Title

An investigation into the relationship between platelet activation (platelet monocyte interaction & microparticles), inflammation and microvascular dysfunction in coronary artery disease

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Declaration

I, Dr Chrysostomos Mavroudis confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Thesis Abstract

Introduction

Inflammation and platelet activation play a pivotal role in the pathogenesis of acute coronary syndrome (ACS). Mounting evidence indicates that microparticles (MP) are potent pro-coagulant molecules modulating inflammatory processes.

Aims

The aim of my study was to investigate the intracoronary and systemic microparticle expression in patients with symptomatic coronary heart disease (CHD) and their relationship with markers of inflammation, platelet activation and microvascular dysfunction (MvD).

Methods

Forty eight patients with CHD (37 with ACS [23 patients with ST segment myocardial infarction (STEMI) and 14 with non-ST segment elevation myocardial infarction (NSTEMI)] and 11 with stable angina (SA) treated with percutaneous coronary intervention (PCI) were recruited. Blood samples were aspirated sequentially from the right atrium (RA) and the coronary (CO) artery (distal to the culprit lesion). AnnexinV+ MP (AnV+MP) from platelet poor plasma were measured using fluorescent monoclonal antibodies and 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 measured using ELISA. MvD was assessed by measuring the index of microvascular resistance (IMR) and coronary wedge pressure (Pw).

Results

The main novel findings of this study are the demonstration of: 1) differences between MP levels in patients with varying severity of CHD i.e. MP levels were overall higher in those with ACS (more specifically the highest MP levels were observed in the STEMI patients) compared with SA; 2. differential local and systemic AnV+ MP expression in human coronary artery disease; 3. differential local and systemic expression of inflammatory markers in ACS patients; 4. differential local and systemic expression of markers of platelet activation in ACS patients; 5. positive correlation between AnV+MP and markers of inflammation, platelet activation and myocardial necrosis in ACS patients both in the CO and RA; and 6. positive correlation between invasive markers of microvascular dysfunction and PMA in human coronary artery disease.

Conclusions

High levels of AnV+MP occur in the coronary artery of patients with ACS. Levels of AnV+MP correlate with severity of the ischaemic lesion since higher MPs were 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 and cell specific AnV+MP. The interaction between activated platelets and monocytes with endothelial cells and the subsequent formation of AnV+MP and PMA during ACS would be compatible with a direct pathogenic link between inflammatory and prothrombotic pathways in the pathogenesis of ACS and myocardial necrosis. This may suggest a novel role for MPs as effectors of the inflammatory response and cellular injury in ACS. Our observations also support the hypothesis that PMA formation may be important

determinants of platelet activation, inflammation and microvascular dysfunction in coronary artery disease. Whether these MP merely reflect the severity of the ischaemic lesion, and/or are active participants in the pathogenesis of ACS now warrants further study, since these could be important, novel therapeutic targets.

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Abbreviations

Abbreviation	Definition
ACS	Acute Coronary Syndrome
ACT	Activated Clotting Time
ADP	Adenosine di-Phosphate
AMI	Acute Myocardial Infarction
AnV	Annexin V
AnV+MP	Annexin V+ Microparticle
AO	Aorta
CD	Cluster of differentiation
CAC	Circulating angiogenic cells
CHD	Coronary Heart Disease
CFR	Coronary Flow Reserve
CK	Creatine Kinase
CO	Coronary Artery
COX-1	Cyclooxygenase-1
CV	Coefficient of Variation
CVD	Cardiovascular diseases
CXCL10	C-X-C motif chemokine 10
EDTA	Ethylenediaminetetra-acetic acid
EMP	Endothelial derived Microparticle
FITC	Fluorescein Isothiocyanate
FFR	Fractional Flow Reserve
HDL	High Density Lipoprotein
hs-CRP	Highly sensitive C-Reactive Protein
ICAM-1	Intercellular Cell Adhesion Molecule-1
INF-γ	interferon-γ
IL	Interleukin
IMR	Index of Microvascular Resistance
IQR	Inter Quartile Range
LDL	Low Density Lipoprotein
Left ventricular hypertrophy	LVH
LPS	Lipopolysaccharide (Endotoxin)
mAb	Monoclonal Antibody
Mac-1	Macrophage 1 Antigen
MMP	Monocyte derived Microparticle
MMPr	metalloproteinase enzymes
MVD	Microvascular dysfunction
MVO	Microvascular obstruction
MPV	Mean platelet volume
MCP-1	Monocyte Chemotactic Protein- 1
MP	Microparticle
MPO	Myeloperoxidase
NSTEMI	Non-ST Elevation Myocardial Infarction
NF-kB	Nuclear Factor Kappa light chain enhancer of activated B cells

NO	Nitric Oxide
NMP	Neutrophils derived Microparticle
Pa	aortic pressure
PAF	Platelet-activating factor
PAI-1	Plasminogen Activator Inhibitor 1
PAR	Protease-activated Receptors
PCI	Percutaneous Coronary Intervention
Pd	Distal Coronary pressure
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll-Protein Complex
PerCP-Cy5	Peridinin-Chlorophyll-Protein Complex with cyanine-5.5
PF4	Platelet factor 4
PLT	Platelet
PMA	Platelet-Monocyte Aggregates
PMP	Platelet derived Microparticle
pPCI	Primary Percutaneous Coronary Intervention
PS	Phosphatidylserine
PSGL-1	P selectin Glycoprotein Ligand 1
Pv	Central venous pressure
Pw	Coronary wedge Pressure
RA	Right Atrium
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
SA	Stable Angina
SMCs	smooth muscle cells
s-P-Selectin	Soluble P-Selectin
SSA	serum amyloid-A
SSC	Side-scatter
STEMI	ST Elevation Myocardial Infarction
TF	Tissue Factor
TFPI	tissue factor pathway inhibitor
TGF-β	transforming growth factor-β
TH1	Type 1 T Helper Cell
TH2	Type 2 T Helper Cell
TIMI	Thrombolysis in myocardial infarction
T_m	Mean transit time
TNF-α	Tumour Necrosis Factor-α
Trop T	Troponin T
TXA_2	thromboxane A_2
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor

Chapter 1: Introduction

1.1 Platelet activation in coronary heart disease

1.2 Platelet monocyte interactions

1.3 Microparticle

1.4 Role of inflammation in coronary heart disease

1.5 Microvascular dysfunction in coronary heart disease

1.6 Aims of this thesis

1.1 Platelet activation in coronary heart disease

1.1.1 Role of platelet activation and aggregation in haemostasis

Platelets are anucleate membrane particles produced from fragmented megakaryocytes of the bone marrow. Once they are released into the circulation, platelets have a life span of 7 to 10 days. Platelets contain only m-RNA and as such have limited ability to synthesize proteins. Platelet production is regulated mainly by thrombopoietin, a glycoprotein synthesized in the liver and kidneys (1). Platelets play a crucial role in the transformation of the ruptured or eroded atherosclerotic plaque to an unstable lesion and the initiation of thrombosis at the site of the complicated atherosclerotic plaque (2). Rupture, erosion or ulceration of an atherosclerotic plaque exposes the subendothelial thrombogenic extracellular matrix (collagen and tissue factor bearing microparticles) to circulating blood. The first step in thrombus formation is platelet adhesion to collagen via the platelet glycoproteins GP Iβ (α2β1) and GP VI which bind directly to collagen and platelet glycoprotein GPIIβ/IIIα via the von Willebrand factor (vWF) which acts as a bridge between platelet's receptor GPIIβ/IIIα and collagen. Von Willebrand factor is synthesized by endothelial cells (storage in Weibel-Palade bodies) and megakaryocytes (alpha granules). It assembles into multimers that range from 550 to over 10,000 kDa (3). There is considerable difference in reactivity between circulating and anchored (in the arterial sub-intima) vWF which is the result of the variation of vWF conformation. Most of the vWF released from storage enters the circulation in a warped conformation that does not allow contact of its platelet-binding domain to vWF receptors on the platelet surface. Contrary to the above vWF released from the abluminal surface of endothelial cells accumulates in the subendothelial matrix, where it binds collagen through its A3 domain and platelets through its A1 domain which is exposed (4). vWF multimers

act as the adhesive that tethers platelets to the subendothelial matrix. Large vWF multimers provide additional binding sites for collagen, and enhance platelet adhesion because platelets have more vWF receptors than collagen receptors (5). Platelet adhesion to collagen or vWF initiates signalling pathways that result in platelet activation which is the next step in platelet plug formation. These pathways induce cyclooxygenase-1 (COX-1)–dependent synthesis and release of thromboxane A_2 (TXA_2), and the release of ADP from storage granules. Both TXA_2 and ADP amplify platelet activation through binding to their platelet receptors. This process results in platelet recruitment and further expansion of the platelet plug. Thromboxane receptor (TR) is found on platelets and on the endothelium also (6) which explains why TXA2 is a potent vasocontrictive molecule. ADP platelet receptors are G protein–coupled receptors on the platelet membrane (7). The most important ADP receptor is $P2Y_{12}$ which is the thienopyridine's target, but $P2Y_1$ also contributes to ADP-induced platelet activation. Maximal ADP-induced platelet activation requires activation of both receptors. A third ADP receptor, $P2X_1$, is an ATP-gated calcium channel. The final effect on platelets of TR and the ADP receptors signal is the increase in intracellular calcium concentration. Platelet activation leads to (a) morphological shape change via cytoskeleton rearrangement of the platelet which increases the platelet surface area on which thrombin generation can occur (b) degranulation of the platelet alpha and dense granules, releasing TXA_2, ADP, serotonin, p-selectin and other platelet aggregatory and chemoattractant agents (c) increased platelet membrane expression of the GPIIβIIIα receptor followed by a conformational change of the receptor that enhances its affinity for fibrinogen and (d) platelet aggregation, in which fibrinogen binds to the activated platelet fibrinogen inhibitor GPIIβIIIα, causing a growing platelet plug. Part of the platelet activation

process is the release of platelet derived microparticle (MP formation is discussed in section 1.3). At the same time with platelet plug formation, activation of coagulation cascade occurs. Activated platelets promote coagulation by expressing phosphatidyl serine on their surface; an anionic phospholipid that is highly thrombogenic and a perfect substrate on which coagulation factors become activated. Once these clotting factor complexes are assembled they generate thrombin. In addition to converting fibrinogen to fibrin, thrombin enhances platelet recruitment and activation via the thrombin receptors (protease-activated receptors types 1 and 4 [PAR1 and PAR4] respectively) thereby promoting expansion of the platelet plug. This final step of clot formation is facilitated by the stabilizing effects of factor XIII. Platelet aggregation is the final step in the formation of the platelet haemostatic plug. GPIIb/IIIa expressed on the activated platelet mediates these platelet-to-platelet linkages. Divalent fibrinogen and multivalent vWF molecules serve as bridges and bind adjacent platelets together. Once bound to GPIIb/IIIa, fibrinogen and vWF trigger signals that augment platelet activation and result in the activation of additional GPIIb/IIIa receptors, creating a positive feedback loop. Because GPIIb/IIIa acts as the final effector in platelet aggregation, it is a logical target for potent antiplatelet drugs. Fibrin, the ultimate product of the coagulation system, tethers the platelet aggregate together and fix them to the site of injury. Therefore, an orchestrated response of activated platelets and coagulation cascade occur that results in fibrin network formation from thrombin action which helps anchoring the platelet-fibrin net plug formation at the site of injury. Activated platelets also release adhesive proteins, such as vWF, thrombospondin, and fibronectin which amplify platelet adhesion at sites of injury, as well as growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) both which promote wound healing.

1.1.2 P-selectin and platelet activation

P-selectin belongs to the selectin family of transmembrane proteins. It has a 140 KDa weight and is stored in the Webel-Palade bodied of the platelets and the endothelial cells (8). P-selectin consists of three domains: N-terminal, trans-membrane and cytoplasmic domains (9). Upon activation, endothelial cells and platelets express P-selectin on their surface which binds to the P-selectin ligand (PSGL-1) on the leukocytes through which P-selectin exerts its biological activities. P-selectin mediates platelet-leukocyte interaction via the P-selectin-PSGL-1 bond which leads to leukocyte activation and recruitment at the site of inflammation (10). Hahne et all have shown that inflammatory mediators like as tumour necrosis factor-α (TNF-α), lipopolysaccharide (LPS) and interleukin-4 (IL-4) increase P-selectin production from endothelial cells in addition to upregulating P-selectin at the cell surface within minutes after stimulation with TNF-α, both the above effects are additive (11). Soon after P-selectin is expressed on the platelet or endothelial cell membrane, it undergoes enzymatic cleavage at the junction between the N-terminal portion and the transmembrane domain. The N-terminal domain is released in the circulation and represents the soluble P-selectin (s-P-selectin) (12;13). It has been shown that soluble P-selectin has the same biological activities as the attached to the cells P-selectin (14). P-selectin plays an essential role in the initial recruitment of leukocytes to the site of injury during inflammation. Endothelial cells activated by molecules such as histamine or thrombin during inflammation express P-selectin on the endothelial cell surface. P-selectin then interacts with PSGL on the monocytes/and leukocytes facilitating the recruitment and activation of inflammatory cells (15). P-selectin is also very important in the recruitment and aggregation of platelets at areas of

vascular injury promoting platelet aggregation and activation through platelet-fibrin and platelet-platelet binding.

1.1.3 Platelet activation and coronary thrombosis in acute coronary syndrome

Although the pathophysiology of acute coronary syndrome (ACS) is multifactorial, atheromatous plaque rupture or erosion with superimposed thrombus is the most common cause of ACS. Other coexisting pathophysiologic processes contributing to the development of ACS include 1) dynamic obstruction of the epicardial coronary artery due to a) artery spasm in the presence of surplus, in the ACS setting, of vasoconstrictive and prothrombotic molecules released from the activated platelets, b) peripheral embolization of debris and c) endothelial dysfunction which almost always exist in these patients 2) severe luminal narrowing caused by progressive coronary atherosclerosis 3) restenosis after coronary interventions 3) intracoronary and systemic inflammation 4) coronary ischaemia related to decreased oxygen supply (anaemia) or increased oxygen demand (fever and tachycardia) of the myocardium. The following observations support the central role of coronary artery thrombosis in the pathogenesis of ACS: (a) the frequent finding of thrombus with coronary angioscopy (b) the demonstration with coronary angiography, intravascular ultrasound and optical coherence tomography of plaque ulceration or irregularities suggesting a ruptured plaque or thrombus (c) autopsy findings that coronary artery thrombi usually localized at the site of the ruptured or eroded coronary plaque (16) (d) the elevation of several serum markers of platelet activation and fibrin formation and (e) antiplatelet and antithrombotic therapy improves the clinical outcomes.

1.2 Platelet-monocyte interactions

1.2.1 Platelet monocyte aggregate formation

Activated platelets accumulate at the sites of atheromatous plaque rupture (17) via interaction with the vWF of the subendothelial collagen as described above. During platelet activation, P-selectin expression on their surface facilitates platelet binding and interaction with leukocytes, via the PSGL-1 on the leukocyte surface, leading to platelet-monocyte aggregate formation (PMA). This interaction between activated platelet and leukocyte may alter leukocyte migration, recruitment and activation patterns (18). It has also been shown that activated platelets lose their surface P-selectin despite the fact they continue to circulate and function (19). Therefore PMA measurement may represent a more robust marker of platelet activation than detection of P-selectin on their surface (20-22).

1.2.2 The pathophysiologic significance of platelet-monocyte aggregate formation in coronary heart disease

The role of PMA formation in the pathophysiology of coronary heart disease (CHD) remains unknown. Whether PMA formation is just a bystander of the inflammatory and thrombotic process occurring in the setting of ACS or whether the PMA is an effector of disease remains unclear. Nevertheless P-selectin on the surface of activated platelets induces monocyte TF expression and binding of P-selectin to monocytes in the setting of a ruptured atheromatous plaque during ACS which may trigger or contribute to the cascade of thrombosis (23). In addition, pro-inflammatory cytokines e.g. TNF-α and IL-1 also partially regulate the expression of TF on endothelial cells and monocytes (24).

Neumann et al showed that in patients with acute myocardial infarction (AMI) thrombin stimulated platelets induce monocyte cytokines, IL-1β, IL-8, and monocyte chemotactic protein-1 (MCP-1) expression, a process that is regulated by NF-kB activity. It has also been shown that secretion of these chemokines is not, however, directly signalled by P-selectin. Instead, tethering of the monocytes by P-selectin is required for their activation by a platelet chemokine, RANTES (25). Weyrich et al have demonstrated that adhesion of human monocytes to P-selectin, the most rapidly expressed endothelial tethering factor, increased the secretion of MCP-1 and TNF-α by the leukocytes when they were stimulated with platelet-activating factor (PAF). Increased cytokine secretion was specifically inhibited by G1, an anti P-selectin mAb that prevents P-selectin from binding to its ligand, PSGL-1, on myeloid cells (25).

Activated platelets also express CD40 ligand (CD154), a molecule critical to adaptive immune responses, which has been implicated in platelet-mediated modulation of innate immune responses and inflammation. Binding of CD40L to CD40 on monocytes leads to monocyte activation and secretion of pro-inflammatory cytokines including IL-6 which is associated with unstable angina (26;27), and often associated with increased C-reactive protein (CRP) (28). Binding of non-activated platelets does not affect receptor expression, cytokine production, NF-kB activation, chemotactic responses, or apoptosis. In contrast binding of activated platelets does trigger pro-inflammatory responses in monocytes. Bournazos et al, in line with the above, showed that in the absence of overt platelet activation P-selectin-PSGL-1 dependent monocyte-platelet interaction represents a normal physiological process with little impact on the potential of monocytes to cause vascular injury (29). They concluded that high levels of P-selectin on the surface of activated platelets or binding of multiple platelets per monocyte are required to trigger monocyte

activation via the P- selectin-PSGL-1 pathway. In addition they also suggest that release of a range of cytokines, including IL-1ß, IL-6 and IL-12 after platelet activation might provide additional signals that lower the threshold for monocyte responsiveness.

The extent of PMA formation depends mainly on platelet activation status rather than the monocyte activation (30). It has been shown that prevention of platelet adhesion to monocytes by interfering with the binding of platelet P-selectin to PSGL-1 reduces inflammation (31). Platelet binding to monocytes via the P-selectin dependant pathway increases the adhesive properties of monocytes by up-regulating the expression and functionality of beta1 and beta2 integrins (32;33). This process is facilitated by the presence of RANTES and CXCL10 chemokines produced by platelets on to the monocytes (34). Binding of platelets to monocytes induce TF expression on the monocyte surface in addition to increasing the release of pro-inflammatory chemokines. P-Selectin-PSGL-1 interaction is important but not exclusively responsible for this process. TF expression on the PMA is found to be reduced by a P-selectin blocking antibody (35).

In summary, activated platelet-monocyte interaction leads to monocyte activation which consequently transforms the monocyte into a more pro-inflammatory cell through the production of chemokines such as IL-6 and TNF-α and more pro-coagulant cell, via TF expression on the monocyte surface.

1.2.3 Clinical significance of platelet-monocyte aggregate in coronary heart disease

Increased PMA levels have been observed in patients with stable CHD compared with normal control subjects (36). Furman et al have also found higher circulating PMA in patients with AMI who presented within 4 hours of symptoms compared to unstable angina patients concluding that PMA formation is an early marker of AMI (37). Similarly Sarma et al studied 13 patients with AMI, twelve patients with unstable angina and 27 patients with non-cardiac chest pain. They found significantly elevated PMA in patients with AMI and unstable angina compared with those with non-cardiac chest pain. They also reported that calcium independent PMA formation is significantly higher in AMI group (38).

Ray et al demonstrated elevated markers of platelet activation including PMA and soluble P-selectin in patients with CHD prior to percutaneous coronary intervention (PCI) (20). Furthermore increased levels of PMA before PCI were associated with increased expression of P-selectin on the platelet. They found that patients with higher levels of PMA prior to PCI were more likely to develop an elevation of cardiac troponin I during the first 24h after PCI and that PMA levels may identify patients at risk for troponin elevation following PCI (20).

PMA have also been studied in patients with ACS who underwent PCI with concurrent administration of Glycoprotein IIb/IIIa inhibitors (20;39). Both studies have shown that inhibition of platelet-platelet adhesion and activation via glycoprotein IIb/IIIa inhibitors leads to PMA formation reduction in addition to the reduction of platelet activation and degranulation.

Suboptimal angiographic results following coronary interventions, where PCI achieves epicardial coronary perfusion but not myocardial perfusion occurs in a

proportion of patients and is called ‘no-reflow phenomenon’. No reflow phenomenon is a poorly understood complication of PCI and is defined as transient cessation of blood flow, due to microvascular obstruction, in the epicardial coronary artery during PCI despite the removal of the luminal obstruction. Recently experimental and clinical data have shown that no-reflow phenomenon occurs after reperfusion with a variable prevalence ranging between 5 to 20% (40).

Notably the incidence of post-AMI complications including arrhythmias, adverse left ventricular remodelling with heart failure and overall mortality is higher in patients with no reflow phenomenon. No reflow phenomenon has a multifactorial pathogenesis including distal embolization, ischemia injury, reperfusion injury and individual predisposition of coronary microcirculation to injury. Distal embolization can occur either from coronary thrombus formed in-situ or debris from the ruptured atherosclerotic plaque during ACS (41). Ischemia related injury involves endothelial cells swelling and protrusion in the artery lumen, myocardial cells swelling and interstitial oedema. All the above factors lead to compromise of coronary microcirculatory flow (42;43).

Reperfusion myocardial injury is mediated via leukocytes and platelets infiltrating the injured reperfused myocardium which become activated. Activated neutrophils release a variety of cytotoxic agents (i.e. oxygen free radicals, proteolytic enzymes) and pro-inflammatory mediators that cause tissue and endothelial damage. Activated platelets also bind leukocytes forming PMA which can cause mechanical plugging of the capillaries blocking the flow (44;45). Additionally activated platelets, endothelial cells and monocytes release vasoconstrictive substances contributing to local vasoconstriction of the coronary microcirculation compromising further the

perfusion (46). Activated platelets express P-selectin on their surface which bind to monocyte via the p-selectin ligand as described earlier in this chapter.

PMA formation of at the site of plaque rupture with subsequent monocyte activation and cytokine release may play an unfavourable role in the restoration of microcirculatory flow. Ko et al have found increased levels of soluble CD40 ligand, IL-6, serotonin, TF and factor VII in the culprit coronary artery compared to those in peripheral blood (47). Also a positive relationship between inflammatory markers (IL6 and CRP) and markers of platelet activation like P-selectin and PMA has been demonstrated by Wang et al (48). Therefore the possibility that PMA may contribute in the pathophysiology of microvascular dysfunction via exaggerating and sustaining the coagulation cascade in addition to perpetuating local coronary inflammation exist.

Takahashi et al have showed that neutrophilia on admission is associated with impaired microvascular reperfusion and poor functional recovery in patients with STEMI treated with primary PCI. Similar finding were found by Sezer et al (49). In this study the authors studied 41 patients with anterior STEMI treated with primary PCI. They assessed the MvD invasively using Coronary Flow Reserve (CFR), the index of Microvascular Resistance (IMR) and coronary wedge Pressure (Pw). Thermodilution-derived technique and a pressure-temperature sensor-tipped guide-wire in the left anterior descending artery, within 48 h after primary PCI, was used to calculate the CFR and IMR. Increased neutrophil count and higher mean platelet volume are found to be associated with higher IMR lower CFR and higher coronary wedge pressure. Therefore the association of mean platelet volume (MPV) and impaired microvascular perfusion can represent platelet-leukocyte complex and platelet-platelet aggregate mediated microvascular injury and endothelial dysfunction

in coronary arterioles and capillaries. Individual predisposition of coronary microcirculation to no-reflow can be genetic and/or acquired. Particularly diabetes mellitus has been associated with impaired microvascular reperfusion after primary PCI, while hypercholesterolemia in the animal model aggravates reperfusion injury by enhancing endothelial oxidative stress (50).

1.2.4 Differential expression of platelet-monocyte aggregate in the coronary artery and peripheral circulation

PMA formation is increased in CHD in both stable angina and unstable coronary syndromes as discussed above (36;51). In addition patients who have had coronary interventions, there is increased PMA expression (20). It has been suggested that increased platelet-monocyte interaction at the site of the ruptured atheromatous plaque may well be a contributing factor in the pathogenesis of no-reflow phenomenon. Botto et al have demonstrated increased platelet-monocyte interaction and PMA formation in blood samples aspirated from the infarct related artery (IRA) compared with the peripheral circulation in patients presented with AMI (52). The concept of an active involvement of PMA in ACS has been supported with the observations by Patel et al who demonstrated trans-coronary gradient of platelet-leukocyte formation in patients with ACS. They found increased PMA level in the coronary sinus samples compared to the aortic samples in both patients with ACS and stable angina (21). The increment was 22% in ACS group and 16% in the stable angina group.

In conclusion PMA is a sensitive marker of platelet activation but also may be the link between platelet activation, thrombosis and inflammation via monocyte activation in patients with CHD. The downstream effect of the increased expression

of PMA at the site of the ruptured plaque appears to be the link between platelet activation, thrombosis and coronary inflammation in patients with CHD.

1.3 Microparticles

1.3.1 Introduction

Microparticles (MP) were first described in 1967 when Wolf reported platelet membrane fragments in human plasma (53). He called these fragments 'platelet dust' which are essentially cell vesicles with diameter of less than 0.1μm. MP are submicron membrane vesicles 100-1000nm derived from virtually any eukaryotic cell during biological processes like cell activation, differentiation and apoptosis. Microparticles differ in size and membrane formation including the phospholipids and protein composition and their biological effects depend on the initial stimulus which led to their formation. Mounting evidence indicates that MP are potent pro-coagulant molecules affecting vascular endothelial function and regulate the inflammatory process (54;55). In healthy individuals MP formation represents a physiological process, a fine balance between MP formation and clearance. However a variety of diseases, mainly thrombotic, disorders including atherothrombosis (56), diabetes mellitus, preeclampsia and systemic inflammatory conditions are associated with elevated circulating MP in the peripheral blood (57;58). The question as to whether MP is implicated in the pathogenesis of atherothrombotic diseases or just a bystander in these clinical disorders remains to be answered. Platelet derived microparticles (PMP) represent 70-80% of circulating MP (59). Endothelial derived (EMP), neutrophil derived (NMP), monocyte derived (MMP), and red blood cells account for the rest of the MP population. While many questions about the function of MP remained to be answered, in general MP propagates the actions of their parental cells, the activated platelets in the case of PMP.

1.3.2 Microparticle formation

The initial step in MP formation is plasma membrane budding. This step requires a disruption of the asymmetrical distribution of lipids between the inner and outer surface of the plasma membrane and cytoskeleton protein reconstruction (60). At rest the predominant phospholipids of the outer surface of the cell membrane is phosphatidylcholine and sphingomyelin whilst phosphatidylserine (PS) is located in the inner monolayer of the cell membrane (61). During cell activation or apoptosis, the combination of the activation of the 'floppase' protein' and the inhibition of the 'flippase' protein' of phospholipid transporters (62) triggered by the increased concentration of the intracellular calcium, lead to a rapid and overwhelming externalization of the PS (63). The 'floppase' (an ATP-dependent trans-membrane protein that specifically transfers PS from the inner to exterior surface) mediates the rapid outward translocation that is directly responsible for the disruption of asymmetry when cells are activated (64). 'Flippase' is a trans-membrane protein which maintains the normal resting phospholipid distribution with PS being the main phospholipid of the inner membrane and phosphatidylcholine and sphingomyelin the phospholipids of the external layer of the membrane (65). The above step has been named as the flip-flop phenomenon and is the most crucial step in the MP formation cascade. In addition, the integrity of the cell membrane, supported by the sub-membrane cytoskeleton, is believed to contribute to the maintenance of its asymmetry and therefore the cell shape. Cytoskeleton reorganization could therefore help membrane budding and MP formation. Proteolytic cleavage of the cytoskeleton proteins via calcium-activated enzymes (calpains in activated cells or caspases in apoptotic cells) results in membrane destabilization and eventually MP formation (66). The combined membrane phospholipids and cytoskeleton reconstruction in

response to cell activation has been labelled as ‘membrane remodelling’. Membrane remodelling of the cells undergoing activation or apoptosis with PS exposure appears to be a universal feature and an underlying feature of MP release by these cells (67). It is important to underline that the flip-flop phenomenon with rapid externalization of PS is a naturally pro-coagulant process. PS borne by MPs provides an excellent substrate for the assembly of the pro-thrombinase complex (which consists of the coagulant factors Xa the cofactor Va and calcium), leading to thrombin generation and clot formation (68).

As discussed above MP release from activated cells is generally a pro-coagulant process. Whether PS negative MP without pro-coagulant activity exists or not is a matter of debate. It has been reported that the majority of platelet-derived MPs circulating in the platelet poor plasma (PPP) of healthy donors fail to bind AnnexinV (AnV) and as such lack phospholipid-dependent pro-coagulant activity (69). Nevertheless the nature of the platelets or endothelial cells derived AnV negative MP detected by flow cytometry (69;70) and the mechanisms facilitate their release is poorly understood. These findings suggest that cell-derived MPs are not only potent procoagulant effectors in atherothrombosis but can also convey a variety of different molecules, such as, cytokines, enzymes, bioactive lipids and mRNA to the neighbouring cells.

1.3.3 Microparticle composition

MP composition is an important determinant of their biological effects. MP lipid and protein composition may vary depending on the parental cell and the stimulus which triggered their formation. For example, the phospholipid composition of MPs isolated from patients with chronic inflammatory arthritis differs from that of MPs

from platelet poor plasma (PPP) of healthy subjects (71). In addition, the degree of phospholipid oxidation varies depending on the stimulus initiating the MP release (72).

1.3.4 Biological effects of microparticles

Coagulation, inflammation and endothelial dysfunction are the main biological effects of MP. Those mechanisms also play a central role and contribution to the evolution of the atheromatous plaque in patients with CHD. Besides the above mentioned deleterious MP effects, MP can transfer biological information in the form of proteins, adhesion molecules [receptors and integrins], nucleic acids and lipids. For those reasons they have been recently described as protagonists of intercellular communications and signalling (57;58;73). In addition MP are involved in the "cellular waste process" because they contain increased (compared with parental cell) concentrations of chemotherapeutics, oxidized phospholipids, or caspase 3 (58). Caspase 3, an intracellular cleavage enzyme, accumulates in endothelial cells. EC which undergo apoptosis release caspases 3–containing MP (74) indicating that the release of MP is part of a protective mechanism to prevent the intracellular accumulation of caspase 3 at dangerously high levels (75).

1.3.4.1 Coagulation

The procoagulant effect of cell-derived MP is the most described characteristic. The negatively charged phospholipids layer of the MP (mainly phosphatidylserine) provides an excellent substrate for the assembly of the prothrombinase complex (which consists of the coagulant factors Xa the co-factor Va and calcium), leading to thrombin generation and clot formation. Interestingly, PMP surfaces have 50- to 100-

fold higher pro-coagulant activity compared with the normal platelet surface (76). Also circulating TF bearing MP can initiate and propagate the TF-dependant coagulation cascade (77;78). The TF exposed on the MP appears to be in an inactive, non-coagulant form as Damiant et al showed in patients with type 2 DM (79). However this inactive form of TF may become procoagulant after the contact of TF-bearing MP with the developing thrombus (80), an interaction which is believed to be mediated by P-selectin, expressed on the activated platelets (81). This is supported by the finding that those patients with ST-elevation ACS who had the highest plasma level of TF-exposing MP were characterized by a procoagulant phenotype, as reflected by increased plasma levels of thrombin-antithrombin complexes (82). The complexity of the involvement of MP in the coagulation cascade is supported by evidence showing the divergent coagulant effects of MP. For example in healthy individuals PPP derived MP exert an anticoagulant activity via activation of protein C, which, in turn, inhibits additional thrombin generation by inactivating the coagulation factors Va and VIIIa (83). Contrary to the above anti-thrombotic effects of MP in the healthy population, in pathological situations the observed increased levels of TF-bearing MP eliminates their anticoagulant effect resulting in a net procoagulant effect.

1.3.4.2 Inflammation

It is now widely accepted that atherosclerotic disease is a chronic inflammatory process (84). Patients with high inflammatory markers like CRP including those with diabetes mellitus, metabolic syndrome and chronic inflammatory arthritis (psoriatic or rheumatoid arthritis) are at high risk of developing CHD (85). The exact mechanisms are not well understood however the evidence suggest various pathways

through which MP formation may act as an inflammatory mediator. For instance in vitro leukocytes derived MP can stimulate the expression of pro-inflammatory genes in endothelial cells, leading to the formation of leukocyte– endothelial cell adhesion molecules and pro inflammatory cytokines, like IL-6 (86). Also platelet-derived MP delivers arachidonic acid (AA) in the endothelial cells leading to up-regulation of ICAM-1 (intercellular adhesion molecule-1, CD54) which mediates monocyte-endothelial cells adhesion (87). In addition oxidative stress is a well-recognized phenomenon in atherosclerosis and coronary heart disease. Oxidized modification of low density lipoproteins (LDL) appears to play a key role during all the stages of atherosclerosis via activation, migration and transformation of monocytes into macrophages and scavenger cells within the atheromatous plaque (72). It has been shown that MP derived from oxidative stress-treated-endothelial cells contains oxidized phospholipids which can activate monocytes and neutrophils and adherence with EC via expression of adhesion molecules. The exact mechanism by which oxidized phospholipids are involved in this process is not clear however PAF receptors expressed on leukocytes and platelets has been postulated as a possible pathway (87).

1.3.4.3 Endothelial dysfunction

Endothelium is a multifunctional organ. Apart from being the mechanical barrier between the blood cells and the subendothelial tissue preventing the coagulation cascade activation, endothelial cells exert a variety of characteristics including anti-inflammatory, vascular tone control, vessel wall permeability and cell growth (87). The above functions render endothelium an important player in the pathophysiology of CHD. Impaired endothelial function has been reported in cardiovascular diseases

like atherosclerosis (84;88) ACS (89), heart failure (90), hypertension (91) and pre-eclampsia and eclampsia syndromes (92). Mechanism by which MP may cause endothelial dysfunction include a) alteration of the endothelial nitric oxide (NO) synthase activity and the balance between NO availability and reactive oxygen species production and 2) the post-receptor NO transduction pathway. The latter has been shown by Boulanger et al. who found that circulating MP from patients with acute MI cause severe endothelial dysfunction in healthy blood vessels by affecting the post-receptor nitric oxide (NO) transduction pathway (93). Mostefai et al showed in vitro that MP from T-lymphocytes decrease NO production and increase oxidative stress in endothelial cells (94). These effects are associated with a reduction of endothelial NO synthase activity, which depends on phosphatidylinositol-3-kinase (PI3K), extracellular signal–regulated kinase 1/2, and nuclear factor-B pathways (94). In vivo data also indicate that circulating MP from patients with type II DM (95) and chronic renal failure (96) are associated with vascular endothelial dysfunction.

Interestingly, in contrast to the above findings, in some situations MP can improve endothelial dysfunction. The protective role of MP has been shown by Mostefai and co-workers providing evidence that increased circulating MP are protective against vascular hypo-reactivity accounting for hypotension in patients with septic shock (97). In line with these findings Soriano et al have shown that elevated levels of EMP and PMP predict a more favourable outcome in severe sepsis in terms of mortality rate and organ dysfunction (98).

In summary, MP can have both detrimental and favourable effects on endothelial function by altering the balance between NO and ROS production and release depending on the clinical setting. It appears that the expression of those effects

depends on the specific stimulus underlying the release of MP by their parent cells (75).

1.3.4.4 Angiogenesis

As mentioned above atherosclerosis is a chronic inflammatory process of the vascular wall with monocyte infiltration, lipid accumulation and modification with subsequent focal thickening of the intima of arteries. Evolution of the atheromatous plaque leads to vulnerable atherosclerotic plaques prone to rupture. Those vulnerable plaques are characterized by an enlarged necrotic core containing apoptotic macrophages, increased number of vasa vasorum, and more frequent intra-plaque haemorrhage (99). Researchers have found that MPs isolated from human atherosclerotic lesions express CD40L and stimulate endothelial cells after CD40 ligation promoting in vivo angiogenesis. Therefore, MPs could represent a major determinant of intra-plaque neovascularisation and plaque vulnerability (100). Circulating angiogenic cells (CACs) are believed to represent a cell population enriched in monocytes and exert their angiogenic effects via paracrine and signalling mechanisms (101). Recent reports suggested that the neo-vascularization-related capacities of CACs are impaired in atherosclerotic patients. Also PMPs were reported to augment the re-endothelialization capacity of CACs (102). Ohtsuka et al isolated mononuclear cells and PMPs from peripheral blood of patients with atherosclerotic heart disease. They generated PMP-pretreated CACs (PMP-CACs) by co-culturing of the mononuclear cells and PMPs. They found that although the migration capacity of PMP-CACs was similar to that of CACs, the adhesion capacity of PMP-CACs was greater. This augmented adhesion and neo-vascularization capacities by PMP-CACs were canceled out by a RANTES neutralizing antibody.

They concluded that PMP-secreted RANTES may play a role in the augmenting adhesion and neo-vascularization capacities of CACs (102).

1.3.5 Clinical significance of microparticle formation in patients with coronary heart disease

Clinical studies in patients with CHD or conventional risk factors for CHD have shown that MP levels are elevated compared with healthy volunteers (56;103;104). Endothelial dysfunction is the main contributing factor in the pathophysiology of atherosclerosis and subsequently stable or unstable CHD. Also in ACS, where platelet activation and thrombus formation dominates, markers of platelet activation like total MP and PMP are expected to be markedly elevated. Therefore the interactions between a dysfunctional endothelium, activated platelets and monocytes may play an important role in the pathogenesis of ACS. Singh et al studied the PMP level in 6 patients with UA. Blood samples were drawn from the patients every 4 h over a 24 h period and PMP were estimated using flow cytometry. There was a marked, six-fold increase of PMP levels in the unstable angina patients ($p=0.001$) compared with 6 healthy, volunteers (105). Stepien et al determined the levels of PMP, EMP, MMP and s-P-selectin in 12 AMI patients, 10 stable angina (SA) patients and 9 healthy controls. They found that patients with AMI displayed higher levels of total MP, PMP and TF+MP than patients with SA (104). In addition Bernal-Mizrachi et al found that CD31+EMP and CD51+EMPs were significantly higher in ACS than SA. Among patients with first MI, CD31+EMP was higher in patients with MI than in patients with UA and was significantly higher than in patients with recurring MI. CD51+EMP did not discriminate ACS from SA and PMPs did not discriminate patients with SA from control subjects (106).

Whether elevated MP levels play a contributory role to the pathophysiology of CAD or the consequence of CAD or even represent a bystander is still matter of debate. Several clinical studies have shown a correlation between MP levels and total mortality, cardiovascular mortality, major cardiac and cerebro-vascular events, myocardial damage and indices of myocardial dysfunction (107-111). Montoro-Garcia et al compared MP (total MP, PMP, EMP and MMP) levels in patients with STEMI, NSTEMI, SA and healthy individuals. They found that in NSTEMI patients, EMP and MMP were independently predictive for future admissions related to heart failure. They concluded that small-size MP could be potentially implicated in the modulation of the post-ACS reparative response to injury, with prognostic implications (111). Sinning et al determined the number of CD31+EMP in 200 patients with stable CHD and their correlation with cardiovascular outcomes. The median follow-up for major adverse cardiovascular and cerebral event (MACCE)-free survival was 6.1 years. MP levels were significantly higher in patients with MACCE compared to patients without MACCE (p=0.004). In multivariate analysis high MP levels were associated with a higher risk for cardiovascular death (HR 4.0; p=0.04), the need for revascularization (HR 2.4; p=0.005), and the occurrence of a first MACCE (HR 2.3; p=0.001). Inclusion of the MP level into a classical risk factor model substantially increased c-statistics from 0.637 to 0.702 (p=0.03). They concluded that the level of circulating AnV+CD31+EMP is an independent predictor of cardiovascular events in stable CHD patients and may be useful for risk stratification (108). Amabile and his team examined whether or not increases in circulating MP levels could predict the all-cause and cardiovascular mortality (fatal myocardial infarction, stroke, acute pulmonary edema and sudden cardiac death) in 81 stable haemodialysed patients with end-stage renal disease (ESRD). Kaplan-

Meier analysis demonstrated significantly higher probability of all-cause ($p<0.001$) and cardiovascular mortality ($p<0.0001$) between the lower and upper EMP tertiles even after adjustment for confounding factors. They concluded that increased plasma levels of EMP is a robust independent predictor of severe cardiovascular outcome in end-stage renal failure patients (107). Jung et al tried to correlate the levels of EMP or PMP in 36 patients with STEMI with the myocardium at risk and infarct size which were determined by cardiac MRI (CMR) imaging one week after the index event. They found that EMP and PMP correlated to myocardium at risk, but not to infarct size suggesting that MP levels reflect the severity of the endothelial injury and platelet activation during myocardial ischemia (109). Biasucci et al looked at the AnV+MP, EMP and PMP levels in 33 patients with SA and 43 patients with ACS undergoing PCI. They found that in the ACS group, Day 2 AnV+ MP correlated with peak Troponin T levels (112). In line with the above findings Mavroudis et al showed a positive correlation between systemic arterial total MP and EMPs with markers of myocardial damage in patients with STEMI treated with pPCI (113). Recently Porto et al studied 78 STEMI patients undergoing successful pPCI following STEMI. They measured the systemic (aortic) and local (in the culprit coronary artery) levels of PMP and EMP and their relation to indices of microvascular obstruction (MVO) like Thrombolysis in myocardial infarction (TIMI) flow, thrombus score (TS), corrected TIMI frame count (cTFC), myocardial blush grade (MBG). Both PMP and EMP levels were significantly higher in the intracoronary than in the aortic blood samples. They found that intracoronary EMP and both systemic and intracoronary PMP levels correlated with TS. Moreover, the correlation of intracoronary MP with indices of microvascular dysfunction suggests a possible direct role of MP in the pathogenesis of MVO in addition to the role of MP

as markers of ongoing thrombosis (110). Endothelial derived MPs have been also correlated with angiographically high risk coronary artery lesions. Bernal-Mizrachi et al studied 43 patients undergoing coronary angiography. Fifteen had presented with AMI, 20 with unstable angina (UA), 5 with SA and 3 with congestive heart failure. Coronary angiography was reviewed and coronary lesions were classified using the Ambrose classification as high or low risk. They found that high EMP was associated with high-risk angiographic lesions like eccentric, multiple irregular or thrombotic lesions. They concluded that EMP may be a useful marker in detecting endothelial injury and risk of ACS as defined by angiography (114). The levels of circulating MP have also been linked with the remote ischemic conditioning. Nagy et al found that PMP level is significantly elevated in patients treated electively with PCI compared to subjects with diagnostic catheterization alone. They showed that at 15 minutes after the completion of PCI the levels of PMPs, the platelet P-selectin expression and the ratio of platelet-monocyte heterotypic aggregates were significantly ($p<0.05$) elevated in the PCI group compared to the non-stented subjects, but no difference was found in soluble P-selectin values. They concluded that the observed cellular changes are early and sensitive markers to detect the platelet-activating effect of stent implantation (115). Jeanneteau et al hypothesized that the circulating MPs are the effectors of remote ischemic conditioning. Remote ischemic conditioning was induced by 5-min inflation and 5-min deflation of a blood-pressure cuff causing limb ischemia. They found that CD54+EMP and procoagulant AnV+MP are markedly increased after remote ischemic conditioning. However, MP release did not appear to be a biological vector of remote ischemic conditioning in their model (116).

1.3.6 Lifespan of circulating microparticles

Another interesting aspect of the MP generation in stable and unstable CHD is the life span of these cell fragments. Skeppholm et al measured PMP levels in 51 patients with ACS and 61 sex- and age-matched healthy controls on admission, within 24 hours (before coronary angiography), and six months later. Plasma concentrations of PMP were elevated on admission ($p<0.001$ compared to controls), decreased significantly 24 hours later following initiation of treatment with clopidogrel and subcutaneous anticoagulation ($p<0.001$), and decreased even further six months later ($p<0.01$). However, PMP were still almost 2-fold higher than in healthy controls ($p<0.001$). They concluded that PMP concentrations follow the pattern of platelet activation during and after an ACS. Also PMP levels decreased after initiation of antithrombotic treatment, but were still elevated after six months compared to healthy control levels (117). In line with the above findings Biasucci et al did serial measurements of AnV+MP, EMP and PMP in 33 patients with SA and 43 patients with ACS undergoing PCI. They found that AnV+MP increased until Day 2 ($P=0.001$), while EMP and PMP peaked on Day 1 ($p<0.01$) then decreased to baseline values (112).

1.3.7 Microparticle and inflammatory markers

Ueba et al looked at the levels of inflammatory markers IL-6, hs-CRP and their correlation with PMP as a marker of platelet activation in 464 healthy Japanese volunteers. They found that plasma level of IL-6 was associated with plasma level of PMP after adjustment for diastolic blood pressure, platelet count, and high sensitivity C-reactive protein in men ($p<0.001$) and associated with plasma level of PMP after adjustment for platelet count in women ($p<0.001$) (118). Biasucci et al in 33 patients

with SA and 43 patients with ACS undergoing PCI found that EMP and PMP 24 hours after the admission correlated with hs-CRP ($r=0.37$, $p=0.04$ and $r=0.33$, $p=0.05$; respectively) (112).

I have described in the above paragraphs the mechanisms underlie the MP formation, composition and their biological effects which are mainly coagulation, inflammation and vascular dysfunction. Our understanding that the above processes are also crucial in the pathogenesis of CHD together with the correlation between elevated MP in individuals with CHD or risk factors for CHD with markers of platelet activation and inflammation, leads to the view that circulating MP are harmful, contributing to CHD and risk of CHD. Furthermore, whether cell-derived MP is a cause, consequence, or both of CHD remains to be established. Also the clinical question whether MP is marker of CHD risk and whether they can be used as independent predictors of CHD outcome remains unanswered. In addition, it is fair to state that the data from the clinical studies referenced, which are looking into MP levels and clinical outcomes in patients with clinically manifested CHD, have potentially important limitations as the number of patients recruited is small. As such firm conclusions cannot be drawn.

Recently, the association of the Framingham risk score and numbers of platelet-, leukocyte- and endothelium derived MP were reported in two retrospective (119;120) and one prospective study (121). The addition of endothelial CD144+MP to the Framingham risk model improved the classification of risk and appeared as a significant and independent predictor of future CVD events in a high-risk population (121). The above results are promising, but more prospective studies are needed to further detail the prognostic value of cell-derived MP in individuals at high risk for cardiovascular disease (CVD) (75).

1.4 Role of inflammation in coronary heart disease

Atherosclerosis has both chronic and acute manifestations, affects both mid and large-size arteries and can cause either focal or diffuse stenosis of the affected vessels. Over the last twenty years, data have emerged showing that immune cells are involved in the pathogenesis of formation and evolution of the atherosclerotic plaques. Understanding of the anatomic structure and function of the normal artery and its native cell types is essential for the understanding of the pathophysiology of atherosclerosis.

1.4.1 Normal artery structure

Normal artery is a tripetalous structure, the inner layer-tunica intima, the tunica media and the outer layer-adventitia (122). The intima consists of a monolayer of endothelial cells adjacent to basal lamina which is a basement membrane containing non-fibrillar collagen types, such as type IV collagen, laminin, fibronectin, and other extracellular matrix molecules. The internal elastic lamina lies between the intima and tunica media. The tunica media consist of smooth muscle cells (SMCs) and layers of extracellular matrix (123). The composition of the extracellular matrix and organization of the smooth muscle cells differs according to artery size. The external elastic lamina lies between the tunica media and the adventitia. The adventitia contains collagen fibrils like the intima but in a looser arrangement. It also contains vasa vasorum and nerve endings. The existence of SMCs in the adventitia is very little compared with the other layers (124).

1.4.2 Endothelial cells

Endothelial cells (EC) of the arterial intima represent the natural barrier between blood and the prothrombotic subendothelial matrix. Arterial endothelial cells are effectors of many important haemostatic mechanisms including anticoagulant, fibrinolytic activities and platelet inhibition. EC is the main anti-coagulant by regulating thrombin generation. Amongst the various mechanisms through which endothelial cells exert these effects are: 1) production of heparan sulphate proteoglycans, which bind anti-thrombin III and tissue factor pathway inhibitor (TFPI). Heparan sulphate-antithrombin complex increases speed the rate at which anti-thrombin inhibits thrombin and other coagulation enzymes (125) while TFPI is a natural inhibitor of coagulation after it binds heparan sulphate on the endothelial cell (126), and 2) Expression on the endothelial cells surface thrombomodulin (127). Thrombomodulin binds thrombin and this complex activates the anti-coagulant factor protein C and S. Activated protein C inhibits the activated factors V and VIII which are involved in process of thrombin generation (128). Endothelial cells also exhibit fibrinolytic properties by producing and releasing tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA), should the clot formation cascade begin. Both t-PA and u-PA can initiate fibrinolysis by converting plasminogen to plasmin (129). Endothelium also releases type 1 plasminogen activator inhibitor (PAI-1), the main regulator of t-PA and u-PA. Thus, net fibrinolytic activity of endothelium depends on the dynamic balance between the release of plasminogen activators and plasminogen activator inhibitor 1 (PAI-1). As a result, healthy coronary arteries actively oppose platelet and clotting cascade activation and prevent thrombotic events (129). Endothelial cells produce and release into the blood prostacyclin and NO that are not only effective platelet inhibitors but also potent

vasodilators. In addition, endothelial cells regulate the platelet activation (130) via the molecule CD39, an ADPase expressed on the platelet surface. CD39 degrades ADP, a platelet agonist.

1.4.3 Smooth muscle cells

Smooth muscle cells are important cells of the normal artery wall with many pivotal functions contributing to the normal vascular homeostasis, and also in the pathogenesis of arterial diseases. SMC, apart from regulating blood flow through contraction and relaxation of the coronary arteries, synthesize the extracellular matrix that plays a crucial role in normal vascular homeostasis and in the development and evolution of atheromatous plaques. These cells also can migrate and proliferate, contributing to the formation of intima thickness, atherosclerosis and stent restenosis after PCI. Imbalance between synthesis by SMCs and degradation by MMPs of extracellular matrix can promote either stabilization or destabilization of atheromatous plaques. In the former case the patient will present with stable angina contrary to the latter where the presentation will be ACS.

1.4.4 Initiation and evolution of atheromatous plaque

Although the initial step in the pathophysiology in human artery atherosclerosis is still a matter of debate, observations of tissues obtained from young humans combined with the results of experimental studies in animals suggest that the first step in the pathophysiology of atherosclerosis is small lipoprotein particles (LDL) accumulation in the intima (129). These LDL particles become susceptible to oxidative and other chemical alterations after they bind to extracellular matrix. Alterations of these lipoproteins, including oxidation and glycation, promote the release of pro-inflammatory cytokines from SMCs. These cytokines amplify the

expression of leukocyte and monocyte adhesion molecules like MCP-1 and chemoattractant molecules that result in monocyte attachment and migration into the intima (131;132). MCP-1 is produced by endothelial cells and SMCs in response to oxidized lipoprotein and other inflammatory stimuli. MCP-1 selectively promotes the directed migration of monocytes. Increased levels of MCP-1 are expressed on human atherosclerotic lesions compared with uninvolved vessels. Several other cytokines including IL-8 bind on leukocytes, and interferon-γ which induces the genes encoding T-cell chemo-attractants (123), contribute to monocyte recruitment during atherogenesis. Normally there is minimum interaction between healthy endothelium and leukocytes. In atherogenesis, as described above, leukocyte migration, recruitment and accumulation within the intima is a crucial step and occurs in the early stages. Monocytes adhere to the endothelium and move through endothelial cell junctions or even can penetrate endothelial cells and enter the intima, where they begin to accumulate lipids and become foam cells (133). Specific leukocyte adhesion molecules expressed on the surface of the endothelial cell governs the interaction and adherence of monocytes and T cells to the endothelium (134). Those are: 1) members of the immunoglobulin family which mainly promote tighter adhesive interactions and immobilization of leukocytes; and 2) selectins which tend to promote less tight and rolling locomotion of leukocytes over the endothelium. The first family includes vascular cell adhesion molecule 1 (VCAM-1 or CD106) and ICAM-1 which are the main adhesion molecules mediating interaction between endothelium and leukocytes. CD106 plays an important role in the early stages of atherogenesis whilst the interaction of endothelial cells via CD54 with monocytes occurs throughout the whole process of atherogenesis. Selectins represent the second broad category of leukocyte adhesion molecules. The main representative of selectins is E-selectin

(CD62E), which recruits neutrophils. Other members of this family, including P-selectin may play a greater role in leukocyte recruitment involved in atherogenesis. CD62P is expressed on endothelial cells overlying human atheromatous plaques. Experimental studies in genetically altered mice have proven roles for VCAM-1 and P-selectin (including both platelet- and endothelium-derived P-selectin) in experimental atherosclerosis (135;136). During the last decade, the combination of basic and clinical research has demonstrated the crucial role of inflammation in atherothrombosis. Cell-cell interactions play a pivotal role in the evolution of atheromatous plaque. Antigen-presenting cells like macrophages, dendritic cells and endothelial cells allow the presented antigen to interact with and activate T cells. Activated T cells subsequently release pro- and anti-inflammatory cytokines that can modulate atherogenesis. There are two types of CD4+ T cells (T helper), Th1 and Th2. Th1 has pro-inflammatory properties and they can secrete cytokines like interferon-γ (INF-γ), TNF-α and CD40 ligand. This group of cytokines can in turn activate immune and non-immune vascular cells and coordinate changes of the plaque homeostasis that can lead to plaque destabilization and susceptible to thrombosis. In contrast, Th2 elaborate anti-inflammatory cytokines, such as interleukin-10 (137). Cytotoxic T cells (CD8+ T cells) promote cytolysis and apoptosis of macrophages, endothelial cells and SMCs. The death or apoptosis of these cell types can occur within the atheromatous plaque and may contribute to plaque progression and complication.

1.4.5 Evolution of plaque

SMCs, under the chemoattractant effect of PDGF, migrate into the intima from the tunica media. PDGF is a potent SMC attractant secreted by activated monocytes and

is vividly expressed in human atherosclerosis. SMCs in the atherosclerotic intima are involved in the progression of atheromatous plaque including SMCs proliferation. There are differences at sub-cellular level between SMCs of the normal arterial tunica media and those in the intima of an evolving atheromatous plaque (138). Intima SMCs contain rougher endoplasmic reticulum and fewer contractile fibres than the SMCs of the normal tunica media do. Intriguingly there is a non-linear pattern of SMCs proliferation during atherosclerosis evolution and growth of the intima. In fact bursts of SMCs migration and replication more likely to occur. Besides SMC proliferation, death or apoptosis (programmed cell death) of the SMCs may also be involved in the progression of the atheromatous plaque (139;140). Apoptosis of SMCs in the atheromatous plaque is facilitated by inflammatory cells and mediated by death receptors like Fas. More specifically migrated T cells express on their surface the Fas ligand while SMC under the influence of the pro-inflammatory cytokines express Fas. Interaction between Fas ligand and Fas initiates the process of the programmed cell death (141).

Although the anatomic and functional changes (at cellular and molecular level), during the evolution of the atheromatous plaque in patients with CHD, follow a continuous pattern (progressive disease), the clinical manifestations of each patient with CHD are probably determined by distinct genetic differences. Reilly et al have shown that specific genetic predispositions promote the development of coronary atherosclerosis whereas others lead to myocardial infarction in the presence of coronary atherosclerosis (142). Therefore, signalling pathways predisposing to atherosclerosis probably differ from those contributing to plaque vulnerability. That explains why many patients with angiographically proven CHD remain stable on

medical management, while in other patients myocardial infarction represents the first manifestation of CHD.

1.4.6 Arterial extracellular matrix

Extracellular matrix (ECM) which accounts most of the plaque volume is also equally important in the evolution of the atherosclerotic plaque. Collagens (types I and III), proteoglycans and elastin fibres are the most important extracellular matrix molecules of the atheromatous plaque. ECM is normally produced by the SMCs of atheroma and this process is part of the natural development and maintenance of the arterial wall. PDGF and TGF-β secreted by activated platelet and other cell types in the atheromatous lesions are the main stimulants of SMCs for extracellular matrix production. Extracellular matrix turnover and maintenance is the result of the biosynthesis (by SMCs) on the one hand, and ECM breakdown by the metalloproteinase (MMPr) enzymes on the other hand. Extracellular matrix breakdown certainly plays a role in SMCs migration into the intima from the media. In injured arteries, overexpression of MMPr inhibitors (known as tissue inhibitors of metalloproteinases, or TIMPs) can delay smooth muscle accumulation in the intima of injured arteries (143). Extracellular matrix synthesis and degradation also plays a role in arterial wall remodelling that accompanies lesion growth. During the early stages of atheromatous lesion development, plaque grows outwardly, in a way that would lead to luminal stenosis. This outward growth of the intima leads to an increase in the external diameter of the entire artery and is called positive remodelling or compensatory enlargement. Luminal stenosis tends to occur only after the plaque burden exceeds some 40% of the cross-sectional area of the artery.

1.4.7 Angiogenesis in atheromatous plaques

Atherosclerotic plaques develop their own microcirculation as they grow, a process named neovascularisation. Histologic examination with endothelial markers of the evolving plaque reveals a rich network of capillary vessels which develop in response to angiogenic factors (vascular endothelial growth factor [VEGF], placental growth factor [PlGF], and oncostatin M) expressed in atheromatous plaques. These micro-vessels within plaques have significant functional implications. They provide a conduit so leukocytes can enter and exit the atheromatous plaque. In advanced human atherosclerotic plaque, microvascular endothelium displays mononuclear-selective adhesion molecules such as VCAM-1 much more prominently than does the macrovascular endothelium overlying the plaque. The neovascularisation of the atheromatous plaques also allow growth of the plaque by supplying oxygen and nutrients. Alongside with this view, administration of inhibitors of angiogenesis to mice with experimentally induced atherosclerosis limits the growth lesion. Also, the plaque micro-vessels may be friable and prone to rupture leading to in situ thrombosis which can promote SMC proliferation and ECM accumulation in the area immediately adjacent to the microvascular disruption.

1.4.8 Plaque mineralization

Plaques often develop areas of calcification as they evolve, a process which shares many mechanisms with bone formation. SMC subpopulations contribute to this by increased secretion of cytokines such as bone morphogenetic proteins, homologues of TGF-β. Receptor activator of NF-κB ligand (RANKL), a member of the tumour necrosis factor family, appears to promote SMC mineral formation through a bone morphogenetic protein 4 (BNP4)–dependent pathway (134).

1.4.9 Arterial stenoses and their clinical implications

The sections above have considered the initiation, formation and evolution of the atherosclerotic plaque. These phases last many years, during which the affected individual is often asymptomatic. When the plaque burden exceeds the capability of outward remodelling, advance of the atheromatous plaque within the arterial lumen begins. This chronic asymptomatic or stable phase of lesion growth probably occurs with bursts of rapid progression and periods of relative quiescence. Ultimately, the stenosis may progress to a degree that limits the coronary artery flow. Lesions that produce more than 60% stenosis can cause flow limitation under conditions of increased demand. This type of atheromatous disease commonly leads to stable angina pectoris. However in many cases of acute myocardial infarction there is no history of stable angina prior to the acute event. Several clinical observations suggest that many myocardial infarctions result from no-flow limiting lesions. It is now widely accepted that many cases of acute coronary syndromes occur because of thrombosis complicating a non-occlusive plaque (144). This has been supported by studies showing that the culprit lesion in patients who had myocardial infarction was less than 50% during the coronary angiography months before the acute event. Serial angiographic studies have shown that lesions with stenosis severity more than 60% from a previous coronary angiography account only for approximately 15% of the acute myocardial infarctions. The above findings imply that mild to moderate lesions cause most of the myocardial infarctions. Undoubtedly high-degree stenosis cause myocardial infarction and critical stenoses are more likely to cause acute myocardial infarction than do non-occlusive lesions.

1.4.10 Atheromatous plaque inflammation, rupture and thrombosis

Atherothrombosis is considered as the most important pathophysiologic mechanism in the progression and transition of the chronic atherosclerosis to acute coronary syndromes. We now recognize that disruption of the atheromatous plaque causes in situ coronary thrombosis (145). Although there are several mechanisms of plaque disruption and coronary thrombi formation, the most common mechanisms are fracture and erosion of the fibrous cap of the plaque. Rupture of the atheromatous plaque fibrous cap is the result of the dynamic balance between the forces that encroach on the cap and the mechanical stability and strength of the fibrous cap to resist. Extracellular matrix and SMCs are the factors responsible for most of the biomechanical resistance to disruption of the fibrous cap. Therefore, production and catabolism of the above ingredients probably play a main role in regulating the susceptibility to disruption of the plaque. Stimuli that trigger decreased collagen synthesis by SMCs or increase collagen metabolism can damage their capability to maintain the integrity of the plaque's fibrous cap. For instance, INF-γ, a lymphocyte derived cytokine, can effectively stop SMC collagen production. In contrast, as previously mentioned, inflammatory mediators like TGF-β and PDGF released from activated platelets increase collagen synthesis by SMCs and strengthen the plaque's fibrous stability. Furthermore, increased catabolism of the extracellular matrix can also contribute to weakening of the plaque's fibrous cap and render it susceptible to rupture. Matrix metalloproteinase and cathepsins, macrophage-derived matrix-degrading enzymes, catalyse the breakdown of the collagen and elastin of the arterial ECM (2;143) contributing to weakening of the fibrous cap. The result of reduced collagen synthesis and increased degradation is thinning of the plaque's fibrous cap, a feature of the so called vulnerable plaque. Histologic studies (145-149) have shown

that rupture of atherosclerotic plaques with thin fibrous caps have caused fatal myocardial infarction. Another characteristic of the vulnerable atherosclerotic plaque and especially regions of local inflammation within the plaque is the relative scarcity of SMCs. As explained above, both soluble and lymphocytes associated inflammatory factors, can provoke programmed death of SMCs. Given that SMCs is the source of ECM collagen necessary to preserve the matrix of the fibrous cap, lack of SMCs may contribute to weakening of the fibrous cap and hence the propensity of that plaque to rupture (150). Another micro-anatomic characteristic of the vulnerable atherosclerotic plaque is the accumulation of macrophages and a large lipid pool. Activated macrophages produce pro-inflammatory cytokines and the matrix-degrading enzymes responsible for the regulation of the matrix metabolism and SMC apoptosis. Apoptotic macrophages and SMCs generate TF-bearing MP, a potential trigger of coronary vascular thrombosis after spontaneous or iatrogenic plaque rupture. Also from a mechanical viewpoint, a large lipid pool can transmit the biomechanical forces on the shoulders of the plaque, which are common sites of disruption of the fibrous cap. The success of lipid-lowering therapy in reducing the incidence of acute coronary syndromes in patients at risk may result from a reduced accumulation of lipid in addition to decrease the inflammatory drive and plaque thrombogenicity. Animal studies (151;152) and monitoring of peripheral markers of inflammation in humans (153;154) support this concept. Therefore, the strength of the plaque's fibrous cap goes through a dynamic regulation, linking the inflammatory response in the intima, the metabolism of ECM macromolecules as the determinants of plaque stability, the response of the SMCs to inflammation and hence thrombotic complications of the atheromatous plaque.

1.4.11 Inflammation regulates the thrombogenicity of plaques

Tissue factor expression by plaque macrophages appears essential in triggering thrombosis that complicates plaque disruption. Even though expression of tissue factor by a subset of plaque macrophages was well established, the stimulus to tissue factor expression on these cells remained uncertain (155;156). Soluble cytokines associated with atherosclerotic plaques, including IL-1 and TNF-α, weakly induced TF expression by human macrophages. CD40-ligand (CD154), a cell-surface-associated inflammatory cytokine, promptly triggers tissue factor production by human monocyte/macrophages (157).

1.4.12 Superficial erosion and coronary thrombosis

Fracture of the plaque's fibrous cap accounts for 60-70% of fatal acute myocardial infarctions. Around 20-25% of these events result from a superficial erosion of the intima rather than from true fracture of the fibrous cap but. Superficial erosion appears particularly important in patients with dyslipidaemia, and in women (145). The pathogenesis of plaque fibrous cap erosion is less well understood compared to that of rupture. One possible mechanism is apoptosis of the luminal endothelial cells exposing platelets to the prothrombotic subendothelial collagen. Potential direct toxins of the endothelial cells include hypochlorous acid, produced by myeloperoxidase (MPO). MPO is an enzyme localized in plaques and associated with acute myocardial infarction (158). Hypochlorous acid is also a potent trigger of TF production by endothelial cells. Activated endothelial and SMCs also produce enzymes capable of degrading extracellular matrix, including MMPr as mentioned above. Overproduction of active forms of the MMPr could damage the adherence of endothelial cells and the basement membrane, facilitating their desquamation

providing substrate for coronary thrombosis (143). In this regard, inflammation may promote superficial erosion and rupture of the plaque's fibrous cap.

Thus, inflammation controls not only the evolution of atherosclerosis but also the propensity of plaques to complicate via rupture or erosion by altering matrix composition through the mechanisms described above. Inflammation also increases the thrombogenicity of the plaque's interior, promoting thrombus formation of the ruptured/eroded plaque.

1.4.13 Diffuse and systemic nature of plaque vulnerability and inflammation in atherogenesis

The concept that mainly vulnerable atheromatous plaques cause coronary thrombosis and ACS encouraged many to find ways of recognizing and treating such high-risk atherosclerotic coronary lesions. Recent data, though, suggest that more than one such high-risk plaque often exists in a given coronary tree (159;160). In addition, the inflammation thought to be localized in the so-called vulnerable plaques appears to extent in more than one lesion in many cases after careful analysis of angiograms of individuals with acute coronary syndromes. Studies using various imaging modalities have highlighted the multiplicity of such high-risk plaques (134;159). The above has been also shown by angioscopic studies where multiple sites of coronary thrombosis have been found in patients with ACS. Intravascular ultrasound, optical coherence tomography and computed tomographic angiography have contributed to the better understanding of the plaque morphology that cause acute coronary syndrome (160). It has been found that patients demonstrating positively remodelled coronary lesions on CT angiography were at a higher risk of ACS developing over time when compared with patients having lesions without these characteristics (161).

1.4.14 Inflammatory markers and myocardial necrosis in acute coronary syndrome

Inflammation is defined as a complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants (162). It is a stereotyped response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen (163).

During ACS with or without ST-elevation there is a degree of myocardial damage/necrosis accompanied by ischemia reperfusion injury. Therefore, an immediate inflammatory response to the above stimuli which is part of the myocardium healing process takes place in the post-infarction period. In addition, and more interestingly the inflammatory process taking place within the atheromatous plaque as described above may well be the primary inflammatory triggering factor of coronary instability. Mounting evidence support an important association between increased circulating inflammatory markers (CRP, serum amyloid-A (SSA), IL-6, and IL-1 receptor antagonist) and adverse clinical outcomes, both in-hospital and short-term follow up (164-169). It has been demonstrated by Liuzzo et al that in patients with ACS who had similar ischemic burden, high serum CRP is discriminator of adverse short-term prognosis compared to those patients with normal CRP levels (170). Also high CRP levels in ACS patients at discharge associated with recurrent admissions and re-infarction (167). Elevated CRP levels do not appear to correlate with the extent and burden of CHD as only 20% of patients with severe CHD have raised CRP (171;172). In contrast, patients with ACS have a high prevalence of elevated CRP irrespective of the CHD ischemic burden, affirming the specificity of systemically detectable inflammation in ACS. In line with above,

patients with severe enough peripheral vascular disease warranting revascularization have similar CRP levels to the levels observed in patients ACS and single-vessel CAD (173). Although high levels of CRP are not a pre-requisite for ACS and unstable angina, CRP is elevated on admission in virtually all patients with AMI who experienced unstable angina. Multiple studies have shown that various systemic inflammatory markers, such as CRP, increase in patients at risk for acute coronary syndromes. Furthermore, inflammation precedes the ACS, as shown by profiling of the platelet transcriptome. Researchers have found that elevated serum levels of MRP-8/14 (inflammatory marker) predicts the risk of a first cardiovascular event in apparently healthy women, and also two of the most elevated mRNA transcripts from platelets of patients with ACS compared to that from patients with stable coronary artery disease encode proteins implicated in inflammation (174). Levels of PMA and CRP were significantly increased in patients with non ST-elevation ACS, especially in those with troponin elevation. This increase is strongly related to the risk of in-hospital cardiac events. A panel of PMA, CRP, and Trop I may provide important information additional to current laboratory data for the treatment of ST-elevation ACS (175). Therefore, a combination of imaging studies and investigations using inflammatory markers supports the diffuse and systemic nature of instability of atheromatous plaques in individuals with or at risk of developing ACS.

1.4.14.1 Role of C-reactive protein in coronary heart disease

CRP exerts a variety of pro-inflammatory and pro-coagulant effects such as stimulation of the production of TF by mononuclear cells (176), activation of the complement system (177), and interaction with low density lipoprotein and damaged cell membranes (178). CRP can also induce PAI-1 expression and can suppress NO release from the endothelial cells (179).

Several studies have demonstrated that an elevated CRP in ACS is associated with a worse prognosis (27;165;171). Elevated CRP level in patients with NSTEMI is reported to be associated with cardiac rupture, left ventricular aneurysm and cardiac death at one year (180). After PCI a hyper responsive reaction of the inflammatory system defined by elevated CRP, IL-6 and SAA is considered to be a worse prognostic factor (170). This has been confirmed by showing that persistent elevation of CRP 72 hours after coronary artery stenting identified all patients who suffered a later adverse outcome. In contrast no cardiac events occurred in those with normal levels at one year follow up (181).

MONICA (Monitoring Trends and Determinants in Cardiovascular Disease Study) showed that CRP is a strong predictor of future cardiac events in apparently healthy men (182). Patients with the highest quintile of CRP levels were shown to have a 2.6 fold increased risk of suffering a fatal or nonfatal myocardial infarction or sudden cardiac death. Similarly in the Women's Health Study patients with higher baseline hs-CRP levels than control subjects were found to be associated with cardiovascular events, and patients with highest baseline levels were associated with five and seven fold increase in any vascular events and combined stroke or myocardial infarction respectively (183).

1.4.14.2 Role of serum amyloid antigen in coronary heart disease

Serum amyloid antigen **(**SAA) may play a role in the pathophysiology of atherosclerosis. SAA induces IL-1 and IL-8 production in neutrophils and interferon gamma in lymphocytes. SAA has been shown to be chemotactic for monocytes (184). It also promotes monocyte adhesion and chemotaxis (185-187), induces matrix-metalloproteinase and activates NF-kB in CD4+ lymphocytes (188). These findings raise the question whether these pro inflammatory substances play any pathogenic local role within the coronary artery of the ACS patients. Matsubara et al (189) have shown that the increment of SAA across the coronary circulation correlated positively with the severity of coronary atherosclerosis. Higher adverse outcomes including myocardial infarction, revascularization and death in ACS patients with elevated SAA has been previously reported (171). Song et al have demonstrated that SAA induced higher TF activity in patients with ACS, but not stable angina patients, indicating that SAA may contribute to the procoagulant state in patients with ACS (190). These findings suggest TF response to SAA differs in ACS patients compared to controls, and the difference in induced TF between ACS and stable angina highlights the significance of the prothrombotic properties of SAA. SAA may contribute to the pathology of ACS via the induction of both TF and TNF-α. Song et al have demonstrated upregulation of TNF-α mRNA in SAA-stimulated peripheral blood leukocytes, indicating that TNF is an early response gene product of SAA. They have reported, unlike TF, peripheral blood mononuclear cell-derived TNF-α level is similar in patients and controls, and low dose SAA only, induces TNF-α in cells from ACS patients (190). They concluded that SAA induces more TNF-α in cells from ACS patients than in cells from stable angina or controls, suggesting SAA may be a pro-inflammatory amplifier in ACS (190).

1.4.14.3 Role of IL-6 in coronary heart disease

One of the most widely studied inflammatory markers is IL-6. IL-6 induces the production of CRP from the liver. It increases fibrinogen and PAI-1, and promotes leukocyte adhesion during myocardial reperfusion. It also exerts a negative inotropic effect on myocardium (191). IL-6 is produced by variety of inflammatory cell type and has been shown to remain elevated up to 4 weeks after a myocardial infarction. In patients with ACS elevated IL-6 predicts in-hospital events (192) in addition to future adverse coronary events (193). It has also been reported that circulating IL-6 correlates well with left ventricular remodelling in patients with reperfused AMI (194). It has been reported during the first 48 h after ACS, that IL-6, CRP and TnT levels are high in patients who subsequently had major adverse cardiac events. However one week after admission, this prognostic value of IL-6 had been lost (195). In apparently healthy males elevated levels of IL-6 are associated with increased risk of future myocardial infarction which supports a role of cytokine mediated inflammation in the early stages of atherogenesis (196).

1.4.14.4 Role of TNF-α in coronary heart disease

TNF-α contributes to plaque instability by promoting inflammatory processes and inducing matrix metalloproteinases in atherosclerotic lesions (197). TNF-α mediates a variety of interactions resulting in progressive inflammation, plaque destabilization and prothrombosis. Elevated serum TNF-α levels in CHD predict adverse coronary events in population studies (198). Reperfusion induces TNF-α expression in the coronary microcirculation and TNF-α can impair endothelium- dependent coronary flow reserve (199) which may adversely affect outcome. TNF-alpha can also cause cardiac contractility depression either directly (191) or via the inducible NO synthase

(i-NOS) pathway in cardiomyocytes (200). It is not entirely clear whether TNF-alpha is generated locally in the coronary artery or in the systemic circulation. In experimental and clinical (201) studies, TNF-α did not differ in paired coronary arterial and coronary sinus blood samples, suggesting that TNF-α is not of cardiac, but of peripheral origin. Increased TNF-α concentration contributes to vascular dysfunction. TNF-α induces the expression of cell adhesion molecules such as E-selectin, VCAM-1 and ICAM-1 at the endothelial surface. These molecules mediate the adhesion and migration of leukocytes into the arterial wall. Activated leukocytes, following interaction with platelets, produce TNF-α which induces TF expression on macrophages, neutrophils, and endothelial cells (202-204) influencing the extrinsic pathway of the coagulation cascade via TF (205). TF mediates thrombin formation, leading to fibrin clot formation and intravascular fibrin deposition.

1.5 Microvascular dysfunction in coronary artery disease

1.5.1 Introduction

Microvascular dysfunction (MvD) is a powerful independent predictor of long term outcomes, and mortality in patients presented with ACS and revascularization after PCI. Despite a successful angiographically coronary artery stenting, potential compromise of the coronary microcirculation during or after PCI is associated with "slow flow" or "no flow" with subsequent impaired myocardial tissue oxygenation. A number of different non-invasive and invasive techniques have been developed to evaluate the functional reserves of the coronary microvasculature. Among the invasive methods in the catheter laboratory the 'Index of microvascular resistance' (IMR) which is derived using a Doppler or a pressure-temperature sensing wire, has emerged as a novel index for the assessment of the coronary microcirculation. This has been validated in the experimental model and has been shown to be reproducible.

1.5.2 Coronary circulation and coronary blood flow control

The coronary circulation plays a unique role by not only generating the required arterial pressure to perfuse the systemic circulation but at the same time having its own perfusion hindered during systolic phase of the cardiac cycle. Myocardial contraction is closely connected to coronary blood flow and oxygen delivery. The balance between myocardial oxygen supply and demand is the main determinant of the normal heart function. Therefore any disruption of the coronary flow will disrupt the oxygen-demand balance precipitating a vicious cycle, whereby ischemia-induced contractile dysfunction causes hypotension and further myocardial ischemia.

Coronary arterial blood flow increases during diastole whilst at the same time coronary venous outflow decreases. During systole there is reduction of the diameter

of intra-myocardial micro-vessels (arterioles, capillaries and venules) impeding coronary arterial blood flow, which reaches a nadir, whereas the venous outflow peaks during systole (206). In contrast to other vascular beds, myocardial oxygen extraction is near-maximal at rest reaching close to 75% of the arterial oxygen content (207). As such increase in myocardial oxygen consumption happens predominantly by proportional increases in coronary blood flow and oxygen delivery. Heart rate, systolic pressure (or myocardial wall stress), and left ventricular (LV) contractility are the major determinants of myocardial oxygen consumption (208). Studies in animals have shown that at basal state subendocardial coronary blood flow is auto-regulated and can remain constant as coronary artery pressure reduces to as low as 40 mmHg of mean coronary artery pressure (or 30 mmHg of diastolic pressure) (208). In contrast, the lower auto-regulatory limit for sub-epicardial coronary flow is a mean coronary artery pressure of 25 mmHg. This phenomenon is termed auto-regulation.

The resistance to coronary blood flow can be divided into three major components. R_1 is epicardial arteries resistance, which is normally not significant (<5% pressure drop). R_2 arises from microcirculatory resistance arteries (mainly arterioles with diameter 20 to 200 μm) and R_3 is the compressive resistance which varies during the cardiac cycle that is higher in subendocardial than sub-epicardial myocardium. In hearts without obstructive CHD, R2 is the main contributor to the coronary blood flow resistance. During pharmacologic vasodilation, like intravenous administration of adenosine, the arteriolar resistance (R_2) becomes negligible. Therefore, under the above circumstances, severe epicardial stenosis becomes the main contributor to coronary blood flow resistance. Coronary artery resistances at any segment of the coronary microcirculation represent the integration of anatomical, metabolic and

neural mediators. It has been shown by Kuo et al that the diameter of coronary arterioles respond to changes in local stress, a mechanism termed as flow-mediated vasodilation and is mediated via endothelium-derived relaxing factor (EDRF or NO) and endothelium-dependent hyperpolarizing factor (EDHF) (209). In addition, the capacity of vascular smooth muscle to oppose changes in coronary arterial diameter enables the coronary microcirculatory resistance arteries to relax when coronary arterial pressure decreases and to constrict when coronary arterial pressure increases. The above response has been termed as myogenic regulation and believed to be mediated via L-type Ca++ channels (210). Apart from the physical factors regulating the coronary artery resistance metabolic mediators like hypoxia, acidosis, adenosine and ATP-sensitive K+ channels are also important. In addition the sympathetic and parasympathetic innervation of the coronary arteries and arterioles affecting the vascular smooth muscle tone also contributes to the regulation of the coronary resistance. A wide variety of paracrine vasoactive factors secreted by platelets, endothelial cells and smooth muscle cells also modulate the coronary vascular tone. The most important factors are NO, EDHF, prostacyclin, serotonin, thromboxane A2, ADP, endothelin and thrombin.

1.5.3 Physiologic assessment of epicardial coronary artery stenosis and coronary microcirculation

Physiologic assessment of the epicardial coronary stenosis is an essential element in the clinical decision making process and management of patients with CHD (211). The chronic process of atherogenesis leads to atherosclerotic plaque formation and angiographically epicardial artery stenosis which increases the coronary resistance (R1). This in combination with the impaired coronary microcirculation which is also

common in patients with CHD or risk factors for CHD does reduce maximal myocardial perfusion which can also contribute to causing myocardial ischemia. Separating the role of a stenosis from coronary resistance vessels can be accomplished by simultaneously assessing coronary flow and distal coronary pressure using intracoronary transducers that are currently available for clinical care (212). The relationship between stenosis severity and pressure drop has been validated in animals as well as humans and can be described by the Bernoulli equation which essentially states that the resistance is inversely related with the square of the cross-sectional area at the stenosis point. As such small changes in luminal area can cause significant changes in the stenosis pressure-flow relationship and reduce distal (to the stenosis) coronary pressure which is the main determinant of myocardial perfusion. The length of the stenosis is another factor which contributes to the epicardial coronary resistance. The pressure drop (ΔP) also varies with the square of the flow velocity, which means increased pressure drop across a stenosis during vasodilation (conditions of coronary hyperaemia).

At rest, due to coronary auto-regulation, coronary flow remains stable as stenosis severity increases. As such it is impossible to identify thermodynamically significant stenosis with resting myocardial perfusion imaging in contrast the maximally vasodilated pressure-flow relationship which is much more sensitive in detecting pressure drop and stenosis severity (it is easy to understand this from the Bernoulli equation). Normally there is considerable coronary flow reserve with almost a fivefold increment of the resting coronary flow during conditions of hyperaemia. The increase in epicardial coronary artery resistance is very small until stenosis severity progresses to a 50% internal diameter reduction (75% cross-sectional area). As a result, there is no significant pressure drop across a stenosis until stenosis severity is

more than 50% under circumstances of both resting coronary flow and coronary hyperaemia. As stenosis severity increases further due to the steep curvilinear coronary pressure-flow relationship small increases in stenosis severity will lead to significant increases of pressure drop across the stenosis. The curve becomes even steeper for stenosis >70%. Ultimately the distal coronary pressure reduces with a parallel decrease of the vasodilated coronary flow. In critical stenosis, coronary stenosis of more than 90%, the subendocardial flow reserve is diminished and under these circumstances coronary hyperaemia achieved by pharmacologic vasodilation results in reduction of the distal coronary pressure. This leads to a transmural steal phenomenon where there is redistribution of coronary flow towards the subepicardium.

1.5.4 Fractional Flow Reserve

Fractional flow reserve (FFR) is obtained by calculating the ratio of the driving pressure for microcirculatory flow distal to the stenosis (distal coronary pressure minus coronary venous pressure) to the coronary driving pressure available in the absence of a stenosis (mean aortic pressure minus coronary venous pressure). This technique is based on the principle that the distal coronary pressure measured during vasodilation is directly proportional to maximum vasodilated perfusion and assumes linearity of the vasodilated pressure-flow relationship, which is known to be curvilinear (213). Also it assumes that coronary venous pressure is zero. This results in the simplified FFR index of mean distal coronary pressure/mean aortic pressure (Pd/Pa). FFR measurement as a physiological assessment of a moderate degree stenosis can help in the decision management regarding coronary intervention and is unaffected by alterations in resting flow. FFR can also be used to assess the

functional effects of a residual lesion after PCI. A significant advantage of FFR is that there is now considerable prognostic information. Recent data from a large prospective randomized study, indicating that FFR measurements more than 0.8 are associated with excellent outcomes with deferred rather than prophylactic intervention (214). This study demonstrated that physiologically guided PCI using FFR versus angiographic criteria is safe and cost- effective and reduces the number of major adverse cardiac events at one year (13.2% versus 18.3% in angiographically guided treatment) (214). FFR although simple and clinically useful has several limitations. Contrary to CFR, FFR cannot assess the microcirculatory contribution but can only assess the significance of the epicardial stenosis. Also FFR measurement is dependent on achieving maximal pharmacologic vasodilation (underestimating stenosis severity if maximal vasodilation not achieved) during measurement. In addition the assumption that the vasodilated pressure-flow relationship is linear and that the venous pressure is zero FFR can underestimate the physiological significance of an epicardial stenosis. Besides by inserting the guide-wire across a stenosis can in theory artifactually overestimate stenosis severity caused by the reduction in cross sectional area. By assessing both the physiological significance of epicardial stenosis with FFR and the microcirculatory flow with CFR using a single wire has the potential to identify circumstances where mixed abnormalities contribute to clinical symptoms and discriminate which one is the cause of patients symptoms. Left ventricular hypertrophy (LVH) and impaired endothelial dependant vasodilation are the two most common pathological conditions affecting the microcirculatory resistance independently from the severity of the epicardial stenosis. In the former (acquired LVH) there is increased left ventricular mass with the microcirculatory vessels remain unchanged (215). Also the resting

coronary flow per gram of myocardium remains stable and for this to happen the resting coronary flow increases (more flow to maintain the blood flow per myocardium gram constant). During maximum hyperaemia the coronary flow remains unchanged and as a result of this (relatively increased resting and decreased hyperaemic flow) the coronary flow reserve decreases. In the latter case which is common in almost all risk factors for CHD like diabetes mellitus, hypertension, hypercholesterolemia and smoking, there is inability of the coronary microcirculation to maximum hyperaemia due to impaired NO-mediated endothelial vasodilation. Also the coronary pressure/flow curve shifts towards the right leading to an increment of the minimum coronary pressure required to keep coronary flow stable (216). The net effect of the above is an accentuation of the functional effects of epicardial stenosis rendering myocardium prone to ischemia at higher coronary pressure and less workload (217).

1.5.5 Role of collaterals in coronary microcirculation

Coronary collaterals are a complimentary source of blood supply when an epicardial coronary artery occlusion or stenosis jeopardizes myocardial blood supply. Several studies have demonstrated that collateral blood flow is sufficient to prevent ischemia when collateral flow calculated from mean aortic pressure (Pa), coronary wedge pressure (Pw) and central venous pressure (Pv) exceeds 25% (218). Also during coronary angioplasty and balloon occlusion, ischemia does not develop if FFR (based on coronary wedge pressure during occlusion minus venous pressure) is greater than 0.25 (219). Pressure derived collateral flow index (CFIp) is calculated as (Pw-Pv) / (Pa-Pv). CFI is used as an index of collateral circulation and can be easily measured with a pressure wire during PCI. High collateral flow has a beneficial

effect on the occurrence of future major ischemic events and less severe myocardial ischemia during PCI. On the other hand higher collateral flow also has been associated with increased risk of in-stent restenosis after PCI (220-222) probably due to competing hemodynamic forces for antegrade flow at the traumatised site. A CFI> 0.25 indicates a circulation with good collateral support and CFI< 0.25 indicates a circulation with less collaterals. In a large observational study lower cardiovascular event rate and improved survival was observed in patients with elevated distal coronary pressure arising from recruitable collaterals (223). In patients with stable angina higher CFI indicates the presence of a good collateral circulation as in a non-infarcted heart the pressure signals obtained distal to the occluded artery invariably originates from collateral channels. However, in the acute myocardial infarction setting CFI is unlikely to represent collateral flow but seems to increase with severity of microvascular dysfunction. Yamamoto et al demonstrated higher CFI in acute myocardial infarction is associated with poorer functional recovery. In the acute myocardial infarction, the observed increase in systolic coronary wedge (Pw) pressure may be explained by congested blood flow in the coronary microcirculation. In the non-infracted heart intra-myocardial blood is smoothly squeezed into the venous circulation in systole but this ejection of blood to the venous circulation is impeded owing to the extensive microvascular obstruction in the infarct zone (224).

Collateral circulation develops in response to recurring myocardial ischemia. This is a process dependent on hemodynamic forces and angiogenic growth factors, mainly VEGF. Although several interventions like the use of recombinant growth factors or endothelial progenitor cells have been successful in angiogenesis of capillaries and improve myocardial function, randomized human clinical trials have been disappointing (225). It is believed that relief of ischemia might be expected to lead to

regression of collaterals. Several studies have suggested rapid loss of collateral function after PCI (226-229). Some of these results could be due the fact that during the estimation of CFI either central venous pressure was not measured or adequate hyperaemia was not achieved. Pw alone does not represent collateral flow; right atrial pressure and mean aortic pressure need to be considered to measure CFI accurately. Perera et al showed that assuming a fixed value of right atrial pressure for the measurement of CFI can alter the results substantially (230). The same group have demonstrated coronary collateral flow remains undiminished for at least 24 hours after successful PCI and functional collateral support subsequently declines but does not regress completely (231).

1.5.6 Coronary flow reserve

Coronary flow reserve (CFR) is equal to the ratio of the hyperaemic (during maximum vasodilation) to resting coronary flow. Coronary blood flow increases automatically from resting to a maximum level in response to increase myocardium oxygen demand. CFR reflect both epicardial and microcirculatory contribution though it cannot discriminate the two. CFR can be obtained with non-invasive methods like PET or CMR and invasive methods by measuring the intracoronary flow ($CFR_{Doppler}$) velocity or by thermodilution using a pressure-temperature sensing wire (CFR_{Thermo}) in the catheter laboratory. CFR quantifies the ability of coronary flow to increase above the resting value. In individuals without LVH and normal coronary circulation, CFR varies from 4 to 6. CFR values of less than 2 are pathological and become clinically important (219). Coronary flow reserve is altered by factors that either affects 1) the hyperaemic coronary flow (e.g., heart rate and arterial pressure, stenosis severity and also impaired microcirculatory response) or 2)

the resting coronary flow like haemoglobin, resting arterial pressure, heart rate, and the resting oxygen extraction. Because CFR quantifies the flow reserves of the coronary vascular bed it is impossible to extract conclusions about the importance of an epicardial stenosis and dissociate the magnitude of the stenosis from impaired microcirculatory function which are common in patients with risk factors for CHD like hypertension, diabetes mellitus and hypercholesterolemia. Simultaneous measurements of FFR and CFR give the clinician additional information about the respective contribution of the epicardial vessel and microvasculature to the total resistance to myocardial blood flow. In patients with CHD without a significant pressure drop along the epicardial artery (i.e. normal FFR) a low CFR value indicates microvascular involvement.

1.5.7 Measurement of coronary flow reserve by Doppler flow wire

A Doppler wire is placed in the distal coronary artery to obtain optimal traces. Continuous average peak velocity is recorded at baseline and at maximum hyperaemia. Angiographic views in two orthogonal planes are also taken at baseline and at maximum hyperaemia for measurement of coronary artery diameter. Changes in coronary artery diameter are calculated using an automated QCA edge detection system approximately 2.5 mm distal to the tip of the Doppler wire in a 2.5-5mm length segment. Absolute coronary flow is calculated from the product of corresponding average peak flow velocity (APV) values and QCA derived coronary artery diameter (1/2 x APV x [coronary cross-sectional area]) at baseline and maximal flow. The percentage change in coronary blood flow is obtained from the ratio of baseline and maximal blood flow values (232). Pijls et al studied 103 arteries from 50 patients and demonstrated a fair correlation between CFR_{thermo} and

$CFR_{Doppler}$ (233). They found $CFR_{thermo} = 0.84 \times CFR_{Doppler} + 0.17$ ($r=0.80$; $P<0.001$). The average absolute difference between both indexes was 17±14% (range, 0% to 51%). In 26% of all studies the difference between the both parameters was > 20%. For post-stenotic coronary flow velocity reserve several studies reported strong correlations with myocardial stress perfusion imaging and 2D echocardiographic stress imaging (234). Coronary flow velocity reserve <2 corresponded to reversible ischemia with myocardial perfusion image and stress-induced wall motion abnormality. These studies had high sensitivity (86% to 92%), specificity (89% to 100%), predictive accuracy (89% to 96%) and positive predictive value (94% to 100%) and negative predictive value (77% to 95%) (211;212;235;236).

FFR and CFR have excellent correlation with non-invasive stress test at cut off values of 0.72 to 0.75 for FFR and 1.7 to 2.0 for CFR (211;234;237). Meuwissen et al studied 150 intermediate coronary lesions in 126 consecutive patients with stable angina they found concordant outcomes in 109 lesions (73%) and discordant outcomes in 41 lesions (27%). Out of the 41 discordant cases 26 had FFR <0.75 and CFR >2; 15 had FFR≥ 0.75 and CFR < 2. They also measured a velocity based index of microvascular resistance during hyperaemia (h-MRv) as a ratio of mean distal pressure to average peak flow velocity (APV). Between the two discordant groups h-MRv was significantly higher in the second group in comparison to first. This suggests the observed variability in the microvascular resistance plays a significant role in the discordance between FFR and CFR. Both FFR and CFR are influenced by the combination of coronary artery stenosis and microvascular resistance. In absence of coronary stenosis variability of h-MRv will have little effect on FFR but it will be reflected in CFR (238).

1.5.8 Limitations of coronary flow reserve

As CFR represents a ratio between peak hyperaemic and resting coronary flow, CFR measured by thermodilution has some limitations such as dependency on heart rate, blood pressure, myocardial contractility, previous myocardial infarction, valvular heart disease, LV hypertrophy, age, individual variability between persons and dependency on achieving true hyperaemic coronary blood flow (233;239-241). A previous study (240) has shown that $CFR_{Doppler}$ is significantly reduced by tachycardia and by increased contractility. Although these different methods for assessment of the coronary microcirculation have methodological flaws, the influence of different confounding clinical factors and sometimes poor reproducibility represented limitations for their routine use in clinical practice. With the introduction of a pressure wire with temperature and pressure sensors assessment of coronary microvasculature has become much easier. With the single wire it is now possible to measure CFR, FFR and IMR. IMR has emerged as a novel index of measuring the microcirculation (233;242). This saves the need for a separate Doppler flow wire which is needed to measure the h-MRv.

1.5.9 Measuring the index of microvascular resistance and coronary flow reserve with pressure-temperature sensor wire

IMR and CFR can be measured by a commercially available 0.014 inch floppy pressure wire (pressure wire 3, Radi Medical system) by using the thermodilution technique. The pressure wire has a microsensor at a location 3 cm from the floppy tip, which measures simultaneously pressure and temperature at the location of the sensor with an accuracy of 0.2^0 C. The shaft of the wire can be used as an additional thermistor, providing the input signal at the coronary ostium of any fluid injection

with a temperature difference from blood. All signals can be displayed on the regular catheter laboratory recording system or at a suitable interface (Radi analyzer). In this way the mean transit time of the injected saline down the coronary artery can be calculated from a coronary thermodilution curve. Thermodilution curves in the coronary artery are obtained by short manual injections of 3 mls 0.9% saline at room temperature. Measurements of transit time are calculated 3 times at base line and 3 times at maximum hyperaemia. The mean transit time at baseline and maximum hyperaemia are calculated. Maximum hyperaemia is achieved by adenosine infusion through the femoral vein at a rate 140 microgram/kg/minute. Distal coronary pressure (Pd) is recorded from the pressure wire. Mean aortic pressure (Pa) is measured from the coronary catheter and central venous pressure (Pv) is measured from the right heart catheter at maximum hyperaemia. Coronary wedge pressure (Pw) is also measured at maximum hyperaemia by inflating a semi-compliant balloon (1mm smaller that the vessel diameter) in the coronary artery and the IMR is calculated. In absence of epicardial stenosis (and therefore collaterals) and assuming Pv is close to zero, IMR is calculated as hyperaemic distal coronary pressure divided by inverse of hyperaemic mean transit time (T_m) (a correlate to absolute flow). This can be represented in a simplified manner as a product of distal coronary pressure and mean hyperaemic transit time (Pd × Tm). Fearon et al demonstrated there was significant correlation between mean IMR (distal coronary pressure/Inverse of mean hyperaemic transit time) and true microcirculatory resistance (TMR calculated as a ratio of distal coronary artery pressure and hyperaemic flow measured with external ultrasonic Doppler) in experimental animals (243). Coronary collaterals, in the presence of a coronary epicardial stenosis, might lessen the drop in distal coronary pressure without a change in coronary blood flow and also may increase the coronary

Pw. The combination of the above in turn can lead to an increase in the measured coronary microvascular resistance (243). Therefore, in presence of a coronary stenosis with collaterals, myocardial flow is not equal to coronary flow but exceeds the coronary flow because of collateral flow. Tm is no longer representative of myocardial flow and the contribution of collaterals needs to be considered and the IMR= Pa × Tm × [(Pd-Pw) ÷ (Pa-Pw)] (assuming Pv=0). This could be again represented by IMR=Pa × Tm × FFR_{cor}. In the presence of abnormal Pv the equation should be represented as IMR= (Pa-Pv) × Tm × [(Pd-Pw) ÷ (Pa-Pw)] (Pa=Aortic pressure, Pv =Right atrial pressure, Pw=coronary artery wedge pressure, Pd=distal coronary pressure, all the above measurements during maximum hyperaemia). Clearly the presence of functional collaterals will increase Pw, but there is an argument that Pw below 25 mmHg implies the presence of hemodynamically insignificant collaterals. The cut off of 25mmHg is in line with previous clinical studies found that collateral flow is essentially absent when Pw is lower than 25 mmHg as discussed above. (213;244-247) The vascular volume of the epicardial artery between the ostium and the location where pressure and temperature are measured should remain constant throughout the measurements. This can be achieved by prior administration of 200mcg of intracoronary nitroglycerine. It is of paramount importance to position the sensor at the same distance from the ostium of the coronary artery while measuring the transit time because more distal the sensor is the longer the transit time will be. CFR can be calculated as a ratio of average transit time at baseline and average transit time at maximum hyperaemia provided that the time for analysis of the thermodilution equals at least one cardiac cycle, and variability between values of the 3 transit times < 20%. Flow equals V/Tm, where V represents the vascular volume between injection site of the indicator (tip of the

guiding catheter and the location of sensor). Previous studies have demonstrated transit time of intracoronary room temperature saline derived from thermodilution curves is inversely proportional to coronary flow (233;242;248). Pijls et al, in an experimental model found a strong correlation between the inverse of mean transit time and absolute flow. Using this technique CFR can be measured as a ratio between resting mean transit time and hyperaemic mean transit time and this correlates well with standard CFR measurement (241).

1.5.10 Reproducibility and independence of the index of microvascular resistance from epicardial artery stenosis

The main advantage of IMR over other indices of microcirculatory dysfunction is that it is independent of epicardial artery stenosis, so it reflects true microcirculatory function. Fearon et al in an experimental animal model showed that after collateral flow is taken into account, the microvascular resistance assessed by the IMR is not affected by increasing the epicardial artery stenosis (249). Also Aarnoudse et al studied thirty patients with stable angina scheduled for PCI. They created 10%, 50% and 70% artificial stenosis in the successful stented segment of the coronary artery, by inflating a short compliant balloon with a diameter 1 mm smaller than the stent with increasing pressures. They measured IMR at each degree of stenosis and demonstrated when contribution of collateral flow is properly accounted for, IMR does not change with the epicardial artery stenosis severity (250). Similarly Leyland et al studied forty patients with stable angina undergoing PCI. They showed that when collateral supply is accounted for, epicardial stenosis does not increase microvascular resistance in patients with stable angina (251). Interestingly, Verhoeff et al have shown that Pw adjusted coronary microvascular resistance calculation

(Doppler guided calculation) overestimates the effect of potential collateral flow and is not needed for the assessment of coronary flow-limiting stenosis characterized by a FFR between 0.6 and 0.8 or for non-significant lesions (252).

Compared to CFR, IMR is more reproducible and is independent of hemodynamic perturbations. Ng MKC et al assessed CFR and IMR in 15 patients at baseline and under different hemodynamic conditions like right ventricular pacing at 110 bpm, intravenous nitroprusside injection, and intravenous dobutamine injection. They demonstrated intrinsic variability of IMR is much less in comparison to the CFR. Co-efficient of variation for IMR was 6.9± 6.5% where as for CFR it was 18.6 ± 9.6% ($p<0.01$). Under hemodynamic stress CFR decreased during pacing and dobutamine infusion compared to baseline while IMR remained similar throughout all the above hemodynamic conditions. IMR values during pacing had a significant correlation with the baseline value. As a consequence of increased resting coronary flow with dobutamine infusion CFR values were significantly lower than those at baseline whereas IMR values remained unchanged (253).

1.5.11 Clinical implications of measurement of the index of microvascular resistance

Despite adequate epicardial artery reperfusion a number of patients with STEMI have a poor prognosis because of microvascular damage. IMR helps in assessing the microvascular damage in the setting of pPCI in STEMI patients. Fearon et al demonstrated (254) positive correlation of IMR measured immediately following pPCI for STEMI with peak CK. They also demonstrated that IMR predicted left ventricular function and recovery of left ventricular function, assessed by wall

motion score measured by echocardiography at 3 months follow up. Sezer et al measured IMR two days after pPCI and demonstrated intracoronary streptokinase administration following pPCI was associated with significantly lower IMR compared with the patients who did not receive intracoronary streptokinase following the pPCI. (16.29± 5.06 U vs 32.49 ± 11.04 U. p= <0.002). However at 6 months there was no significant difference in LV size or function between these two groups (255). Payne et al measured at the end of pPCI the IMR in 108 patients presented with STEMI. They found that microvascular resistance measured during pPCI significantly predicts myocardial salvage, infarct characteristics, and left ventricular ejection fraction in patients with STEMI (256). Similar findings were found more recently in patients with anterior STEMI after pPCI (257). Also McGeoch showed that IMR measured acutely was an independent predictor of LV function and infarct volume in patients with STEMI treated with pPCI (258).

In patients with stable angina direct stenting has been suggested to reduce periprocedural microcirculatory injury compared with the stenting that follows pre-dilation of the lesion. Cuisset et al has demonstrated in patients with stable angina who had elective PCI that direct stenting was associated with significantly lower IMR compared to the patients who had pre-dilation with balloon (IMR 13 ± 3 U vs 24± 14 U, p=<0.01). They have also demonstrated that patients who had post PCI troponin release had significantly higher IMR compared to the patients who did not have post PCI troponin release (259). In addition measurement of IMR in patients electively admitted for PCI may allow prospective identification of patients at risk of periprocedural myocardial infarction. Pre-PCI IMR was also the strongest predictor of post-PCI troponin release (260).

1.5.12 Conclusions

Among the different invasive methods of assessing the coronary microcirculation in the catheter laboratory, IMR has emerged as the most robust and independent index. The main advantage of IMR over the other indices is that it exclusively reflects the microcirculatory state and can be measured with a simple technique with the help of a pressure wire. It is also independent of epicardial artery stenosis. IMR is reproducible and is less influenced by the hemodynamic perturbations. Studies have shown worse microcirculatory function assessed by IMR is a predictor of poor LV function in patients with ST elevation myocardial infarction.

1.5 AIMS OF THIS THESIS

The aims of this study were:

1. To investigate the expression of the total AnV+ microparticles (MPs) and their cell-specific phenotype in patients presenting with symptomatic CHD, either ACS or stable angina. The possibility of a trans-myocardial gradient between coronary and periphery of MP release was explored by determining MP levels in the local intracoronary (CO) artery distal to the culprit lesion and in the right atrium (RA) as representative of the systemic circulation.

2. To explore the relationship between local coronary and systemic MP levels, circulating markers of inflammation, and extent of myocardial necrosis in patients with ACS or stable angina undergoing PCI.

3. To compare coronary artery and systemic circulating levels of platelet activation markers, and MPs in patients with symptomatic ACS or SA treated with PCI.

4. To assess the relationship between microvascular dysfunction and the level of PMA expression, intracoronary and systemic (aorta and right atrium) in patients presenting with NSTEMI or stable angina treated with PCI.

Chapter 2: Patients and Methods

2.1 Coronary heart disease patients

2.2 Identification of platelet-monocyte aggregates

2.3 Isolation and analysis of total Annexin V positive microparticle and microparticle subpopulations

2.4 Estimation of the inflammatory markers and Soluble P Selectin

2.5 Measuring the Index of microvascular resistance, coronary flow reserve and coronary wedge pressure

2.6 Recruitment limitations and practical considerations

2.1 Coronary heart disease patients

Eleven patients with stable angina (SA) admitted for coronary angioplasty, fourteen patients presented with non-ST-elevation myocardial infraction (NSTEMI) and twenty one patients presented with ST-elevation myocardial infraction (STEMI) were recruited for the study.

2.1.1 Stable angina patients

Eleven patients with symptoms of stable angina (SA) pectoris who electively admitted for coronary angiography and percutaneous coronary angioplasty (PCI) were recruited for the study. SA patients had been reviewed in the cardiology outpatient clinic with a diagnosis of angina pectoris based on typically ischaemic sounding chest pain and a positive non-invasive test such as exercise tolerance test, stress echocardiography or myocardial perfusion scan (MPI). All SA patients were treated with anti-angina medications and aspirin 75mg od. The decision for diagnostic angiography was made by a cardiologist. SA patients were also treated with Clopidogrel 75mg od per day for 7 days prior to the angioplasty as per hospital protocol. All patients were treated with weight adjusted unfractionated heparin before the intervention to achieve an activated clotting time (ACT) between 200 and 250 sec. Informed consent was obtained prior to the procedure from 25 stable angina patients outside the cardiac catheterisation laboratory. However, only eleven stable angina patients were studied as the other nine had non-significant CHD from the diagnostic coronary angiogram. Ethical permission was granted by the local ethics committee of the Royal Free Hospital, and all participants provided fully informed written consent.

2.1.2 Non ST elevation myocardial infarction patients.

Fourteen patients presented with non-ST elevation myocardial infarction (NSTEMI) and were treated with PCI were recruited for the study. The diagnosis of NSTEMI was based on the history of cardiac chest pain at rest with or without ischemic ECG changes and a 12 hour troponin T value > 0.03 ng/l (261). On arrival at our hospital all NSTEMI patients were treated with 300 mg of Aspirin and 600 mg of Clopidogrel and weight adjusted low molecular weight heparin (enoxaparin 1mg/kg twice daily). Angiography was performed in all NSTEMI patients within 72 hours of onset of chest pain. All NSTEMI patients also received weight adjusted unfractionated heparin in the catheter laboratory before PCI to maintain the ACT between 200 and 250 sec. Informed consent was obtained prior to the procedure from all NSTEMI patients outside the cardiac catheterisation laboratory. Ethical permission was granted by the local ethics committee of the Royal Free Hospital, and all participants provided fully informed written consent.

2.1.3 ST elevation myocardial infraction patients.

Twenty three consecutive patients presented with ST elevation myocardial infarction (STEMI) were recruited for the study. Standard diagnostic criteria were followed for the diagnosis of STEMI according to current guidelines (262). All patients with acute STEMI were treated immediately with primary PCI as the preferred and recommended method of treatment for acute myocardial infarction. All STEMI patients were given 300 mg of Aspirin by the ambulance paramedics and received 600 mg of Clopidogrel on arrival outside the catheterisation laboratory. Intravenous morphine was administered to alleviate symptoms of chest pain as necessary. Patients were given weight adjusted unfractionated heparin to keep the ACT between

200- 250 sec duration pPCI. Informed consent was obtained prior to the procedure from all STEMI patients outside the cardiac catheterisation laboratory. Ethical permission was granted by the local ethics committee of the Royal Free Hospital, and all participants provided fully informed written consent.

2.1.4 Exclusion criteria

Patients with renal failure, prior coronary artery bypass grafts and who had received Glycoprotein IIb/IIIa antagonists prior to sampling were excluded.

2.1.5 Percutaneous coronary interventions (angioplasty)

Standard techniques were followed for the diagnostic coronary angiography via the right femoral artery through a 6F sheath. A 6F venous sheath was also inserted via the right femoral vein. Left coronary artery angiography was performed with a 5F Judkin's left 4 (JL4) diagnostic catheter (Cordis ®, internal diameter 0.11 cm). Right coronary angiography was performed with a 5F Judkin's right 4 (JR4) diagnostic catheter (5F Cordis ®, internal diameter 0.11 cm). Through the femoral vein a 5F multipurpose catheter (5F, Cordis ®, internal diameter 0.11cm) was been placed in the right atrium. Following the diagnostic angiography the culprit lesion was identified and weight adjusted unfractionated heparin was given prior to the PCI. After identification and wiring of the culprit lesion an aspiration catheter (Medtronic ® Export catheter, internal diameter 0.10 cm) was advanced distal to the culprit lesion and 10 ml blood sample was aspirated through the aspiration catheter with a syringe. In the SA and NSTEMI groups blood samples (10 ml from each site) were sequentially aspirated through the diagnostic 5F catheter from the right atrium and the ascending aorta prior to sampling the coronary artery. However in the STEMI

group due to the clinical priority of restoring the coronary blood flow in the infarct related artery, the coronary artery was sampled first and then the right atrium and aorta. In order to maintain similar shear stress during aspiration of the blood samples from the three compartments and minimise shear stress related in vitro platelet activation blood samples from the different sites were aspirated carefully through catheters of similar internal diameter. In NSTEMI and stable angina patients if the advance of the aspiration catheter was difficult initially due to technical reasons, pre-dilation of the lesion with a semi compliant balloon was performed prior to the sampling. All the samples were collected before patients were treated with GP IIb/IIIa antagonists. Angioplasty was performed according to standard procedures following collection of samples.

Blood samples collected from the three sites were used for estimation markers of platelet activation i.e. s-P selectin, platelet-monocyte aggregates (PMA), P-selectin positive (PMA), microparticle (MP) and inflammatory parameters i.e. C-reactive protein (CRP), tumour necrosis alpha (TNF-α), IL-6 and serum amyloid antigen (SSA). Trans-culprit lesion gradient of the above parameters was calculated by the difference between the median values from coronary artery and the right atrium.

2.2 Identification and analysis of platelet-monocyte aggregates

2.2.1 Collection of whole blood for platelet-monocyte aggregate estimation

Ten millilitre of whole blood was collected from the coronary artery and right atrium. Four mls out of the 10mls was poured into a separate sterile vacutainer tubes containing a combination of 3.2% sodium citrate and EDTA for flow cytometric analysis of total PMA and P-selectin positive PMA (CD62P+PMA). Sodium citrate was added to EDTA to stop in vitro calcium dependent PMA formation. Samples

were immediately put onto ice and transferred to the haematology laboratory for preparation for flow cytometry. P-selectin positive PMA implies the presence of activated platelets, expressing p-selectin on their surface, within the PMA. Difference between the median values of the coronary artery and right atrium were calculated to obtain a trans-culprit lesion gradient of the above mentioned parameters.

2.2.2 Preparation of whole blood for platelet-monocyte aggregates estimation

Blood samples collected from the two samples were immediately transferred to the haematology laboratory and prepared for flow cytometry and PMA estimation. Analysis of all the samples was complete within 2 hours of collection. Five µl of anti-CD61 FITC, anti-CD14 PerCP and anti-CD62P PE were added in the two (one tube for each compartment) round bottom polystyrene tubes (BD Falcon 12 x75 mm style). One hundred µl of whole blood was aliquoted into each of the tubes. The samples were incubated in a dark place at room temperature for 20 minutes. After that erythrocytes were lysed by addition of 2 mls of easy lyse™ solution (Dako) (1 in 10 dilution) for 15 minutes at room temperature in dark place. Red blood cells were washed with addition of 1ml of FACS flow and centrifuged at 300g for 5 minutes. After that the supernatant was discarded and the cells resuspended in 500µl of FACS flow for immediate flow cytometric analysis (FACS Calibur equipped with Cell Quest ® software – BD Biosciences, Oxford, UK).

2.2.3 Flow Cytometric identification of platelet-monocyte aggregates

Events were acquired on a 2D dot plot arraying CD14 (logarithmic scale abscissa) and SSC height (linear scale ordinate). Monocytes were identified as CD14 positive

events and distinctive intermediate side scatter height as shown in Figure 2.1. A minimum of 5000 CD14 positive events were acquired from each sample. The monocyte population was gated and named as analysis region (R1). Events within R1 plotted again on a 2D dot-plot arraying CD 61 signal width (linear scale abscissa) and CD14 signal height (logarithmic scale abscissa). To exclude false-positive PMA arising from co-incident analysis of free platelets adjacent but not interacting with monocytes, the events with a narrow CD61 width was drawn (R2 in Fig 2.1b). Events falling within both R1 and R2 regions were subsequently plotted onto another 2D dot-plot arraying CD61 FITC signal height (logarithmic scale abscissa) and CD 62 PE (logarithmic scale ordinate) (Fig 2.1c). Co- incident cells demonstrate longer time of flight to pass through the laser and can be distinguished by eliciting signals which lasts longer than the single complex. This was reflected in the wider width of the signal. Double positive CD14+ and CD61+ events were consider as PMA were expressed as percentage of the total monocytes. Out of the total PMA the events which were expressing CD62P were identified as P-selectin positive PMA and were expressed as percentage of the total PMA. The process was standardised in our laboratory with the percentage of PMAs in the peripheral circulation of normal healthy individuals was found to be 2.57± 0.31 (CV 13.96± 8.30 %).

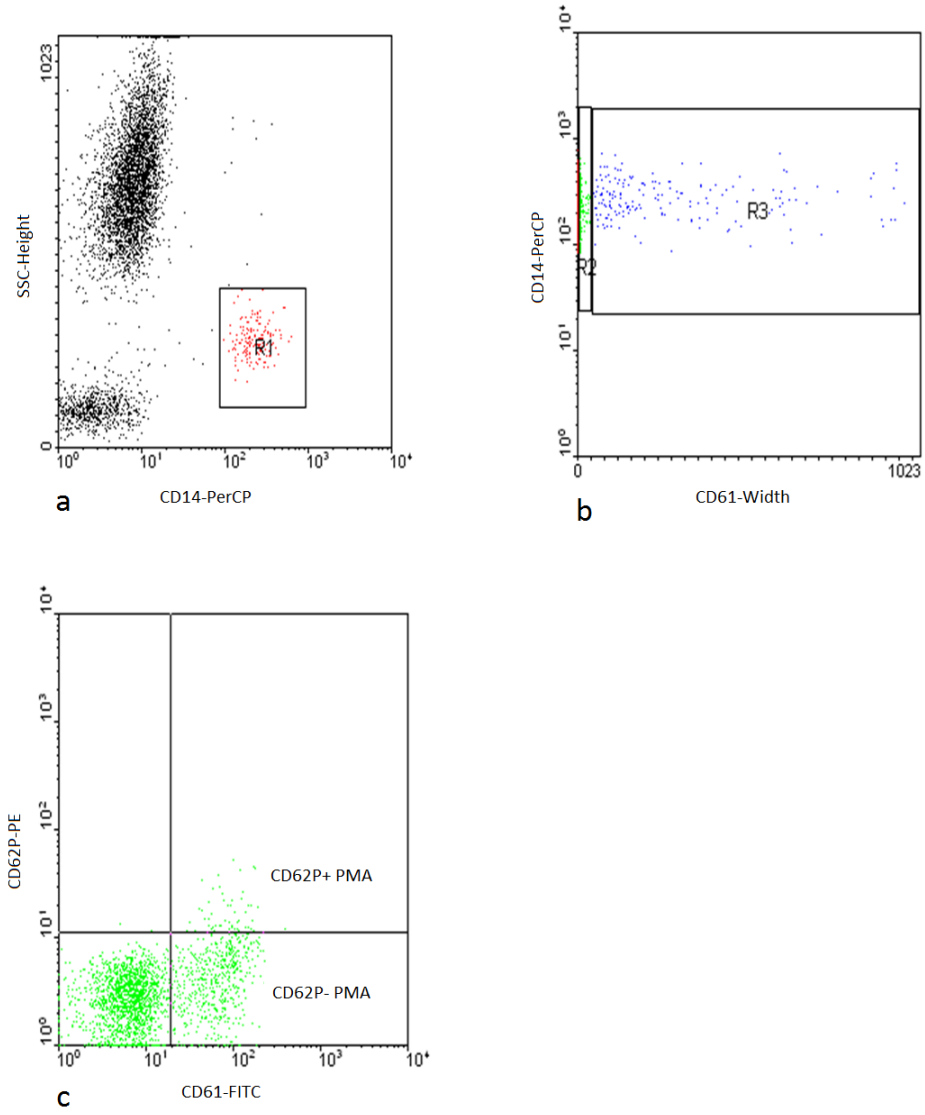


Figure 2.1 Identification of PMA and CD62P+ PMA with flow cytometry

a. Monocyte population was identified by CD14 positivity and distinctive intermediate side scatter. R1 analysis region was drawn around the monocyte population.

b. Events within R1 were plotted again on a 2D dotplot arraying CD61 signal width (linear scale abscissa) and CD14 signal height (logarithmic scale abscissa). To exclude false-positive PMC arising from co-incident analysis of free platelets and monocytes, a region R2 was drawn around the cells with

narrow CD61 width. Cells with wider CD61 width (R3) were identified as co-incidents.

c. Events falling within both R1 and R2 were subsequently plotted onto another 2D dotplot arraying CD6-FITC signal height (logarithmic scale abscissa) and CD62P-PE (logarithmic scale ordinate) (Fig 2.1c). Double positive events for CD14 and CD61 were identified as PMA. PMA positive for CD62P were identified as P-selectin positive complexes (CD62P+ PMA) (right upper quadrant figure- 2.1c).

2.3 Isolation and analysis of total Annexin V positive microparticle and microparticle subpopulations

2.3.1 Preparation of platelet poor plasma

3.5 mls of whole blood was collected into bottles containing 3.2% trisodium citrate (Becton Dickinson). Platelet poor plasma (PPP) was obtained by immediate centrifugation of the whole blood at 5000G for 5 minutes twice. PPP was then stored at –80^{o} Celsius for later analyses of microparticle (MP) estimation and enzyme-linked immunosorbent assay (ELISA) studies (see below).

2.3.2 Isolation of microparticles from platelet poor plasma

Platelet poor plasma was defrosted in a water bath at 37 ^{o} Celcius. Exact volumes of plasma (200 microlitres) were then centrifuged at 17000 G for 60 minutes and the supernatant decanted to obtain the microparticle (MP) pellet. The MP were then reconstituted in 280 microlitres of annexin V buffer (Becton Dickinson), and divided into seven 40-microlitre aliquots plated onto the first 7 wells of a 96 well U-bottomed plate.

2.3.3 Labelling of microparticles with annexin V and monoclonal antibodies

The labelling and quantification of MP was achieved as follows. 5 microlitres of a 1 in 10 dilution of FITC-conjugated (or PE-conjugated for Tissue factor positive monocyte derived MP (TF+MMP) Annexin V in buffer (Becton Dickinson) was added to every well. 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, Becton Dickinson), and the platelet activation marker P-selectin (mouse IgG1 anti-human CD62P-PE, Becton Dickinson). Endothelial surface markers examined were E-selectin (mouse IgG1 anti-human CD62E-PE Becton Dickinson); CD105 ("Endoglin"; mouse anti-human IgG1 CD105-PE, Invitrogen); ICAM-1 (mouse IgG1 anti-human CD54-CYC, Becton Dickinson); and PCAM-1 (mouse IgG1 anti-human CD31-PE, Becton Dickinson). Neutrophil surface marker examined was Integrin Alpha M (mouse IgG1 anti-human CD66b-PE, eBioscience). Monocyte surface markers examined were lipopolysaccharide receptor (LPS-R) (mouse IgG1anti-human CD14- PerCP-Cy5, AbD Serotec) and tissue factor (mouse IgG1 anti-human CD142-FITC, American Diagnosis). Five microlitres of a 1:10 dilution of each antibody was added to individual wells. The final volume of each well was 55 microliters and the final dilution for each of the conjugated antibodies was 1:55. MP were incubated with the labelled antibodies and annexin V for 10 minutes at room temperature with gentle shaking. The incubation was then terminated by adding 200 microlitres of annexin V buffer to each well, and the samples transferred to tubes prior to flow cytometry. Using this methodology it was possible to define the following types of microparticles thus: total AnV+ MP, platelet derived MP (PMP; AnV+CD42a+), endothelial derived MP (EMP; dual positive for AnV+ and CD62E or CD105; or AnV+CD42a-CD31+), neutrophil derived MP (NMP; AnV+CD42a-CD66b+) and tissue factor positive monocyte derived MP (MMP; AnV+CD14+TF+).

2.3.4 Flow cytometric analysis of microparticles

All analysis was performed on a FACS Calibur flow cytometer equipped with Cell Quest ® software (BD Biosciences, Oxford, UK). To obtain optimal forward and side scatter instrument settings for MP, 1 micrometer and 3 micrometer latex beads (Sigma) were run and logarithmic forward and side scatter plots (Fig 2.2a) obtained (263). Gates were then set to include particles less than approximately 1.5 micrometers, 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 labelled annexin V. Particles less than 1.5 micrometers in size and binding annexin V were then gated (Fig 2.2b), and histograms obtained for this gated population for binding to individual monoclonal antibodies to determine the cell of origin of the MP (Fig 2.2c). MP samples were run at medium flow rate with a cut-off time of 30 seconds.

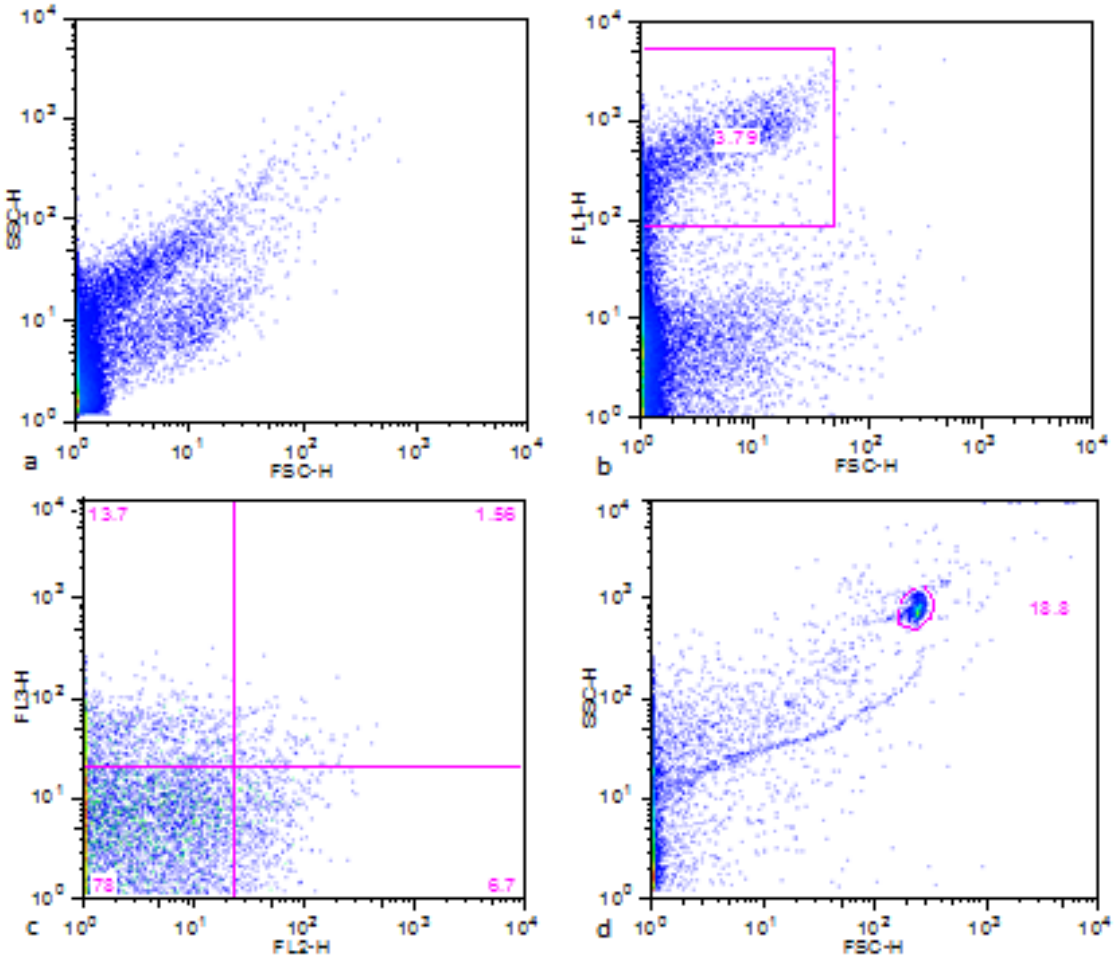


Fig 2.2 Flow cytometric identification and analysis of AnV+ microparticles

a. Logarithmic forward and side scatter plot obtained after cytometric acquisition of a coronary artery stained sample from a patient with NSTEMI.
b. Microparticles were identified, as Annexin V-FITC (Fluorescein Isothiocyanate) (FL1-H) positive events, and gated.
c. Particles less than 1.5 micrometers in size and binding annexin V were then gated and histograms obtained for this gated population for binding to individual monoclonal antibodies (FL2-H and FL3-H colours) to determine the cell of origin of the microparticle.
d. 3 micrometer latex beads were run concurrently with the microparticle samples for conversion of the flow cytometer counts to an estimate of the number of microparticle per ml of plasma.

2.3.5 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 micrometer latex beads (Sigma) were run concurrently with the microparticle samples (Fig 2.2d). 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 (263). The following equation was thus derived to convert flow cytometer counts to an estimate of the number of MP per ml of plasma (Figure 2.3).

Since samples from each individual were run 7 times, microparticle counts from individual subjects were expressed as the mean number per ml of plasma, with standard error of the mean based on 7 measurements. To determine the absolute number of MP derived from different cellular populations (i.e. platelet, or endothelial) the absolute number of total MP derived from the above equation was multiplied by the percentage positivity for that particular marker.

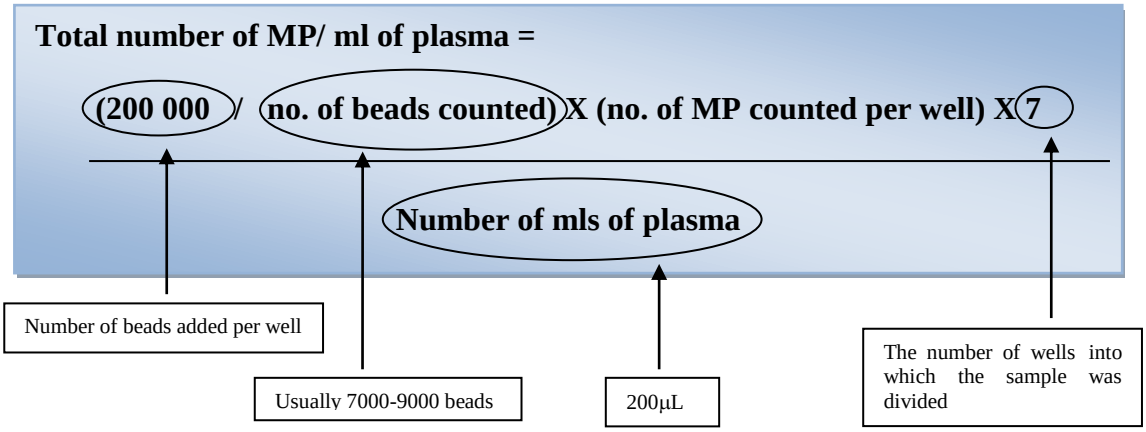


Figure 2.3 Conversion equation for calculation of MP number from flow cytometer counts

Cellular source of MPs	Marker	Alternative name	Clone	Isotype	Company
Platelets					
	CD42a-PerCP-Cy5	GPIX	Beb1	IgG1	BD
	CD62P-PE	P-selectin	AK-4	IgG1	BD
Endothelial cells					
	CD31-PE	PCAM-1	WM59	IgG1	BD
	CD105-PE	Endoglin	SN6	IgG1	Invitrogen
	CD62E-PE	E-selectin	68-5H11	IgG1	BD
	CD54-PE	ICAM	HA58	IgG1	BD
Neutrophils					
	CD66b-PE	Integrin alpha M	CBRM1/5	IgG1	eBioscience
Monocyte					
	CD14-PerCP-Cy5	LPS-R	61D3	IgG1κ	AbD Serotec
	CD142-FITC	Tissue Factor			
Isotype controls					
	PE IgG1		MOPC-21		BD
	PECy5 IgG1		MOPC21	IgG1	BD
	PerCP		X40	IgG1	BD
	Annexin V	FITC			BD

Other reagents:

10 x Annexin-V binding buffer (BD Cat NO: 66121E)

96-Well U-bottom Multiplate (Greiner, polypropylene, Cat 650201)

FACS insert tube (Greiner Cat NO: 101101)

Sarstedt screw cap micro-centrifuge tubes (Simport T338-4S)

Latex beads (Sigma 3 µm:LB30; 1.1 µm: LB11; 0.3 µm: LB3)

Table 2.1 Cell specific monoclonal antibodies and other reagents used

2.4 Estimation of inflammatory markers and soluble p-selectin

2.4.1 Collection and preparation of blood samples

3.5 mls of whole blood was collected into bottles containing 3.2% trisodium citrate (Becton Dickinson). PPP was obtained by immediate centrifugation of the whole blood at 5000G for 5 minutes twice. PPP was then stored at –80^{o} Celcius for later analyses of IL-6 and TNF-α, s P-selectin with ELISA (R&D systems); hs-CRP and SAA using a high sensitivity automated microparticle enhanced latex turbidimetric immunoassay (COBAS MIRA; Roche Diagnostics GmbH).

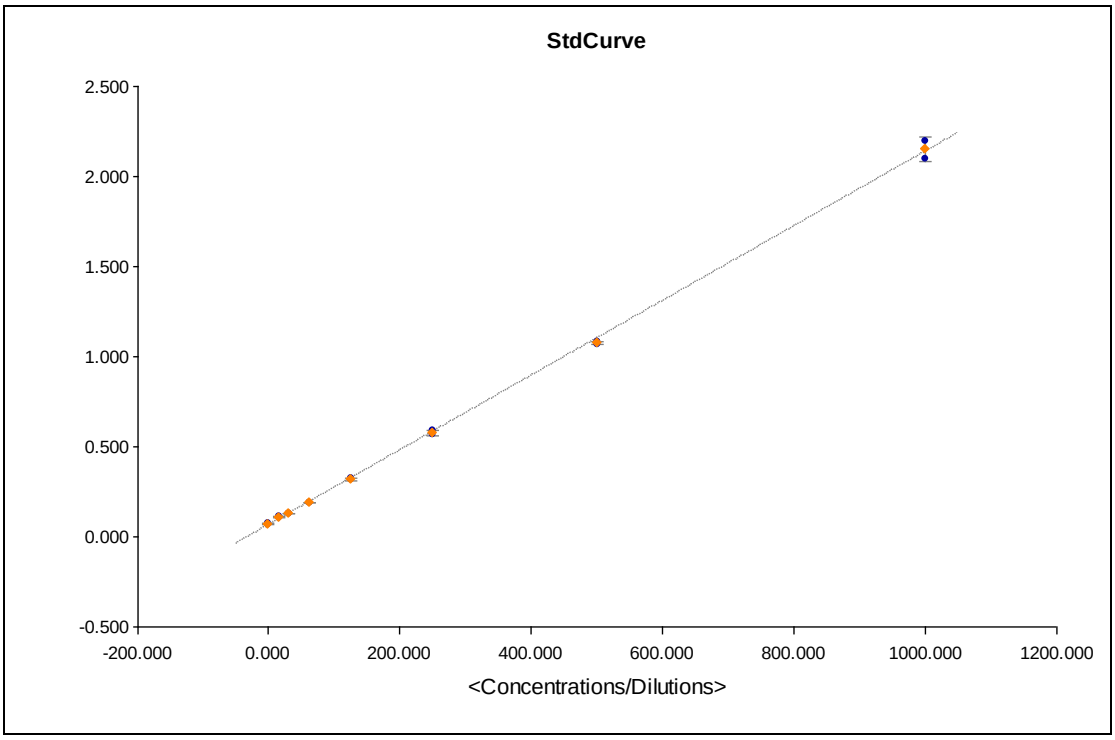
2.4.2 ELISA

2.4.2.1 Estimation of TNF-α and IL-6

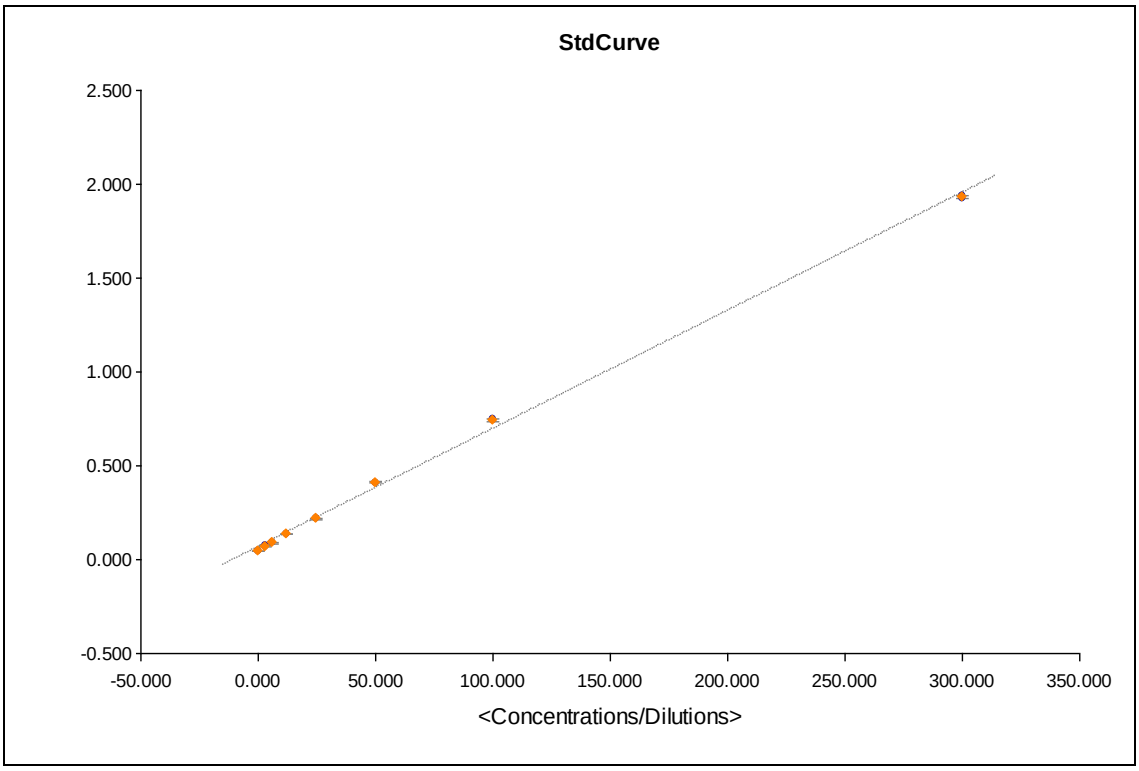
TNF-α and IL-6 were measured using a quantitative enzyme immunoassay technique (R&D Systems). Monoclonal antibobies specific for TNF-α or IL-6 were precoated onto a microplate. Standards and samples were pipetted into the wells and any TNF-α or IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α or IL-6 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of TNF-α or IL-6 bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

A standard curve was constructed by plotting the mean absorbance for each standard on the y axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph. The data was linearized by plotting the log of TNF-α or IL-6 concentrations versus the log of optical density and the best fit line was determined by the regression analysis. The CV of intraassay precision of this

particular TNF-α kit varied between 4.6% and 5.2% and the CV of interassy precision varied between 5.4% and 7.4%. The mean minimal detectable dose of TNF-α was 1.13 pg/ml. The CV of intra assay precision of the IL-6 was between 1.6% and 4.2% whereas CV of interassay precision varied between 3.3% to 6.4%. The minimal detectable dose of IL-6 was typically less than 0.06 pg/ml.



a)



b)

Figure 2.4 Standard curve for TNF-α (a) and IL-6 (b). Standard curve was produced by plotting the log of TNF-α (a) and IL-6 (b) concentrations (x axis) and the log of optical densities (y axis). The best fit line was determined by regression analysis.

2.4.2.2 Estimation of soluble P-selectin

Soluble P-selectin was assayed by the quatitative sandwich immunoassay technique using the R& D Systems kit. A monoclonal antibody specific for s P-selectin was precoated into the wells together with a polyclonal antibody specific for s-P-selectin which has been conjugated to horseradish peroxidase. After removal of unbound conjugated antibody, a substrate was added and colour was developed which was proportional to analyte concentration.

A standard curve was drawn by plotting the mean absorbance for each standard on the y axis against the concentration on the x axis. For samples the concentration

determined from the standard curve was multiplied by 20 (dilution factor). The CV of intra assay precision of this kit was between 4.9 % and 5.6 % and the CV of interassay precion of this test was between 7.9% and 9.9%. Minimal detectable dose of s P-selectin was less than 0.5 ng/ml. Mean value from healthy individual was 29 ng/ml (± 1sd range 18-40 ng/ml).

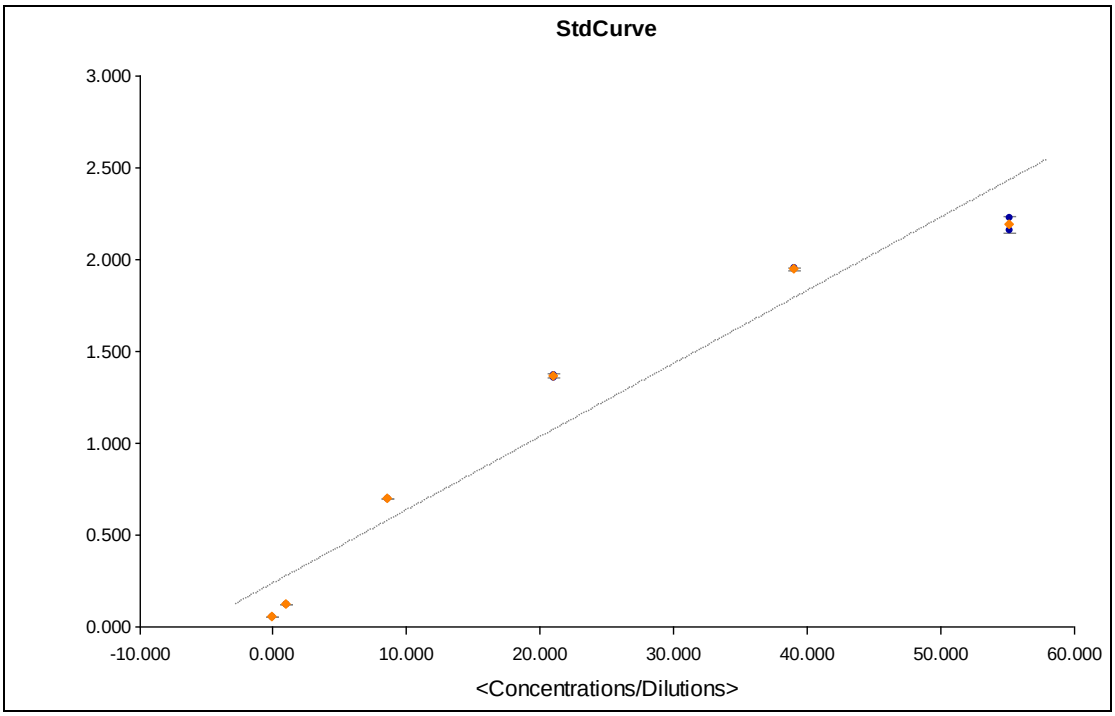


Figure 2.5 Standard curve for soluble P-Selectin (s P-selectin). Standard curve for s P-selectin was drawn by plotting the mean absorbance on the y axis against the concentration on the x axis. Values of the samples obtained from the standard curve were multiplied by the dilution factor of 20 to get the true s P-selectin concentration.

2.4.3 Estimation of high sensitive CRP and SAA

CRP was measured in plasma using a high sensitivity automated microparticle enhanced latex turbidimetric immunoassay (COBAS MIRA; Roche Diagnostics GmbH). The lower limit of detection was 0.2 mg/l with an interassay CV of 4.2% at 4 mg/l and 6.3% at 1 mg/l. SAA was measured in plasma by latex nephelometry (BNII autoanalyser; Dade Behring, Marburg, Germany) (264). The lower limit of detection was 0.7 mg/l, with an inter-assay CV of 2.6% at15 mg/l and 3.7% at 80 mg/l. Standardisation of both CRP and SAA assays was based on the respective WHO International Reference Standards (265;266).

2.5 Measuring the index of microvascular resistance and coronary flow reserve with pressure-temperature thermistor pressure wire

The index of microvascular resistance (IMR) and coronary flow reserve (CFR) were measured in 7 patients with NSTEMI and 7 patients with stable angina using a commercially available 0.014 inch floppy pressure wire (pressure wire 3, Radi Medical system) by using the thermodilution technique. The pressure wire has a microsensor at a location 3 cm from the floppy tip, which measures simultaneously pressure and temperature at the location of the sensor with an accuracy of 0.2^0 C. The shaft of the wire can be used as an additional thermistor, providing the input signal at the coronary ostium of any fluid injection with a temperature difference from blood. All signals can be displayed on the regular catheter laboratory recording system or at a suitable interface (Radi analyzer). In this manner the mean transit time of the injected saline down the coronary artery can be calculated from a coronary thermodilution curve. Thermodilution curves in the coronary artery are obtained by short manual injections of 3 ml 0.9% saline at room temperature. Measurements of transit time are calculated 3 times at base line and 3 times at maximum hyperaemia, provided that the time for analysis of the thermodilution equals at least one cardiac cycle and variability between the values of the 3 transit times was <20%. The mean transit time at baseline and maximum hyperaemia are calculated. Maximum hyperaemia is achieved by adenosine infusion through the femoral vein at a rate 140 microgram/kg/minute. Distal coronary pressure (Pd) is recorded from the pressure wire. Mean aortic pressure (Pa) is measured from the coronary catheter and central venous pressure (Pv) is measured from the right heart catheter at maximum hyperaemia. Coronary wedge pressure (Pw) is also measured at maximum hyperaemia by inflating a semi-compliant balloon (1mm smaller that the vessel

diameter) in the coronary artery and the IMR is calculated. In absence of epicardial stenosis, collaterals and assuming Pv is close to zero, IMR is calculated as hyperaemic distal coronary pressure divided by inverse of hyperaemic mean transit time (T_m) (a correlate to absolute flow). This can be represented in a simplified manner as a product of distal coronary pressure and mean hyperaemic transit time (Pd × Tm). Fearon et al demonstrated there was significant correlation between mean IMR (distal coronary pressure/Inverse of mean hyperaemic transit time) and true microcirculatory resistance (TMR calculated as a ratio of distal coronary artery pressure and hyperaemic flow measured with external ultrasonic Doppler) in experimental animals (243). Coronary collaterals, in the presence of a coronary epicardial stenosis, might lessen the drop in distal coronary pressure without a change in coronary blood flow and also may increase the coronary Pw. The combination of the above in turn can lead to an increase in the measured coronary microvascular resistance (243). Therefore, in presence of a coronary stenosis with collaterals, myocardial flow is not equal to coronary flow but exceeds the coronary flow because of collateral flow. Tm is no longer representative of myocardial flow and the contribution of collaterals needs to be considered and the IMR= Pa × Tm × [(Pd-Pw) ÷ (Pa-Pw)] (assuming Pv=0). This could be again represented by IMR=Pa × Tm × FFR_{cor}. In the presence of abnormal Pv the equation should be represented as IMR= (Pa-Pv) × Tm × [(Pd-Pw) ÷ (Pa-Pw)] (Pa=Aortic pressure, Pv =Right atrial pressure, Pw=coronary artery wedge pressure, Pd=distal coronary pressure, all the above measurements during maximum hyperaemia). Clearly the presence of functional collaterals will increase Pw, but there is an argument that Pw below 25 mmHg implies the presence of hemodynamically insignificant collaterals. The cut off

of 25mmHg is in line with previous clinical studies found that collateral flow is essentially absent when Pw is lower than 25 mmHg (213;244-247).

2.6 Recruitment limitations and practical considerations

There are several recruitment limitations with this project including the difference in the number of patients recruited between the three study groups. As mentioned above 25 SA patients were consented however only 11 were studied (the reasons have been discussed above). Also only 14 out of the 25 ACS patients (NSTEMI and SA) were studied with IMR. The reason being is the fact that the procedure (blood sampling the sites, completing the PCI and then measuring the IMR and CFR) was long, becoming uncomfortable for the patient. In addition there were occasions where I had to stop/interrupt the procedure as a patient with STEMI was admitted and needed to be treated with pPCI. Also the differences in the timing of the blood sampling between the three study groups should be noted as potentially could have affected the MP results (i.e. sampling of the coronary artery in the stable angina and NSTEMI patients after the aorta and the right atrium as opposed to the STEMI patients where the coronary artery was sampled before the aorta and the right atrium).

Lastly, power calculations were not done as the initial intentions of this study were exploratory. Cell derived MP in patients with clinically manifested IHD has not been studied in so much details i.e all cell derived MP, different clinical setting (ACS vs stable angina) and several sites (coronary artery, aorta and right atrium).

Chapter 3: Phenotypic characterisation of local and systemic microparticle expression in patients with acute coronary syndrome and stable coronary artery disease

3.1 Summary

Introduction and aims

Inflammation plays a pivotal role in the pathogenesis of atherosclerotic disease. Increasing evidence indicates that microparticles (MP) are potent pro-coagulant molecules contributing to endothelial dysfunction and modulating inflammatory processes. The aim of this study was to investigate the intracoronary and systemic microparticle expression in patients presenting with symptomatic coronary heart disease (CHD).

Methods

Forty eight patients with CHD (23 patients with ST segment elevation myocardial infarction (STEMI), 14 with non-ST segment elevation myocardial infarction (NSTEMI), and 11 with stable angina (SA) treated with percutaneous coronary intervention (PCI) were recruited. Blood samples were aspirated sequentially from the right atrium (RA) and the coronary (CO) artery (distal to the culprit lesion). AnnexinV+ MP (AnV+ MP) from platelet poor plasma was measured using fluorescent monoclonal antibodies and flow cytometry.

Results

AnV+ MPs in the CO and RA were expressed as medians per ml of plasma with interquartile range. The absolute number of the total AnV+ MP in the STEMI group in the CO and RA were 5.3 (3.0-12) and 1.7 (0.6-5.7) (p=0.01) respectively. In the NSTEMI group the total AnV+ MP in the CO and RA were 2.8 (1.9-4.5) and 0.3 (0.1-1.2) (p=0.004) respectively. In the SA group the total AnV+ MP in the CO and RA were 2.51 (0.9-2.7) and 1.1 (0.1-1.5) (p=0.03) respectively. Platelet derived MP (PMP), endothelial derived MP (EMP) and neutrophil derived MP (NMP) were also higher in the CO vs the RA site in the three groups. Total AnV+ MPs, both in the CO

and RA, were higher in the STEMI group compared with the NSTEMI and SA. Index event to PCI time correlated moderately negatively with PMP and EMP in the NSTEMI group.

Conclusions

High levels of AnV+ MP occur in the coronary artery of patients with ACS. Levels of AnV+MP correlate with severity of the ischaemic lesion since there were higher MPs detected in the STEMI versus the NSTEMI and SA groups. Whether these MP merely reflect the severity of the ischaemic lesion, and/or are active participants in the pathogenesis of ACS now warrants further study.

3.2 Introduction and aims

Atherosclerotic disease is a chronic inflammatory process (123). Over the last twenty years, data have emerged showing that immune cells are involved in the pathogenesis of formation and evolution of atherosclerotic plaques causing either stable angina (SA), or acute coronary syndrome (ACS) (267). Interactions between inflammatory and non-inflammatory cells within the atheromatous plaque may lead to cell activation and differentiation, a process that has been traditionally known to be mediated via interleukins and adhesion molecules (2). Microparticle (MP) release may occur both within the atheromatous plaque and/or systemically (81;268). MP are submicron membrane vesicles (100-1000nm) derived from virtually any eukaryotic cell and platelets during biological processes such as cell activation, differentiation and apoptosis (269). Microparticles differ in surface marker phenotype, and their biological effects depending upon the parent cell of origin and nature of the stimulus which led to their formation (269). Mounting evidence indicates that MP possess potent pro-inflammatory and pro-coagulant properties, and may themselves contribute to endothelial dysfunction via pro-inflammatory mechanisms (54;55). Clinical studies in patients with coronary heart disease CHD or conventional risk factors for CHD have shown that circulating MP levels are elevated compared with healthy volunteers (56;103;104). Also, previous studies suggest a positive correlation between MP levels and clinical outcomes, including: all-cause mortality; cardiovascular mortality; major cardiac events; markers of myocardial damage; and indices of myocardial dysfunction after an ACS (107-112). Whether elevated MP levels play a true contributory role to the pathophysiology of CHD, or represent a purely secondary epiphenomenon is still a matter of debate. In addition, there are

only limited information about MP expression in the coronary artery locally versus systemically in patients with ACS and SA.

I hypothesised that: 1) AnV+MP levels are higher in the ACS versus stable angina patients; 2) AnV+MP expression is higher in the coronary artery versus systemically i.e. the main source of the circulating MP is the complicated atheromatous plaque and/or associated clot. The aim of my study was therefore to investigate the expression of the total AnV+ microparticles (MPs) and their cell-specific phenotype in patients presenting with symptomatic CHD, either ACS or stable angina. The possibility of a trans-myocardial gradient between coronary and periphery of MP release was explored by determining MP levels in the local intracoronary (CO) artery distal to the culprit lesion, and in the right atrium (RA) as representative of the systemic circulation. Lastly, I also studied the relationship between the index event (onset of symptoms/chest pain) to PCI time and MP expression in the NSTEMI patients.

3.3 Patients and methods

3.3.1 Study population

Forty eight patients with CHD were prospectively recruited from our institution (Royal Free Hospital, London, UK) to the study. The CHD patients included 23 consecutive patients presenting with ST segment elevation myocardial infarction (STEMI), 14 patients with non ST segment elevation myocardial infarction (NSTEMI), and 11 patients with stable angina (SA). The standard criteria for the diagnosis of STEMI were followed according to the European Society of Cardiology guidelines i.e. chest pain and ST elevation ≥ 2 mm in ≥ 2 contiguous chest leads, or ≥ 1 mm in ≥ 2 contiguous limb leads on the ECG (262). All STEMI patients were

treated immediately with primary percutaneous coronary intervention (PCI). The diagnosis of NSTEMI was based on the history of chest pain with or without ischaemic ECG changes (other than ST elevation) and a troponin T value > 0.03 ng/l (261). SA patients with symptoms were admitted electively for PCI. SA patients had been reviewed in the cardiology outpatient clinic with a diagnosis of angina pectoris based on typically ischaemic sounding chest pain and a positive non-invasive test such as exercise tolerance test, stress echocardiography or myocardial perfusion scan. The decision for diagnostic angiography was made by a cardiologist as part of routine clinical care. Informed consent was obtained prior to the procedure from all recruited patients outside the cardiac catheterisation laboratory. Ethical permission was granted by the local ethics committee of the Royal Free Hospital, and all participants provided fully informed written consent.

3.3.2 Exclusion criteria

The exclusion criteria have been described in Chapter 2 (Paragraph 2.1.4)

3.3.3 Coronary angiography, coronary angioplasty and blood sampling

Diagnostic coronary angiography and percutaneous coronary intervention (PCI) were performed according to contemporary guidelines. A 6F venous sheath was also inserted via the right femoral vein. Through the femoral vein a 5F multipurpose catheter (5F, Cordis ®, internal diameter 0.11cm) was placed in the right atrium. Left and right coronary artery angiography was performed with a 5F Judkin's Left 4 diagnostic catheter (Cordis ®, internal diameter 0.11 cm) and 5F Judkin's Right 4 diagnostic catheter (5F, Cordis ®, internal diameter 0.11 cm) respectively. Blood samples (10 ml from each site) were aspirated through the diagnostic 5F catheter

from the right atrium. Following the diagnostic angiography the culprit lesion was identified and weight adjusted unfractionated heparin was given prior to the PCI to achieve an activated clotting time (ACT) between 200 and 250 seconds. After identification and wiring of the culprit lesion an aspiration catheter (Medtronic ® Export catheter, internal diameter 0.10 cm) was advanced distal to the culprit lesion and 10 mls blood sample was aspirated through the aspiration catheter with a syringe. Blood samples from the different sites were aspirated carefully through catheters of similar internal diameter in order to maintain similar shear stress during aspiration and minimise shear stress related to platelet activation. In NSTEMI and stable angina patients if the advance of the aspiration catheter was difficult initially due to technical reasons, pre-dilation of the lesion with a semi compliant balloon was performed prior to the sampling of the coronary artery. All the samples were collected before the administration of GP IIb/IIIa antagonists. Angioplasty was performed according to standard procedures following collection of samples. Blood samples from the healthy volunteers were collected from the antecubital vein using a 19G needle.

STEMI patients were given 300 mg of Aspirin by the ambulance paramedics and received 600 mg of Clopidogrel on arrival outside the catheterisation laboratory. Intravenous morphine was administered to alleviate symptoms of chest pain as necessary. NSTEMI patients were treated with 300 mg of Aspirin and 600 mg of Clopidogrel and weight adjusted low molecular weight heparin (enoxaparin 1mg/kg twice daily). SA patients were treated with anti-angina medications; aspirin 75mg od and clopidogrel 75mg od for 7 days prior to the angioplasty as per hospital protocol. The index event to PCI interval was expressed in minutes.

The differences in timing of blood sampling of the three sites between the three groups have been described in chapter 2 (section 2.1)

3.3.4 MP identification and characterization using flow cytometry

MPs were identified by flow cytometry as previously described. Blood was collected in 3.2% buffered citrate and centrifuged at 5,000 *g* for 5 minutes twice to obtain platelet-poor plasma (PPP). MPs were sedimented from 200μL of PPP after centrifugation at 17,000 *g* for 60 minutes and resuspended in An V binding buffer (BD PharMingen, Oxford, UK) prior to incubating with fluorescent monoclonal antibodies: fluorescein isothiocyanate (FITC) or Phycoerythrin (PE) labeled annexin V (BD PharMingen), mouse PE-labeled antihuman CD62E (clone 68-5H11, BD PharMingen), mouse PE-labeled antihuman CD31 (clone L133.1, BD PharMingen), mouse IgG1 anti-human CD66b-PE (clone G10F5, eBioscience), mouse Cy5-labeled antihuman CD14 (clone 61D3,AbD Serotec), mouse FITC-labeled antihuman TF (clone VD8, American Diagnostica), and mouse PE-labeled antihuman P-selectin glycoprotein ligand 1, PSGL-1 (clone KPL1, BD PharMingen). Additional labelling with mouse antihuman CD42a-PERCP (BD PharMingen) to exclude platelet origin was conducted. Using this methodology we defined the following types of microparticles thus: total AnV+ MP, platelet derived MP (PMP; AnV+CD42a+), endothelial derived MP (EMP; dual positive for AnV+ and CD62E or CD105; or AnV+CD42a-CD31+), neutrophil derived MP (NMP; AnV+CD42a-CD66b+) and tissue factor positive monocyte derived MP (MMP; AnV+CD14+TF+). Samples were analysed with a FACS Calibur flow cytometer (BD PharMingen). The flow cytometric gating strategy is summarised in figure 2.3 (chapter 2).

3.3.5 Statistical analyses

Continuous variables were expressed expressed as medians with interquartile range (IQR) Categorical variables were expressed as percentages with 95% confidence intervals (95% CIs). Comparisons of continuous variables between the three groups were performed using Kruskal-Wallis test followed by Mann-Whitney U test for intergroup analyses. Comparisons between the two compartments (CO and RA) were performed using the Mann-Whitney U test. Chi-square test was used for comparing proportions between the three groups. Spearman rank correlation factor r was used for correlations. Differences with a 2-sided p value <0.05 were considered to be statistically significant. All statistical analyses were performed with GraphPad Prism 5 statistical software.

3.4 Results

The demographics and baseline characteristics of the study population are shown in table 3.1. Briefly, the majority of the study population was male (79%) median age (IQR). The SA patients were more likely to have medical history of hypertension compared to the STEMI and NSTEMI groups ($p=0.01$). Also the NSTEMI patients were more likely to have family history of CHD ($p=0.04$).

	STEMI (n=23)	NSTEMI (n=14)	Stable Angina (n=11)	p value
Age (median), years	60	60.5	58	0.7
Male	17	12	9	0.7
Female	6	2	2	0.7
Risk Factors				
Hypertension	11	3	9	0.01
Diabetes	6	3	2	0.8
Dyslipidaemia	10	9	4	0.3
Smoking	9	4	2	0.4
Family History of CHD	7	10	4	0.04
Angiographic characteristics				0.7
1 vessel disease	17	8	6	
2 vessel disease	4	3	3	
3 vessel disease	2	3	2	
Culprit vessel				0.3
Left Anterior Descending	9	5	6	
Left Circumflex	3	4	0	
Right Coronary Artery	11	5	5	
Onset of symptoms to PCI interval, Median (mins)	258.5	4320	N/A	<0.0001

Table 3.1 Baseline characteristics, demographics and angiographic data of the study groups. Chi-square test was used for comparison of proportions between the three groups and Kruskal-Wallis test for continue variables. P values < 0.05 were considered significant. Coronary Heart Disease (CHD).

3.4.1 AnV+ MP expression in the coronary artery in the STEMI, NSTEMI and SA patients

Table 3.2 summarises the CO MP levels between the three patient groups. Total AnV+ MP in the CO were higher in the STEMI population compared with NSTEMI

(p=0.02). AnV+CD42a+ PMP and AnV+CD42a+CD62P+ PMP were also higher in the STEMI group compared with the NSTEMI (p=0.04 and p=0.003 respectively), and SA (p=0.04 and p=0.0008 respectively) groups; Fig 3.1a. Also all types of AnV+ EMPs were higher in the STEMI patients compared with the NSTEMI and the SA patients, Fig 3.1b. NMP and TF+ MMP were also higher in the STEMI population compared with the NSTEMI and SA groups, Fig 3.1c.

	CO			
	STEMI	NSTEMI	SA	Kruskal-Wallis test
	Median (IQR)	Median (IQR)	Median (IQR)	p value
Total AnV+ MP	5.35 (3.00-12.0)	2.85 (1.96-4.50)	2.51 (0.91-5.75)	0.04
CD42a+AnV+ PMP	2.20 (0.72-4.81)	1.05 (0.51-1.71)	0.71 (0.20-1.28)	0.04
CD62P+CD42a+AnV+ PMP	0.95 (0.28-2.45)	0.18 (0.11-0.42)	0.08 (0.02-0.15)	0.0003
CD62E+CD42a-AnV+ EMP	0.69 (0.18-1.70)	0.33 (0.11-0.67)	0.17 (0.07-0.67)	0.02
CD66b+CD42a-AnV+ NMP	0.17 (0.08-0.44)	0.11 (0.04-0.21)	0.04 (0.03-0.20)	0.01
CD54+CD42a-AnV+ EMP	0.64 (0.28-0.88)	0.20 (0.11-0.34)	0.11 (0.05-0.18)	0.0008
CD105+CD42a-AnV+ EMP	0.76 (0.37-1.91)	0.45 (0.11-0.78)	0.38 (0.07-0.77)	0.02
CD31+CD42a-AnV+ EMP	0.75 (0.35-1.40)	0.32 (0.09-0.47)	0.24 (0.10-0.33)	0.0005
CD14+TF+AnV+ MMP	0.15 (0.07-0.31)	0.18 (0.07-0.25)	0.07 (0.03-0.15)	0.04

Table 3.2 Differences of microparticles expression (*10^6 /ml of plasma) in the three groups in the coronary artery (CO). MP levels expressed as medians with interquartile range (IQR). Statistical analysis performed using Kruskal-Wallis test.

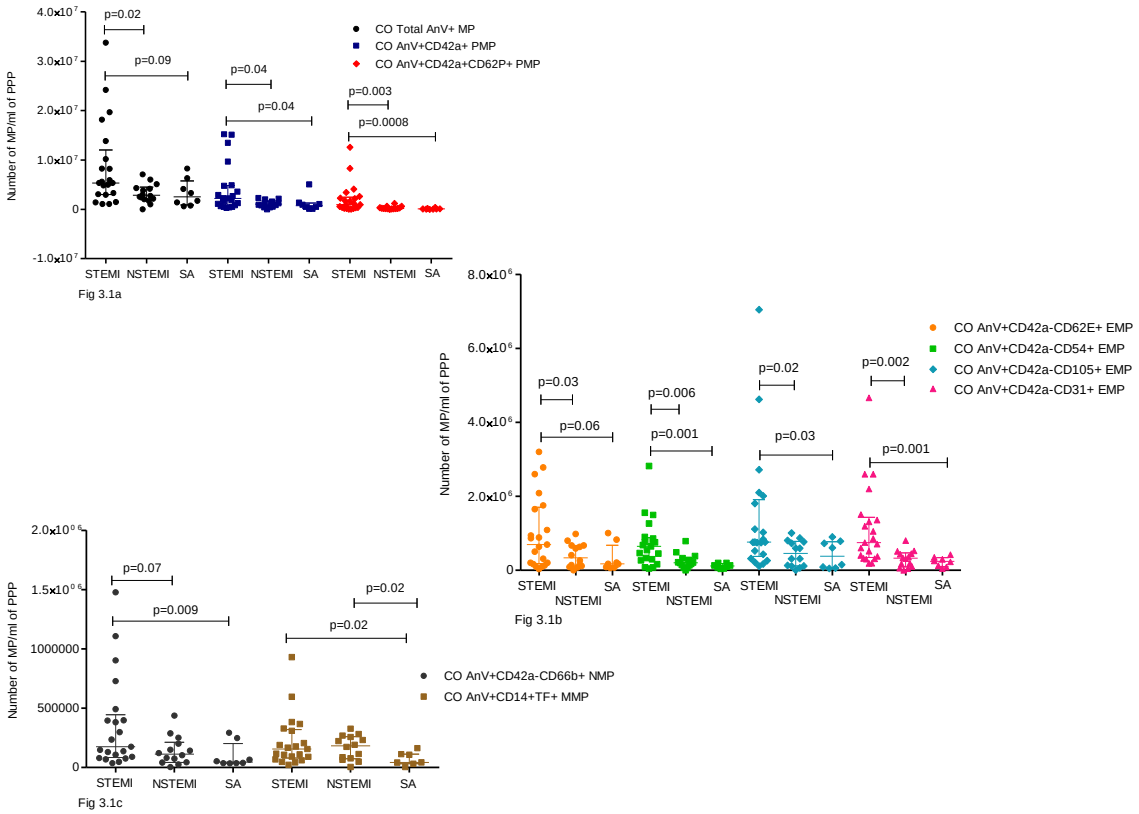


Figure 3.1 Differences of the total AnV+MP and MP subpopulations in the coronary artery (CO) between the three groups of patients. ST elevation myocardial infarction (STEMI), non ST elevation myocardial infarction (NSTEMI), stable angina (SA).

3.4.2 AnV+MP expression in the right atrium in the STEMI, NSTEMI and SA patients

Table 3.3 summarises the RA MP levels between the three patient groups. Total AnV+ MP in the RA were higher in the STEMI population compared with NSTEMI (p=0.02). AnV+CD42a+ PMP and AnV+CD42a+CD62P+ PMP were also higher in the STEMI group compared with the NSTEMI (p=0.02 and p=0.01 respectively) and SA (p=0.03 and p=0.001 respectively) groups; Fig 3.2a. Also all subtypes of AnV+ EMPs were higher in the STEMI patients compared with the NSTEMI and the SA patients; Fig 3.2b. NMP were also higher in the STEMI population compared with

the NSTEMI and SA groups, Fig 3.2c. TF+ MMP were higher in the STEMI group vs the SA but not vs the NSTEMI patients, Fig 3.2

	RA			
	STEMI	NSTEMI	SA	Kruskal-Wallis test
	Median (IQR)	Median (IQR)	Median (IQR)	p value
Total AnV+ MP	1.72 (0.64-5.78)	0.29 (0.11-1.2)	1.11 (0.14-1.49)	0.058
CD42a+AnV+ PMP	0.84 (0.26-3.97)	0.11 (0.03-0.54)	0.28 (0.02-0.60)	0.025
CD62P+CD42a+AnV+ PMP	0.17 (0.07-0.66)	0.03 (0.005-0.14)	0.03 (0.001-0.04)	0.001
CD62E+CD42a-AnV+ EMP	0.23 (0.06-0.44)	0.02 (0.01-0.12)	0.04 (0.009-0.07)	0.009
CD66b+CD42a-AnV+ NMP	0.08 (0.04-0.14)	0.01 (0.004-0.09)	0.01 (0.002-0.02)	0.002
CD54+CD42a-AnV+ EMP	0.07 (0.05-0.16)	0.04 (0.008-0.10)	0.009 (0.004-0.04)	0.007
CD105+CD42a-AnV+ EMP	0.16 (0.06-0.43)	0.04 (0.01-0.09)	0.07 (0.008-0.18)	0.02
CD31+CD42a-AnV+ EMP	0.11 (0.04-0.31)	0.04 (0.01-0.20)	0.05 (0.003-0.09)	0.1
CD14+TF+AnV+ MMP	0.09 (0.02-0.41)	0.05 (0.01-0.13)	0.03 (0.005-0.05)	0.04

Table 3.3 Differences of microparticles expression (10^6 /ml of plasma) in the three groups in the right atrium (RA). MP levels expressed as medians with interquartile range (IQR). Statistical analysis performed using Kruskal-Wallis test

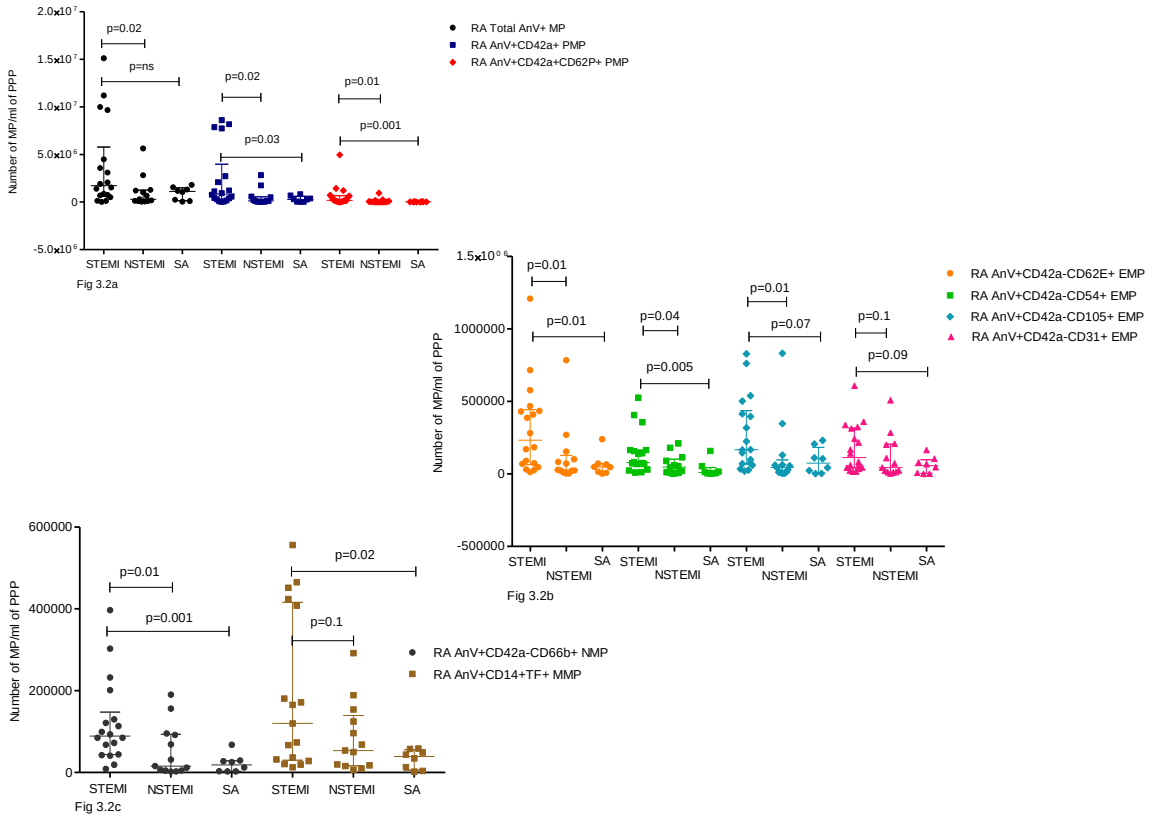


Figure 3.2 Differences of the total AnV+MP and MP subpopulations in the right atrium (RA) between the three groups of patients. (ST elevation myocardial infarction (STEMI), non ST elevation myocardial infarction (NSTEMI), stable angina (SA)

3.4.3 AnV+ MP expression in the coronary and right atrium in the three groups

In the STEMI group the absolute number of the Total AnV+ MP in the CO was $5.35x10^6$/ml (41% PMP, 13% EMP, 3% NMP, 3% TF+ MMP and 40% other AnV+ MP). In the RA site the number of AnV+ MP was $1.72x10^6$/ml (49% PMP, 8% EMP, 5% NMP, 5% TF+ MMP and 33% other AnV+ MP). In the CO site 43% of the PMP were CD62+ PMP vs 20% in the RA site (p=0.0007).

In the NSTEMI group the absolute number of the Total AnV+ MP in the CO was $2.85*10^6$/ml (27% PMP, 8% EMP, 3% NMP, 5% TF+ MMP and 57% other AnV+

MP). In the RA the number of AnV+ MP was $0.29*10^6$/ml (38% PMP, 10% EMP, 4% NMP, 17% TF+ MMP and 31% other AnV+ MP). In the CO 17% of the PMP were CD62+ PMP vs 27% in the RA (p=0.1).

In the SA group absolute number of the Total AnV+ MP in the CO was $2.51*10^6$/ml (28% PMP, 9% EMP, 2% NMP, 3% TF+ MMP and 58% other AnV+ MP). In the RA site the number of AnV+ MP was $1.1*10^6$/ml (25% PMP, 4% EMP, 1% NMP, 3% TF+ MMP and 67% other AnV+ MP). In the CO site 11% of the PMP were CD62+ PMP vs 10% in the RA site (p=ns).

Total AnV+ MP, CD42a+CD62P+ PMP, all EMP subpopulations and AnV+CD42a-CD66b+ NMP were higher in the CO compared to the RA for all patient groups (table 3.4). AnV+CD42a+PMP and TF+MMP were higher in the CO site compared to RA in the NSTEMI group only (table 3.4).

	STEMI			NSTEMI			SA		
	CO	RA		CO	RA		CO	RA	
	Median (IQR)	Median (IQR)	p value	Median (IQR)	Median (IQR)	p value	Median (IQR)	Median (IQR)	p value
Total AnV+ MP	5.35 (3.00-12.0)	1.72 (0.64-5.78)	0.01	2.85 (1.96-4.50)	0.29 (0.11-1.2)	0.004	2.51 (0.91-5.75)	1.11 (0.14-1.49)	0.03
CD42a+AnV+ PMP	2.20 (0.72-4.81)	0.84 (0.26-3.97)	0.06	1.05 (0.51-1.71)	0.11 (0.03-0.54)	0.01	0.71 (0.20-1.28)	0.28 (0.02-0.60)	0.06
CD62P+CD42a+AnV+ PMP	0.95 (0.28-2.45)	0.17 (0.07-0.66)	0.009	0.18 (0.11-0.42)	0.03 (0.005-0.14)	0.01	0.08 (0.02-0.15)	0.03 (0.001-0.04)	0.02
CD62E+CD42a-AnV+ EMP	0.69 (0.18-1.70)	0.23 (0.06-0.44)	0.009	0.33 (0.11-0.67)	0.02 (0.01-0.12)	0.009	0.17 (0.07-0.67)	0.04 (0.009-0.07)	0.007
CD54+CD42a-AnV+ EMP	0.64 (0.28-0.88)	0.07 (0.05-0.16)	<0.0001	0.20 (0.11-0.34)	0.04 (0.008-0.10)	0.002	0.11 (0.05-0.18)	0.009 (0.004-0.04)	0.01
CD105+CD42a-AnV+ EMP	0.76 (0.37-1.91)	0.16 (0.06-0.43)	0.0002	0.45 (0.11-0.78)	0.04 (0.01-0.09)	0.003	0.38 (0.07-0.77)	0.07 (0.008-0.18)	0.02
CD31+CD42a-AnV+ EMP	0.75 (0.35-1.40)	0.11 (0.04-0.31)	<0.0001	0.32 (0.09-0.47)	0.04 (0.01-0.20)	0.02	0.24 (0.10-0.33)	0.05 (0.003-0.09)	0.007
CD66b+CD42a-AnV+ NMP	0.17 (0.08-0.44)	0.08 (0.04-0.14)	0.01	0.11 (0.04-0.21)	0.01 (0.004-0.09)	0.02	0.04 (0.03-0.20)	0.01 (0.002-0.02)	0.007
CD14+TF+AnV+ MMP	0.15 (0.07-0.31)	0.09 (0.02-0.41)	0.2	0.18 (0.07-0.25)	0.05 (0.01-0.13)	0.04	0.07 (0.03-0.15)	0.03 (0.005-0.05)	0.2

Table 3.4 Differences of the total AnV+MP and MP subpopulations (*10^6/ml of plasma) between coronary (CO) and right atrium (RA) in the three groups, ST segment elevation myocardial infarction (STEMI), non-ST segment elevation myocardial infraction (NSTEMI) and stable angina (SA). Statistical analysis performed using the Mann-Whitney test.

3.4.4 MP expression and index event to PCI time

The median (IQR) of the index event to PCI time (defined as time from onset of symptoms to PCI, expressed in minutes) in the STEMI and NSTEMI groups were 285 (116-360) and 4320 (2100-6060) respectively ($p<0.0001$). In the CO of the NSTEMI group there was a moderately negative correlation between the the index event to PCI time and the expression of total and cell specific AnV+MP Fig 3.3.

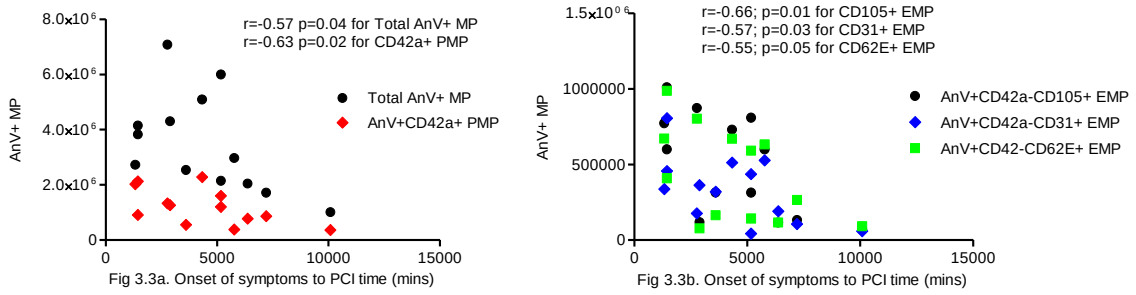


Figure 3.3 Negative correlations between the index event to PCI time and AnV+MPs in the coronary artery in the non ST segment elevation myocardial infarction group suggesting a time dependant mode of MP expression during acute coronary syndrome.

3.5 Discussion

The main novel findings of this study are 1. the demonstration of differences between MP levels in patients with varying severity of CHD: MP levels were overall highest in those with STEMI as compared with NSTEMI and SA; and 2. Differences in local and systemic AnV+MP expression in human coronary artery disease. It is highly likely that the source of these MP was the culprit lesion since total AnV+MP and AnV+MP subpopulations were higher in the coronary artery (CO blood sampled distal to the culprit lesion) compared with the systemic (RA) circulation in the three

study groups. There was also a differential expression of the MP subpopulations in the three patient groups between the CO and RA (table 3.4). Overall these observations confirm that MP release occurs from culprit atheromatous lesions in ACS, and that total AnV+MP number and subpopulation phenotype could relate to clinical severity of ACS. Whilst, the concept that these MP directly contribute to the pathogenesis of ACS cannot be inferred from this study, these findings could have important implications in this context since AnV+MP are known to be both pro-inflammatory and pro-thrombotic (77;78).

In line with previous studies PMP levels were higher compared with the other cell specific MP subpopulations. The above trend was more prominent with respect to the CD62P+PMP in the STEMI patients, especially sampled from the coronary artery. This observation can be explained by the fact that the hallmark of ACS is disruption (rupture, fissure or erosion) of the atheromatous plaque in a coronary tree, with subsequent platelet activation (upregulation of P selectin) and thrombus formation (81). The observation of high levels of AnV+CD62P+PMP in the CO raises the possibility that these could amplify vascular inflammation and thus contribute to lesion progression by further recruitment of inflammatory cells expressing P-selectin glycoprotein ligand (PSGL), which binds CD62P (P-selectin) (29;30). Since high CO AnV+CD62E+ EMP were also detected, almost certainly derived from activated coronary endothelium, it is likely that E-selectin-PSGL binding could further mediate the interaction between inflammatory cells and activated endothelial cells and contribute to lesion progression (270). In addition, the high CO NMP we observed could further amplify vascular inflammation since NMP can bind activated endothelium and contribute to further endothelial activation (271).

As well as amplifying inflammation, MP are also prothrombotic (76). The finding of high CO TF+MMP could implicate their role in clot propagation, since higher levels were observed in STEMI and NSTEMI patients than those with SA. Expressed TF on activated monocytes results in the release of TF+MMP, which promote the clotting cascade (77). Furthermore, irrespective of TF, AnV+MP are themselves prothrombotic (272) and were significantly elevated in the CO of STEMI patients. Thus MP, either total AnV+, and/or TF+ subpopulations could contribute to clot progression in ACS.

Min et al have recently demonstrated locally increased MPs in coronary arteries of patients with STEMI compared to controls. The data from this study now also strongly suggests that MP release in other symptomatic patients with CHD (NSTEMI and stable angina) is mainly locally driven, since CO MP levels at the site of the complicated plaque were higher than systemic (RA) MP levels (110;273). This observation does not, however, preclude the possibility that MP release also takes place in the systemic circulation since atheromatous vascular disease is usually widespread. That said, these findings support the hypothesis that the complicated atheromatous plaque is the predominant source of the circulating microparticles, and that a topographic pattern of microparticle expression appears to exist depending on where you sample the microparticles i.e. the closer to the culprit lesion, the higher the level of microparticles.

A time dependant mode of microparticle expression appears to exist from this study. There was a negative correlation between the index event to PCI time and total MP, PMP, EMP AnV+MP release in the CO in the NSTEMI group. This suggests that, in line with previous studies (112;117), there is a peak in MP release during the first 24-48 hours and thereafter MP levels fall. Arguably, earlier sampling (on admission) of

the NSTEMI patients would have yielded an expression of MP levels similar to that of the STEMI patients. Antiplatelet and anti-coagulation therapy could have in addition influenced MP production.

This study has a number of limitations. Firstly, the study sample was relatively small making the statistical analysis and interpretation of correlations less reliable in addition to statistical error. Also the presence of selection bias cannot be excluded. Secondly, it is difficult to obtain blood samples exclusively from the coronary artery with aspiration catheters therefore contamination with blood from non-affected coronaries or the systemic circulation cannot be excluded. The effects of antiplatelet and anticoagulant therapy on MP production, even though standardised for all patient groups, was not fully analysed and needs further study. In addition sampling of the infarct related artery proximally to the culprit lesion or sampling of a bystander coronary artery could have strengthened my hypothesis that the complicated atheromatous coronary plaque is the predominant source of microparticle release.

3.6 Conclusions

This study demonstrates higher MP levels in patients with STEMI versus those with NSTEMI or SA. In addition, higher MP levels were observed in the CO versus RA in all three patient groups, strongly suggesting that the source of these MP is the culprit atheromatous lesion. We cannot yet conclude that these MP have a direct pathogenic role; however, data from other studies have demonstrated important pro-inflammatory and prothrombotic effects of MP. This leads us to speculate that AnV+ microparticle levels may be indicative of coronary artery plaque instability, and could directly contribute to this pathological process.

Chapter 4 Microparticle expression in patients undergoing percutaneous coronary intervention for acute coronary syndrome and stable coronary heart disease: correlation with intracoronary inflammation and myocardial necrosis

4.1 Summary

Introduction and aims

Inflammation plays a crucial role in the pathophysiology of acute coronary syndrome (ACS). Mounting evidence indicates that microparticles (MPs) released from activated cells are potent pro-coagulant molecules contributing to endothelial dysfunction and modulating inflammatory processes. The aim of this study was to investigate the relationship between MP expression, markers of inflammation, and myocardial necrosis in patients with ACS or stable angina (SA).

Methods

Forty eight patients with coronary heart disease (37 with ACS and 11 with SA) treated with coronary percutaneous coronary interventions (PCI) were studied. Blood samples were aspirated sequentially from the right atrium (RA) and the coronary artery (CO) (distal to the culprit lesion) at the time of PCI. AnnexinV+MPs (AnV+MPs) from platelet poor plasma were measured using fluorescent monoclonal antibodies and flow cytometry. AnV+ MPs in the CO and RA were expressed as medians per ml of plasma with interquartile range. Markers of inflammation: hs-CRP, IL-6, TNF-α, serum amyloid A (SAA) and myocardial injury (Troponin T) were measured using ELISA.

Results

Total and cell specific AnV+MP expression was higher in the ACS versus the SA group in both the CO and RA. CO AnV+MP levels were higher than the RA in both groups. In the ACS group all inflammatory markers except IL-6 were higher in the RA vs CO. There were no differences in the inflammatory markers between patients with ACS or SA (either in the RA or the CO). In the CO and RA of the ACS group, but not the SA group, markers of inflammation correlated positively with AnV+MP.

In the CO, SAA correlated with total AnV+MP (r=0.5; p=0.01), platelet MP (r=0.5; p=0.02) and endothelial MP (EMP) (r=0.6; p=0.005). IL-6 also correlated positively with total AnV+ MP (r=0.6; p=0.004), AnV+EMP (r=0.4, p=0.04) and neutrophil derived AnV+MP (r=0.5; p=0.008). In the RA of the ACS group IL-6 correlated with AnV+EMP (r=0.5; p=0.03) and tissue factor positive AnV+MP (r=0.6, p=0.01). Troponin T levels correlated positively with platelet MP in both CO and RA of the ACS group (r=0.4 for both; p=0.04 and p=0.03 for CO and RA respectively).

Conclusions

During ACS inflammatory markers correlated positively with local CO AnV+MP levels and also to a lesser extent with peripheral circulating AnV+MP. This may suggest a pathogenic role for MPs as effectors of the inflammatory response and cellular injury in ACS.

4.2 Introduction and aims

Inflammation plays a pivotal role in the pathogenesis of coronary heart disease (CHD) and acute coronary syndrome (ACS) (123). Inflammation controls not only the evolution of atheromatous plaque but also its propensity for rupture or erosion, by altering the matrix composition (2). In addition, inflammation, through the activation and differentiation of the inflammatory cells involved, increases the thrombogenicity of the plaque's interior by promoting tissue factor bearing microparticle (MP) release with subsequent thrombus formation of the ruptured/eroded plaque (55;62). Patients with diseases associated with chronic inflammation such as diabetes mellitus and metabolic syndrome, or chronic inflammatory autoimmune diseases (such as psoriatic or rheumatoid arthritis) are at high risk of developing CHD (274). Inflammatory activity has been assessed mainly by measuring the levels of inflammatory markers like C-reactive protein (CRP), serum amyloid antigen (SAA) or pro-inflammatory cytokines such as TNF-α (tumour necrosis factor α) and IL-6 (Interleukin-6). It has also been shown that increased levels of inflammatory markers are associated with adverse outcomes in patients with ACS (171;204;275;276). Whether the increased levels of these inflammatory markers have any causal relationship with the pathogenesis of ACS (277) is not clear. For instance, elevated CRP levels do not correlate with the extent and burden of CHD (171;172). Furthermore, whether the inflammatory markers originate solely from the site of plaque rupture, or represent a systemic response is still a matter of debate. It has been shown that IL-6 is increased locally in the coronary artery compared with the systemic arterial circulation in patients with ACS (278;279). Therefore assessment of inflammatory markers in the peripheral circulation may not truly reflect the burden of the intracoronary inflammatory process. Even though it is

believed that inflammation is a systemic effect of ACS and inflammatory markers are mostly synthesised in the liver, the above evidence supports the concept of an intracoronary production of inflammatory markers, and local paracrine inflammatory effects close to the culprit lesion.

Mounting evidence indicates that microparticles (MP) released from activated cells and platelets are potent pro-coagulant and pro-inflammatory molecules that may contribute to thrombus formation (55), as well as directly causing endothelial activation and dysfunction (54). Locally produced MPs may therefore participate in the local coronary inflammatory processes involved in ACS (68;268;280), although the exact mechanisms are currently poorly understood. Several clinical studies have shown a correlation between circulating MP levels and all-cause mortality, cardiovascular mortality, markers of myocardial damage, and indices of myocardial dysfunction (108;110-112). Previous reports have also suggested a link between circulating pro-inflammatory cytokines and MPs in health and disease. For instance, plasma IL-6 correlated with plasma level of platelet MP in healthy volunteers (118). Furthermore, endothelial and platelet derived MPs correlated with circulating hs-CRP levels in patients with ACS undergoing percutaneous coronary intervention (PCI) (112). Similarly, we have previously demonstrated that tissue factor positive MPs in patients with ST elevation myocardial infarction (STEMI) correlate strongly with circulating IL-6 levels (manuscript in preparation).

I hypothesized that: 1. Local inflammatory processes in the coronary including MP production are important in the pathogenesis of ACS and 2. Local levels of inflammatory mediators including MP correlate with extent of myocardial necrosis.

The aim of this study was therefore to explore the relationship between local

coronary and systemic MP levels, circulating markers of inflammation, and extent of myocardial necrosis in patients with ACS or stable angina undergoing PCI.

4.3 Patients and methods

4.3.1 Study population

Thirty seven patients with ACS (23 patients with STEMI, 14 with NSTEMI) and 11 patients with stable angina (SA) treated with percutaneous coronary intervention (PCI) were recruited for the study. The diagnosis of ACS was based on the history of chest pain with or without ischemic ECG changes and a troponin T value > 0.03 ng/l (261). Patients with STEMI underwent primary PCI and patients with NSTEMI had PCI 3-5 days after the hospital admission. SA patients had been reviewed in the cardiology outpatient clinic with a diagnosis of angina pectoris based on typically sounding chest pain and a positive non-invasive test such as exercise tolerance test, stress echocardiography or myocardial perfusion scan. SA patients admitted electively for PCI. The decision for diagnostic angiography was made by a cardiologist as part of routine clinical care. Informed consent was obtained prior to the procedure from all recruited patients outside the cardiac catheterisation laboratory. Ethical permission was granted by the local ethics committee of the Royal Free Hospital, and all participants provided fully informed written consent.

4.3.2 Exclusion criteria

The exclusion criteria have been described in Chapter 2 (Paragraph 2.1.4)

4.3.3 Coronary angiography, PCI and blood sampling

The methodology of coronary angiography and angioplasty has been described in chapters 2 (section 2.1) and 3 (section 3.3).

4.3.4 Preparation of platelet poor plasma (PPP) MP Cytometric identification and ELISA

3.5 mls of whole blood was collected into bottles containing 3.2% trisodium citrate (Becton Dickinson). Platelet poor plasma was obtained by immediate centrifugation of the whole blood at 5000G for 5 minutes twice. PPP was then stored at –80^{o} Celcius for later analyses of microparticle estimation and enzyme-linked immunosorbent assay (ELISA) studies (see below).

4.3.5 MP identification and characterization using flow cytometry

The methodology of MP identification and characterization has been described above in chapters 2 (paragraph 2.3) and 3 (paragraph 3.3).

4.3.6 Estimation of TNF-α, IL-6, hs-CRP and SAA

TNF-α and IL-6 levels were measured using a quantitive sandwich enzyme immunoassay technique using a kit from R&D systems (Europe). CRP was measured in plasma using a high sensitivity automated microparticle enhanced latex turbidimetric immunoassay (COBAS MIRA; Roche Diagnostics GmbH). SAA was measured in plasma by latex nephelometry (BNII autoanalyser; Dade Behring, Marburg, Germany).

4.3.7 Statistical analyses

Continuous variables were expressed as medians with interquartile range (IQR). Categorical variables were expressed as percentages with 95% confidence intervals (95% CIs). Comparisons between the two groups and the two (coronary and right atrium) compartments were performed using the Mann-Whitney U test. Fisher exact test was used for proportions and Spearman factor r for correlations. Differences with a 2-sided p value <0.05 were considered to be statistically significant. All statistical analyses were performed with GraphPad Prism 5 statistical software.

4.4 Results

The demographics and baseline characteristics of the study population are shown in the table 4.1. Briefly, the majority of the study population was male (79%). The SA patients were more likely to have a medical history of hypertension compared with the ACS group (p=0.01). Otherwise there were no significant differences between patients with ACS and SA.

	ACS	SA	p value
Number of patients (n)	37	11	
Age (mean), years	60	61	0.8
Male	29	9	0.7
Female	8	2	0.7
Risk Factors			
Hypertension	14	9	0.01
Diabetes	9	2	0.9
Dyslipidaemia	19	4	0.4
Smoking	13	2	0.5
Family History of CHD	17	4	0.7
Angiographic characteristics			0.7
1 vessel disease	25	6	
2 vessel disease	7	3	
3 vessel disease	5	2	
Culprit vessel			0.3
Left Anterior Descending	14	6	
Left Circumflex	7	0	
Right Coronary	16	5	

Table 4.1. Baseline characteristics, demographics and angiographic data of the acute coronary syndrome (ACS) and stable angina (SA) groups. Fisher exact test was used for comparison of proportions between the groups.

4.4.1 High levels of local CO AnV+MP are detected in patients with ACS

Table 4.2 summarises the CO MP levels between the two patient groups. Total AnV+ MP in the CO were higher in the ACS population compared with SA (p=0.04). AnV+CD42a+ PMP and AnV+CD42a+CD62P+ PMP were also higher in the ACS group compared with the SA group (p=0.03 and p=0.0008 respectively); Fig 4.1a. Also all types of AnV+ EMPs were higher in the ACS patients compared with the SA patients, Fig 4.1b. NMP and TF+ MMP were also higher in the ACS population compared with the SA group, Fig 4.1c.

	CO			RA		
	ACS	SA		ACS	SA	
	Median (IQR)	Median (IQR)	p value	Median (IQR)	Median (IQR)	p value
Total AnV+ MP	5.22 (3.11-8.22)	2.51 (0.91-5.75)	0.04	1.4 (0.67-4.02)	1.11 (0.14-1.49)	0.1
CD42a+AnV+ PMP	1.61 (0.89-3.23)	0.71 (0.20-1.28)	0.03	0.67 (0.24-2.56)	0.28 (0.02-0.60)	0.02
CD62P+CD42a+AnV+ PMP	0.4 (0.2-1.7)	0.08 (0.02-0.15)	0.0008	0.17 (0.06-0.61)	0.03 (0.001-0.04)	0.001
CD62E+CD42a-AnV+ EMP	0.65 (0.225-1.06)	0.17 (0.07-0.67)	0.03	0.12 (0.03-0.42)	0.04 (0.009-0.07)	0.02
CD54+CD42a-AnV+ EMP	0.42 (0.18-0.76)	0.11 (0.05-0.18)	0.001	0.07 (0.02-0.15)	0.009 (0.004-0.04)	0.008
CD105+CD42a-AnV+ EMP	0.76 (0.29-1.01)	0.38 (0.07-0.77)	0.04	0.11 (0.04-0.38)	0.07 (0.008-0.18)	0.1
CD31+CD42a-AnV+ EMP	0.51 (0.31-1.15)	0.24 (0.10-0.33)	0.005	0.08 (0.02-0.26)	0.05 (0.003-0.09)	0.09
CD66b+CD42a-AnV+ NMP	0.14 (0.07-0.39)	0.04 (0.03-0.20)	0.04	0.08 (0.02-0.12)	0.01 (0.002-0.02)	0.004
CD14+TF+AnV+ MMP	0.17 (0.07-0.27)	0.07 (0.03-0.15)	0.02	0.07 (0.02-0.18)	0.03 (0.005-0.05)	0.02

Table 4.2. Differences of the total AnV+MP and MP subpopulations (*10^6/ml of plasma) between the acute coronary syndrome (ACS) and stable angina (SA) groups in the coronary (CO) and the right atrium (RA). Statistical analysis performed using the Mann-Whitney U test.

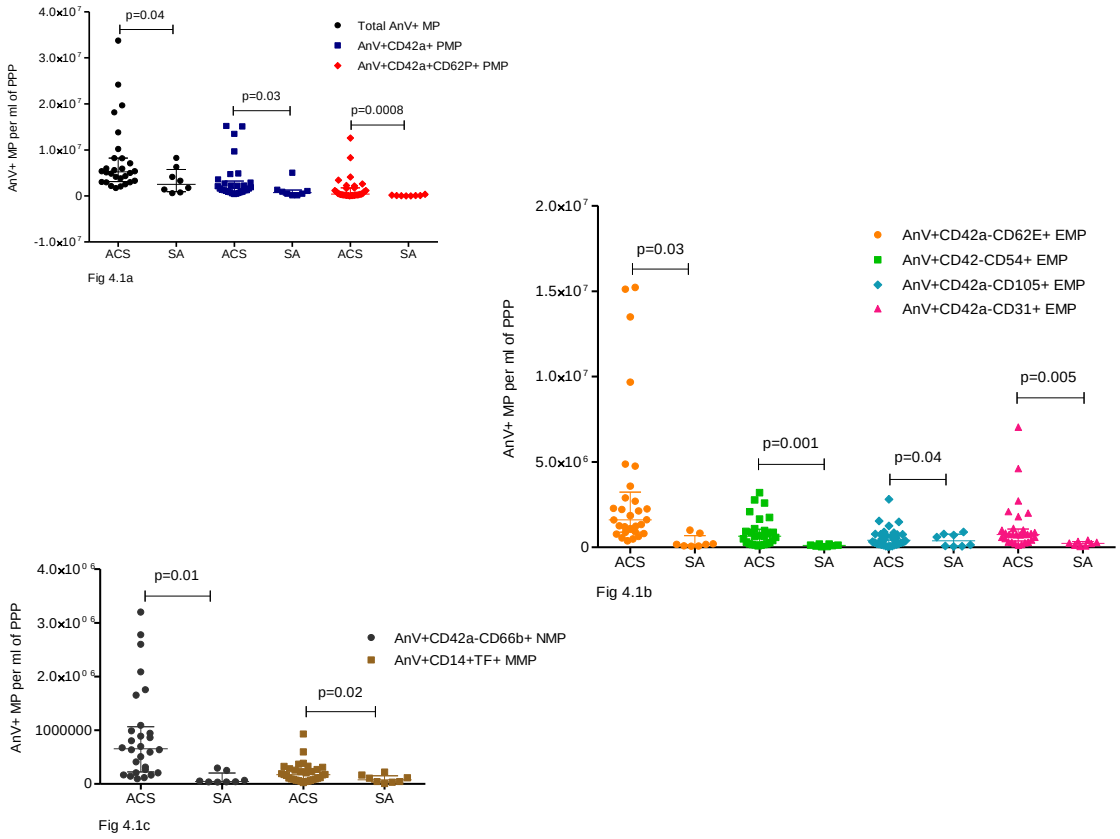


Fig 4.1 Differences of the total AnV+MP and MP subpopulations in the coronary artery (CO) between the two groups, acute coronary syndrome (ACS) and stable angina (SA). Statistical analysis performed using the Mann-Whitney U test.

4.4.2 Platelet, endothelial and leucocyte MP levels are higher in the systemic (RA) circulation of patients with ACS compared with SA

Table 4.2 summarises the RA MP levels between the two patient groups. There was no difference in the total AnV+ MP levels in the RA in the ACS group compared with the SA group. However, AnV+CD42a+ PMP and AnV+CD42a+CD62P+ PMP were higher in the ACS group compared with the SA group (p=0.02 and p=0.001 respectively); Fig 4.2a. Also AnV+ EMPs (CD62E+ and CD54+ EMP) were higher in the ACS patients compared with the SA patients, Fig 4.2b. NMP and TF+ MMP were also higher in the ACS population compared with the SA group, Fig 4.2c.

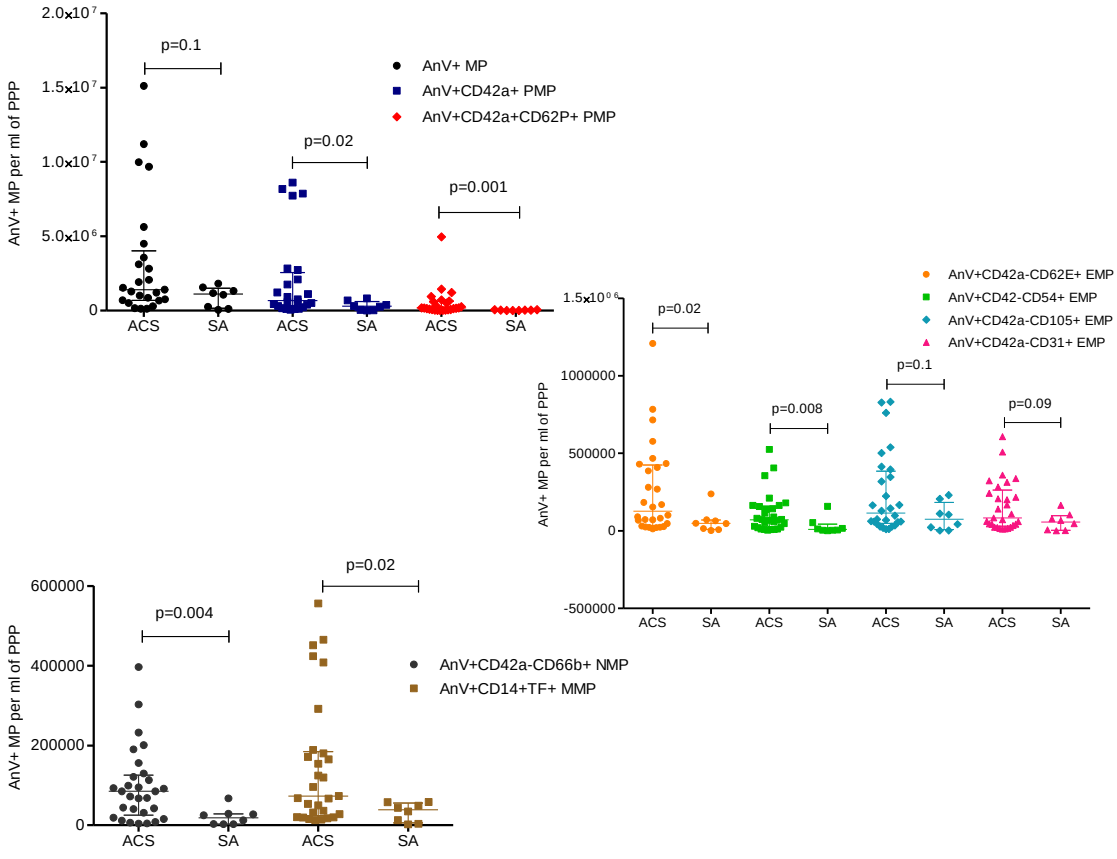


Fig 4.2. Differences of the total AnV+MP and MP subpopulations in right atrium (RA) between the acute coronary syndrome (ACS) and stable angina (SA) groups. Statistical analysis performed using the Mann-Whitney U test

4.4.3 High coronary AnV+MP levels and IL-6 are observed in ACS patients

The absolute numbers of the total AnV+MP and MP subpopulations were higher in the CO versus the right atrium in both patient groups (ACS and SA), but particularly so for those with ACS as shown in table 4.3.

	ACS			SA		
	CO	RA		CO	RA	
	Median (IQR)	Median (IQR)	p value	Median (IQR)	Median (IQR)	p value
Total AnV+ MP	5.22 (3.11-8.22)	1.4 (0.67-4.02)	0.0002	2.51 (0.91-5.75)	1.11 (0.14-1.49)	0.03
CD42a+AnV+ PMP	1.61 (0.89-3.23)	0.67 (0.24-2.56)	0.01	0.71 (0.20-1.28)	0.28 (0.02-0.60)	0.06
CD62P+CD42a+AnV+ PMP	0.4 (0.2-1.7)	0.17 (0.06-0.61)	0.006	0.08 (0.02-0.15)	0.03 (0.001-0.04)	0.02
CD62E+CD42a-AnV+ EMP	0.65 (0.225-1.06)	0.12 (0.03-0.42)	<0.0001	0.17 (0.07-0.67)	0.04 (0.009-0.07)	0.007
CD54+CD42a-AnV+ EMP	0.42 (0.18-0.76)	0.07 (0.02-0.15)	<0.0001	0.11 (0.05-0.18)	0.009 (0.004-0.04)	0.01
CD105+CD42a-AnV+ EMP	0.76 (0.29-1.01)	0.11 (0.04-0.38)	<0.0001	0.38 (0.07-0.77)	0.07 (0.008-0.18)	0.02
CD31+CD42a-AnV+ EMP	0.51 (0.31-1.15)	0.08 (0.02-0.26)	<0.0001	0.24 (0.10-0.33)	0.05 (0.003-0.09)	0.007
CD66b+CD42a-AnV+ NMP	0.14 (0.07-0.39)	0.08 (0.02-0.12)	0.002	0.04 (0.03-0.20)	0.01 (0.002-0.02)	0.007
CD14+TF+AnV+ MMP	0.17 (0.07-0.27)	0.07 (0.02-0.18)	0.03	0.07 (0.03-0.15)	0.03 (0.005-0.05)	0.2

Table 4.3. Differences of the total AnV+MP and MP subpopulations (*10^6/ml of plasma) between coronary (CO) and right atrium (RA) in the acute coronary syndrome (ACS) and stable angina (SA) groups. Statistical analysis performed using the Mann-Whitney U test

Table 4.4 summarizes the differences between the inflammatory markers in the CO and RA in the two patient groups. In patients with ACS IL-6 was higher in the CO vs the RA (p=0.01); in contrast, SAA, hs-CRP and TNF-α were higher in the RA vs the CO (p=0.003, p=0.0005 and p=0.03 respectively). In the SA patients there were no differences in any of the inflammatory markers between the CO and RA.

There were also no differences in the inflammatory marker levels in the CO between ACS and SA patients. However, hs-CRP and SAA levels in the RA of the ACS group were higher than the SA group (p=0.03 for both hs-CRP and SAA respectively).

TNF-α and IL-6 levels in the RA did not differ between ACS and SA groups.

	ACS			SA		
	RA	CO		RA	CO	
	Median (IQR)	Median (IQR)	p value	Median (IQR)	Median (IQR)	p value
IL-6 (pg/mL)	5.03 (1.53-13.06)	6.3 (3.7-13.6)	0.01	2.29 (1.53-6.26)	2.01 (0.38-16.1)	ns
SAA (ml/L)	5.5 (2.6-12.8)	3.3 (1.8-9.5)	0.003	2.5 (1.6-2.8)	2.4 (1.1-2.72)	ns
hs-CRP (mg/L)	2.8 (0.9-6.1)	2.1 (0.77-5.82)	0.0005	0.8 (0.7-2.1)	0.8 (0.57-1.55)	ns
TNF-α (pg/ml)	18.77 (7.4-33.92)	14.62 (5.9-28.86)	0.03	22.77 (8.61-38.98)	9.16 (6.92-30.83)	ns

Table 4.4 Differences of the inflammatory markers (medians (IQR)) between the acute coronary syndrome (ACS) and stable angina (SA) patients in the coronary (CO) and right atrium (RA). Statistical analysis performed using the Wilcoxon test. IL-6 (interleukin 6), SAA (serum amyloid A), TNF-α (tumour necrosis alpha) and hs-CRP (high sensitivity C-reactive protein).

4.4.4 Coronary inflammatory markers correlate with MP levels and troponin T in ACS

In the CO markers of inflammation correlated moderately positively with total AnV+MP, PMP, EMP and NMP. SAA correlated with total AnV+MP (r=0.5; p=0.01), AnV+CD42a+PMP (r=0.5; p=0.02), AnV+CD42a-CD62E+EMP (r=0.6; p=0.005) and AnV+CD105+EMP (r=0.7; p=0.002), Fig 4.3. Similarly, IL-6 levels in the CO correlated positively with total AnV+MP, AnV+CD42a-CD62E+EMP and AnV+CD66b+NMP. There was also a positive correlation between troponin T and CO AnV+CD62P+PMP (r=0.4; p=0.03); Fig 4.3.

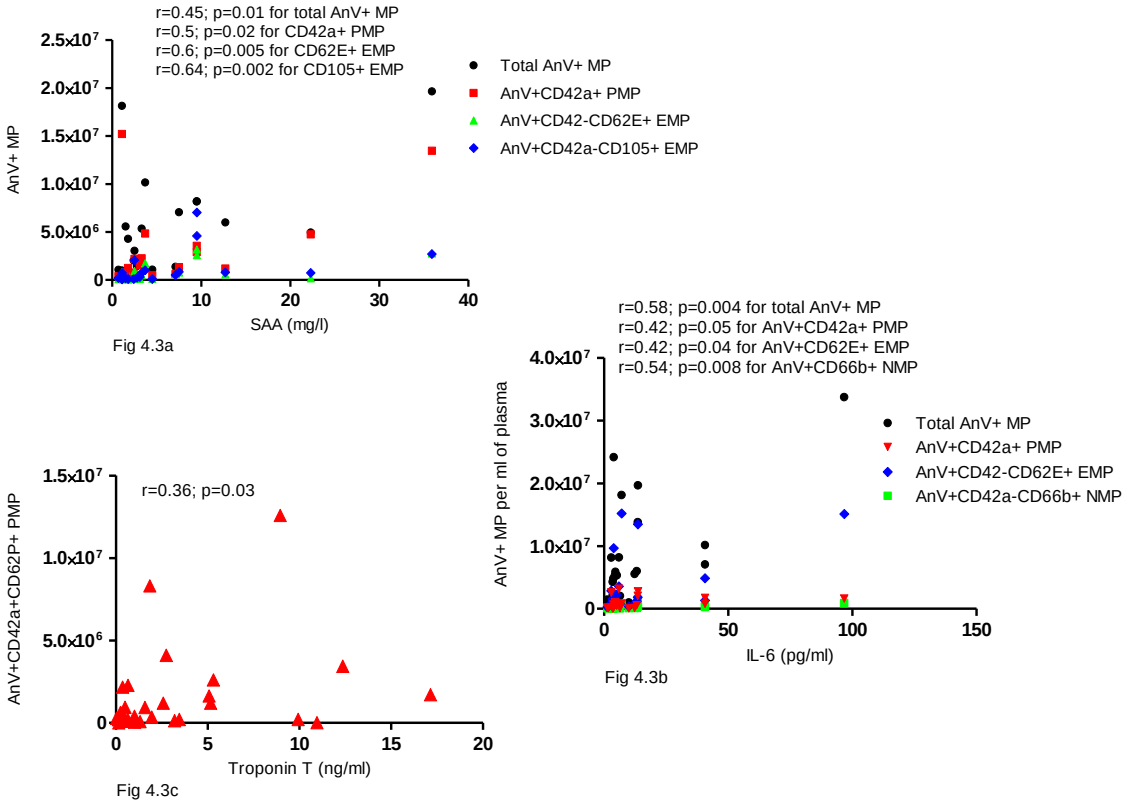


Fig 4.3 Positive correlation between AnV+MP and markers of inflammation (IL-6 and SSA) and myocardial injury in the coronary in the acute coronary syndrome (ACS) groups. Spearman factor r was used for correlations.

4.4.5 Right atrial IL-6 levels correlate with MP levels and troponin T in ACS

In the RA of the ACS group there was a moderately positive correlation between IL-6 and AnV+CD31+EMP (r=0.5; p=0.03) and AnV+TF+MMP (r=0.6; p=0.01). Troponin T levels correlated positively with RA AnV+CD62P+PMP (r=0.4; p=0.04), (Fig 4.4).

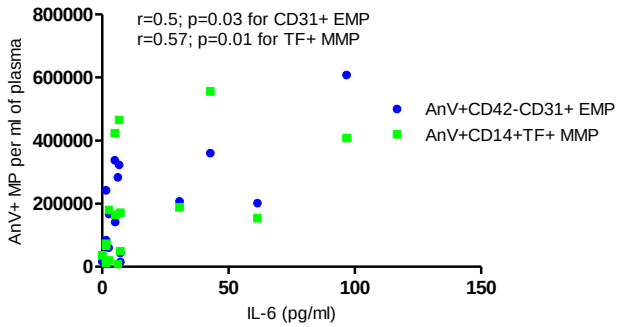


Fig 4.4a

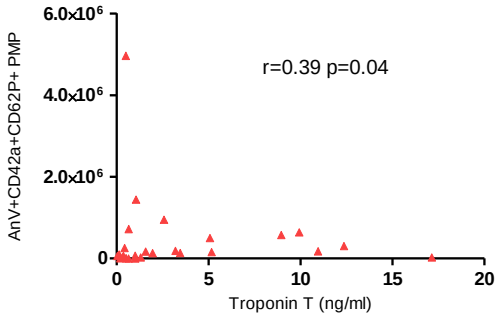


Fig 4.4b

Fig 4.4 Positive correlation between AnV+MP and markers of inflammation and myocardial injury in the right atrium (RA) in the acute coronary syndrome (ACS) groups. Spearman factor r was used for correlations.

In contrast to the above, in the SA patients markers of inflammation and troponin T levels did not correlate with total AnV+MP, or cell specific AnV+MP.

4.5 Discussion

The data from chapter 3 showed that high levels of AnV+MP occur in the coronary artery of patients with ACS and that levels of AnV+MP correlate with severity of the ischaemic lesion since the highest MPs levels were detected in the STEMI patients. Total AnV+MP and MP subpopulations were higher in ACS patients compared with SA patients in both the CO and RA. In particular, platelet, endothelial and leucocyte MP (NMP and MMP) levels were higher in the systemic (RA) circulation of ACS patients with compared with SA patients. CO IL-6 was also higher than RA IL-6 in ACS patients. Overall, coronary inflammatory markers correlated with MP levels and myocardial injury in ACS (as assessed by troponin T levels); in addition RA IL-6 levels correlated with MP levels and troponin T in ACS. Taken together, these results suggest that patients with more severe ACS have higher levels of local and

systemic inflammation, and higher levels of local and systemic circulating MP. Whilst these data suggest that these indices correlate with extent of myocardial injury, and that it is indeed likely that these are causally linked, I cannot yet infer from these data that a causal association is definite.

These data are in agreement with previous reports demonstrating that during ACS, circulating platelet and endothelial MPs are elevated (104-106). This study has also now demonstrated that the same is true for leukocyte (neutrophil and monocyte) MP, since higher levels were observed in ACS compared to SA patients. Furthermore, these data demonstrate that although the magnitude of MP expression occurs predominantly locally at the site of the culprit atheromatous plaque, there is also a significant systemic response with high levels of MP in the RA. The increased MP in the systemic circulation are likely to be derived the local culprit CO atheromatous lesion, with additional contribution from systemic release secondary to the systemic inflammatory response observed in the ACS patients.

In addition to changes in MP levels, the second important finding of this study is the differential expression of inflammatory markers in the CO and in the systemic circulation during ACS. In line with previous reports (278;279) the data from this study has shown that during ACS, local intracoronary IL-6 is increased compared with the systemic circulation. This IL-6 is likely to be derived from coronary endothelium (281), and/ or from plaque leucocytes. The rest of the inflammatory markers hs-CRP, SAA and TNF-α were higher in the peripheral circulation, which is unsurprising since CRP and SAA are produced and released from the liver; while TNF-α is produced from leukocytes and therefore is likely to have a predominant

(although not exclusive) systemic source. In the SA group there were no differences of the inflammatory markers between CO and RA. In addition, the higher levels of SAA and hs-CRP in the RA which has been observed in the ACS group compared with SA group are consistent with an exaggerated systemic inflammatory response in ACS patients.

A potentially important finding is the positive correlation between total AnV+ microparticles and MP subpopulation with markers of inflammation. Intracoronary inflammatory markers correlated with total AnV+ microparticles and MP subpopulations (including platelets, endothelial and leukocytes derived MP) whilst in the RA markers of inflammation correlated only with endothelial and monocyte derived TF+MMP. The fact that in SA the markers of inflammation did not correlate with MP formation could suggest a pivotal role of intracoronary inflammation as a driver of MP release from the culprit plaque in the pathogenesis of ACS. However, these data do not yet allow us to definitively prove a causal association.

The finding of this study, that SAA correlates with PMP and EMP may also provide a novel explanation for the previously reported pro-coagulant and pro-inflammatory amplification properties of SAA (190). The association between NMP and IL6 could be consistent with the previous suggestion that NMP can induce endothelial IL6 production by inducing endothelial cell activation (271). Furthermore, it has been shown that platelet MP deliver arachidonic acid to endothelial cells leading to the up-regulation of the intercellular adhesion molecule-1 (ICAM-1, or CD54) which mediates monocyte-endothelial cell adhesion (87). Ueba et al found that plasma IL-6 correlated with PMP levels in healthy Japanese volunteers (118). In addition

Biasucci et al reported a positive correlation between hs-CRP and microparticles in patients with ACS undergoing PCI 24 hours after the admission (112). Matsubara et al have shown that the increment of SAA across the coronary circulation correlated positively with the severity of coronary atherosclerosis (189). Higher adverse outcomes including myocardial infarction, revascularization and death in ACS patients with elevated SAA has also been previously reported (204).

Another important finding from this study is the positive correlation between p-selectin positive AnV+ PMP with marker of myocardial necrosis. Interestingly in both CO and RA, troponin T correlated with P-selectin positive PMP only, emphasizing the crucial role of platelet activation in the pathogenesis of ACS and myocardial damage during ACS. These observations could therefore suggest the pathogenic role of MP as effectors linking inflammatory and prothrombotic pathways in ACS.

The study limitations have been described in chapter 3 (paragraph 3.5) with the main one being the small number of patients. This can potentially explain the unexpected observation that there were no differences of the measured inflammatory markers in the coronary artery between ACS and stable angina patients.

Conclusions

Markers of 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. The interaction between activated platelets and monocytes with endothelial cells and the subsequent formation of AnV+ MP during ACS would be

compatible with a direct pathogenic link between inflammatory and prothrombotic pathways in the pathogenesis of ACS and myocardial necrosis. Future studies should now address potential MP mediated pathogenic mechanisms in ACS to investigate whether these are directly pathogenic, or merely epiphenomena.

Chapter 5 Microparticle expression in human coronary heart disease: relationship to platelet-monocyte interaction and platelet activation

5.1 Summary

5.2 Introduction and aims

5.3 Patients and methods

5.4 Results

5.5 Discussion

5.6 Conclusions

5.1 Summary

Introduction and aims

Platelets play a pivotal role in the pathogenesis of acute coronary syndrome (ACS). Mounting evidence indicates that microparticles, released from activated cells, are potent pro-coagulant and pro-inflammatory molecules. The aim of this study was to investigate the relationship between markers of platelet activation and microparticle (MP) expression in patients presenting with ACS or stable angina.

Methods

Forty eight patients with coronary heart disease (37 with ACS and 11 with stable angina (SA) treated with percutaneous coronary intervention (PCI) were recruited for the study. Blood samples were obtained sequentially from the right atrium (RA) and the coronary artery (CO) (distal to the culprit lesion). AnnexinV+MP (AnV+MP) and platelet-monocyte aggregates (PMA) were estimated using flow cytometry. Soluble p-selectin (s-P-selectin) was measured using ELISA.

Results

Total and cell specific AnV+MP were higher in the ACS versus the SA group in both the CO and RA. CO AnV+MP levels were higher than the RA levels in both groups. CD62P+PMA were higher in ACS vs the SA group in both CO ($p=0.0003$) and RA ($p=0.04$). S-P-selectin was also higher in ACS vs SA in the CO ($p=0.01$). In the CO and RA of the ACS group, but not the SA group, markers of platelet activation correlated positively with AnV+MP. In the CO s-P-selectin correlated positively with total AnV+MP ($r=0.61$; $p=0.001$), platelet derived AnV+CD42a+MP ($r=0.6$; $p=0.002$), endothelial derived AnV+CD62E+MP, ($r=0.62$; $p=0.001$) and neutrophil derived AnV+CD66b+MP ($r=0.54$; $p=0.006$). CD62P+PMA also correlated positively with platelet derived AnV+CD42a+MP and endothelial derived

AnV+CD62E+MP (r=0.5; p=0.04 for both). In the RA soluble P-selectin correlated positively with total AnV+MP (r=0.81; p=0.001), platelet derived AnV+CD42a+MP (r=0.77; p=0.003), endothelial derived AnV+CD62E+MP (r=0.78; p=0.002) and tissue factor positive AnV+CD66b+MP (r=0.72; p=0.007).

Conclusions

Markers of platelet activation correlated positively with total AnV+MP, platelet, endothelial and leukocyte derived MP, both at the site of the culprit lesion and in the systemic circulation during ACS. These novel observations suggest a potential important interaction between activated platelets, monocytes and endothelial cells via MP formation. Future studies are now required to explore how MP could be directly involved in the pathogenesis of ACS, since these could be important, novel therapeutic targets.

5.2 Introduction and aims

Platelets play a pivotal role in the pathogenesis of ACS, with or without ST elevation. Recent data has demonstrated the crucial role of platelets in the pathology of atherothrombosis (282;283). Atherosclerotic disease is a chronic inflammatory process involving complex interactions between immune (leukocytes) and non-immune cells (platelets and endothelial cells) (283). Many traditional risk factors for coronary heart disease (CHD) such as diabetes mellitus, hypertension and hyperlipidaemia are associated with endothelial dysfunction and endothelial cell activation (284-288).

Upon activation, endothelial cells secret P-selectin (CD62P) and von-Willebrand factor (vWF), both stored in Weibel-Palade bodies, mediating platelet-leukocyte and platelet-endothelial cell interactions respectively. In addition E- selectin (CD62E) is expressed early in the process of atherosclerosis on the endothelial cell surface. The P-selectin-PSGL-1 bond leads to platelet monocyte aggregate (PMA) formation, which is a sensitive marker of platelet activation (21). Leukocytes also interact with endothelial cells via the P-selectin-PSGL-1 pathway facilitating the inflammatory process within the vessel wall (10;18;289). This process of leukocyte rolling on the activated endothelial cells activates the leukocyte integrin Mac-1, a process mediated by PAF-4 (290). The above enhance the firm leukocyte adhesion to the endothelial cells through binding to fibrinogen, a molecule that is also bound to platelets via the integrin IIb/IIIa (291). This leukocyte-endothelial cell interaction and adhesion leads to further endothelial activation and expression of intracellular and vascular adhesion molecules (ICAM (CD54) (87) and VCAM (CD106) respectively (135). P-selectin undergoes enzymatic cleavage at the junction between the n-terminal portion and the

trans-membrane domain leading to release of the N-terminal domain in the circulation which represents the soluble P-selectin (s-P-selectin) (292;293). It has been shown that s-P-selectin has the same biological activities as the P-selectin attached to the cells (294). During the adhesion to endothelium, platelets become activated and, in addition to pro-thrombotic molecules, release a variety of pro-inflammatory cytokines such as P-selectin, CD40L, RANTES protein (CCL5) and platelet factor 4 (PAF-4 or CCL4) (295).

It is obvious from the above that platelets are important modulators of the early and late stages of atherosclerosis, ultimately including atherothrombosis, the hallmark of ACS (267;296;297). Activated and/ or apoptotic cells (platelets, endothelium and leukocytes) release microparticles (MP) with important pro-thrombotic and pro-inflammatory effects (55;271;298). Consequently, mounting evidence indicates that MP may directly contribute to the pathogenesis of ACS by providing an important link between pathological thrombogenicity and vascular inflammation (299;300).

I therefore hypothesized that: 1) markers of platelet activation and MP expression in patients with ACS would be higher in the coronary versus the systemic circulation; and 2) markers of platelet activation may correlate causally with microparticle levels. The aims of this study were therefore to compare coronary artery and systemic circulating levels of platelet activation markers, and MPs in patients with symptomatic ACS or SA treated with PCI.

5.3 Patients and methods

5.3.1 Study population

Thirty seven patients with ACS [23 patients with ST segment elevation myocardial infarction (STEMI), 14 with non-ST segment elevation myocardial infarction (NSTEMI)] and 11 patients with stable angina (SA) treated with percutaneous coronary intervention (PCI) recruited for the study. The study population has been described in sections 3.3.1 (chapter 3) and 4.3.1 (chapter 4).

5.3.2 Exclusion criteria

The exclusion criteria have been described in Chapter 2 (Paragraph 2.1.4)

5.3.3 Coronary angiography, PCI and blood sampling

The coronary angiography and angioplasty procedures have been extensively described in chapters 2 (2.1) and 3 (section 3.3).

5.3.4 Preparation of whole blood for PMA Cytometric identification

The methodology of PMA cytometric identification and analysis has been described in chapter 2 (section 2.2).

5.3.5 Preparation of platelet poor plasma (PPP) MP Cytometric identification and ELISA

3.5 mls of whole blood was collected into bottles containing 3.2% trisodium citrate (Becton Dickinson). Platelet poor plasma was obtained by immediate centrifugation of the whole blood at 5000G for 5 minutes twice. PPP was then stored at –80^{o} Celcius for later analyses of microparticle estimation and enzyme-linked immunosorbent assay (ELISA) studies (see below).

5.3.6 MP identification and characterization using flow cytometry

MPs were identified by flow cytometry as previously described in chapter 2 (section 2.3), chapter 3 (section 3.3) and chapter 4 (section 4.3).

5.3.7 Estimation of soluble P-selectin (s P-selectin)

Soluble p-selectin was measured using a quantitive sandwich enzyme immunoassay (ELISA) technique using a kit from R&D systems (Europe) as described in chapter 2 (section 2.4.2.3).

5.3.8 Statistical analyses

Continuous variables were expressed as medians with interquartile range (IQR). Categorical variables were expressed as percentages with 95% confidence intervals (95% CIs). Comparisons between the two groups and the two (coronary and right atrium) compartments were performed using the Mann-Whitney U test. Fisher exact test was used for proportions and Spearman factor r for correlations. Differences with a 2-sided p value <0.05 were considered to be statistically significant. All statistical analyses were performed with GraphPad Prism 5 statistical software.

5.4 Results

The demographics and baseline characteristics of the study population are shown in the table 4.1, section 4.4 (chapter 4).

5.4.1 High levels of local CO AnV+MP are detected in patients with ACS

Table 4.2 in chapter 4 summarises the CO MP levels between the two patient groups. Total AnV+MP, AnV+PMP, AnV+EMPs, AnV+NMP and TF+MMP in the CO were higher in the ACS population compared with SA (Figure 4.1, chapter 4).

5.4.2 Platelet, endothelial and leucocyte MP levels are higher in the systemic (RA) circulation of patients with ACS compared with SA

Table 4.2 in chapter 4 summarises the RA MP levels between the two groups. There was no difference in the total AnV+ MP level in the RA in the ACS group compared with SA. However AnV+PMP, AnV+EMPs, AnV+NMP and TF+MMP in the CO were higher in the ACS population compared with SA (Figure 4.2, chapter 4).

5.4.3 High CD62P+PMA (in both CO and RA) and high CO s-P-selectin were observed in the ACS group.

Figure 5.1 illustrates the total PMA and CD62P+PMA in the two groups. CD62P+PMA were higher in the ACS group vs the SA group in both the CO ($p=0.0003$) and RA ($p=0.04$). Total PMA in the CO and RA did not differ between ACS and SA. Also, CD62P+PMA in the CO were higher vs the RA in the ACS group ($p=0.001$) but not in the SA group. There was no difference between total PMA in the CO vs the RA in both groups.

Coronary, but not RA, s-P-Selectin was higher in the ACS group vs the SA (Fig 3). There was no difference between CO and RA s-P-selectin in both groups.

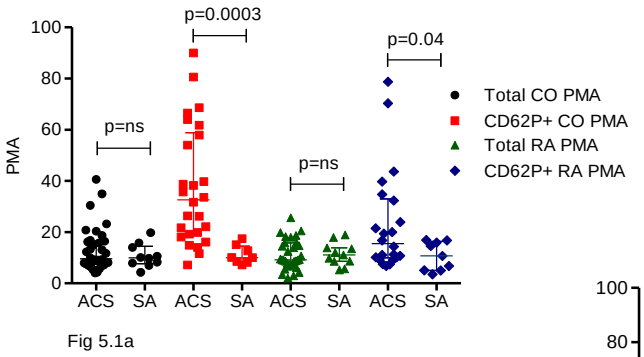


Fig 5.1a

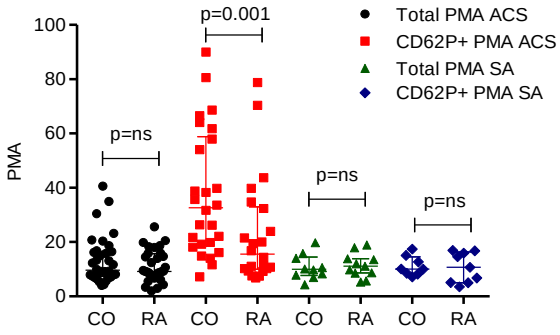


Fig 5.1b

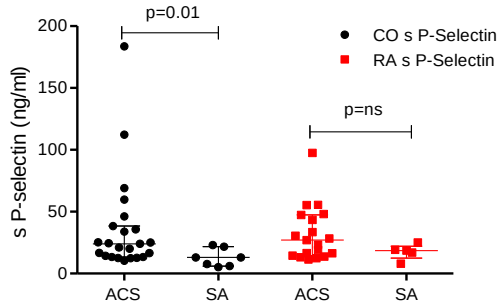


Fig 5.1c

Figure 5.1. Differences in total PMA, CD62P+PMA and soluble p-selectin in the acute coronary syndrome (ACS) and stable angina (SA) groups. Total PMA were expressed as percentage of the total monocytes. P-selectin positive PMA (CD62P+PMA) were expressed as percentage of the total PMA. Statistical analysis performed using the Mann-Whitney test.

5.4.4 Coronary AnV+MP correlate with markers of platelet activation in ACS

In the ACS group markers of platelet activation in the coronary artery correlated moderately positive with total AnV+MP, PMP, EMP and NMP. Soluble p-selectin correlated with total AnV+MP (r=0.61; p=0.001), AnV+CD42a+PMP (r=0.6; p=0.002) and AnV+CD42a+CD62P+PMP (r=0.64; p=0.0009) Table 5.1. Similarly there was a moderate positive correlation between soluble p-selectin with AnV+CD62E+EMP, AnV+CD105+EMP, AnV+CD54+EMP and AnV+CD31+EMP (r=0.62; p=0.001, r=0.56; p=0.005, r=0.48; p=0.02 and r=0.61;

p=0.001 respectively). There was also a positive correlation between CD62P+PMA and AnV+CD42a+PMP (r=0.5; p=0.04), AnV+CD42a-CD62E+EMP (r=0.5; p=0.04) and AnV+CD42a-CD31+EMP (r=0.5; p=0.03) Table 5.1.

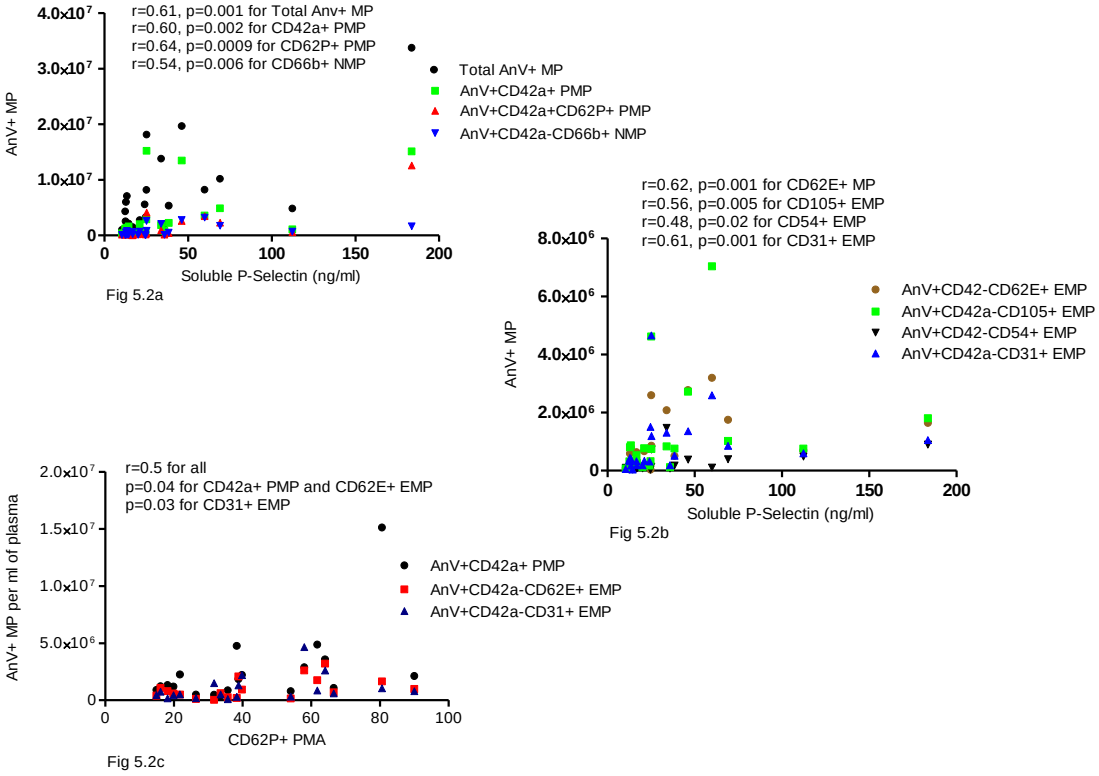


Fig 5.2. Positive correlation between AnV+MP and markers of platelet activation (platelet monocyte complexes (PMA) and soluble P-Selectin) in the coronary (CO) in the acute coronary (ACS) group. Spearman factor r was used for correlations.

Coronary samples	Soluble P-selectin		CD62P+ PMA	
	Spearman r	p value	Spearman r	p value
Total AnV+ MP	0.61	0.0016	0.28	0.230
CD42a+ MP	0.60	0.0020	0.46	0.044
CD62P+ MP	0.64	0.0009	0.40	0.089
CD62E+ MP	0.62	0.0016	0.46	0.046
CD66b+ MP	0.54	0.0067	0.21	0.374
CD54+ MP	0.48	0.0202	0.15	0.513
CD105+ MP	0.56	0.0052	0.39	0.096
CD31+ MP	0.61	0.0018	0.49	0.030
TF+ MP	0.22	0.3034	0.008	0.970

Table 5.1. Spearman correlations between AnV+MP and markers of platelet activation (platelet monocyte complexes (PMA) and soluble P-Selectin) in the coronary (CO) in the acute coronary syndrome (ACS) group.

5.4.5 Right atrial soluble P-selectin correlate with MP levels in ACS

In the RA of the ACS group there was a strong positive correlation between soluble p-selectin and total AnV+MP, AnV+CD42a+PMP and AnV+CD42a+CD62P+PMP. (r=0.81; p=0.001, r=0.77; p=0.003 and r=0.69; p=0.01 respectively) (Fig 5.3) There was also a strong correlation between soluble p-selectin and the EMP (Fig 5.3). AnV+TF+MMP correlated positively with soluble P-selectin r=0.72; p=0.007 (Fig 5.3).

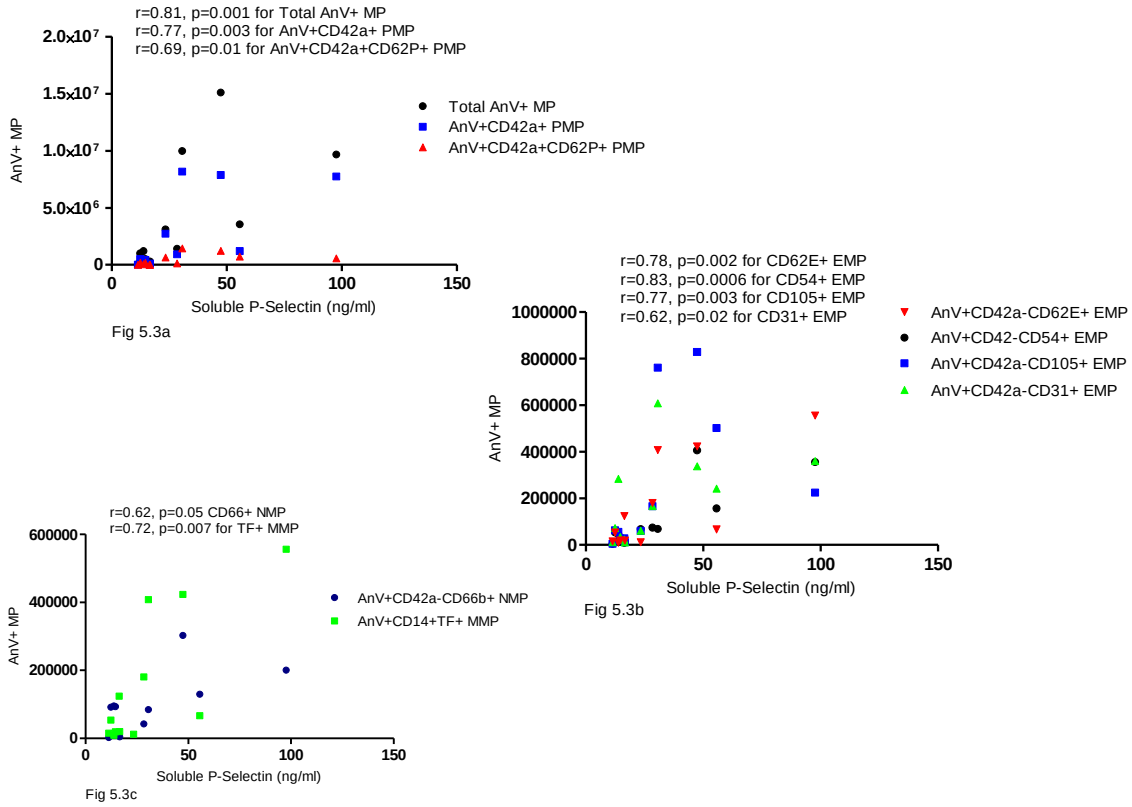


Fig 5.3 Positive correlation between AnV+MP and soluble P-selectin in the right atrium (RA) in the acute coronary (ACS) group. Spearman factor r was used for correlations.

In contrast to the above in the SA group markers of platelet activation (total PMA and soluble P-selectin) did not correlate with total or cell specific AnV+MP.

5.5 Discussion

In chapters 3 and 4, higher expression of total AnV+MP and MP subpopulations in patients with ACS compared with stable angina patients both locally at the site of the culprit lesion and in the systemic circulation have been shown. Furthermore, coronary inflammatory markers correlated with MP levels and myocardial injury in ACS (as assessed by troponin T levels); in addition RA IL-6 levels correlated with MP levels and troponin T in ACS. These observations suggest that patients with

more severe ACS have higher levels of local and systemic inflammation, and higher levels of local and systemic circulating MP.

The novelty of the study described in this chapter is the positive correlation between total AnV+MP and MP subpopulations with markers of platelet activation in ACS patients, both at the site of the culprit lesion and the systemic circulation. Previous research has shown positive correlation between soluble p-Selectin and platelet, endothelial and monocyte derived MP in patients with hypertension and diabetes mellitus (301). However this is the first study to show that a positive correlation between MP formation and markers of platelet activation exist in both the coronary artery and right atrium in ACS patients but not in patients with stable angina.

The role of PMA formation in the pathophysiology of CHD remains unknown. Activated platelets express P-selectin on their surface which mediates the platelet-leukocyte interaction via the P-selectin-PSGL-1 bond leading to PMA formation with leukocyte activation, migration and recruitment at the site of inflammation (10;18). The platelet-monocyte interaction via the P-selectin-PSGL-1 pathway increases further the adhesive and pro-thrombotic properties of monocytes by up-regulating the expression and functionality of beta1 and beta2 integrins (32;33) and by TF expression on the monocyte surface respectively (23;35). It has been shown that activated platelets lose their surface P-selectin despite the fact they continue to circulate and function (19). Therefore PMA measurement may represent a more robust marker of platelet activation than detection of P-selectin on their surface (21;22). Increased PMA levels have been observed in patients with stable angina (36;51), ACS patients (37) and also after coronary interventions (20). The extent of

PMA formation depends mainly on platelet activation status rather than the monocyte activation (30). Also a positive relationship between inflammatory markers (IL6 and CRP) and markers of platelet activation like P-selectin and PMA has been demonstrated by Wang et al (48). Studies in patients with ACS who underwent PCI after administration of glycoprotein IIb/IIIa inhibitors have shown that inhibition of platelet adhesion and activation via glycoprotein IIb/IIIa inhibitors leads to PMA formation reduction in addition to the reduction of platelet activation and degranulation (20;39). It has also been shown that prevention of platelet adhesion to monocyte by blocking the P-selectin-PSGL-1 interaction reduces inflammation (31). Patel et al have demonstrated a trans-coronary gradient of platelet-leukocyte formation in patients with ACS. They found increased PMA in the coronary sinus compared with the aortic samples in patients with ACS (21). The above observations support an active involvement of PMA in the pathophysiology of ACS possibly via exaggerating and sustaining the coagulation cascade in addition to perpetuating local coronary inflammation.

The data from this study has shown higher CD62P+PMA in patients with ACS compared with SA in both CO and the RA, in addition to the fact that CD62P+PMA were higher in the CO vs the RA only in ACS (Fig 5.1). In contrast, there was no difference in total PMA expression between ACS and SA in both CO and RA sites. In addition, CD62P+PMA correlated with AnV+ MP (platelet and endothelial MP) only in the coronary sample in the ACS group. These findings arguably support the hypothesis that platelets can trigger a pro-inflammatory monocyte response, via platelet-monocyte interaction and PMA formation, only when platelets are activated (platelets expressing P-selectin). Also the correlation between CD62P+PMA, a

marker of platelet activation and a link between platelet activation and inflammation, and endothelial MP underlines the crucial role of endothelial cells in the pathophysiology of ACS. Previous reports have shown that the presence of hemodynamically significant coronary stenosis (lesion with fractional flow reserve (FFR) ≤ 0.75) does not alter platelet reactivity in patients with stable coronary artery disease, nor the platelet-monocyte complexes in the systemic circulation (302). In addition, Bournazos et al have reported that in the absence of overt platelet activation, P-selectin-PSGL-1 dependent platelet-monocyte interaction represents a normal physiological process with little impact on the potential of monocytes to cause vascular injury (303). They concluded that high levels of P-selectin on the surface of activated platelets or binding of multiple platelets per monocyte are required to trigger monocyte activation via the P-selectin-PSGL-1 pathway. Also, it has been shown that intravenous administration of a P-selectin antagonist (inclacumab) in patients with ACS scheduled for PCI reduces myocardial damage (304). They found that peak Troponin I and creatine kinase reduced by 23%. This raises the possibility that PMA may contribute to the pathophysiology of ACS possibly via exaggerating and sustaining platelet activation and coagulation cascade in addition to perpetuating local coronary inflammation. Therefore, targeting PMA formation in ACS may interrupt the platelet-monocyte interactions and limit intra-coronary inflammation and thrombus formation.

Increased levels of s-P-selectin in the plasma have been previously demonstrated in patients with CHD, peripheral vascular disease and hypertension, with some relationship to prognosis (305;306). The observations from this study have shown higher CO s-P-selectin and platelet MP in the ACS vs SA with a positive correlation

between CO s-P-selectin and platelet MP underlining the importance of intracoronary platelet activation in the pathophysiology ACS. Also the strong correlation between endothelial MP and s-P-selectin emphasizes the role of the activated endothelial cells and endothelial MP as inflammatory mediators in pathogenesis of ACS.

Interestingly TF+MP is the only MP subpopulation which did not correlate with markers of platelet activation in the CO despite the fact that TF+MP was higher in ACS vs SA in both CO and RA. Nevertheless there was a positive correlation between TF+MP and s-P-selectin in the systemic circulation (right atrium). The above could be due to the fact that the interaction between activated platelet and monocytes via the P-selectin-PSGL-1 pathway with subsequent PMA formation and TF expression on the monocyte is relatively delayed as opposed with the imminent platelet activation and s-P-selectin in the coronary during ACS. It could also be due to a systematic response of the platelet activation and inflammation with PMA and MP formation in the systemic circulation.

The above observations raise the possibility that MP and PMA may contribute to the pathophysiology of ACS possibly via exaggerating and sustaining platelet activation, coagulation and intracoronary inflammation. Therefore, targeting MP release and PMA formation in ACS may limit intracoronary inflammation and thrombus formation.

5.6 Conclusions

Markers of platelet activation correlated positively with total AnV+MP, platelet, endothelial and leukocyte derived MP, both at the site of the culprit lesion and in the systemic circulation during ACS. These novel observations suggest a potential important interaction between activated platelets, monocytes and endothelial cells via MP formation. Future studies are now required to explore how MP could be directly involved in the pathogenesis of ACS, since these could represent important novel therapeutic targets.

Chapter 6: P-Selectin Activated Platelet Monocyte Aggregates and Microvascular Dysfunction in patients with stable angina and non-ST elevation myocardial infarction undergoing PCI

6.1 Summary

Introduction and aims

Microvascular dysfunction (MvD) is a powerful independent predictor of outcomes in patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI). Activated platelets interact with leukocytes forming platelet monocyte aggregates (PMA). PMA represent a sensitive marker of platelet activation. Our aim was to assess the relationship between MvD and PMA expression in patients with non-ST elevation myocardial infarction (NSTEMI) and stable angina (SA) treated with PCI.

Methods

Twenty five patients (14 patients with NSTEMI and 11 patients with SA were recruited. Blood samples were aspirated sequentially from the right atrium (RA), ascending aorta (AO) and coronary artery (CO) distal to the culprit lesion. MvD was assessed by measuring the index of microvascular resistance (IMR), coronary flow reserve (CFR) and the coronary wedge pressure (PwC) in 14 patients (7 NSTEMI and 7 SA). Monoclonal fluorescent antibodies and flow cytometry were used for platelet monocyte estimation (PMA).

Results

Total PMA and CD62P+ PMA did not differ between CO, AO and RA in the two groups. CD62P+ PMA expression in the CO and AO of the ACS group was higher compared to the SA group (p=0.005 and p=0.03 respectively). CwP (median) was higher in the ACS group (52.5 mmHg) compared to the SA group (18 mmHg) p= 0.01. IMR did not differ between ACS and SA. CwP and IMR in both groups correlated positively with the total PMA in the three compartments (r^2=0.3; p=0.009

and r^2=0.4; p=0.01 for CwP and IMR respectively in ACS; r^2=0.2; p=0.03 and r^2=0.3; p=0.01 for CwP and IMR respectively in SA).

Conclusions

Elevated CD62P+ PMA is a hallmark of ACS. PMA correlate with measured microvascular dysfunction in both stable angina and ACS patients. This study supports the hypothesis that PMA formation may be important determinants of platelet activation, inflammation and microvascular dysfunction in coronary disease.

6.2 Introduction and aims

Microvascular dysfunction (MvD) is a powerful independent predictor of long term outcomes, left ventricular function and mortality in patients presenting with acute coronary syndrome (ACS) and undergoing revascularization with PCI. Despite angiographic successful coronary artery stenting potential compromise of the coronary microcirculation during or after PCI is associated with "slow flow" or "no flow" with subsequent impaired myocardial tissue oxygenation. A number of different non-invasive and invasive techniques have been developed to evaluate the coronary microvasculature. Among the invasive methods in the catheter laboratory the 'Index of microvascular resistance' (IMR) which is derived using a Doppler or a pressure-temperature sensing wire, has emerged as a novel index for the assessment of the coronary microcirculation (233;240;243).

Activated endothelial cells and platelets express P-selectin on their surface which mediates the platelet-leukocyte interaction via the P-selectin-PSGL-1 bond leading to platelet monocyte aggregate (PMA) formation with leukocyte activation, migration and recruitment at the site of inflammation (10;18). It has been shown that inflammatory mediators such as tumour necrosis factor-α (TNF-α) and LPS increase P-selectin production from the endothelial cells in addition to up-regulating P-selectin at the cell surface (11). It has also been shown that activated platelets lose their surface P-selectin despite the fact they continue to circulate and function (19). Therefore PMA measurement may represent a more robust marker of platelet activation than detection of P-selectin on their surface. In addition, P-selectin on the surface of the activated platelets, within the PMA complex, induces monocyte TF expression which, in the ACS setting with a ruptured atheromatous plaque, may trigger or contribute to the cascade of athero-thrombosis (23). The amount of PMAs,

both at the site of the coronary lesion and systematically, has been described as a sensitive marker of platelet activation and inflammation. Increased PMA levels have been observed in both stable angina and (36;51) ACS patients (37). However, the role of PMA formation in the pathophysiology of ACS remains unknown. Whether PMA is just a bystander of the inflammatory and thrombotic process occurring in the setting of ACS or whether the PMA is an effector of the disease remains unclear. Also recent data suggest that platelet monocyte interaction is a normal physiological process in the absence of overt platelet activation with little impact on the potential of monocytes to cause vascular injury (29). This subpopulation of PMA was identified as P-selectin positive PMAs (CD62+PMAs). I hypothesized that 1) PMA is higher in the ACS group compared with the stable angina and 2) a positive correlation should exist between PMA expression and markers of microvascular dysfunction. The aim of my study was to assess the relationship between microvascular dysfunction and the level of PMA expression, intracoronary and systemic (aorta and right atrium) in patients presenting with NSTEMI or stable angina treated with PCI.

6.3 Patients and methods

6.3.1 Study population

Twenty five patients (14 patients with NSTEMI and 11 patients with stable angina (SA) presenting to our hospital with symptomatic coronary heart disease and treated with PCI were recruited for the study. The diagnosis of NSTEMI was based on the history of chest pain with or without ischaemic ECG changes (other than ST elevation) and a troponin T value > 0.03 ng/l (261). SA patients with symptoms were admitted electively for PCI. SA patients had been reviewed in the cardiology

outpatient clinic with a diagnosis of angina pectoris based on typically ischaemic sounding chest pain and a positive non-invasive test such as exercise tolerance test, stress echocardiography or myocardial perfusion scan. The decision for diagnostic angiography was made by a cardiologist as part of routine clinical care. Informed consent was obtained prior to the procedure from all recruited patients outside the cardiac catheterisation laboratory. Ethical permission was granted by the local ethics committee of the Royal Free Hospital, and all participants provided fully informed written consent.

6.3.2 Exclusion criteria

The exclusion criteria have been described in Chapter 2 (Paragraph 2.1.4)

6.3.3 Coronary angiography, coronary angioplasty and blood sampling

The coronary angiography, angioplasty procedures and blood sampling have been extensively described in chapters 2 (2.1) and 3 (section 3.3).

6.3.4 Measuring IMR and CFR with pressure-temperature sensor wire

Thermodilution technique (233) using a 0.014 inch floppy pressure wire (pressure wire 3, Radi Medical system) was used to measure IMR and CFR, in 14 patients of the study group (7 NSTEMI and 7 SA). The pressure wire has a microsensor which measures simultaneously pressure and temperature at the location of the microsensor. The shaft of the wire can be used as an additional thermistor, providing the input signal at the coronary ostium of any fluid injection with a temperature difference from blood. In this manner the mean transit time of the injected saline down the coronary artery can be calculated from a coronary thermodilution curve.

Thermodilution curves in the coronary artery are obtained by short manual injections of 3 mls 0.9% saline at room temperature. Measurements of transit time are calculated 3 times at base line and 3 times at maximum hyperaemia. The mean transit time (Tm) at baseline and maximum hyperaemia were calculated. Maximum hyperaemia was achieved by adenosine infusion through the femoral vein at a rate 140 µg/kg/min. Distal coronary pressure (Pd) is recorded from the pressure wire. Mean aortic pressure (Pa) is measured from the coronary catheter and central venous pressure (Pv) is measured from the right heart catheter at maximum hyperaemia. Coronary wedge pressure (Pw) is also measured at maximum hyperaemia by inflating a semi-compliant balloon (1mm smaller that the vessel diameter) in the coronary artery. IMR measurements were calculated after the completion of the PCI. Previous research has shown that Pw below 25 mmHg implies that collateral flow is essentially hemodynamically insignificant (213;244-247). Therefore the complete IMR formula, which takes into account the contribution of the coronary collaterals, was used to calculate the IMR i.e. IMR= Pa × Tm × [(Pd-Pw) ÷ (Pa-Pw)] as described before (243). All the above measurements were taken during maximum hyperaemia. CFR can be calculated as a ratio of the average transit time at baseline and average transit time at maximum hyperaemia provided that the time for analysis of the thermodilution equals at least one cardiac cycle, and variability between values of the 3 transit times < 20%.

6.3.5 Flow Cytometry and PMA methodology

The methodology of PMA cytometric identification and analysis has been described in chapter 2 (section 2.2) and 5 (section 5.3.4).

6.3.6 Statistical analysis

Continuous variables were expressed as medians with interquartile range (IQR). Categorical variables were expressed as percentages with 95% confidence intervals (95% CIs). Comparisons between the two groups and the two (coronary and right atrium) compartments were performed using the Mann-Whitney U test. Fisher exact test was used for proportions. Linear regression was used to correlate the markers of microvascular dysfunction and PMA. A 2-sided p value <0.05 were considered to be statistically significant. All statistical analyses were performed with Graph Pad Prism 5 statistical software.

6.4 Results

The demographics and baseline characteristics of the study population are shown in table 6.1. Briefly the majority of the study population was male (83%). The SA patients were more likely to have past medical history of hypertension compared to the NSTEMI group. Also the NSTEMI patients were more likely to have family history of CHD. The study population was well balanced with respect to other risk factors, diabetes mellitus, dyslipidaemia and smoking.

	NSTEMI	Stable Angina	p value
Number of patients (n)	14	11	
Age (mean), years	62	61	0.8
Male	11	9	0.7
Female	2	2	0.7
Risk Factors			
Hypertension	3	9	0.01
Diabetes	3	2	0.8
Dyslipidaemia	9	4	0.3
Smoking	4	2	0.4
Family History of CHD	9	4	0.04
Angiographic characteristics			
1 vessel disease	8	6	0.8
2 vessel disease	3	3	0.8
3 vessel disease	2	2	0.9
Culprit vessel			
Left Anterior Descending	5	6	0.8
Left Circumflex	4	0	N/A
Right Coronary Artery	4	5	0.8
Onset of symptoms to PCI interval (median), (mins)	4320	N/A	<0.0001

Table 6.1 Baseline characteristics, demographics and angiographic data of the NSTEMI (non ST elevation myocardial infarction) and stable angina groups. Fisher exact test was used for comparison of proportions between the groups.

6.4.1 Total PMA expression in the CO, AO and RA sites in ACS and SA groups

Within NSTEMI and SA groups there were no difference seen in the expression of total PMA and CD62P+ PMA levels between the three sites (CO, AO and RA). In comparing total PMA expression in the CO, AO and RA sites between the NSTEMI and SA groups no difference was seen (Fig 6.1). However the expression of CD62P+ PMA in the CO site of the NSTEMI patients was higher compared to SA patients (p=0.005) Fig 6.1a. Similarly in the AO site of the NSTEMI patients the expression of the CD62P+ PMA was higher compared to SA (p=0.03) Fig 6.1b. There were no

differences in the expression CD62P+ PMA in the RA site between the two groups Fig 6.1c.

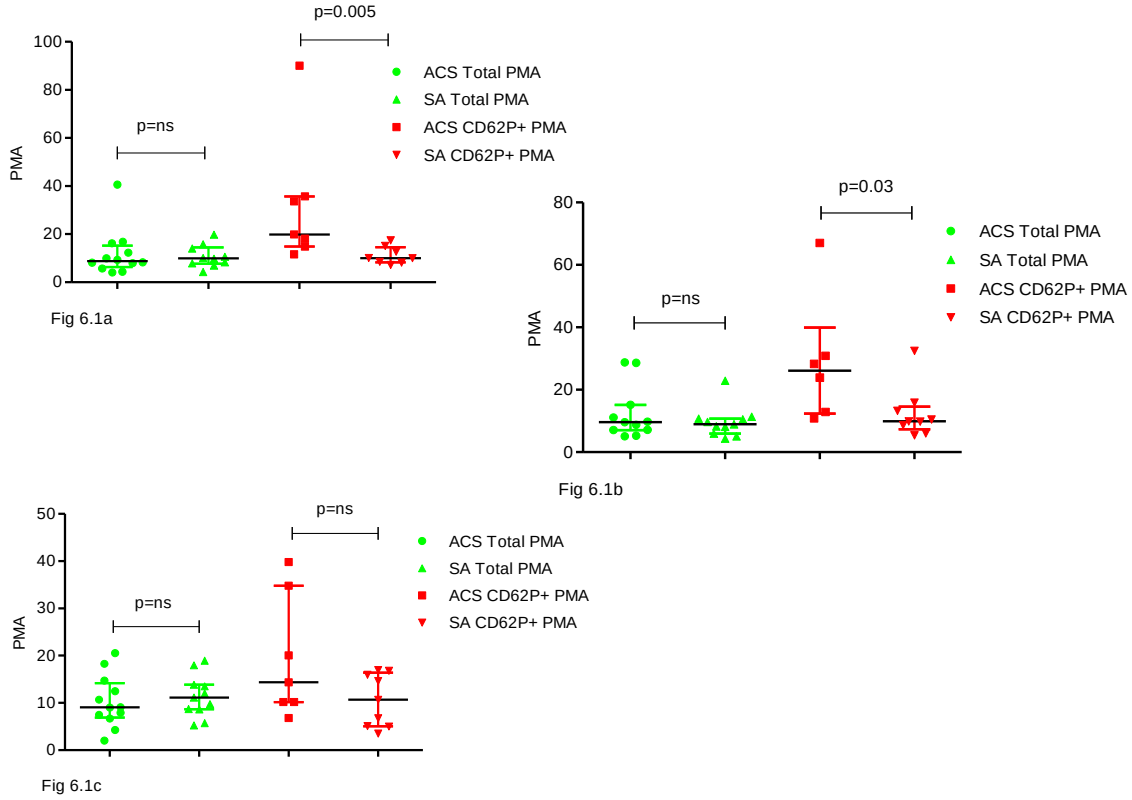


Fig 6.1 Total PMA and CD62P+ PMA in the coronary artery (CO) Fig 1a, aorta (AO) Fig 6.1b and the right atrium (RA) Fig 6.1c (total PMA expressed as % of monocytes and CD62P+ PMA as % of total PMA). PMA (platelet monocyte complexes), ACS (acute coronary syndrome), SA (stable angina).

6.4.2 IMR, CFR and Pw in the two groups

Pw (median (IQR) was higher in the NSTEMI group [52.5 mmHg (34.2-65.5)] compared with the SA group [18 mmHg (17-32)] p= 0.01] Fig 2. IMR (median (IQR) did not differ between NSTEMI [19.6 mmHg*sec (14.4-34.2)] and SA group

[19.9 mmHg*sec (17.6-26.8)] Fig 2. CFR also did not differ between the two groups (data not shown).

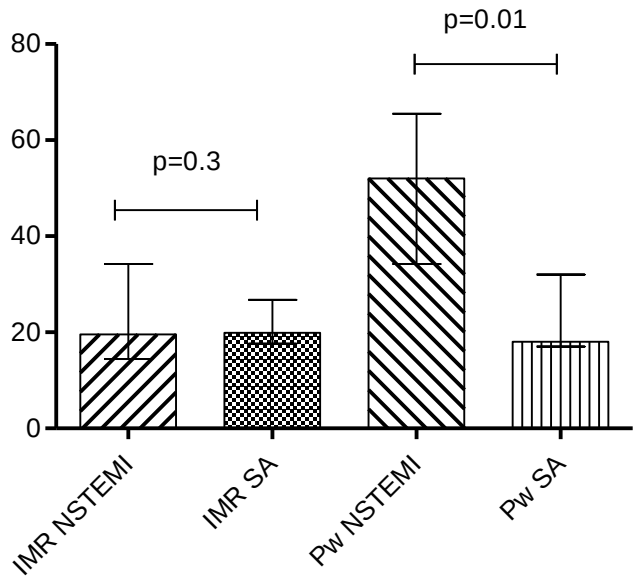


Fig 6.2 Differences of IMR and Pw in patients with NSTEMI and SA (IMR in mmHg*sec, Pw in mmHg). IMR (index of microvascular dysfunction), Pw (coronary wedge pressure), NSTEMI (non ST elevation myocardial infarction), SA (stable angina).

6.4.3 Correlations between IMR, Pw and PMA

Using linear regression analysis Pw and IMR in the NSTEMI correlated positively with the total PMA levels in the three compartments (r^2=0.3; p=0.009 and r^2=0.4; p=0.01 for Pw and IMR respectively). In addition there was a strong positive correlation between Pw and CD62P+ PMA (r^2=0.5; p=0.007).

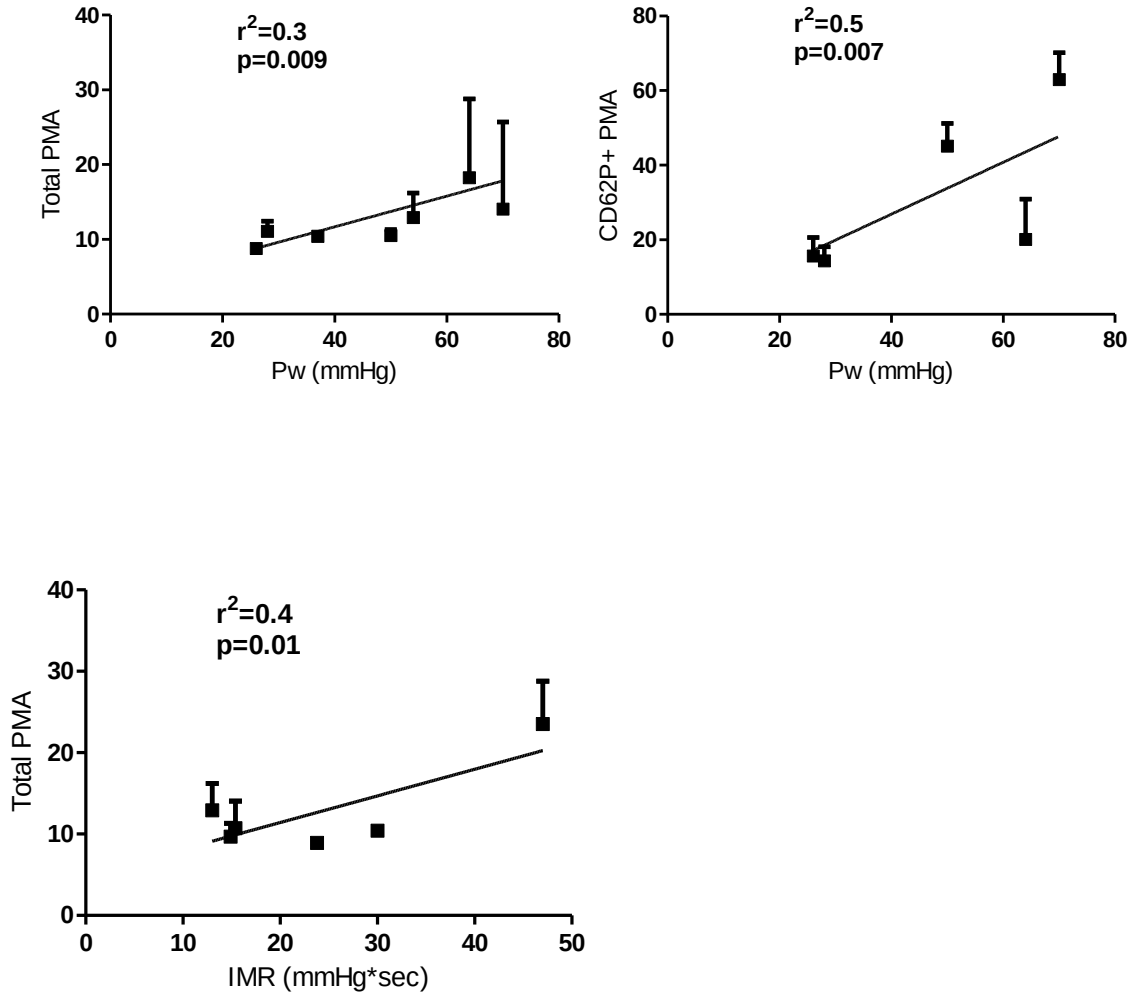


Fig 6.3 Correlation between Pw and IMR with PMA in NSTEMI patients. IMR (index of microvascular dysfunction), Pw (coronary wedge pressure), NSTEMI (non ST elevation myocardial infarction), PMA (platelet monocyte aggregates).

In the SA group there was a weak to moderate correlation between Pw and IMR with the total PMA (r^2=0.2; p=0.03 and r^2=0.3; p=0.01 for CwP and IMR respectively). CD62P+ PMA in the SA did not correlate with Pw or IMR.

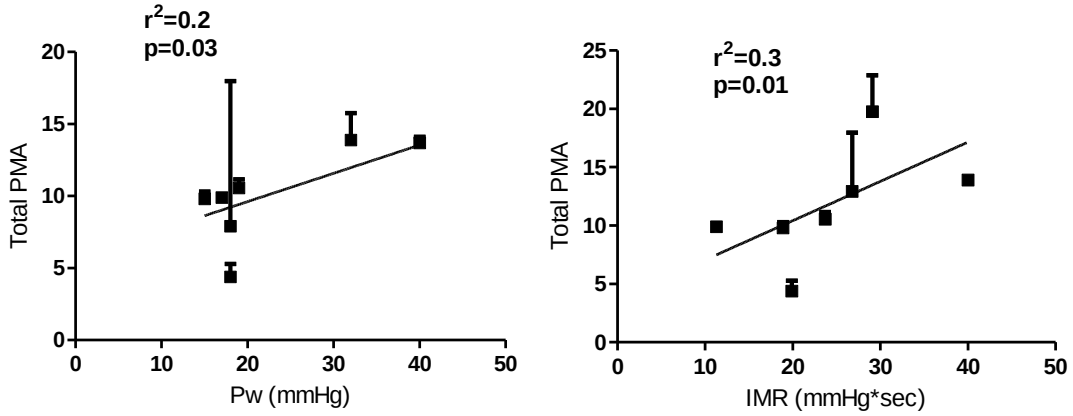


Fig 6.4 Correlation between Pw and IMR with PMA in SA patients. IMR (index of microvascular dysfunction), Pw (coronary wedge pressure), PMA (platelet monocyte aggregates), SA (stable angina).

6.5 Discussion

The unique finding of this study is the positive correlation between invasive markers of microvascular dysfunction and PMA in patients with NSTEMI or stable angina. In line with previous studies CD62P+ PMA in the coronary, were higher in ACS patients compared with stable angina. The above findings support the knowledge that platelet activation is pivotal in ACS and as such CD62P+ PMA which is a sensitive marker of platelet activation is the hallmark of ACS. It also supports the notion that PMA formation is dependent on platelet activation and that PMA expression and platelet activation follow the same pattern of expression. Furthermore these observations strengthen our understanding that PMA formation in the setting of acute coronary syndrome occurs mainly intracoronary, at the site of the ruptured plaque, rather than in the peripheral circulation. The fact that the NSTEMI patients were studied, blood sampling and IMR measurements, 2-4 days after the onset of symptoms whilst receiving treatment with anti-platelet and anti-coagulant medications may have altered the expression of total PMA and contributed to our

observations that total PMA did not differ between the 2 groups. This may also explain the fact that only Pw was higher in the ACS group compared to the SA group whilst IMR and CFR did not differ between the 2 groups.

During ACS, PMA formation at the site of plaque rupture with subsequent monocyte activation and cytokine release may contribute to the impairment of microcirculatory flow. Ko et al have found increased levels of soluble CD40 ligand, IL-6, serotonin, TF and factor VII in the culprit coronary artery compared to those in peripheral blood (307). Also a positive relationship between inflammatory markers (IL6 and CRP) and markers of platelet activation like P-selectin and PMA has been demonstrated by Wang et al (48). Takahashi et al have showed that neutrophilia on admission is associated with impaired microvascular reperfusion and poor functional recovery in patients with myocardial infarction treated with primary PCI. Similar finding were found by Sezer et al (49) who showed that neutrophilia and higher mean platelet volume were found to be associated with higher IMR, lower CFR and higher coronary wedge pressure in patients with ACS treated with PCI. The results of this study are in line with the above findings. Both total PMA and CD62P+ PMA correlated positively with the coronary wedge pressure in the ACS patients. Also IMR correlated positively with total PMA. Contrary to the above, in the SA group IMR and Pw correlated positively with total PMA only but not with CD62P+ PMA.

Clinical studies in animal models, with coronary artery occlusion and reperfusion, have shown that administration of recombinant monoclonal PSGL-1 antibodies targeting the P-selectin-PSGL interaction results in inhibition of leukocyte-platelet and leukocyte-endothelial binding with subsequent improvement of reperfusion in addition to the reduction of the infarct size (308-310). However in human beings administration of recombinant PSGL in patients with STEMI as an adjunct to

thrombolysis have failed to improve clinical outcomes like TIMI flow (Thrombolysis in myocardial infarction), infarct size and mortality (311). This can be explained by the fact that in both studies the recombinant antibody was given intravenously and as such may have failed to reach the coronary circulation. This is because the recombinant antibody was human antibody and as such was probably absorbed by the antibody receptors expressed on the macrophages in the liver and spleen. Recently, Tardif et al have shown that intravenous administration of a P-selectin antagonist (inclacumab) in patients with ACS scheduled for PCI reduced myocardial damage (304). They found that peak Troponin I and creatine kinase reduced by 23%. The findings from this study may explain the mechanism behind the above positive findings. This raises the possibility that PMA may contribute to the pathophysiology of microvascular dysfunction via exaggerating and sustaining platelet activation and coagulation cascade in addition to perpetuating local coronary inflammation. Therefore, targeting PMA formation in ACS may interrupt the platelet-monocyte interactions and limit intra-coronary inflammation and thrombus formation.

As discussed in the chapters above the main limitation of this study is the number of patients making the statistical analysis and the interpretation of correlations difficult. Also the presence of patient's selection bias cannot be excluded.

6.6 Conclusions

In conclusion PMA are increased in patients with ACS compared with stable angina with CD62P+ PMA being a hallmark of ACS. PMA and CD62P+ PMA correlated positively with indices of microvascular dysfunction. Hence PMA may represent a marker of both platelet activation and microvascular dysfunction.

Chapter 7: General discussion and Conclusions

7.1 General discussion

7.2 Conclusions

7.1 General discussion

The studies outlined in this thesis have investigated the intracoronary and systemic microparticle expression in patients presenting with ACS and SA and their relationship with markers of inflammation, platelet activation and myocardial necrosis. The relationship between PMA, a marker of platelet activation, and MvD was also studied.

There are important limitations to the data presented in this thesis many of which have been discussed in the chapters above in addition to many unanswered questions. This chapter will highlight these limitations and proposes areas of future experiments that may address some of these unresolved issues. There had also been many difficulties running this project from designing the study to recruiting and collecting the blood samples and preparing them for flow cytometry.

The main novel findings of this study are the demonstration of: 1) differences between MP levels in patients with varying severity of CHD i.e. MP levels were overall higher in those with ACS (more specifically the highest MP levels were observed in the STEMI patients) compared with SA; 2. differential local and systemic AnV+MP expression in human coronary artery disease; 3. differential local and systemic expression of inflammatory markers in ACS patients; 4. differential local and systemic expression of markers of platelet activation in ACS patients; 5. positive correlation between AnV+ MP and markers of inflammation, platelet activation and myocardial necrosis in ACS patients both in the CO and RA; and 6. positive correlation between invasive markers of microvascular dysfunction and PMA in human coronary artery disease.

In chapter 3 higher levels of total AnV+MP and MP subpopulations were demonstrated in patients with ACS compared with SA patients in both the CO and RA. It is highly likely that the source of these MP was the culprit lesion since total AnV+MP and MP subpopulations were higher in the coronary artery (blood sampled distal to the coronary culprit lesion) compared with the systemic (RA) circulation in the study groups. Overall these observations confirm that MP release occurs from culprit atheromatous lesions in ACS, and that total AnV+MP number and subpopulation phenotype could relate to clinical severity of ACS. These findings, which are consistent with previous reports (110;273), strongly suggest that MP release in symptomatic patients with CHD is mainly locally driven, since CO MP levels at the site of the complicated plaque were higher than systemic MP levels. Whilst a causal relationship cannot be established from these data, that these MP directly contribute to the pathogenesis of ACS, the above findings could have important implications in this context since AnV+MP are known to be both pro-inflammatory and pro-thrombotic (77;78). These observations do not, however, preclude the possibility that additional contribution of MP release also takes place in the systemic circulation since atheromatous vascular disease is usually widespread and a systemic inflammatory response is observed during ACS. That said the findings from this study support the hypothesis that the complicated atheromatous plaque is the predominant source of the circulating microparticles, and that a topographic pattern of microparticle expression appears to exist depending on where you sample the microparticles i.e. the closer to the culprit lesion, the higher the level of microparticles.

The limitation of this project had been discussed in chapter 3. However it is worthwhile mentioning in this chapter the difficulty of obtaining blood samples exclusively from the coronary artery distal to the culprit lesion with aspiration catheter. Therefore contamination with blood from non-affected coronaries or systemic circulation cannot be excluded. The effects of antiplatelet and anticoagulant therapy on MP production, even though standardised for all patient groups, was not fully analysed and needs further study. In addition sampling of the infarct related artery proximally to the culprit lesion or sampling of a bystander coronary artery could have strengthened my hypothesis that the complicated atheromatous coronary plaque is the predominant source of microparticle release.

PMP levels were higher compared with the other cell specific MP subpopulations but particularly so in with respect to the CO P-selectin positive PMP in the STEMI patients. This observation can be explained by the fact that the hallmark of ACS is disruption of the atheromatous plaque in the coronary tree, with subsequent platelet activation and thrombus formation (81). The observation of high levels of CD62P+PMP in the CO raises the possibility that these could amplify vascular inflammation and thus contribute to lesion progression by further recruitment of inflammatory cells expressing P-selectin glycoprotein ligand (PSGL), which binds CD62P (P-selectin) (29;30). Since, high CO CD62E+EMP, almost certainly derived from activated coronary endothelium, was also detected, it is likely that E-selectin-PSGL binding could further mediate the interaction between inflammatory cells and activated endothelial cells and contribute to lesion progression (270). In addition, the high CO NMP which were observed could further amplify vascular inflammation

since NMP can bind activated endothelium and contribute to further endothelial activation (271).

As well as amplifying inflammation, MP are also prothrombotic (76). The finding of high CO TF+MMP could implicate their role in clot propagation, since higher levels were observed in ACS patients than those with SA. Expressed TF on activated monocytes results in the release of TF+MMP which promote the clotting cascade (77). Furthermore, irrespective of TF, AnV+MP are themselves prothrombotic (272) and were significantly elevated in the CO of STEMI patients. Thus MP, either total AnV+, and/or TF+ subpopulations could contribute to clot progression in ACS.

A time dependant mode of microparticle expression appears to exist from this study. There was a negative correlation between the index event to PCI time and total MP, PMP and EMP in the CO in the NSTEMI group. This suggests that, in line with previous studies (112;117), there is a peak in MP release during the first 24-48 hours and thereafter MP levels fall. Arguably, earlier sampling (on admission) of the NSTEMI patients would have yielded an expression of MP levels similar to that of the STEMI patients. Antiplatelet and anti-coagulation therapy could have in addition influenced MP production.

In chapters 4 and 5 the study population was divided in 2 groups (ACS, which includes STEMI and NSTEMI patients, vs SA). The observation, that total AnV+MP and MP subpopulations are elevated in patients with ACS compared with SA patients in both the CO and RA have been extensively discussed at the beginning of this chapter. CO IL-6 was higher than RA IL-6 in ACS patients, contrary to the other

inflammatory markers which were studied. Overall, coronary inflammatory markers correlate with MP levels and myocardial injury in ACS (assessed by troponin T levels); in addition RA IL-6 levels correlated with MP levels and troponin T in ACS. Taken together, these results suggest that patients with more severe ACS have higher levels of local and systemic inflammation, and higher levels of local and systemic circulating MP. Whilst these data suggest that these indices correlate with extent of myocardial injury, and that it is indeed likely that these are causally linked, I cannot yet infer from our data that a causal association is definite.

These data are in agreement with previous reports demonstrating that during ACS, circulating platelet and endothelial MPs are elevated (104-106). This pattern appears to exist for leukocyte (neutrophil and monocyte) derived MP, since higher levels were observed in ACS compared to SA patients. The observed increased MP in the systemic circulation are likely to be derived the local culprit CO atheromatous lesion, with additional contribution from systemic release secondary to the systemic inflammatory response we observed in the ACS patients.

In addition to the differences in MP expression, the second important finding from chapter 4 is the differential expression of inflammatory markers in the CO and in the systemic circulation during ACS. In line with previous reports (278;279) increased intracoronary IL-6 was observed during ACS, compared with the systemic circulation. This IL-6 is likely to be derived from coronary endothelium (281), and/or from plaque leucocytes. The rest of the inflammatory markers i.e. hs-CRP, SAA and TNF-α were higher in the peripheral circulation, which is unsurprising since CRP and SAA are produced and released from the liver; while TNF-α is produced from

leukocytes. In the SA group there were no differences of the inflammatory markers between CO and RA.

The unexpected observation that there were no differences of the measured inflammatory markers in the coronary artery between ACS and stable angina patients have been discussed in chapter 4.

Positive correlation between total AnV+ MP and MP subpopulations with markers of inflammation in ACS patients was also shown in **chapter 4.** Intracoronary inflammatory markers correlated with total AnV+MP and MP subpopulations (including platelets, endothelial and leukocytes derived MP) whilst in the RA markers of inflammation correlated only with endothelial and monocyte derived TF+MMP. The fact that in the SA patients inflammatory markers did not correlate with MP formation could suggest a pivotal role of intracoronary inflammation as a driver of MP release from the culprit plaque in the pathogenesis of ACS.

The finding from this study that SAA correlates with platelet and endothelial MP may also provide an explanation for the previously reported pro-coagulant and pro-inflammatory amplification properties of SAA (190). Matsubara et al have shown that the increment of SAA across the coronary circulation correlated positively with the severity of coronary atherosclerosis (189). Higher adverse outcomes including myocardial infarction, revascularization and death in ACS patients with elevated SAA has also been previously reported (204).

Another potentially important finding from my study is the positive correlation between P-selectin positive PMP with markers of myocardial necrosis both in the CO and RA, emphasizing the crucial role of platelet activation in the pathogenesis of ACS. These data could therefore suggest a pathogenic role of MP as effectors linking inflammatory and prothrombotic pathways in ACS.

In chapter 5 a positive correlation between total AnV+MP and MP subpopulations with markers of platelet activation was reported in ACS patients, both at the site of the culprit lesion and the systemic circulation. Previous research has shown positive correlation between soluble p-Selectin and platelet, endothelial and monocyte derived MP in patients with hypertension and diabetes mellitus (301). However this is the first study to show that a positive correlation between MP formation and markers of platelet activation exist in both the coronary artery and right atrium in ACS patients but not in patients with stable angina.

The mechanism of PMA formation, their biological effects their role in the pathophysiology of CHD has been extensively discussed in **chapters 2, 5 and 6**. Higher CD62P+PMA in patients with ACS compared with SA in both CO and the RA, in addition to the fact that CD62P+PMA were higher in the CO vs the RA only in ACS have been shown in this chapter. Furthermore, CD62P+PMA correlated with AnV+MP (platelet and endothelial MP) only in the coronary sample in the ACS group. The correlation between CD62P+PMA (a marker of platelet activation and a link between platelet activation and inflammation) and endothelial MP underlines the crucial role of endothelial cells in the pathophysiology of ACS.

Increased levels of s-P-selectin in the plasma has been previously demonstrated in patients with CHD, peripheral vascular disease and hypertension, with some relationship to prognosis (305;306). Coronary s-P-selectin was higher in the ACS vs SA. The positive correlation between CO s-P-selectin and MP (platelet and endothelial) underlines the importance of local intracoronary platelet and endothelial activation with MP formation as inflammatory mediators in the pathophysiology ACS. Interestingly TF+MMP are the only MP subpopulation which did not correlate with markers of platelet activation in the CO. Nevertheless there was a positive correlation between TF+MMP and s-P-selectin in the systemic circulation (right atrium). The above could be due to the fact that the interaction between activated platelet and monocytes via the P-selectin-PSGL-1 pathway with subsequent PMA formation and TF expression on the monocyte is relatively delayed compared with early platelet activation via s-P-selectin in the circulation.

In chapter 6, a positive correlation between invasive markers of microvascular dysfunction and PMA in patients with NSTEMI or stable angina was observed.

Takahashi et al have showed that neutrophilia on admission is associated with impaired microvascular reperfusion and poor functional recovery in patients with STEMI treated with primary PCI. Similar findings were found by Sezer et al (49) who showed that neutrophilia and higher mean platelet volume were found to be associated with higher IMR, lower CFR and higher coronary wedge pressure in patients with ACS treated with PCI. The results of this study are in line with the above findings. Both total PMA and CD62P+PMA correlated positively with the coronary wedge pressure in the ACS patients. Also IMR correlated positively with

total PMA. Contrary to the above, in the SA group IMR and Pw correlated positively with total PMA only but not with CD62P+PMA.

Possible explanations of the failure, of the clinical studies of recombinant PSGL administration in patients with STEMI as an adjunct to thrombolysis (311), to improve clinical outcomes like TIMI flow, infarct size and mortality have been also discussed in chapter 6. The observations in chapters 5 and 6 may explain the mechanism of the positive findings reported by Tardif et al. They have shown that intravenous administration of a P-selectin antagonist (inclacumab) in patients with ACS scheduled for PCI reduce myocardial damage (304) by 23% peak Troponin I and creatine kinase. This raises the possibility that PMA may contribute to the pathophysiology of microvascular dysfunction via exaggerating and sustaining platelet activation and coagulation cascade in addition to perpetuating local coronary inflammation.

The observations from this study raise the possibility that MP and PMA may contribute to the pathophysiology of ACS possibly via exaggerating and sustaining platelet activation, coagulation and intracoronary inflammation. Therefore, targeting MP release and PMA formation in ACS may limit intracoronary inflammation and thrombus formation.

7.2 Conclusions

The study described in this thesis demonstrates higher MP levels in patients with STEMI versus those with NSTEMI or SA. In addition, higher MP levels were observed in the CO versus RA in all patient groups, strongly suggesting that the source of these MP is the culprit atheromatous lesion. Moreover, markers of inflammation and platelet activation in ACS patients, both at the site of the culprit lesion and in the systemic circulation, strongly correlated with total AnV+, platelet, endothelial, and leukocyte MP. CD62P+PMA also expressed higher in patients with ACS compared with stable angina and correlated positively with invasive indices of microvascular dysfunction in both ACS and stable angina. The interaction between activated platelets and monocytes with endothelial cells and the subsequent formation of AnV+MP and PMA during ACS would be compatible with a direct pathogenic link between inflammatory and prothrombotic pathways in the pathogenesis of ACS and myocardial necrosis. I cannot yet conclude that these MP have a direct pathogenic role; however, data from other studies have demonstrated important pro-inflammatory and prothrombotic effects of MP. This leads us to speculate that AnV+MP levels may be indicative of coronary artery plaque instability, and could directly contribute to this pathological process. Also PMA may represent a marker of both platelet activation and microvascular dysfunction. Future studies are now required to explore how MP and PMA could be directly involved in the pathogenesis of ACS, since these could represent important novel therapeutic targets.

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Publications from this thesis

1. Mavroudis C, Eleftheriou D, Majumder B, Sapsford R, North J, Lowdell M, Brogan P, Rakhit R. Demonstration of intracoronary microparticle expression and their association with activated platelet monocyte aggregate in human ST elevation myocardial infarction Heart 2012; 98: A127

2. Mavroudis C, Majumder B, Lowdell M, Rakhit R. Platelet Monocyte Aggregates Correlate with Indices of Microvascular Dysfunction (Coronary Wedge Pressure and Index of Microvascular Resistance) During PCI for Stable Angina and Non-ST Segment Elevation Acute Coronary Syndrome. Circulation 2011; 124: A11630

3. Mavroudis C, Majumder B, Lowdell M, Rakhit R. Platelet monocyte aggregates are determinants of microvascular dysfunction during percutaneous coronary intervention for stable angina and non-ST segment elevation myocardial infarction. Heart 2011; 97: A20

Manuscripts submitted pending review

- Phenotypic characterisation of local and systemic microparticle expression in patients with acute coronary syndrome and stable coronary artery disease

- Microparticle expression in patients undergoing PCI for acute coronary syndrome and stable coronary disease: correlation with intracoronary inflammation and myocardial necrosis

- Microparticle expression in human CHD: relationship to platelet-monocyte interaction, and platelet activation

- P-Selectin activated platelet monocyte aggregates and microvascular dysfunction in patients with stable angina and non-ST elevation acute coronary syndrome undergoing PCI