day 1post PCI, Time 4 – day 7 post PCI, Time 5 – day 30 post PCI and Time 6 – day 180 post PCI.

4.4.13.1(d) Comparisons with control group

The final set of analyses compared the thrombin parameters at the first time-point between the study groups; two patient groups and a control group. A summary of the analysis of results is given in the next table (Table 28). Normally distributed variables were summarised by the mean and standard deviation, whilst variables with a skewed distribution were summarised by median and inter-quartile range. P-values indicating the significance of the overall group difference are also reported.

Table 28. Comparison of TGA parameters between all three groups. Summary statistics are Mean (SD) or Median [IQR]

TGA parameter	ACS	SA	Control	P-value
Lag time	16.8 (7.1)	15.7 (6.5)	20.5 (7.1)	0.31
Thrombin	254 [226, 339]	255 [219, 328]	132 [57, 252]	**0.006**
Vel index	20.9 [12.3, 26.2]	35.4 [21.7, 45.6]	11.9 [4.6, 30.8]	**0.02**
AUC	3411 (685)	3726 (751)	2334 (1319)	**0.006**

The results suggested that there were overall differences between the three groups for all of thrombin, velocity index and AUC. The three groups did not significantly differ for lag time. Additionally, specific comparisons between pairs of groups were made, and the p-values from these comparisons are summarised in the next table (table 29). The p-values were given a Bonferroni adjustment to allow for multiple testing.

Table 29. Comparison of TGA parameters between all three groups

Variable	ACS vs. SA P-value	ACS vs. Control P-value	SA vs. Control P-value
Lag time	1.00	0.74	0.41
Thrombin	1.00	**0.006**	**0.02**
Vel index	0.59	0.18	**0.01**
AUC	1.00	**0.04**	**0.006**

There was a significant difference in thrombin values between the control group and each of the two patient groups. The values were significantly lower in the control group, with a median of 132 compared to a median of over 250 in the other two groups. There was a significant difference in velocity index between the control group and the SA group, with significantly lower values in the control group. However, no difference between the control and ACS groups was observed. AUC values were found to be significantly lower in the control group than in both patient groups. The two patient groups (ACS and SA) did not significantly vary for any of the thrombin parameters.

4.4.14 Correlation between MPs and TGA

The results suggested significant positive correlation between thrombin parameters and MPs from the first venous sample obtained pre PCI. In ACS cohort significant correlation was noted between total MPs with velocity index (r= 0.672, p=0.035). In SA cohort there was significant correlation between total MPs with AUC (r= 0.636, p=0.035), CD62P with thrombin (r= 0.627, p=0.039 and AUC (r= 0.755, p=0.007), CD62E, CD54 and SMMP with AUC (r= 0.655, p= 0.029; r= 0.664, p=0.02; r= 0.809, p=0.003) respectively (Figure 50).

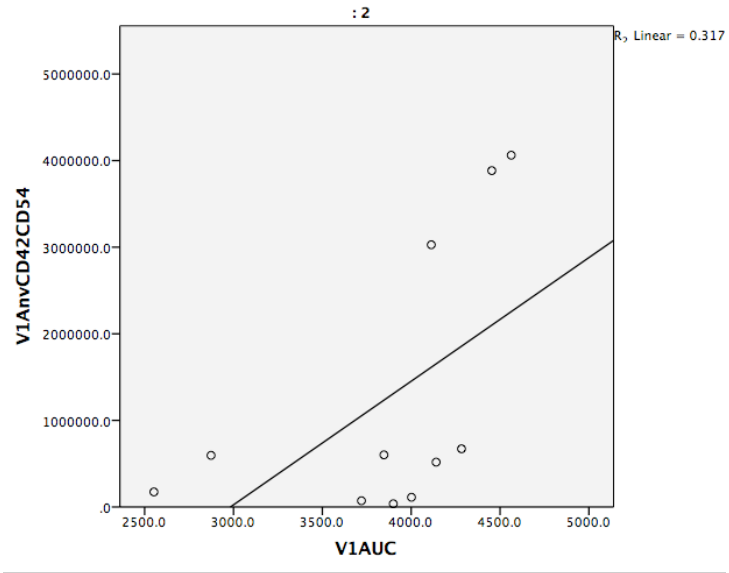


Figure 50. Correlation between CD54+EMPs and AUC pre PCI in SA cohort

4.5 Discussion

MPs were previously shown to be elevated in CAD; more so in acute coronary syndromes than stable angina and are associated with adverse outcomes (64,140,175). MPs were also shown to correlate with inflammatory markers such as IL-6 and CRP in previous studies (49,140,178). This, in addition to lab based studies demonstrating the capability of MPs to act as transporters for micro RNAs, lends further credence to their role in CAD (19,179,180). Although MPs are known to be elevated at the time of the index event in ACS patients (48), no information exists regarding the balance on production and clearance post ACS. Studies in the past quantified the levels of MPs post PCI but not

beyond 48 hours (48,67). By carrying out serial quantification of MPs to 6 months I have demonstrated that production and clearance of MPs is dynamic in patients with CAD. MPs were higher following index event in ACS patients though statistically not significant when compared to the SA cohort. This finding perhaps reflects the chronic inflammatory state seen in CAD, with an elevated baseline level of MPs secondary to on-going apoptosis or cellular/platelet activation. Following the index event in ACS, a spike in MPs occurs due to activation of various cells. This is evident by the fact that in the SA cohort total and individual phenotypes of various MPs returned to near baseline levels in the longer term. It is not possible to assume a similar situation in the ACS cohort as the baseline was not known given the event had already occurred a few days prior to obtaining blood samples for MP measurement. Furthermore, the attenuating effects of potent antiplatelet drugs and low molecular weight heparin / direct thrombin inhibitors may have had resulted in the reduction of MP levels in ACS cohort. However, we observed the levels of MPs to be dynamic at various time points. The most striking finding and to the best of my knowledge demonstrated for the first time, was higher individual phenotypes of MPs in the ACS cohort at 6 months when compared to the SA cohort despite antiplatelet drugs and high dose statins. The individual phenotypes of MPs that were elevated at 6 months in the ACS cohort when compared to the SA cohort were CD54+ EMPs, CD62E+ EMPs and CD62P+PMPs. These findings suggest on-going endothelial injury across both cohorts of patients, though significantly more in the ACS cohort. Previous studies demonstrated that constitutively expressed EMPs (CD31+, CD105) were elevated in relation to apoptosis where as inducible EMPs (CD62E, CD54 & CD106) were elevated in activation (181). Thus CD62E EMPs expressed on activated endothelium (182) is likely to be reflective of on-going endothelial injury in ACS patients, either directly related to the index lesion causing the ACS, and/or from areas of activated endothelium remote from the index lesion. Similarly, CD54 or intercellular adhesion cell molecule (ICAM-1), expressed on activated endothelial cells and activated monocytes, is reflective of underlying inflammation. CD31 or platelet endothelial adhesion cell molecule (PECAM) is expressed on platelets, monocytes and on endothelial cells particularly in apoptosis, has a role in angiogenesis and in neutrophil recruitment (183). The role of angiogenesis and neutrophil recruitment in plaque instability is well established; angiogenesis and resultant

formation of friable neovessels can lead to intraplaque haemorrhage (168) and matrix metalloproteinases released by neutrophils in atherosclerotic plaque can lead to degradation of collagen / extracellular matrix(184). Furthermore, the role of CD 31+ MPs in amplifying the expression of the selected miRNAs in patients with vulnerable CAD was also noted (185). Another novelty in my study was characterisation of SMMPs. To the best of my knowledge this was not carried out before in subjects with CAD. The levels of SMMPs were clearly numerically higher in the ACS cohort at all time points apart from post PCI. The importance of this need to be explored further. The clinical importance of elevated activated PMPs and certain phenotypes of EMPs at 6 months need to be explored further in a large cohort of patients as data relating to prognostic importance of activated platelets and certain phenotypes of MPs following acute coronary event is emerging (55,186,187).

Another interesting observation of note was the massive spike in total and individual phenotypes of MPs following PCI. The spike was particularly large in the SA cohort reflecting the fact the iatrogenic endothelial injury by balloon angioplasty in a non-primed environment whereas in the ACS cohort there was preceding smouldering endothelial injury and inflammation. By stabilising the culprit plaque with mechanistic PCI therapy, the resultant passivation most likely led to decreased levels of MPs and thrombin parameters as seen on day 1 in my study. However, a rather curiously rebound phenomenon of MPs and thrombin parameters were seen at 6 months suggesting the role of underlying inflammation. In their respective studies Biasucci et al & Inoue et al also noted similar response(48,67).

The abnormal TGA was also worthy of note: thrombin generation remained abnormal at 6 months with shorter lag time and higher thrombin in the ACS cohort but did not discriminate between ACS and SA. Previous studies have shown enhanced thrombin generation in patients with CAD, though correlation with MP was not carried out. Borissoff et al showed positive independent association between severe coronary atherosclerosis and in vivo thrombin generation (188). In my study TGA was abnormal in both groups at index event and at 6 months, though intuitively one would expect abnormal thrombin generation assay in ACS cohort. This could be explained by the fact that multi-

vessel disease was seen in a similar percentage of patients from both groups; and by the observation that optical coherence tomography studies demonstrate incidental healed ruptured plaques in the SA cohort (albeit less frequently; data from our optical coherence tomography sub study – manuscript in preparation). Another relatively old study from the era when Aspirin was still the mainstay of therapy following AMI showed enhanced thrombin generation for up to 2 years following myocardial infarction (189). My study has shown that this is still the case despite patients being treated with potent secondary prevention regimes. Figueras et al demonstrated greater thrombin generation at 10 days in patients who develop angina following AMI or UA when compared to other who attain early stability (190). The explanation for the findings in my study and observed correlations is uncertain. It may be secondary to activated PMPs that are rich in procoagulant phosphatidylserine (191). Furthermore, TF present on activated endothelium as reflected by higher number of CD54 and CD62E+ EMPs may also be contributing. Whilst the comparable thrombograms between ACS and SA at the time of index event could be attributed to the attenuating effects of low molecular weight heparin treatment given to ACS patients, the comparable thrombograms at 6 months remain unexplained. This may well be explained by the fact that silent atherothrombotic events are common in stable CAD when compared to annual incidence of myocardial infarction (159).

4.6 Limitations

Limitations associated with a prospective observational study involving small sample size at a single centre are worthy of note. However, the study design allowed us to carry out robust analysis of MPs and TGA at various time points giving valuable information. The study is underpowered with a limited follow up to observe any hard end points. Thus, elevated MPs and abnormal TGA at 6 months remains an observation and if they have any association with recurrent cardiovascular events is not established. Furthermore, the pattern of MP clearance in our study also remains an observation and whether underlying inflammation and treatment modulates MP release and clearance is not proven. Another limitation is use of platelet poor plasma for TGA. Platelet poor plasma is a pool of all MP phenotypes thus it was not possible to pinpoint which MP fraction contributed most to the thrombogenesis.

4.7 Conclusions

My study was successful in demonstrating altered levels and signature of MPs at different time points following index event. Furthermore, my study for the first demonstrated persistent excess thrombin generation, with good correlation with some individual MP phenotypes, which may explain the prolonged prothrombotic state that exists after ACS. My study also questions the adequacy of current preventive therapy. Studies with large number of participants are required to further explore if MPs have a role as biomarker and therapeutic target in CAD.

Chapter 5. Association Between Circulating Microparticles and Optical Coherence Tomography Derived Coronary Atherosclerotic Plaque Characteristics

5.1 Summary

Introduction and aim

Microparticles (MPs) are implicated in the pathogenesis of ACS. I set out to determine whether circulating MPs correlate with high-risk coronary atherosclerotic plaque phenotype and to assess if vulnerable plaque characteristics have any relation to the thrombogenic potential of MPs after the index event.

Patients and methods

I characterized coronary atherosclerotic plaque burden and phenotype by three-vessel optical coherence tomography (OCT) in 25 patients [13 ACS & 12 Stable angina (SA)] undergoing PCI. Endothelial (EMPs), platelet (PMPs), Neutrophil (NMPs), tissue factor (TFMPs) and smooth muscle (SMMPs) were measured at various time points for up to 6 months. Pre and post PCI MPs measured were correlated with OCT parameters. MPs and their thrombogenicity was compared between patients with and with out thin cap fibroatheroma (TCFA).

Results

OCT analysis revealed ACS patients had significantly higher lipid volume and thinner fibrous cap in culprit and non-culprit coronary arteries. Levels of MPs were similar between groups at index event and up to 1 month. However, at six months CD54+EMPs [Median 9.9 (IQR 3.8,18.7) Vs. 2.6 (0.7, 5.2); p=0.008], CD62E+ EMPs [11.9 (4.7, 29.0) vs. 3.4 (1.8, 6.7); p=0.02] and CD62P+PMPs [3.6 (1.2, 6.7) vs. 0.7 (0.4, 2.7); p=0.03] were significantly elevated in the ACS cohort. Furthermore there was significant correlation between EMPs and

SMMPs with ruptured plaques (% frames) (r=0.4, p=0.03) in the ACS cohort. Patients with TCFA did not show a difference in MP expression but exhibited enhanced thrombogenecity as evidenced by higher endogenous thrombin potential (AUC - 0.744, p value – 0.039).

Conclusion

ACS patients exhibit more vulnerable plaque features; have higher microparticle load at follow up and are pro-thrombotic despite secondary prevention including dual anti-platelet therapy. MPs may have a role as novel biomarkers in identifying vulnerable patients. Patients with TCFA expressed enhanced thrombogenecity irrespective of clinical presentation suggesting the association of this plaque phenotype with vulnerability on a patient level

5.2 Introduction

Microparticles (MPs) are pro-inflammatory and pro-thrombotic submicron phospholipid vesicles derived from eukaryotic cells up on activation or apoptosis (18,179). Following activation the plasma membrane of cells such as platelets, leukocytes and endothelial cells forms into blebs, which are then cleaved by caspases into MPs (16). The stimulus for activation is usually by processes such as inflammation or sepsis (80). Atherosclerosis is an inflammatory process and it has been previously shown that MPs are elevated in the circulation of patients with coronary artery disease (CAD) and appear to correlate with the severity of disease (175,192). Sinning et al demonstrated high levels of circulating CD31+/Annexin V+ (AnV+) MPs as an independent predictor of cardiovascular events in stable CAD patients with a potential role for risk stratification (64). Lee et al established association of CD62E+ MPs with cardiovascular events in patients with prior stroke history, suggesting their role in ongoing systemic endothelial activation and inflammation, which in turn increases the risk of cardiovascular events (63). The levels of circulating MPs in the blood are modulated in disease states and, therefore, might be useful as biomarkers for cardiovascular diseases. Traditional scoring systems do not predict future cardiovascular events accurately (193-195). Nozaki et al previously demonstrated how better risk stratification of cardiovascular events can be achieved by assessing EMP mediated endothelial dysfunction and

incorporating it into multiple biomarker model (196). MPs fit the role of an ideal biomarker in CAD as they indicate inflammatory status as well as endothelial dysfunction and damage (53). Optical coherence tomography (OCT) allows for a detailed analysis of coronary atherosclerotic plaque characteristics in humans and thereby provides valuable in vivo information regarding plaque stability (107). Given that circulating concentrations of MPs may reflect vulnerable plaque phenotype, we assessed coronary atherosclerotic plaque characteristics by OCT and measured the levels of various relevant circulating MPs and their procoagulant potential. The aim was to establish association of circulating MPs with coronary atherosclerotic plaque characteristics particularly vulnerable plaque phenotype and to test the MPs serially for up to 6 months so that a reliable peripheral signal can be identified.

5.3 Patients & methods

13 patients with ACS scheduled for invasive angiography and percutaneous coronary intervention (PCI) were included in the study. 12 patients with SA were recruited as a comparator group. A 3rd group consisting of 10 age-matched subjects with no evidence of CAD were recruited as controls for TGA aspect of study. The ACS group included patients with non-ST elevation–myocardial infarction (NSTEMI) and unstable angina (UA). Patients with prior coronary artery bypass graft surgery (CABG), renal failure with estimated glomerular filtration rate < 50 and those that were not able to consent were excluded. Ethical approval was obtained from London – Stanmore research ethics committee. Informed consent was obtained from every patient prior to participation in the study. The methodology for MP quantification, thrombin generation assay and coronary OCT image acquisition is as described in chapters 3 & 4.

5.3.1 Statistical analysis

Normal data is presented as mean ± SD. Non-parametric data are reported as median and interquartile range (IQR). Categorical variables were compared with chi-square or Fisher's exact test, as appropriate. We analysed the strength of association between the microparticle measurements and the OCT variables in each of the ACS and SA subgroups separately. Due to the skewed

distribution of the microparticle measurements (and some of the OCT variables), all analysis was performed using Spearman's rank correlation. We then examined if the differences in the associations between the OCT and microparticle variables varied between subgroups (ACS vs. SA & TCFA vs. non TCFA). These were performed using a test of independent correlations.

5.4 Results

5.4.1 Patient characteristics

Majority of the patients across both groups are of male sex. More number of patients in ACS group was on statins. Both groups were evenly matched on other variables (Table-30).

Table 30 – Baseline characteristics

	ACS (n=13)	SA (n=12)	p value
Age (mean ± SD)	60.1 ± 8.14	56.6±8.9	0.32
Male (n, %)	12 (92.3)	11 (91.7)	0.79
Diabetes (n, %)	5 (38.4)	2 (16.7)	0.67
Hypertension (n, %)	5 (38.5)	4 (33.3)	1
Hyperlipidaemia (n, %)	12 (92.3)	8 (67)	0.15
Smoking (n, %)	3 (23.1)	4 (33.3)	0.4
Family History of CAD (n, %)	1 (7.7)	3 (25)	0.65
Previous PCI (n, %)	2 (15.3)	0	0.15
Creatinine (Median, IQR)	82.5 (76.25 – 88.50)	84 (75.2-87.5)	0.6
Troponin (Median, IQR)	0.24 (0.2 - 3.8)		
CRP (Median, IQR)	4 (1 - 6.25)		
LVEF (mean ± SD)	58.3±5.3	57.8±8.5	0.6
Medications:			
ACE- I / ARB (n, %)	3 (23.1)	4 (33.3)	0.82
Beta-blockers (n, %)	4 (31)	4 (33.3)	1
Aspirin (n, %)	7 (54)	4 (33.3)	0.69
Clopidogrel (n, %)	11(85)	12 (100)	0.8
Ticagrelor (n, %)	2 (15)	0 (0)	0.1
Statin (n, %)	10 (77)	4 (33.3)	0.08
Distribution of CAD (n, %):			
1 vessel disease	5 (38.5)	7 (58.3)	0.2
2 vessel disease	4 (31)	2 (17)	0.3

3 vessel disease	4 (31)	3 (25)	0.8

M – Male, CAD – Coronary artery disease, PCI – Percutaneous coronary intervention, CRP - C-reactive protein, LVEF – left ventricular ejection fraction, ACE – I – Angiotensin converting enzyme inhibitors, ARB – Angiotensin receptor blockers, LAD – Left anterior descending, LCX – Left circumflex and RCA – Right coronary artery.

5.4.2 Microparticles, Thrombin generation assay & Troponin

Total AnV+ MPs and individual phenotypes were numerically higher in ACS cohort but statistically not significant. Furthermore no significant gradient was noted between aortic and venous compartments. Post PCI a similar significant spike in MPs were seen across venous and aortic compartments in both ACS and SA cohorts. At six months CD54+EMPs [Median 9.9 (IQR 3.8,18.7) Vs. 2.6 (0.7, 5.2); p=0.008], CD62E+ EMPs [11.9 (4.7, 29.0) vs. 3.4 (1.8, 6.7); p=0.02] and CD62P+PMPs [3.6 (1.2, 6.7) vs. 0.7 (0.4, 2.7); p=0.03] were significantly elevated in the ACS cohort (detailed results presented in Chapter 5). Thrombin generation was abnormal in the study groups when compared to controls. The study groups demonstrated shorter lag time, higher thrombin generation, higher velocity index and bigger area under curve (AUC). However no significant difference was noted between above parameters in ACS and SA cohorts. Positive correlation was noted between pre PCI venous total AnV+ MPs and high sensitive troponin T levels in the ACS cohort (Figure 53). Correlation of Troponin with EMPs and NMPs was borderline.

5.4.3 OCT analysis

OCT analysis revealed ACS patients had more lipid plaque burden when compared to SA cohort. This was evident by longer lipid length, 20mm (11,35) in ACS vs. 13mm (2.25, 20.75) in SA; p = 0.014, wider maximum lipid arc, 165.1 (10.9.4, 253.3) in ACS vs. 125.3 (78.5, 161.7) in SA; p = 0.024 and higher lipid volume, 1819.5 (694.5, 3638.8) in ACS vs. 860.3 (314.4, 1620.6) in SA; p=0.14. Further more the fibrous cap was thinner in ACS patients. The % frames of neovessles were numerically higher in SA but statistically not significant [5.3 (1.5, 14.7) vs. 1.9 (0, 8.2); P=0.1]. Ruptured plaques not related to culprit lesion were seen in both SA (50%) and ACS (37%) cohorts (detailed results presented in chapter 4).

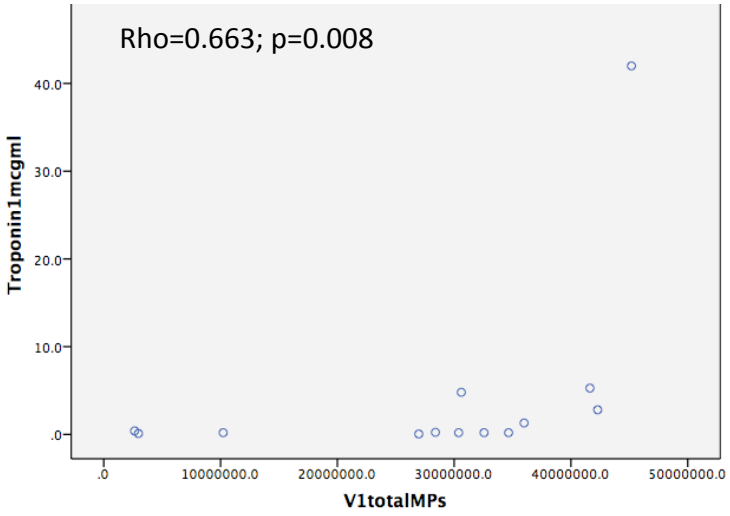


Figure 51. Correlation of Troponin with total AnV+MPS

5.4.4 Correlations of circulating MPs with plaque phenotype

In the samples obtained from venous compartment prior to PCI significant positive correlation was noted between NMPs and lipid plaque in the ACS cohort. In the samples obtained from aortic compartment prior to PCI significant positive correlation was noted between CD105 EMPs and lipid plaque in the ACS cohort. In the SA cohort significant positive correlation was noted between activated PMPs, CD62E EMPs, NMPs and SMMPs with calcific plaque from the samples obtained from aorta prior to PCI.

5.4.5 Correlation of circulating MPs with distinct plaque characteristics

SMMPs from venous samples and CD54 EMPs from aortic samples obtained pre PCI showed good correlation with ruptured plaques in ACS cohort. Significant positive correlation was also noted between CD105 EMPs, PMPs, CD31 EMPs and SMMPs with neovessels from aortic samples obtained prior to PCI in SA cohort. Positive correlation was also noted between activated PMPs, CD62E EMPs, NMPs, and SMMPs with calcific plaque from aortic samples in SA cohort (Table 29, Figures 52-62). Detailed analysis looking at association of individual MPs with atherosclerotic plaque characteristics are presented in Table 32.

Table 31. Correlation between OCT variables and individual MPs

Time	Micro-particle	OCT variable	ACS		SA		Group
			r	p	r	p	p-value
T1	NMP	Lipid volume	0.56	**0.05**	-0.21	0.51	0.07
(V1)	SMMP	Ruptured plaques	0.60	**0.04**	-0.03	0.93	0.13
T2	CD105	Lipid volume	0.71	**0.02**	-0.10	0.80	0.05
(A1)	CD105	Neo vessels % frames	0.27	0.42	0.70	**0.04**	0.24
	PMP	Calcium volume	0.44	0.18	0.73	**0.03**	0.35
	PMP	Neo vessels % frames	0.15	0.67	0.78	**0.01**	0.07
	CD62E	Calcium volume	0.21	0.69	0.75	**0.02**	0.13
	NMP	Calcium volume	0.37	0.26	0.77	**0.02**	0.21
	CD54	Ruptured plaques	0.66	**0.04**	0.21	0.59	0.24
	CD31	Neo vessels % frames	0.21	0.53	0.93	**<0.001**	**0.003**
	SMMP	Calcium volume	0.15	0.65	0.82	**0.007**	0.05
	SMMP	Neo vessels % frames	-0.24	0.47	0.87	**0.002**	**0.002**

NMP – Neutrophil microparticles, CD105/CD54/CD31/CD62E – Endothelial microparticles, PMP – platelet microparticles stained positive for CD62P, SMMP – smooth muscle cell microparticles.

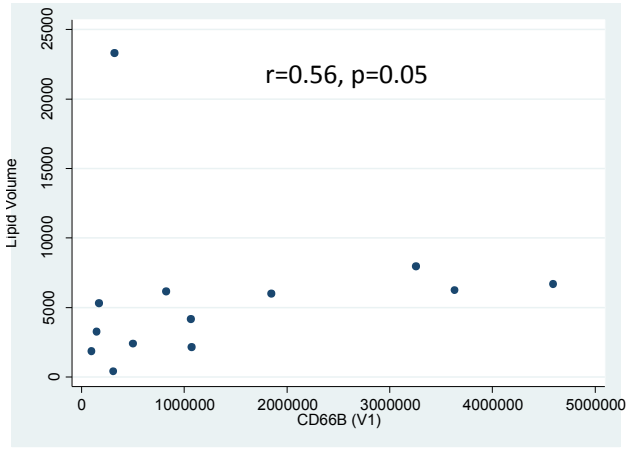


Figure 52. Correlation of CD66B NMPs with lipid volume in ACS cohort

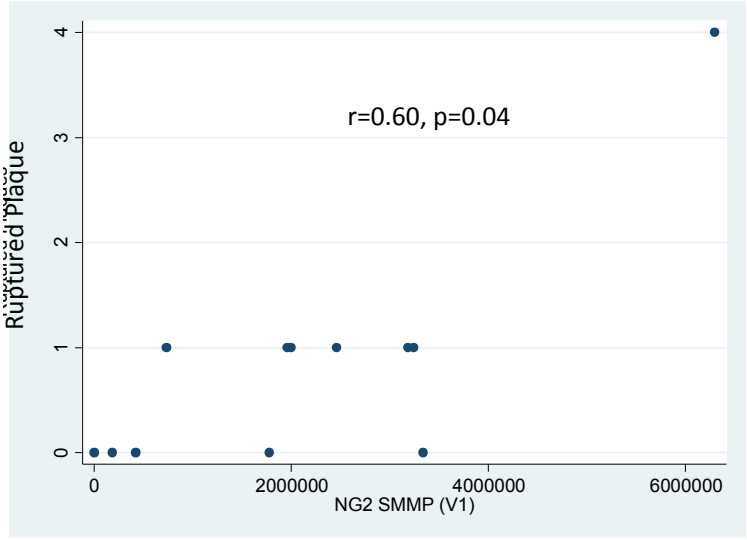


Figure 53. Correlation of SMMPs with ruptured plaques in ACS cohort (ruptured plaque in % of OCT frames)

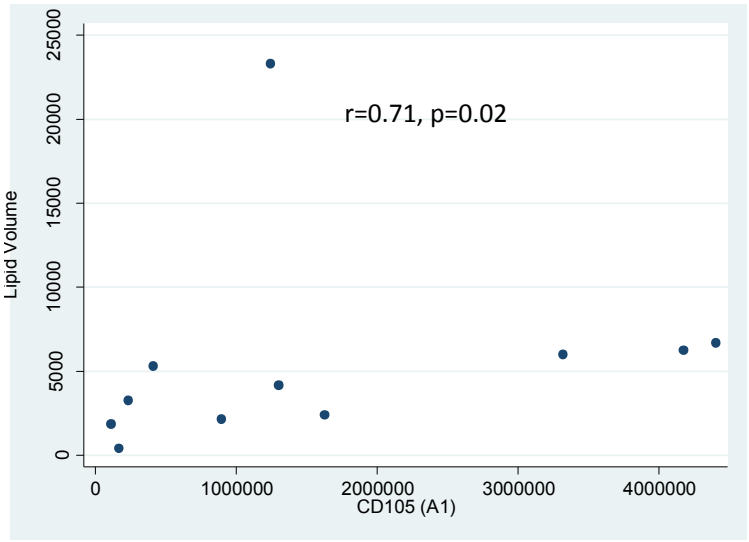


Figure 54. Correlation of CD105 EMPs with lipid volume in ACS cohort

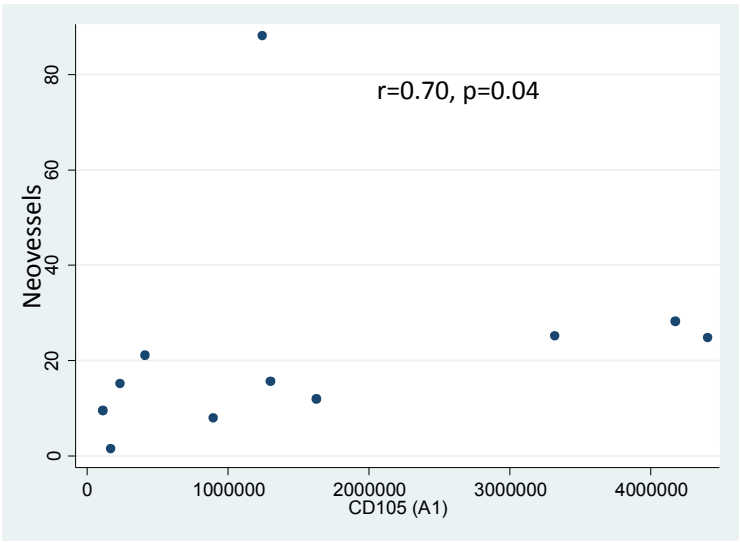


Figure 55. Correlation of CD105 EMPs with neovessels in SA cohort (neovessels in % of OCT frames)

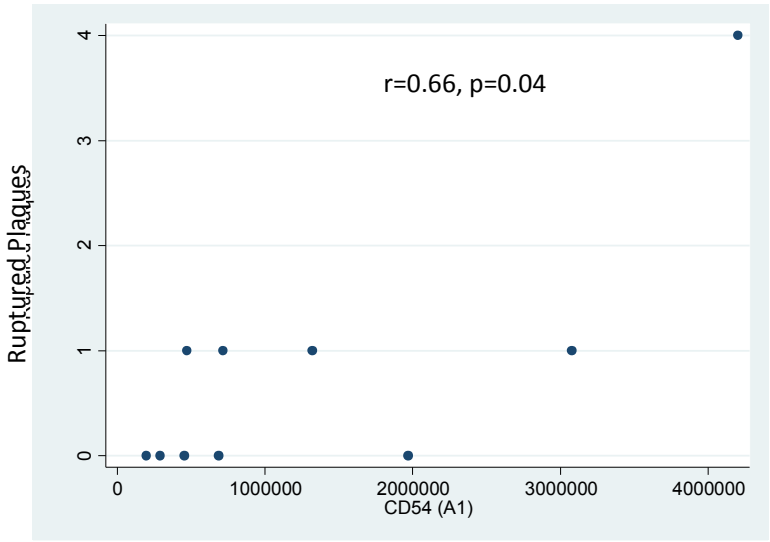


Figure 56. Correlation of CD54 EMPs with ruptured plaques in ACS cohort (ruptured plaque in % of OCT frames)

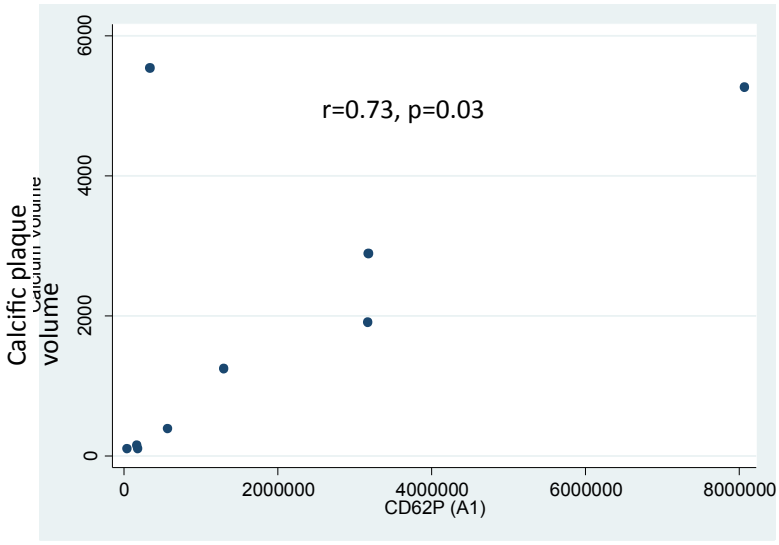


Figure 57. Correlation of CD62P PMPs with calcific plaque volume in SA cohort

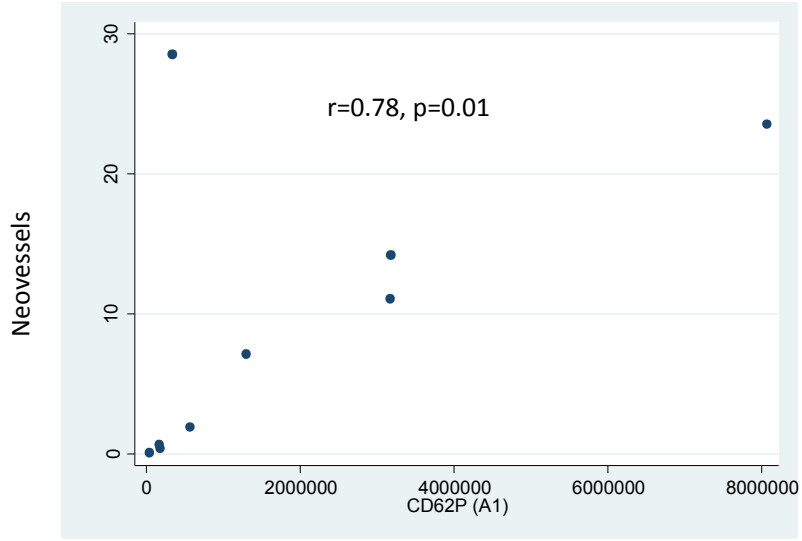


Figure 58. Correlation of CD62P PMPs with neovessels in SA cohort

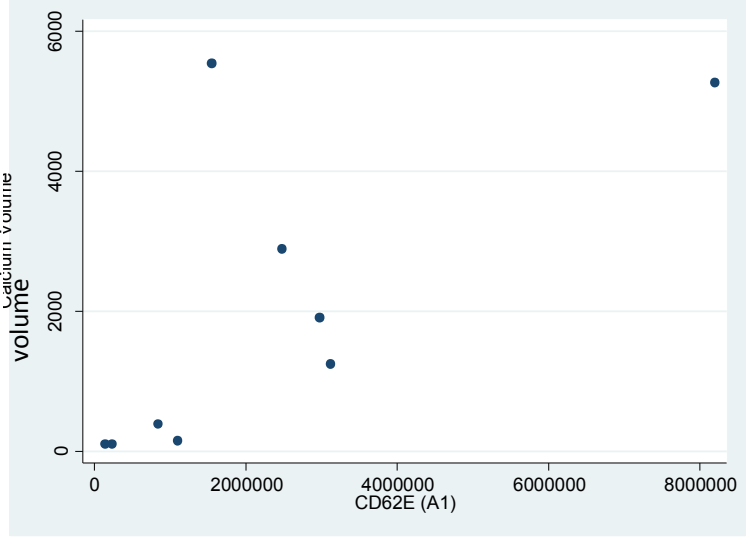


Figure 59. Correlation of CD62E EMPs with calcific plaque in SA cohort

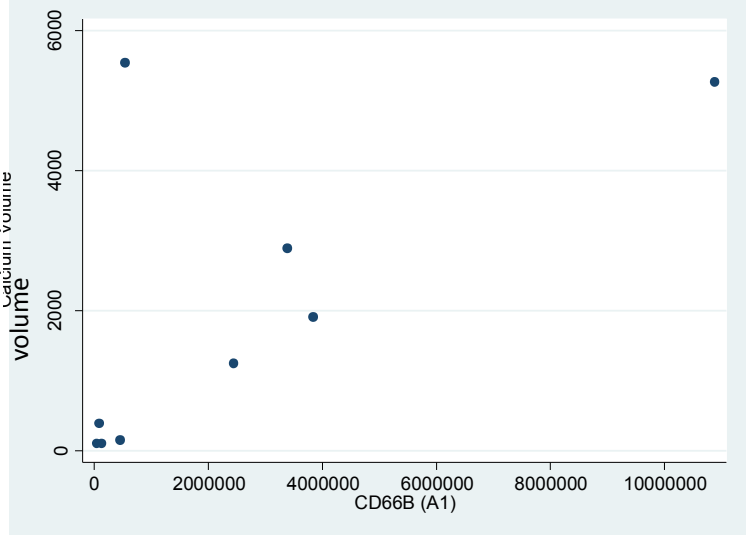


Figure 60. Correlation of CD66B NMPs with calcific plaque in SA cohort

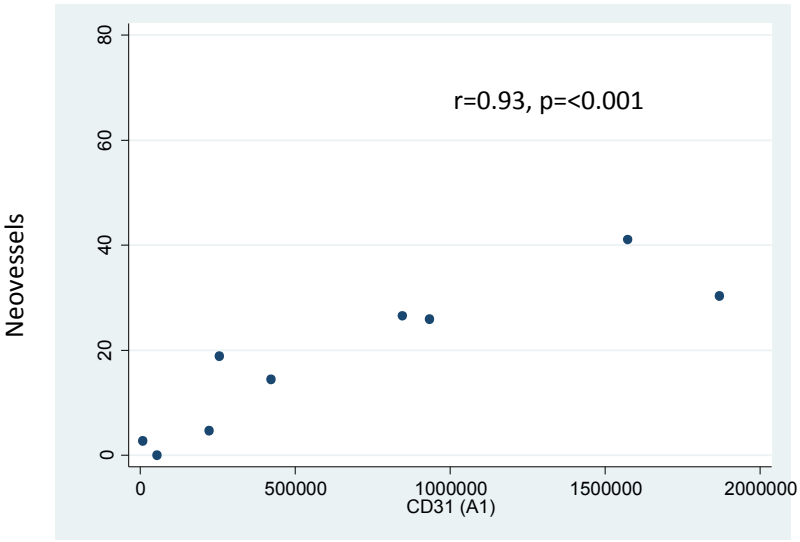


Figure 61. Correlation of CD31 EMPs with neovessels in SA cohort

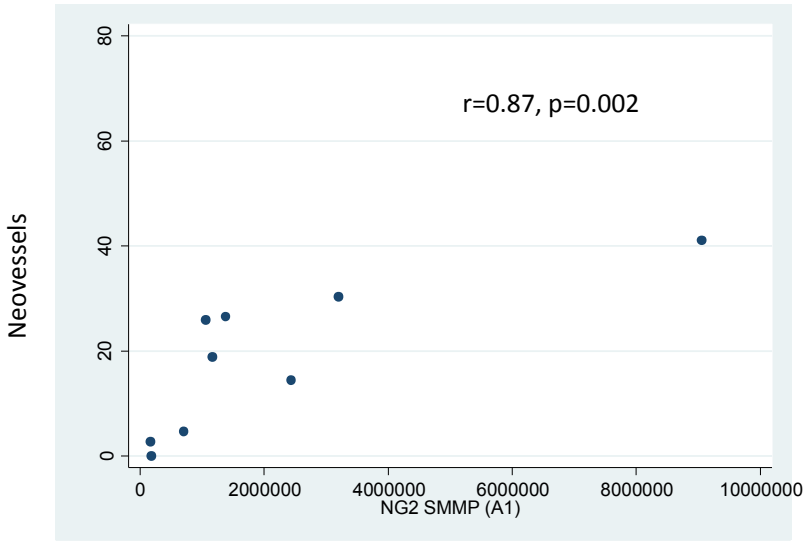


Figure 62. Correlation of SMMPs with neovessels in SA cohort

Table 32. Correlation of all MPs with OCT variables pre and post PCI in venous and aortic compartments

Time	Micro-particle	OCT variable	ACS		SA	
			r	p	r	p
V1	CD 105	Lipid volume	0.456	0.117	-0.224	0.484
		Lipid relative vol.	0.308	0.306	-0.035	0.914
		Calcium volume	-0.162	0.596	0.238	0.457
		Calcium relative vol.	-0.223	0.464	0.252	0.430
		Macrophages	0.517	0.07	-0.245	0.443
		Neovessels	0.143	0.64	0.140	0.665
		Cholesterol crystals	-0.118	0.700	0.304	0.337
		Thrombus	0.234	0.441	-0.005	0.496
		TCFA	0.311	0.301	-0.218	0.496
		Ruptured plaques	0.346	0.270	0.084	0.796
	CD 62P	Lipid volume	0.390	0.188	-0.392	0.208
		Lipid relative vol.	0.286	0.344	-0.202	0.527
		Calcium volume	0.044	0.886	0.287	0.366
		Calcium relative vol.	0.006	0.986	0.308	0.331
		Macrophages	0.305	0.310	0.301	0.342
		Neovessels	-0.006	0.986	0.084	0.795
		Cholesterol crystals	-0.292	0.333	0.416	0.179
		Thrombus	0.372	0.211	-0.263	0.408
		TCFA	-0.308	0.306	-0.178	0.580
		Ruptured plaques	0.288	0.364	0.084	0.796
	CD62E	Lipid volume	0.335	0.263	-0.252	0.430
		Lipid relative vol.	0.170	0.578	-0.014	0.966
		Calcium volume	0.077	0.802	0.294	0.354
		Calcium relative vol.	0.017	0.957	0.308	0.331
		Macrophages	0.261	0.388	-0.126	0.697
		Neovessels	0.017	0.957	0.063	0.846
		Cholesterol crystals	.066	0.829	0.229	0.474
		Thrombus	0.186	0.543	0.005	0.987
		TCFA	0.129	0.675	-0.160	0.620
		Ruptured plaques	0.475	0.119	0.139	0.667
	CD66B	Lipid volume	0.560	0.046	-0.210	0.513
		Lipid relative vol.	0.418	0.156	-0.245	0.443
		Calcium volume	0.066	0.830	0.042	0.897
		Calcium relative vol.	0.022	0.943	0.000	1.000
		Macrophages	0.495	0.085	0.252	0.430

		Neovessels	0.223	0.464	-0.049	0.880
		Cholesterol crystals	-0.147	0.631	0.054	0.867
		Thrombus	0.498	0.083	-0.285	0.369
		TCFA	0.012	0.969	0.000	1.000
		Ruptured plaques	0.428	0.165	0.028	0.931
	CD54	Lipid volume	0.516	0.071	-0.231	0.471
		Lipid relative vol.	0.324	0.280	-0.084	0.795
		Calcium volume	0.110	0.720	0.210	0.513
		Calcium relative vol.	0.050	0.872	0.217	0.499
		Macrophages	0.459	0.114	-0.196	0.542
		Neovessels	0.113	0.714	0.007	0.983
		Cholesterol crystals	0.023	0.940	0.391	0.209
		Thrombus	0.346	0.248	-0.005	0.987
		TCFA	0.141	0.647	-0.222	0.489
		Ruptured plaques	0.529	0.077	0.195	0.543
	CD31	Lipid volume	0.044	0.887	-0.441	0.152
		Lipid relative vol.	-0.143	0.642	-0.210	0.513
		Calcium volume	0.228	0.453	0.217	0.499
		Calcium relative vol.	0.184	0.547	0.238	0.457
		Macrophages	-0.162	0.596	0.000	1.000
		Neovessels	0.140	0.648	0.035	0.914
		Cholesterol crystals	0.211	0.489	0.237	0.458
		Thrombus	0.416	0.157	-0.124	0.702
		TCFA	-0.114	0.712	-0.076	0.814
		Ruptured plaques	0.556	0.060	0.084	0.796
	CD14+	Lipid volume	0.489	0.090	-0.028	0.931
	TF+	Lipid relative vol.	0.544	0.055	0.168	0.602
		Calcium volume	-0.187	0.541	-0.119	0.713
		Calcium relative vol.	-0.171	0.577	-0.113	0.681
		Macrophages	0.487	0.091	-0.476	0.118
		Neovessels	0.462	0.112	-0.049	0.880
		Cholesterol crystals	-0.046	0.881	0.241	0.450
		Thrombus	-0.149	0.628	0.382	0.221
		TCFA	0.329	0.272	-0.080	0.805
		Ruptured plaques	-0.202	0.528	0.251	0.432
	CD14-	Lipid volume	0.495	0.086	0.385	0.217
	TF+	Lipid relative vol.	0.462	0.112	0.542	0.131
		Calcium volume	-0.039	0.901	0.217	0.499
		Calcium relative vol.	-0.028	0.929	0.168	0.602
		Macrophages	0.413	0.161	-0.357	0.255
		Neovessels	0.300	0.320	0.021	0.948

		Cholesterol crystals	-0.384	0.195	0.216	0.948
		Thrombus	0.156	0.611	0.640	0.025
		TCFA	-0.185	0.544	-0.418	0.177
		Ruptured plaques	0.078	0.810	0.585	0.046
	NG2+	Lipid volume	0.462	0.112	-0.280	0.379
		Lipid relative vol.	0.253	0.405	-0.203	0.527
		Calcium volume	0.069	0.823	0.056	0.863
		Calcium relative vol.	0.019	0.950	0.084	-.0795
		Macrophages	0.338	0.258	-0.140	0.665
		Neovessels	0.220	0.470	-0.182	0.572
		Cholesterol crystals	0.040	0.896	0.175	0.587
		Thrombus	0.353	0.237	-0.183	0.570
		TCFA	0.91	0.531	0.073	0.822
		Ruptured plaques	0.599	0.040	-0.028	0.931
A1	CD105	Lipid volume	0.709	0.015	-0.100	0.798
		Lipid relative vol.	0.645	0.032	0.250	0.516
		Calcium volume	0.318	0.340	0.667	0.050
		Calcium relative vol.	0.282	0.410	0.667	0.050
		Macrophages	0.497	0.120	0.117	0.765
		Neovessels	0.269	0.424	0.700	0.036
		Cholesterol crystals	-0.084	0.807	0.139	0.722
		Thrombus	0.500	0.117	0.000	1.000
		TCFA	0.167	0.623	-0509	0.162
		Ruptured plaques	0.422	0.224	0.104	0.791
	CD62P	Lipid volume	0.564	0.071	-0.200	0.606
		Lipid relative vol.	0.582	0.060	0.100	0.798
		Calcium volume	0.436	0.180	0.733	0.025
		Calcium relative vol.	0.445	0.170	0.733	0.025
		Macrophages	0.210	0.536	0.667	0.050
		Neovessels	0.146	0.669	0.783	0.013
		Cholesterol crystals	-0.216	0.713	0.218	0.573
		Thrombus	0.300	0.370	-0.137	0.725
		TCFA	-0210	0.535	-0.525	0.146
		Ruptured plaques	0.147	0.684	0.207	0.593
	CD62E	Lipid volume	0.382	0.247	-0.247	0.576
		Lipid relative vol.	0.227	0.502	0.217	0.576
		Calcium volume	0.209	0.537	0.750	0.020
		Calcium relative vol.	0.136	0.689	0.750	0.020
		Macrophages	0.150	0.659	0.300	0.433
		Neovessels	0.200	0.555	0.850	0.004
		Cholesterol crystals	0.205	0.546	0.050	0.899

		Thrombus	0.500	0.117	0.000	1.000
		TCFA	0.296	0.376	-0.458	0.215
		Ruptured plaques	0.724	0.018	0.104	0.791
	CD66B	Lipid volume	0.273	0.417	-0.133	0.732
		Lipid relative vol.	0.236	0.484	0.223	0.546
		Calcium volume	0.373	0.259	0.767	0.016
		Calcium relative vol.	0.336	0.312	0.767	0.016
		Macrophages	-0.050	0.884	0.267	0.488
		Neovessels	-0.132	0.699	0.733	0.025
		Cholesterol crystals	0.000	1.000	0.109	0.780
		Thrombus	0.400	0.223	0.000	1.000
		TCFA	-0.206	0.544	-0.661	0.053
		Ruptured plaques	0.422	0.224	0.207	0.593
	CD54	Lipid volume	0.491	0.125	-0.183	0.637
		Lipid relative vol.	0.382	0.247	0.183	0.637
		Calcium volume	0.164	0.631	0.817	0.007
		Calcium relative vol.	0.100	0.770	0.817	0.007
		Macrophages	0.342	0.304	0.483	0.187
		Neovessels	0.005	0.989	0.867	0.002
		Cholesterol crystals	0.005	0.989	0.020	0.960
		Thrombus	0.500	v0.117	0.000	1.000
		TCFA	0.206	0.544	-0.576	0.104
		Ruptured plaques	0.663	0.037	0.207	0.593
	CD31	Lipid volume	0.164	0.631	-0.183	0.637
		Lipid relative vol.	0.018	0.183	0.183	0.637
		Calcium volume	0.400	0.223	0.600	0.088
		Calcium relative vol.	0.327	0.326	0.600	0.088
		Macrophages	0.082	0.811	0.633	0.067
		Neovessels	0.214	0.527	0.933	0.000
		Cholesterol crystals	0.279	0.406	-0.198	0.610
		Thrombus	0.300	0.370	-0.137	0.725
		TCFA	0.167	0.623	-0.322	0.398
		Ruptured plaques	0.415	0.223	-0.104	0.791
	CD14+	Lipid volume	0.236	0.484	-0.209	0.589
	TF+	Lipid relative vol.	0.309	0.355	0.285	0.458
		Calcium volume	0.136	0.689	0.728	0.026
		Calcium relative vol.	0.145	0.670	0.728	0.026
		Macrophages	0.269	0.424	0.008	0.983
		Neovessels	0.556	0.076	0.536	0.137
		Cholesterol crystals	0.270	0.422	0.050	0.899
		Thrombus	-0.300	0.370	0.550	0.125

		TCFA	0.406 0.215	-0.579 0.103
		Ruptured plaques	-0.389 0.267	0.520 0.152
	CD14-	Lipid volume	-0.173 0.612	0.267 0.488
	TF+	Lipid relative vol.	-0.263 0.484	0.233 0.546
		Calcium volume	0.218 0.519	0.067 0.865
		Calcium relative vol.	0.164 0.631	0.067 0.865
		Macrophages	-0.132 0.699	-0.467 0.205
		Neovessels	-0.046 0.894	-0.183 0.637
		Cholesterol crystals	-0.158 0.642	0.366 0.332
		Thrombus	0.100 0.770	0.411 0.272
		TCFA	-0.196 0.564	-0.220 0.569
		Ruptured plaques	-0.013 0.971	0.311 0.416
	NG2	Lipid volume	0.400 0.223	-0.183 0.637
		Lipid relative vol.	0.427 0.190	0.183 0.637
		Calcium volume	0.155 0.650	0.817 0.007
		Calcium relative vol.	0.118 0.729	0.817 0.007
		Macrophages	0.118 0.729	0.483 0.187
		Neovessels	-0.241 0.474	0.867 0.002
		Cholesterol crystals	-0.456 0.159	0.020 0.960
		Thrombus	-0.300 0.370	0.000 1.000
		TCFA	-0.249 0.461	-0.576 0.104
		Ruptured plaques	0.429 0.216	0.207 0.593
A2	CD105	Lipid volume	0.406 0.244	-0.430 0.214
		Lipid relative vol.	0.297 0.405	-0.176 0.627
		Calcium volume	0.006 0.987	0.491 0.150
		Calcium relative vol.	0.018 0.960	0.527 0.117
		Macrophages	0.370 0.293	0.018 0.960
		Neovessels	0.176 0.626	0.273 0.446
		Cholesterol crystals	-0.231 0.520	0.052 0.886
		Thrombus	0.043 0.906	-0.164 0.650
		TCFA	0.253 0.481	-0.295 0.407
		Ruptured plaques	0.112 0.775	0.038 0.917
	CD62P	Lipid volume	-0.118 0.603	-0.564 0.090
		Lipid relative vol.	-0.321 0.365	-0.467 0.174
		Calcium volume	0.345 0.328	0.321 0.365
		Calcium relative vol.	0.382 0.276	0.358 0.310
		Macrophages	-0.285 0.425	0.188 0.603
		Neovessels	0.024 0.947	0.152 0.676
		Cholesterol crystals	0.225 0.532	0.231 0.521
		Thrombus	-0.112 0.757	-0.355 0.315
		TCFA	-0.006 0.986	-0.209 0.562

		Ruptured plaques	-0.037 0.924	0.038 0.917
	CD62E	Lipid volume	0.079 0.829	-0.406 0.244
		Lipid relative vol.	-0.103 0.777	-0.188 0.603
		Calcium volume	0.224 0.533	0.442 0.200
		Calcium relative vol.	0.212 0.556	0.467 0.174
		Macrophages	-0.018 0.960	0.079 0.829
		Neovessels	0.085 0.815	0.333 0.347
		Cholesterol crystals	0.056 0.877	0.052 0.886
		Thrombus	0.061 0.868	-0.182 0.615
		TCFA	0.149 0.681	-0.240 0.504
		Ruptured plaques	0.242 0.530	0.038 0.917
	CD66B	Lipid volume	0.030 0.934	-0.148 0.229
		Lipid relative vol.	-0.152 0.676	-0.212 0.556
		Calcium volume	0.309 0.385	0.503 0.138
		Calcium relative vol.	0.345 0.328	0.527 0.117
		Macrophages	0.030 0.934	0.164 0.651
		Neovessels	0.158 0.663	0.345 0.328
		Cholesterol crystals	0.200 0.579	0.142 0.696
		Thrombus	0.078 0.831	-0.182 0.615
		TCFA	0.208 0.565	-0.314 0.377
		Ruptured plaques	0.149 0.702	0.114 0.754
	CD54	Lipid volume	0.370 0.293	-0.382 0.276
		Lipid relative vol.	0.236 0.511	-0.273 0.446
		Calcium volume	0.079 0.829	0.382 0.276
		Calcium relative vol.	0.091 0.803	0.394 0.260
		Macrophages	0.248 0.489	0.188 0.603
		Neovessels	0.195 0.590	0.333 0.347
		Cholesterol crystals	0.269 0.453	0.142 0.696
		Thrombus	0.138 0.703	-0.199 0.582
		TCFA	0.415 0.233	-0.160 0.659
		Ruptured plaques	0.242 0.530	0.114 0.754
	CD31	Lipid volume	-0.188 0.603	-0.661 0.038
		Lipid relative vol.	-0.382 0.276	-0.576 0.082
		Calcium volume	0.358 0.310	0.224 0.533
		Calcium relative vol.	0.309 0.385	0.248 0.489
		Macrophages	-0.224 0.533	0.515 0.128
		Neovessels	-0.213 0.555	0.248 0.489
		Cholesterol crystals	0.444 0.199	0.157 0.666
		Thrombus	-0.208 0.565	-0.372 0.290
		TCFA	0.156 0.668	0.154 0.671
		Ruptured plaques	0.130 0.738	0.038 0.917

	CD14+	Lipid volume	0.248 0.489	-0.188 0.603
	TF+	Lipid relative vol.	0.127 0.726	-0.224 0.533
		Calcium volume	0.261 0.467	-0.139 0.701
		Calcium relative vol.	0.236 0.511	-0.188 0.603
		Macrophages	0.358 0.310	-0.430 0.244
		Neovessels	0.407 0.243	-0406 0.244
		Cholesterol crystals	0.225 0.532	0.186 0.606
		Thrombus	-0.225 0.532	0.337 0.340
		TCFA	0.545 0.103	0.043 0.906
		Ruptured plaques	-0.149 0.702	0.570 0.086
	CD14-	Lipid volume	-0.236 0.511	0.394 0.260
	TF+	Lipid relative vol.	-0.236 0.511	0.103 0.777
		Calcium volume	-0.164 0.651	0.030 0.934
		Calcium relative vol.	-0.212 0.556	-0.055 0.882
		Macrophages	-0.067 0.855	-0.345 0.328
		Neovessels	-0.024 -0.947	-0.152 0.676
		Cholesterol crystals	-0.288 0.420	-0.112 0.758
		Thrombus	-0.104 0.775	0.510 0.132
		TCFA	-0.039 0.915	-0.160 0.659
		Ruptured plaques	-0.112 0.775	0.570 0.086
	NG2	Lipid volume	0.176 0.627	-0.442 0.200
		Lipid relative vol.	-0.042 0.907	-0.309 0.385
		Calcium volume	-0.067 0855	0.394 0.260
		Calcium relative vol.	-0.091 0.803	0.418 0.229
		Macrophages	0.164 0.651	0.200 0.580
		Neovessels	0.024 0.947	0.309 0.385
		Cholesterol crystals	-0.075 0.837	0.142 0.696
		Thrombus	-0.017 0.962	-0.227 0.439
		TCFA	0.441 0.202	-0.215 0.550
		Ruptured plaques	0.466 0.206	0.038 0.917
V2	CD105	Lipid volume	0.464 0.151	-0.227 0.502
		Lipid relative vol.	0.491 0.125	-0.173 0.612
		Calcium volume	0.210 0.536	0.191 0.574
		Calcium relative vol.	0.200 0.555	0.155 0.650
		Macrophages	0.424 0.194	-0.100 0.770
		Neovessels	0.210 0.536	0.409 0.212
		Cholesterol crystals	-0.076 0.824	-0.074 0.829
		Thrombus	0.040 0.906	0.027 0.937
		TCFA	0.388 0.239	-0.014 0.967
		Ruptured plaques	0.221 0.539	0.129 0.705
	CD62P	Lipid volume	-0.45 0.894	-0.064 0.853

		Lipid relative vol.	0.100 0.770	-0.055 0.873
		Calcium volume	0.346 0.297	0.164 0.631
		Calcium relative vol.	0.364 0.270	0.127 0.709
		Macrophages	-0.128 0.709	0.100 0.770
		Neovessels	-0.292 0.384	0.336 0.312
		Cholesterol crystals	0.010 0.978	-0.147 0.665
		Thrombus	-0.418 0.201	-0.054 0.875
		TCFA	-0.075 0.828	0.089 0.796
		Ruptured plaques	-0.080 0.825	0.129 0.705
	CD62E	Lipid volume	0.227 0.502	-0.173 0.612
		Lipid relative vol.	0.282 0.401	-0.155 0.650
		Calcium volume	0.497 0.120	0.255 0.450
		Calcium relative vol.	0.478 0.137	0.218 0.519
		Macrophages	0.087 0.800	0.091 0.790
		Neovessels	-0.087 0.800	0.409 0.212
		Cholesterol crystals	0.019 0.956	-0.032 0.927
		Thrombus	0.040 0.906	-0.121 -0.722
		TCFA	-0.070 0.839	-0.079 0.817
		Ruptured plaques	0.382 0.276	0.065 0.850
	CD66B	Lipid volume	-0.091 0.790	0.209 0.537
		Lipid relative vol.	0.036 0.915	0.191 0.574
		Calcium volume	0.282 0.400	0.055 0.873
		Calcium relative vol.	0.292 0.384	0.036 0.915
		Macrophages	-0.018 0.958	0.036 0.915
		Neovessels	-0.260 0.441	0.373 0.259
		Cholesterol crystals	-0.048 0.889	-0.326 0.327
		Thrombus	-0.351 0.290	-0.121 0.722
		TCFA	-0.055 0.873	0.219 0.517
		Ruptured plaques	-0.201 0.578	-0.194 0.568
	CD54	Lipid volume	0.327 0.326	-0.064 0.853
		Lipid relative vol.	0.373 0.259	-0.055 0.873
		Calcium volume	0.355 0.284	0.164 0.631
		Calcium relative vol.	0.337 0.311	0.127 0.709
		Macrophages	0.118 0.729	0.100 0.770
		Neovessels	-0.018 0.958	0.336 0.312
		Cholesterol crystals	-0.114 0.738	-0.147 0.665
		Thrombus	0.189 0.578	-0.054 0.875
		TCFA	0.089 0.794	0.089 0.796
		Ruptured plaques	0.302 0.397	0.129 0.705
	CD31	Lipid volume	-0.209 0.537	-0.045 0.894
		Lipid relative vol.	-0.282 0.401	-0.191 0.574

		Calcium volume	0.487 0.174	-0.136 0.689
		Calcium relative vol.	-0.301 0.369	-0.173 0.612
		Macrophages	-0.301 0.369	0.145 0.670
		Neovessels	-0.155 0.649	0.309 0.355
		Cholesterol crystals	0.391 0.235	-0.263 0.434
		Thrombus	-0.189 0.578	-0.067 0.844
		TCFA	0.40 0.908	0.546 0.083
		Ruptured plaques	0.261 0.466	0.000 1.000
	CD14+	Lipid volume	0.082 0.811	-0.064 0.853
	TF+	Lipid relative vol.	0.082 0.811	-0.036 0.915
		Calcium volume	0.264 0.432	0.164 0.631
		Calcium relative vol.	0.273 0.416	0.100 0.631
		Macrophages	0.014 0.968	-0.564 0.071
		Neovessels	0.196 0.564	0.055 0.873
		Cholesterol crystals	0.277 0.410	0.032 0.927
		Thrombus	-0.499 0.118	0.364 0.271
		TCFA	0.457 0.157	-0.303 0.365
		Ruptured plaques	-0.221 0.539	0.3232 0.333
	CD14-	Lipid volume	0.045 0.894	0.673 0.023
	TF+	Lipid relative vol.	-0.027 0.937	0.500 0.117
		Calcium volume	-0.009 0.979	0.082 0.811
		Calcium relative vol.	-0.027 0.936	0.009 0.979
		Macrophages	0.105 0.659	-0.555 0.077
		Neovessels	0.150 0.659	-0.155 0.650
		Cholesterol crystals	-0.153 0.654	-0.242 0.473
		Thrombus	0.013 0.969	0.674 0.023
		TCFA	0.114 0.738	-0.033 0.924
		Ruptured plaques	0.141 0.698	0.452 0.163
	NG2	Lipid volume	0.264 0.433	-0.200 0.555
		Lipid relative vol.	0.264 0.433	-0.182 0.593
		Calcium volume	0.328 0.325	0.127 0.709
		Calcium relative vol.	0.310 0.354	0.082 0.811
		Macrophages	0.132 0.699	-0.055 0.873
		Neovessels	0.087 0.800	0.382 0.247
		Cholesterol crystals	0.010 0.978	-0.053 0.878
		Thrombus	-0.108 0.752	-0.054 0.875
		TCFA	0.229 0.499	-0.037 0.913
		Ruptured plaques	0.241 0.502	0.065 0.850

5.4.6 Comparison of MPs and Thrombin parameters based on presence or absence of thin cap fibroatheroma (TCFA):

Next we interrogated if presence of TCFA but not clinical presentation made any difference to MPs and TGA associations. Patients with TCFA did not differ from patients with out TCFA with respect to base line characteristics that included troponin (table 33).

Table 33. Comparison of clinical characteristics and pharmacological therapies in patients with and with out TCFA

	Patients with TCFA (n=12)	Patients with out TCFA (n=13)	p value
Age (mean ± SD)	59.17 ± 9.84	58.77 ±8.8	0.43
Male (n, %)	11 (91.7)	12 (92.3)	0.90
ACS (n,%)	6 (50)	7 (53.8)	0.85
Diabetes (n, %)	4 (33.3)	3 (23)	0.56
Hypertension (n, %)	3 (25)	6 (46)	0.14
Hyperlipidaemia (n, %)	8 (66.7)	12 (92.3)	0.49
Smoking (n, %)	4 (33.3)	3 (20.7)	0.32
Family History of CAD (n, %)	2 (16.7)	2 (15.3)	0.69
Creatinine (Median, IQR)	84 (78 – 87)	78 (71.5 - 91)	1
Troponin (Median, IQR)	0.85 (0.2 - 3.42)	0.20 (0.10-4.80)	0.28
TC (Median, IQR)	5 (4.8 - 5.8)	4.1 (3.1 – 5.6)	0.22
LDL (Median, IQR)	2.7 (2.4 – 3.1)	2.4 (1-8 – 2.8)	0.08
LVEF (mean ± SD)	60.83 (60 – 67.5)	55.8 (50 – 60)	0.09
Medications:			
ACE- I / ARB (n, %)	4 (33.3)	3 (23.1)	0.114
Beta-blockers (n, %)	4 (33.3)	4 (31.3)	0.114
Aspirin (n, %)	5 (41.7)	6 (46.1)	0.1
Statin (n, %)	5 (41.7)	9 (69.2)	0.32
Distribution of TCFA (n, %):			
LAD	9 (75)		
LCX	4 (33.3)		
RCA	4 (33.3)		

TCFA – thin cap fibroatheroma, ACS – acute coronary syndrome, TC – total cholesterol, LDL – low density lipoproteins, LVEF – left ventricular ejection fraction, ACE – I – Angiotensin converting enzyme inhibitors, ARB – Angiotensin receptor blockers, LAD – Left anterior descending artery, LCX – Left circumflex artery and RCA – Right coronary artery.

Total AnV+MPs and individual phenotypes of MPs did not differ between the patients with and with out TCFA (table 34).

Table 34. Comparison of total and individual MPs phenotypes between TCFA +ve and TCFA –ve patients pre PCI in arterial and venous compartments

TCFA + ve				TCFA - ve			
	Median	IQR		Median	IQR		p value
TotalMPs	3.15	2.3	4.2	1.8	0.3	2.9	0.13
CD105	0.2	0.04	0.4	0.1	0.01	0.3	0.53
CD62P	0.1	0.03	0.3	0.04	0.01	0.3	0.29
CD62E	0.16	0.1	0.3	0.1	0.03	0.3	0.18
CD66B	0.1	0.03	0.3	0.01	0.006	0.06	0.05
CD54	0.15	0.08	0.3	0.06	0.01	0.3	0.09
CD31	0.06	0.01	0.08	0.01	0.007	0.04	0.17
CD14-TF+	0.03	0.01	0.2	0.03	0.005	0.1	0.76
CD14+TF+	0.23	0.06	0.7	0.02	0.06	1	0.74
NG2+	0.2	0.07	0.3	0.09	0.02	0.3	0.35
Total & Individual MPs (x 10^6) in venous compartment pre PCI							
TotalMPs	2.7	0.9	3.8	1.9	0.8	4.4	0.97
CD105	0.12	0.03	0.3	0.14	0.01	0.3	1
CD62P	0.08	0.02	0.2	0.05	0.01	0.2	0.87
CD62E	0.15	0.04	0.3	0.13	0.04	0.3	0.81
CD66B	0.1	0.02	0.24	0.05	0.01	0.35	0.7
CD54	0.1	0.05	0.27	0.1	0.03	0.3	0.93
CD31	0.03	0.01	0.1	0.06	0.01	0.14	0.81
CD14-TF+	0.05	0.02	0.2	0.03	0.02	0.1	0.44
CD14+TF+	0.16	0.07	0.9	0.05	0.14	1.4	0.31
NG2+	0.06	0.03	0.2	0.11	0.03	0.3	0.87

Total & Individual MP phenotypes (x10^6) pre PCI in arterial compartment

TCFA – Thin cap fibroatheroma.

Amongst TGA parameters endogenous thrombin potential reflected by AUC from venous sample pre PCI was larger in patients with evidence of TCFA.

Furthermore receiver operating characteristics curve showed a sensitivity of 0.667 for AUC value of 3527 to predict presence or absence of TCFA (Table 35 & Fig 63).

Table 35. Comparison of thrombin generation assay parameters between patients with and with out TCFA

	TCFA +ve	TCFA -ve	P value
V1 lag time	14.2 (8.5 – 20.75)	15.5 (12-23.5)	0.429
V1 thrombin	249.6 (227 – 364)	255.6 (150 – 342)	0.514
V1 velocity index	22.7 (17.3 – 36.8)	27.5 (10 – 40.4)	1.000
V1 AUC	3950 (3542 – 4240)	3240 (2410 – 3827)	**0.039**
A1 lag time	16 (12 - 20)	17 (12.7 - 21)	0.423
A1 thrombin	258.6 (158.1 – 312.9)	172.5 (143.6 – 278.9)	0.305
A1 velocity index	27.1 (15 – 44.3)	10.9 (10.1 – 28.7)	0.138
A1 AUC	3458.7 (2874 – 4522.6)	3103.9 (2560.7 – 3796.3)	0.160

TCFA – thin cap fibroatheroma, AUC – area under curve.

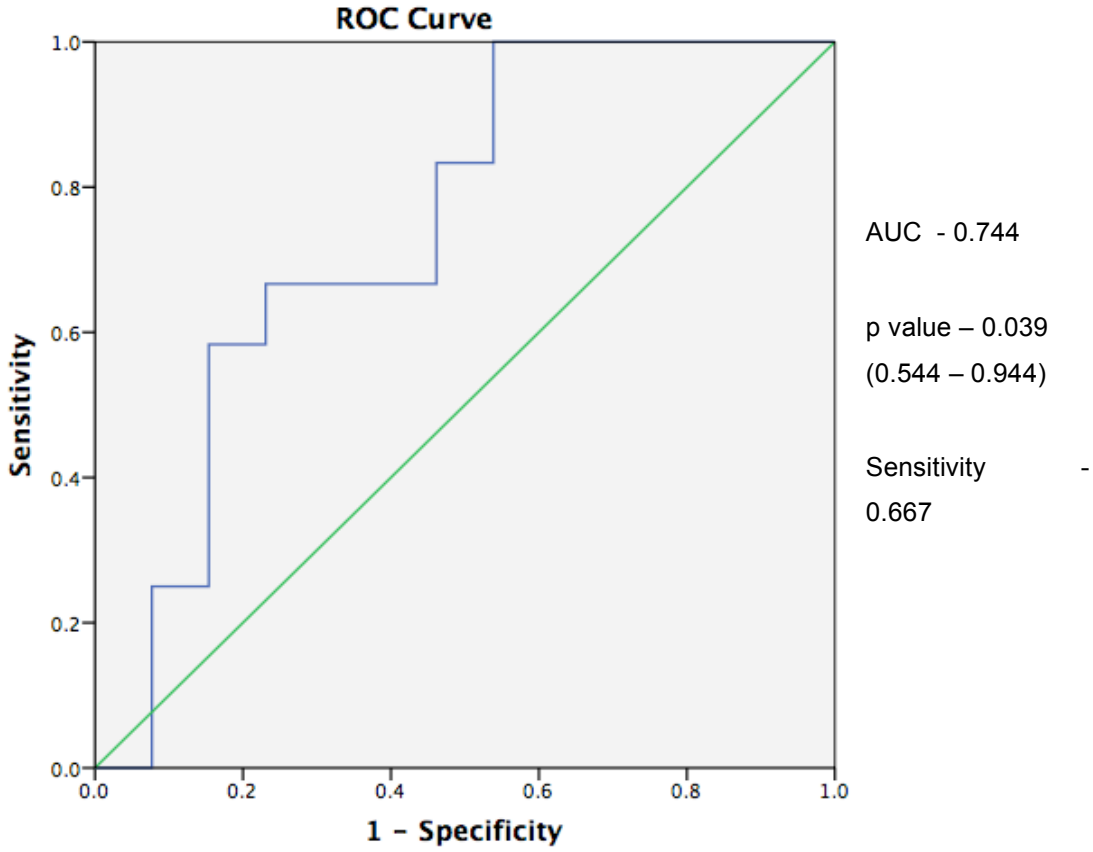


Figure 63. ROC curve of AUC in predicting TCFA

5.5 Discussion

The results of my study demonstrated several important findings. The total and individual phenotypes of circulating levels of MPs were numerically higher in ACS patients. Strong correlation was noted between total MPs in pre PCI venous sample with Troponin in ACS cohort. This finding may indicate the utility of using MPs in assessing myocardial injury. TGA was reliably assayed in both the cohorts and compared with controls of no documented CAD. Although TGA was abnormal in the study patients, it did not discriminate between ACS and SA. This lack of discriminatory value could be explained by the fact that ACS cohort was on treatment with potent antiplatelet drugs such as Ticagrelor and anticoagulants. It was also striking to note the thrombogenicity of blood in SA group. While the causes for this remain elusive the inflammatory process involved in atherosclerosis and presence of vulnerable plaque (TCFA) may account. By studying features of vulnerable plaque and assaying MPs at the same time we demonstrated the association of MPs with various atherosclerotic

plaque characteristics. The association of individual phenotypes of MPs with plaque characteristics varied according to clinical presentation. In ACS cohort strong correlation was noted between NMPs, CD105 EMPs with lipid plaque and SMMPs, CD54 EMPs with ruptured plaques. In SA cohort percentage of frames with neovessels were numerically higher and had strong correlation with SMMPs, CD105 EMPs, CD62P PMPs and CD31EMPs. Further correlation was also noted between calcific plaque and NMPs, CD62E EMPS, NMPs and CD62P PMPs. Associative data between neovessels with CD31 EMPs and SMMPs in SA cohort was statistically significant when compared to ACS cohort. Correlation of CD54 EMPs and SMMPs with ruptured plaques was only noted in ACS but not SA suggesting the indolent nature of incidental ruptured plaques seen in non-culprit coronary arteries of SA cohort. Furthermore, this finding may also reflect the dynamic nature of expression of CD54 EMPs and SMMPs. It was established from previous studies that CD54 EMPs are released following activation of endothelial cells (181). The role of PMPs in stimulating angiogenesis by vascular endothelial growth factor and the role of EMPs in endothelial dysfunction has been shown before in laboratory-based models (26,53). Thus the association of various MPs with neovessels and presence of more neovessles in SA suggest ongoing endothelial injury, apoptosis and the chronicity of disease. On long term testing we have found that CD54 EMPs, CD62E EMPs and CD62P PMPs were elevated in ACS cohort when compared to SA cohort at 6 months. Of the three MP phenotypes CD54 EMPs correlated well with ruptured plaques. SMMPs though not elevated at 6 months in ACS cohort when compared to SA cohort discriminated between ruptured plaques in ACS and SA. Thus these two individual phenotypes of MPs may have roles as biomarkers and studies aimed at this should be conducted. It was interesting to note no clear trend towards higher circulating MP load in patients with TCFA, which is a marker of vulnerability. However, interestingly the blood in patients with TCFA was more thrombogenic when compared to those with out TCFA as reflected by high endogenous thrombogenic potential. Measuring procoagulant potential of MP subpopulation may find an answer to this conundrum. This also gives further support to the concept of vulnerable patient in which vulnerable plaque and vulnerable blood are key components (174). There were several novelties in our study. To the best of our knowledge this is the first study that looked at associating MP levels with OCT derived atherosclerotic plaque

characteristics. There were previous attempts correlating MPs with imaging based assessment of coronary plaque morphology. High EMP was shown to be associated with coronary angiography identified high-risk lesions (192). Bernard et al. demonstrated high EMP in diabetic ACS patients and association with non-calcific coronary plaques(197). No such association was noted with PMPs. Our findings are also similar in that CD105 EMPs correlated well with lipid plaque in ACS patients where as PMPs did not have that association. Min PK et al attempted to correlate intra vascular ultrasound identified target lesion morphology in stable angina patients with circulating MPs (CD31, CD146 and CD42b) and found elevated levels of circulating MPs to be associated with necrotic core (198). Another novelty was attempts to measure and correlate SMMPs with OCT derived plaque characteristics. SMMPs may originate from activation of smooth muscle cells present in coronary vessel wall following endothelial injury and vascular remodeling. SMMPs may explain the prothrombotic blood in both cohorts at 6 months and enhanced thrombogenicity of blood in patients with TCFA. This is possible as greater numbers of SMMPs express TF constitutively as opposed to being induced in endothelial cells (199). We did not come across any attempts to characterize SMMPs in CAD before. Further studies associating MPs with plaque characteristics in a large cohort with particular emphasis on CD54 EMPs and SMMPs might help in establishing their role as a biomarker

5.6 Limitations

Limitations associated with a small observational study are worthy of note. We did not carry out correlation with the traditional inflammatory marker hsCRP as the values were by and large normal except in two patients. For TGA we used platelet poor plasma and did not distinguish between individual phenotypes of MPs. Thus it is possible that abnormal TGA may be due to other prothrombotic factors besides microparticles that could induce thrombin formation.

5.7 Conclusions

This small observational study has shown strong association of MPs with OCT derived vulnerable plaque characteristics. The blood in CAD patients is prothrombotic particularly in those with vulnerable plaque irrespective of usage

of antiplatelet drugs. Further studies including larger cohort of patients is recommended to establish our findings before incorporating MPs into traditional risk scoring systems to improve their discriminatory value.

Chapter 6. General Discussion and Conclusion

Coronary artery disease (CAD) remains a leading cause of mortality and morbidity in the UK. Despite current evidence based preventive therapy, CAD imposes a considerable burden with high prevalence, morbidity and mortality (200). CAD is projected to remain leading cause of mortality for the next decade on a global scale (201). Furthermore, recurrent coronary events are common in patients with preexisting CAD (202). These issues emphasize the necessity for an ideal biomarker in order to predict coronary events and carry out effective risk stratification. Traditional cardiovascular risk scoring systems underestimate cardiovascular risk at an individual patient level (203). Wang et al investigated the utility of integrated biomarker model in predicting the risk of death and major cardiovascular events in a large community based cohort. The role of ten biomarkers including high-sensitivity C-reactive protein (hs-CRP), B-type natriuretic peptide (BNP) and homocysteine was interrogated in a Framingham offspring cohort. The C statistics computed by the integrated biomarker model only increased the predictive power of an individual at risk of future cardiac events moderately. Traditional biomarkers such as hs-CRP and BNP showed lot of promise and were studied extensively in single and multiple biomarker studies (204,205). Where as BNP was good at predicting death and cardiovascular events (205), hs-CRP predicted death alone (203). However, in patients with established CAD higher CRP levels at the time of coronary intervention were predictive of death and myocardial infarction at 10 years (206). Nozaki et al. interrogated integrated biomarker model (BNP, hs-CRP and endothelial microparticles (EMP)) in patients with and without documented CAD and found that EMP can independently predict future cardiovascular events (196). Cardiac Troponins, specifically high sensitive Troponin T and I (TnT and TnI) have a very important role in the early diagnosis of ACS and also in prognostication (207,208). Oemrawsingh et al analysed the role of cardiac Troponins in stable CAD and assessed their relation with intravascular ultrasound (IVUS) derived coronary atherosclerotic plaque characteristics (209). Elevated circulating levels of hs TnT were associated with IVUS identified thin cap fibroatheroma (TCFA) lesions. However, the mechanism behind elevated hsTNT in stable CAD is not clear and is possibly secondary to sub clinical plaque rupture (209). Thus it is clear that traditional risk scoring systems and

traditional biomarkers have their own set of limitations. It is now well established that atherosclerosis is driven by chronic low-grade inflammation (2,100,156). Atheromatous plaque formation driven by inflammation interrupts the laminar blood flow in the coronary vessels. Ensuing low shear stress has been demonstrated to cause endothelial dysfunction, which further augments the process of inflammation and atherosclerosis (210). Thus a biomarker that is reflective of underlying inflammation, endothelial dysfunction and vascular injury is required to predict future cardiovascular events more accurately. Microparticles (MPs), released on activation or apoptosis of endothelial cells, platelets and leukocytes carry all the biological functions of the parent cell (179). The role of MPs in promoting inflammation, coagulation and endothelial dysfunction is well established (179). However, the role of MPs as a biomarker in CAD and ACS is not well explored apart from few observational studies demonstrating elevated levels in ACS (48). Sinning et al previously demonstrated high levels of circulating CD31+/Annexin V+ (AnV+) MPs as an independent predictor of cardiovascular events in stable CAD cohort with a potential role for risk stratification (64). Similar data pertaining to the association of MPs and cardiovascular events in ACS cohort is sparse. Furthermore, it is also not clear if elevated levels of MPs in CAD and ACS are just an epiphenomenon. The role of MPs in ACS can be established by two possible methods; 1. Correlating vulnerable coronary plaque characteristics and microparticles, 2. By promoting coronary atherosclerotic plaque formation in lab based animal models through MPs isolated from human ACS samples. I chose the first pathway as intravascular imaging by optical coherence tomography (OCT) yields high-resolution images and thus well-defined plaque characteristics (83,85) can be correlated with various subtypes of relevant MPs. As the study protocol entails in chapter 3 my aim was to associate a panel of MPs with OCT defined vulnerable coronary atherosclerotic plaque characteristics. The collection of MPs and analysis is done at the time of PCI following index coronary event and for up to 6 months to identify a clear indicator in the peripheral blood. To the best of my knowledge this is the first time a study is carried out associating MPs with OCT derived plaque characteristics. Further strengths are, I have assessed global plaque burden in the whole coronary tree rather than limited segments in the culprit coronary artery. Along with global plaque burden correlation was also done with

vulnerable plaque characteristics such as lipid burden, presence of macrophages, neovessels and TCFA. A recently reported IVUS based study in stable angina patients revealed good correlation of necrotic core in culprit lesion with MPs (198). My study demonstrated several important results. OCT analysis showed ACS patients harboring more lipid plaque in culprit and non-culprit arteries when compared to stable angina. Similarly total and individual MPs were elevated in ACS patients at the time of index event when compared to SA cohort. Thrombin generation assay (TGA) was abnormal in both ACS and SA cohorts when compared to controls. A significant correlation was noted between various MPs and plaque characteristics. Furthermore, the association of MPs with plaque characteristics varied depending on which compartment the blood was drawn from; aortic vs. venous. In the samples obtained from venous compartment significant positive correlation was noted between NMPs and lipid plaque in ACS cohort. In the samples obtained from aortic compartment significant positive correlation was noted between CD105 EMPs and lipid plaque in the ACS cohort. In the SA cohort significant positive correlation was noted between PMPs, CD62E EMPs, NMPs and SMMPs with calcific plaque from the samples obtained from aorta. SMMPs from venous samples and CD54 EMPs from aortic samples obtained showed good correlation with ruptured plaques in ACS cohort. Significant positive correlation was also noted between CD105 EMPs, PMPs, CD31 EMPs and SMMPs with neovessels from aortic samples obtained pre PCI in SA cohort. Positive correlation was also noted between PMPs, CD62E EMPs, NMPs, and SMMPs with calcific plaque from aortic samples in SA cohort. Total AnV+MPs and individual phenotypes of MPs did not differ between the patients with and without thin cap fibroatheroma (TCFA). Amongst TGA parameters endogenous thrombin potential reflected by AUC from venous sample pre PCI was larger in patients with evidence of TCFA. This is interesting given the fact that hypercoagulability and markers of clotting activity were shown to be associated with atherogenesis in lab based models and human beings (211). Furthermore, there are also reports on pleiotropic effects of novel oral anticoagulant (NOAC) group of drugs(212-215). NOACs are direct thrombin inhibitors and are used in prophylaxis against thromboembolic events in patients with atrial fibrillation (216). The safety and efficacy of Dabigatran was established in “randomized evaluation of long-term anticoagulation therapy” (RE-LY) clinical trial (217). Dabigatran was one of the

first NOACs that found clinical utility in stroke prevention in patients with Atrial Fibrillation (AF). Sub analysis of RE-LY trial showed reduction in plasma apolipoprotein B (apoB) levels over and above those achieved with high dose statin (218). It is well established that ApoB is atherogenic and altered apolipoprotein metabolism due to competitive inhibition of carboxyesterase by Dabigatran as the possible explanation for reduction in ApoB levels. Similar protective effects were also noted with Rivaroxoban – another direct Xa inhibitor used for stroke prevention in AF – in experimental atherosclerosis models(219). Furthermore, two recent large randomized clinical trials have shown benefit of continuing with long term dual antiplatelet treatment following ACS. The prevention of cardiovascular events in patients with prior heart attack using ticagrelor compared to placebo on a background of aspirin – thrombolysis in myocardial infarction 54 (PEGASUS TIMI 54) trial showed reduction in cardiovascular death, MI and stroke when compared to placebo(220). Similarly dual antiplatelet therapy (DAPT) trial also showed benefit of long term antiplatelet therapy with P2Y12 receptor blocking drugs along with Aspirin when compared to placebo in reduction of non-fatal ischemic events(221). The benefits seen in both the trials are understandable given that coronary events are platelet centric and that the atherothombosis is a dynamic process (161). Vorapaxar, a protease – activated receptor (PAR-1) blocking drug was also effective in reducing MACE when compared to standard therapy though at increased bleeding risk (222,223). Interestingly the interaction of thrombin with PAR-1 receptor enhances the pro inflammatory and procoagulant properties of the former. Dabigatran has been shown to attenuate the above effects of thrombin in lab-based model of atherosclerosis (219). In my study elevated levels of CD54 & CD 62E EMPs and CD62P PMPs were shown in ACS cohort when compared to SA cohort at six months. I have also noted excellent correlation between CD31 EMPs and neovessels in SA cohort ($r=0.93$, $p<0.001$). This observation along with that of numerically more neovessles on OCT analysis of SA cohort suggests potential role of EMPs in proliferation of neovessles secondary to various stimuli. Furthermore, the thrombograms were also abnormal despite current evidence based secondary preventive therapy. This it is also evident from the role played by direct thrombin and PAR-1 receptor blocking drugs a multi directional approach is required to prevent future coronary events rather than relying on cyclo-oxygense path way and P2Y12

receptor based platelet inhibition. The above approach may impart stability to vulnerable patient from vulnerable blood point of view but not from vulnerable plaque point of view. Previous OCT based studies have shown that lipid rich plaques are associated with coronary events; atherosclerotic plaques with longer lipid length, wider lipid arc, minimal luminal area and discontinuation of statins were independent predictors(224). My study has also shown ACS patients on the whole and non-culprit arteries in the ACS cohort to have higher lipid burden. Thus there may also be role for plaque stabilizing drugs such as high dose statin (225) and proprotein convertase subtilisin/kexin type 9 (PCSK-9) inhibitor (226). PCSK-9 inhibitor, Evolocumab is a novel monoclonal antibody shown to further reduce low density lipoprotein (LDL) levels when compared to standard therapy and in reducing cardiovascular death, heart attack, stroke, hospitalization for angina and revascularization (227).

Limitations:

My study has several limitations. Limitations associated with a small observational study are worthy of note. In choosing patients with low incidence of vascular disease other than CAD I may have inadvertently contributed towards selection bias and thus selected lower risk cohort than anticipated. However, by being selective I have ensured that the elevated MPs in my study are secondary to CAD rather than other comorbid conditions. I carried out anonymisation of the study patients and a serial number was ascribed in the order of recruitment. Given the small number of study patients and relative ease at remembering phenotype I may have contributed towards bias in analysis of OCT studies. However, two independent clinicians carried out OCT analysis and only findings that were agreed up on were included. Though CD54 EMPs, CD62 E EMPs and CD62P PMPs were observed to be elevated at six months in the ACS cohort, lack of repeat imaging of coronaries with OCT limits this to a mere observation as the coronary phenotype may have changed by then. However, subjecting clinically well patient to repeat coronary angiography and three vessel OCT would have caused issues related to patient consent.

6.1 Future directions

With FOAM study I have demonstrated that correlation exists between subtypes of MPs and atherosclerotic plaque characteristics seen on OCT. The preliminary nature and the possibility that these observations represent epiphenomena is emphasized. The causative role of MPs, if any, in vulnerable plaque has not been established. Role of selective MPs with excellent correlation such as CD31 EMPs need to be explored further in lab based animal models. Furthermore, studies including large cohort of patients is required to see if CD54 EMPs, CD62 E EMPs and CD62P PMPs can be used as biomarker in identifying an individual at high risk of coronary events and if there is any merit in developing a specific therapeutic target to these MPs or at adopting a treatment strategy that involves targeting various pathway of platelet activation. Larger validatory studies to confirm my observations are now needed, a significant challenge as the assays required and described in this thesis and three vessel OCT imaging are non-routine, time consuming and expensive. However, longitudinal studies in a large number of patients may yield a higher event rate at follow up which can then be justified for repeat coronary angiography and OCT imaging. The plaque characteristics analyzed on repeat imaging can then be compared with the initial imaging to assess if there is any alteration in the plaque phenotype. Any correlation obtained with MPs then will have much better discriminatory potential between subtypes of CAD.

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