AN INVESTIGATION INTO THE ROLE OF PLATELET – MONOCYTE INTERACTION AND INFLAMMATION IN CORONARY ARTERY DISEASE

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DECLARATION

I Bikash Majumder confirm that the work presented in this thesis is my own.Help and contribution of others to this work are specified in the acknowledgement section. Where information has been derived from other source, I confirm that this has been indicated in the thesis .

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ABSTRACT

Introduction: -

Platelet monocyte complex (PMC) formation has been widely reported as a marker of platelet activation in vascular disease states and several studies have shown heightened systemic expression of PMC in stable and acute coronary disease. However, the relationship between intracoronary platelet and monocyte activation status and local intracoronary inflammation in acute coronary syndrome (ACS) remains unclear.

Method:-

Fifteen ST elevation myocardial infarction (STEMI), 8 non ST elevation myocardial infarction (NSTEMI) and 7 stable angina patients were recruited. PMC, P selectin positive PMC (activated platelet within the complex), tissue factor (TF) positive PMC (activated monocyte within the complex) were estimated with flow cytometry from blood samples aspirated from the coronary artery (distal to the lesion), aorta and right atrium . Plasma CRP, SAA, TNF – alpha and IL-6 were also measured.

Results:-

In ACS patients no significant transculprit lesion gradient of PMC expression was observed but significant gradients were found with P-selectin positive PMC (p=

0.01) and TF positive PMC expression (p=0.04). Overall median P- selectin positive PMC expression in ACS patients was significantly higher compared to stable angina (p= 0.006). Intracoronary P-selectin positive PMC was also found to be higher in the ACS group compared to stable group (p=0.003). Overall median CRP (p= 0.001), SAA (p=0.0007) and IL-6 (p= 0.03) levels were significantly higher in ACS.

In STEMI intracoronary PMC correlated positively with intracoronary TNF-alpha (r= 0.68, p= 0.03). Positive correlation was also observed between intracoronary TF positive PMC (% monocyte) with TNF-alpha and IL-6 (r=0.66, p=0.05 & r=0.71, p= 0.05 respectively).

Conclusion:-

The work outlined in this thesis has demonstrated the importance of platelet and monocyte activation status of the PMC as a determinant of intracoronary inflammation. Beyond a local intracoronary role, PMC may contribute to systemic inflammation through P-selectin expression and local intracoronary inflammation through increased P-selectin and tissue factor expression.

To my wife Jayita,my son Ishaan and my parents.

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ABBREVIATIONS

1. PMCs- Platelet Monocyte Complexes
2. ACS- Acute Coronary Syndrome
3. PSGL-1 P Selectin Glycoprotein Ligand- 1
4. RANTES- Regulated on Activation Normal T cell Expressed and Secreted
5. NF- kB – Nuclear Factor Kappa light chain enhancer of activated B cells.
6. IL- Interleukin
7. TNF- Tumour Necrosis Factor
8. CXCL-10- C-X-C Motif Chemokine 10
9. TF- Tissue Factor
10. MCP-1- Monocyte Chemotactic Protein- 1.
11. mAb- Monoclonal Antobody
12. LDL- Low DensityLipoprotein
13. TH1- Type 1 T Helper Cell
14. TH2- Type 2 T Helper Cell
15. ADP- Adenosine di Phosphate
16. TRAP- Thrombin Receptor Activating Peptide
17. CK- Creatine Kinase
18. AMI- Acute Myocardial Infarction
19. PAC- 1 Procaspase Activating Compound 1
20. MPV- Mean Platelet Volume
21. IMR- Index of Microvascular Resistance
22. CFR- Coronary Flow Reserve
23. Mac- 1- Macrophage 1 Antigen
24. s P Selectin- Soluble P Selectin

25. hs CRP- High Sensitive C Reactive Protein
26. EDTA- Ethylenediaminetetraacetic Acid
27. EGTA – Ethyleneglycol- Bis- (β – Aminoethylether) Tetraacetate
28. PPACK - D- Phenylalanine- L- propyl-L-arginine chloromethyl ketone
29. CTAD- Citrate Theophylline Adenosine Dipyridamole
30. SAA- Serum Amyloid A
31. PF4- Platelet Factor 4
32. JAM 3- Junctional Adhesion Molecule 3
33. HDL- High Density Lipoprotein
34. CAD- Coronary Artery Disease
35. ERK- Extracellular Signal- Regulated Kinase
36. MAPK- Mitogen Activated Propetin Kinase
37. TIMI- Thrombolysis in Myocardial Infarction
38. NO- Nitirc Oxide
39. VCAM- Vascular Cell Adhesion Molecule
40. ICAM- Intercellular Cell Adhesion Molecule
41. STEMI- ST Elevation Myocardial Infarction.
42. NSTEMI- Non ST Elevation Myocardial Infarction
43. PAI 1- Plasminogen Activator Inhibitor 1
44. Tn T- Troponin T
45. TSP- 1 Thrombospondin 1
46. PPCI- Primary Percutaneous Coronary Intervention.
47. ACT- Activated Clotting Time.
48. PCI- Percutaneous Coronary Intervention.
49. PerCP- Peridinin-Chlorophyll-Protein Complex

50. FITC- Fluorescein Isothiocyanate
51. PE- Phycoerythrin.
52. SSC- Sidescatter
53. CV- Coefficient of Variation.
54. F- French
55. IQR- Inter Quartile Range
56. PDGF-Platelet Derived Growth Factor
57. CO- Coronary artery
58. AO- Aorta
59. RA- Right atrium

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PUBLICATIONS CONTAINING WORK UNDERTAKEN IN THIS THESIS

ABSTRACTS:-

- **Majumder B**, Lowdell M, Lachman H, Gallimore R, Coghlan JG, Shiu M F, Rakhit RD.Positive Correlation of Intracoronary C- Reactive Protein with Platelet-Monocyte Complexes and Serum Amyloid A Protein Suggests Local Interrelationship Between Platelet Activation and Inflammation in the Acute coronary Syndrome Patients. Circulation, Supplement 2010; 122: A288.
- **B. Majumder**, M. Lowdell, R. Sapsford, J. Hurst, JG. Coghlan, MF. Shiu, R. Rakhit. Tissue factor expression by platelet bound monocytes correlates with intracoronary inflammation in ST elevation myocardial infarction patients. European Heart Journal (2010) 31 (Abstract Supplement), 977.
- **B. Majumder**, M Lowdell, R Sapsford, J Coghlan, M F Shiu, J Hurst, R Rakhit. Platelet monocyte complex formation is associated with increased intracoronary P-selectin expression and correlates positively with TNF alpha in ST elevation myocardial infarction. Cardiovascular Medicine. 2010; 13(5) supplement 18, p166.
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- **SUBMITTED ARTICLES:-**

  B Majumder; MW Lowdell; JG Coghlan; MFShiu; RD Rakhit. Intracoronary platelet and monocyte activation status within platelet-monocyte complexes (PMC) are determinants of inflammation in ST elevation myocardial infarction (STEMI). Thrombosis and Haemostsasis.

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CHAPTER 1

INTRODUCTION

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1.1INTRODUCTION:-

Traditionally the role of platelets in the pathogenesis of ACS is believed to be in thrombus formation upon rupture of atherosclerotic plaques. Formation of PMCs has generated much interest in the recent years in understanding the pathophysiological process of platelet activation and inflammation in ACS. PMCs may be the link between thrombogenesis and inflammation. Although PMC formation is a well known phenomenon in ACS the contribution of platelet and monocyte interaction to both plaque instability and the progression to ACS is unknown. It is also unclear whether PMC formation is a systemic phenomenon or plays a local role in the pathogenesis of local intracoronary inflammation in ACS patients. How far the formation of PMCs and local inflammatory burden contribute to microvascular dysfunction and determine myocardial salvage following percutaneous intervention in the ACS patients are yet to be determined.

1.2 PLATELET – MONOCYTE COMPLEXES : -

1.2.1 PATHOPHYSIOLOGIC SIGNIFICANCE OF PLATELET- MONOCYTE COMPLEX FORMATION:-

Activated platelets deposit at the sites of plaque rupture (1). Binding of platelets via P-selectin expressed on the surface of the PSGL-1 may alter leukocyte recruitment and activation patterns (2-4). The measurement of PMCs may represent a more robust signal of platelet activation than detection of surface P-

selectin on individual platelets because degranulated platelets rapidly lose surface P-selectin *in vivo* yet continue to circulate (5).The pathophysiological significance of PMC formation is unknown. Whether it is just an epiphenomenon of the inflammatory and thrombotic process occurring within the setting of ACS or whether the PMC is an effector of disease remains unclear. Neither it is clear whether PMC formation represents an initial step in the phagocytotic clearance of spent platelets by monocytic cells. Nevertheless P-selectin on the surface of activated platelets induces TF from monocytes, and binding of P-selectin to monocytes in the area of vascular injury may be an initiator of thrombosis (6). Apart from this, pro-inflammatory cytokines e.g.TNF and IL-1 also partially regulate the expression of TF on endothelial cells and monocytes (7).

It has been shown that thrombin stimulated platelets induce monocyte cytokine expression (2). It has also been shown that synthesis of monocyte chemokines can be regulated by platelet surface P-selectin in conjunction with the platelet chemokine RANTES (8). It is now known that activated platelets express CD 40 ligand on their surface (9). Binding of CD40L to CD 40 on monocytes leads to monocyte activation and production of cytokines including IL-6 which is associated with unstable angina (10, 11) and often associated with increased CRP (12). In a recent study Bournazas et al. have found that in the absence of overt activation PSGL-1, P- selectin dependent platelet binding to monocytes represents a normal physiological process with little impact on the potential of monocytes to cause vascular injury. Binding of unstimulated platelets does not affect receptor expression, cytokine production, NF-kB activation, chemotactic responses, or apoptosis. In contrast they suggest binding of activated platelets does trigger pro-inflammatory responses in monocytes. They conclude 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 PSGL-1. 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 (13). Monocyte expression of IL-1*β* and TNF- alpha are substantially upregulated when monocytes are co-incubated with thrombin stimulated platelets but not with unstimulated platelets. 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. The extent of PMC formation is mostly dependent on platelet activation (14) and to a limited extent to monocyte activation (15).

P-selectin dependent platelet monocyte interaction induces L-selectin shedding from the monocyte surface and this interaction also increases the monocyte expression of α4ß1 and αMß2-integrins (16) (17). The presence of RANTES and CXCL10, deposited by platelets on to the monocytes, augments ß2-integrin avidity upon PSGL-1 cross linking (18). Monocyte binding to platelets is also associated with increased pro-inflammatory mediator release and TF expression. P-Selectin-PSGL-1 interaction is important but not exclusively responsible for this process. TF expression is found to be reduced by a P-selectin blocking antibody (19). Monocyte expression of chemokines, induced by thrombin activated platelets is regulated by NF-kB activity. Ligation of monocyte PSGL-1 together with RANTES induces NF-kB activity and subsequent secretion of MCP-1 (monocyte chemotactic protein -1), TNF-alpha and IL-8. (8) (20). Weyrich et al have demonstrated P-selectin on the platelet surface is relatively stable and sustains the platelet-monocyte contacts for hours. They have demonstrated

monocytes in the basal condition do not release MCP-1 whereas when monocytes are incubated with thrombin stimulated platelets there is significant increase in MCP-1 release by the monocytes. Thrombin activated platelets show increased surface expression of P-selectin which suggests P-selectin expression is required for the platelet-induced monokine secretion. This study also demonstrates the $F(ab')_2$ fragment of blocking mAb to P-selectin, G1,significantly attenuated MCP- 1 secretion in platelet-monocyte mixtures (8). In summary platelet monocyte interaction results in monocyte activation which subsequently renders the monocyte more adhesive, more procoagulant (via TF expression) and more inflammatory through the production of cytokines such as IL-6 and TNF- alpha. However, the pathological effect may not only depend on the formation of PMCs but may be dependent on the activation status of platelets and monocytes within the complex. It is a possibility that not all the platelets and monocytes within the complex are equally active. The activation status of platelets and monocytes within the complexes may have a differential site specific distribution. Activation status of the platelet and monocytes within the complex may be higher in the coronary circulation than the peripheral circulation of patients with coronary artery disease and acute coronary syndrome. P-selectin expression on the PMC indicates the platelet activation status and TF expression on the PMC indicates the monocyte activation status within the complexes. In summary the monocyte undergoes phenotypic changes after coming into contact with activated platelets resulting in the release of pro-inflammatory cytokines such as IL-6 ,TNF- alpha and TF which may have important consequences in the pathogenesis of ACS. These changes may not occur when monocytes come into contact with unstimulated platelets.

1.2.2 CLINICAL SIGNIFICANCE OF PMCS IN CORONARY ARTERY DISEASE:-

Furman et al have shown increased PMC in stable angina patients compared with control subjects (14). They have studied 19 patients with stable angina and 19 normal control subjects. Anticoagulated peripheral blood samples of patients with stable angina are also shown to form more PMC than control on stimulation with 1µmol/litre of ADP or 5µmol/litre of TRAP.

In a separate study Furman et al have also demonstrated higher numbers of circulating PMCs in patients with AMI compared with unstable angina patients. They have measured circulating PMCs in 211 consecutive patients with chest pain (61 patients with AMI diagnosed with greater than 3 times of control CK-MB levels and 150 patients without AMI). In the patients with AMI PMCs appear in the peripheral circulation earlier than routine markers of myocardial necrosis such as CK-MB. Of the 61 patients with AMI, 35 demonstrated normal CK isoenzyme ratio at the time of presentation but were found to have high circulating PMCs. They concluded that PMCs may reflect platelet activation caused by intracoronary plaque disruption, fissuring and erosion (21).

Sarma et al have examined PMCs in 52 patients (12 patients with unstable angina, 13 patients with AMI and 27 patients with non-cardiac chest pain) and have reported significantly elevated PMCs in the ACS patients compared to those with non-cardiac chest pain. Apart from higher total PMC frequencies in the AMI patients their report also suggests that calcium independent PMC formation is significantly higher in AMI patients. They have come to a conclusion that identification and quantification of PMCs in patients with chest pain may provide key early in vivo evidence of vascular injury responses and offer

opportunities for novel therapeutic intervention strategies in the treatment of acute coronary syndrome (22).

Ray et al have demonstrated increased pre intervention PMC levels are associated with increased troponin I level following PCI. They have collected blood samples from 40 patients before percutaneous intervention and 10 minutes after abciximab administration. Compared to healthy individuals, patients with coronary artery disease are found to have elevated PMC, P- selectin and PAC- 1 prior to PCI. Increased levels of pre PCI PMC are found to be associated with increased expression of P-selectin on the platelet surface. Their findings also reveal that abciximab therapy reduces platelet- monocyte aggregates but have no effect on platelet surface P- selectin (23). Therefore, increased pre PCI PMC may be a determinant of PCI related myocardial damage.

Ashman et al have studied stable patients on ambulatory peritoneal dialysis and haemodialysis patients without any evidence of coronary artery disease. Platelet expression of CD 62P and PMC formation has been found to be significantly increased in haemodialysis patients. Increased PMC is associated with reduced leukocyte PSGL-1 expression irrespective of dialysis modality. Their findings have also demonstrated higher PMC in dialysis patients is associated with increased cardiovascular events (24).

No reflow and “slow flow” phenomena following percutaneous coronary intervention is a problem and often associated with poor outcome. This phenomenon is observed in 5 to 25% of patients after PCI. There are multiple factors may be responsible for this phenomenon. The most likely cause is an acute microcirculatory disturbance due to distal embolisation of thrombus and/or plaque debris by mechanical intervention. An alternative explanation can be the

presence of a pro-inflammatory environment created by plaque disruption; either spontaneous or secondary to intervention. This process may generate activated platelet and initiate PMC generation. Increased production of PMCs at the site of plaque rupture may play an adverse role by activating the monocyte dependent inflammatory cascade and cytokine release. Ko et al have demonstrated increased levels of soluble CD40 ligand, IL-6, serotonin, TF and factor VII in the culprit coronary artery compared to the peripheral blood (25). Kotani et al have demonstrated no reflow phenomenon in 19.1 % of the patients who underwent a PCI procedure for acute coronary syndrome. Microscopic analysis of the aspirate at the time of no reflow has detected plaque elements including foam-cell macrophages, agitated platelets, cholesterol crystals as well as thrombi in the debris. Therefore it is probable that no reflow following PCI is not only due to distal embolisation of thrombi but also to plugging of the coronary microvasculature with plaque components (26). In Particularly activated platelets are known to release vasoactive substances and also to enhance PMC formation. PMC formation in the coronary tree may play a role in thrombus formation and perpetuation of coagulation and may also be linked with microvascular dysfunction by mediating ischaemia and reperfusion injury. During reperfusion PMCs may plug the capillaries in the coronary microcirculation and monocyte mediated inflammatory substance release and TF expression may result in the no reflow phenomenon, with loss of coronary vascular flow reserve. Sezer et al have demonstrated absolute and relative neutrophilia and higher MPV are independently associated with impaired microvascular perfusion in patients with anterior myocardial infarction treated with primary PCI. They have studied 41 patients with anterior wall myocardial infarction successfully treated

with primary PCI. They have measured leukocyte count, neutrophil count and MPV on admission. They have also measured CFR and IMR with the help of a fibreoptic pressure temperature sensor tipped guidewire in the left anterior descending artery within 48 hours after the PPCI. Increased neutrophil count and higher MPV are found to be associated with higher IMR lower CFR and higher coronary wedge pressure (27). Higher MPV may correspond to an increased number of both platelet- leukocyte and platelet- platelet aggregates. Therefore the association of MPV and impaired microvascular perfusion can represent platelet- leukocyte complex and platelet- platelet aggregates mediated microvascular injury and endothelial dysfunction in coronary arterioles and capillaries.

1.2.3 PMC FORMATION; SYSTEMIC OR LOCAL INTRACORONARY PHENOMENON ?

The presence of increased circulating PMCs have been demonstrated in patients with stable angina (14) unstable angina (28), AMI (2) and also in the patients who underwent PCI (29). Botto et al have demonstrated increased leukocyte-platelet functional interaction at the site of plaque rupture relative to the systemic circulation (30). They have studied 10 patients with AMI and aspirated intracoronary blood during PCI and compared leukocyte platelet adhesion index with peripheral blood. Their findings have demonstrated higher leukocyte-platelet adhesion index from the samples taken from the coronary occlusion site compared to peripheral circulation. They have also demonstrated an upregulation of CD18 adhesion molecule on monocytes and neutrophils in

coronary blood compared to peripheral blood. Their study suggests that increased leukocyte- platelet functional interaction in the blood at the site of plaque rupture may be one of the pathogenic mechanisms for no reflow in AMI patients.

In another study Patel et al have studied 39 patients (23 unstable angina and 16 stable angina patients). Trans-coronary gradient of leukocyte platelet aggregates are calculated from the coronary sinus and aortic root samples; trans-coronary gradient of platelet and leukocyte activation are also measured. Their findings have demonstrated 22% increase in neutrophil-platelet aggregates in the coronary sinus of patients with unstable angina compared to the aortic root samples and a 92% increment in CD 62 expressing platelets. In stable angina the increments are found to be 16% and 49% respectively. They have concluded that a transcoronary gradient of platelet- neutrophil aggregates suggests a local role of these complexes (31).

Elevated monocyte-platelet interaction at the site of the plaque rupture may play an adverse role in distal myocardial reperfusion by activating further inflammation. Increased levels of soluble CD40 ligand, IL-6, serotonin, TF and factor VII have been demonstrated in the culprit coronary artery compared to those in peripheral blood (25). These highlight the interaction between inflammation and thrombotic states at the site of the ruptured plaque. Wang et al. have shown a positive correlation of PMC with systemic IL-6, s P-selectin and CRP in stable angina patients suggesting relationship between PMC formation and systemic inflammation and platelet activation (32).

1.2.4 METHODOLOGICAL CONSIDERATIONS OF PMC ESTIMATION WITH FLOW CYTOMETRY:-

Different flow cytometric methods have been described for the estimation of PMCs. The high sensitivity of the assay leaves it vulnerable to artefactual in vitro activation. A number of factors e.g choice of anticoagulation, sample collection and processing technique potentially affects PMC estimation and frequencies vary widely amongst published data (33-35).

Unfractionated heparin activates platelets and increase PMC formation by a P-selectin dependent mechanism. Cation chelation with EDTA and EGTA markedly reduces in vitro platelet leukocyte interaction (22) . The Direct thrombin inhibitor PPACK is found to be reliable and does not cause cation chelation or platelet activation (36). Pearson et al have used CTAD (0.109 M buffered sodium citrate, 15 mM theophylline, 3.7 mM Adenosisne and 0.198 mM dipyridamole) or sodium citrate (0.106 M) (37) and they have found that CTAD prevents in vitro platelet activation following venepuncture.

Harding et al have demonstrated that PMCs increase in a time dependent manner in vitro irrespective of anticoagulant used. The rate of increase in PMCs appears to be slower in samples anticoagulated with citrate. It is therefore important to process the samples as soon as possible. Therefore in situations where there is a possibility of substantial delay prior to immunostaining and fixation it may be appropriate to use citrate as anticoagulant. They have also demonstrated following immunostaining and fixation that if the samples are stored at 4^0 c they remain stable at 24 hours (36).

There are differences of opinion regarding erythrocyte lysis during the processing of samples. Li et al have suggested that red cell lysis can lead to artificial increase in platelet leukocyte aggregation(34). On the other hand in abundance of erythrocyte flow cytometric analysis of PMCs become difficult and cumbersome. Barnard et al have demonstrated erythrocyte lysis allows efficient and accurate discrimination of leukocyte subpopulations and flow cytometry can be performed easily. Red cell lysis along with immediate fixation does not increase platelet- monocyte aggregation and is less time consuming to perform (36).

The method of blood collection also affects the PMC aggregation. PMC frequency increases significantly with time if samples are obtained through an intravenous cannula where as it does not change with time if samples are obtained through venepuncture (36) .

A consistent problem of overestimation of the platelet-monocyte complexes remains due to coincident events (38). Two colour flow cytometry using monoclonal antibodies specific for monocytes and platelet is widely used for PMC estimation; platelet-monocyte complexes appear as double positive events. However double positive events can arise not only from true complexes but also from non-interacting coinciding platelets and monocytes. As the concentration of platelets is higher than that of monocytes the chances of having one or multiple platelets close to but not attached to a monocyte are high and when a non-conjugated monocyte have a transit through the laser adjacent to (but not conjugated to) one or more platelets the cells can be detected as one event and appears as a PMC (39) (40).

It is imperative to develop a flow cytometry technique which can address these issues and is able to exclude the coincidence effectively and is capable of determining the true conjugates.

1.3 ROLE OF TISSUE FACTOR IN CORONARY ARTERY DISEASE:-

TF, formerly known as thromboplastin, is a 47-kDa glycoprotein expressed in both vascular and nonvascular cells. TF triggers the extrinsic pathway of the coagulation cascade. The classic view of coagulation implies that upon disruption of the vessel wall, TF sequestered in the adventitia is exposed to flowing blood with consequent activation of the coagulation cascade and thrombus formation. This view has been challenged by demonstration of circulating TF. Palmerini et al. have demonstrated *in vitro* that circulating TF plays a pivotal role in thrombus formation on stents. Monocytes appear to be the main source of TF depositing in the thrombus. They have also demonstrated that only monocytes attached to platelets stained positive for TF expression (41). On the contrary Weyrich et al have found thrombin activated platelets do not elicit TF activity on the monocytes (8). In the vessel wall TF is expressed in sub-endothelial cells, such as vascular smooth muscle cells, leading to rapid initiation of coagulation when the vessel is damaged (42). Endothelial cells and monocytes do not express TF under physiological conditions. TF expression in monocytes can be induced by inflammatory stimuli such as CRP or CD40 ligand (43) together with PDGF-BB, angiotensin II, and oxidized LDL (44),(45) have also been observed to induce TF in monocytes. Endotoxin is one of the most extensively studied stimuli which is known to induce TF in monocytes (46) (47).

TH1 but not TH2 cells secrete mainly pro-inflammatory mediators such as TNF-alpha and interferon,which are involved in macrophage activation (48). Cytokines derived from TH1 cells as well as cell-to cell contact with TH1 cells induce TF

expression in monocytes whereas TH2-derived mediators such as IL-4, IL-10 and IL-13 prevent TH1-induced TF expression (49). TF expression is increased once monocyte derived macrophages transform into foam cells (50). Infiltration of the monocyte in the intimal layer and then transformation into macrophages and foam cells are the hallmark of the inflammatory nature of atherosclerosis (48). Cytokines such as TNF- alpha and interleukins are released and induce expression of TF in this inflammatory environment. Enhanced TF expression is observed in monocytes not only in the early stage but (42) at later stages, TF expression is also detected in foam cells, endothelial cells, and smooth muscle cells (42) (51). Increased levels of TF antigen and activity are detected in atherectomy specimens from patients with unstable angina or myocardial infarction as compared with those with stable angina (52).

Maier et al have demonstrated in ACS plasma concentration of inflammatory cytokines such as TNF alpha and interleukins are increased at the site of coronary artery occlusion to such an extent that TF is induced in vascular cells (53). In patients with ACS vascular cells as well as circulating leukocytes and aggregating platelets may be a source of the elevated levels of circulating TF (54). Exposure of highly pro-coagulant plaque content following plaque rupture in acute coronary syndrome patients may also contribute to the elevated TF plasma levels (55). Virmani et al have demonstrated higher plasma TF levels in patients with unstable angina compared to those with stable angina (56) and elevated TF plasma levels may even predict future cardiovascular events in patients with unstable angina(56). Because a substantial number of patients with acute myocardial infarction have coronary artery thrombi on top of a superficial erosion, increased TF plasma levels in these patients may also originate from endothelial

erosions of atherosclerotic lesions(56). Interestingly, several polymorphisms of the TF gene are known, and certain data suggest that certain genetic variations in the TF gene as well as the TF promoter may be associated with a worse outcome in patients with acute coronary syndrome, possibly through increased monocyte TF expression(57) (58). Increased TF levels have a negative prognostic value with regard to the development of restenosis after percutaneous coronary angioplasty with or without stenting (59).This effect may well be related to the pro-migratory and pro-proliferative action of TF on vascular smooth muscle cells, which are known to contribute to the development of restenosis (60) (61) (62).

Monocytes are one of the principal sources of circulating TF. Human megakaryocytes neither express TF mRNA nor protein and it has been hypothesised that growing platelets may take up TF only from other cells. Indeed, TF containing microparticles, shed from monocytes and possibly polymorphonuclear leukocytes, are taken up by mature platelets via a CD15 and P-selectin dependent interaction (63, 64). In addition, activated endothelial cells release TF containing microparticles, which could potentially be transferred to platelets as well (65, 66).

1.4 PSELECTIN:-

1.4.1 ROLE OF P SELECTIN IN CORONARY ARTERY DISEASE:-

P- selectin is a member of the selectin family and is localised in the alpha granule of platelets and the Weibel-Palade bodies of endothelial cells (67). P-

selectin is the largest of the selectins with a mass of 140 k Da. P- selectin has an N- terminal lectin domain, an epidermal growth factor motif, in general nine regulatory protein repeats, a transmembrane section and a short intracytoplasmic tail (68). P- selectin on the activated platelets is an aid to leukocyte or endothelial adhesion, and inhibition with monoclonal antibodies to P- selectin is able to achieve de-aggregation (69).

Apart from the cellular form of P- selectin soluble P- selectin has also been identified. Soluble P- selectin lacks the cytosolic/transmembrane domain. Plasma sP-selectin mostly appears from active cleavage from the cell surface, presumably by a non specific enzyme or other mediators that arise both in leukocytes and/or endothelium (70, 71).Though the activated endothelial cell is also known to be a source of s P- selectin it is believed the majority arises from platelets (72). It has been suggested that sP-selectin, like its membrane counterpart has biological activities of its own as it posses the lectin and epidermal growth factor domains required to bind the physiologically relevant P-selectin receptor PSGL-1(73). Blood levels of sP- selectin are found to correlate with the progression of atherosclerosis in humans (74, 75).

Wollard et al have demonstrated s P- selectin is biologically active and induces neutrophil activation through engagement of PSGL-1, independent of any contribution from the membrane bound platelet p selectin. They suggest s P-selectin may promote leukocyte recruitment to the site of vessel wall injury in patients with vascular disease. Soluble P- selectin binding to PSGL-1 on leukocytes initiates a signalling cascade to activate Mac-1, enabling it to bind to activated platelets and/or endothelial cells (76, 77).

1.4.2 INFLAMMATION AND SOLUBLE P- SELECTIN:-

Although sP-selectin predominantly originates from activated platelets it is unclear whether it has any causal relationship in inflammation associated with coronary artery disease. While IL-6 has been shown to be a stimulator of platelet function there is no evidence to suggest it results in increased membrane or sP-selectin (78). Schumacher et al have demonstrated increased levels of IL-6, TNF- alpha and all soluble adhesion molecules in patients with coronary artery disease compared to matched controls. Their observations fail to show any significant multivariate correlations between soluble P- selectin, CRP, IL-6 and TNF alpha. These observations fail to support the hypothesis that levels of sP-selectin are responsive to inflammatory cytokines (79). Soluble P- selectin is not a surrogate marker of platelet cell surface P- selectin. Gurbel et al has shown no correlation between soluble and cell surface P- selectin in patients with non cardiac chest pain, unstable angina, acute myocardial infarction and congestive heart failure (80). They also hypothesise soluble P- selectin reflects antecedent events (platelet activation) but may not reflect simultaneous platelet activation. It is not clear whether there is any correlation between platelet activation on the PMC (P- selectin positive PMC) and sP- selectin levels (80).

1.5 ROLE OF INFLAMMATION IN THE PATHOGENESIS OF ACUTE CORONARY SYNDROME:-

In ACS inflammation plays a significant role in the disease process and increased levels of inflammatory parameters e.g CRP and SAA (81) have been associated with adverse outcomes in the patients with ACS (82),(83),(84). Mulvihill and colleagues have also found that a CRP concentration of < 3mg/l has a negative predictive value of 97% for a major adverse cardiovascular event within six months. Conversely a CRP concentration of > 3 mg/l is found to have a sensitivity of 96% for predicting adverse cardiovascular events, with a specificity of 52 %. It is not clear whether increased levels of these inflammatory markers have any causal relationship with the pathogenesis of ACS (85).

1.5.1 INTERRELATIONSHIP BETWEEN INFLAMMATION AND PMC FORMATION:-

There is an interrelationship between inflammation, platelet activation and platelet monocyte complex formation. An increased state of inflammation is paralleled by activation of platelets with phosphorylation of the membrane protein p-selectin in the platelet alpha granule membrane (86). Upon activation from TNF- alpha and IL-1 the granule moves to the platelet surface exposing P-selectin and tethering the platelet with the monocyte via PSGL-1 to form PMCs. Formation of PMCs is important in regulating monocyte expression and secretion of TF and inflammatory cytokines. There is evidence to suggest that inflammation may precede myocardial injury; Hillis and colleagues have found

increased expression of CD11b/ CD 18 (Mac-1, CR3) on circulating neutrophils and monocytes of patients with unstable angina, this appears to occur in some patients in the absence of any detectable cardiac troponin I (87). Other studies have indicated that neutrophils and monocyte activation occur in unstable angina, and this may happen in the coronary circulation (88), (89). An unstable and subsequently ruptured atherosclerotic coronary plaque with superimposed thrombosis constitutes the most common, general and pathological background of the ACS (90).To elucidate the role of inflammation in the pathogenesis of ACS, many studies have focused on the sensitive and specific serum biomarkers of inflammation for vulnerable plaques (91).

Upon activation platelets also express CD40L. This transmembrane protein is structurally related to TNF- alpha. CD40L is rapidly expressed by activated platelets and induces the expression of chemokines and cell adhesion molecules by endothelial cells thus provoking cell attraction, activation and migration into the arterial wall (9, 10). Danenberg et al (92) have reported incubation of human blood with recombinant human CRP doubles PMC formation. CRP-induced PMC formation is calcium and P-selectin dependent. CRP modulates PMC both ex vivo in human blood, and in vivo in mice transgenic to the human CRP gene. These results further implicate PMCs as an inflammatory-thrombotic link that may help mediate the pro-thrombotic activity of CRP. CRP increases platelet activation. As monocytes are recruited at sites of endothelial injury, increased PMC levels may also result in more activated platelets being recruited to sites of plaque instability, further increasing the risk of thrombosis.

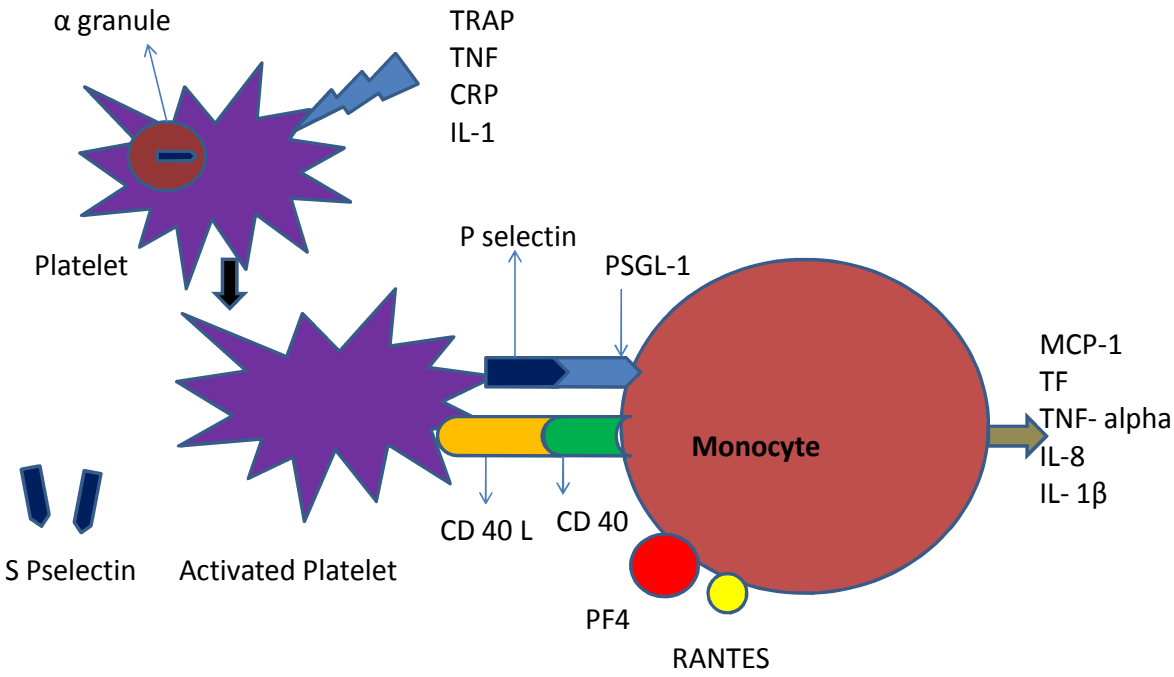


Figure 1.1:- Interrelationship between platelet monocyte and inflammatory parameters Adapted from van Gils JM et al. (93)**.** Upon activation (sometimes by different inflammatory markers e.g TNF alpha, hs CRP etc) the platelet alpha granule degranulates and P- selectin is expressed on the activated platelet surface. Platelets get attached to the monocyte through the PSGL-1. Apart from this P-selectin/ PSGL-1 mediated conjugation there are several other integrin dependent mechanisms by which platelet monocyte complexes are formed. Another important interaction is between CD40 Ligand on the platelet surface and CD40 on the monocyte. P -selectin from the platelet surface also gets shed and circulates as sP- selectin. Platelet chemokine RANTES and platelet factor 4 (PF4) also play roles in monocyte activation. Activated monocytes undergo certain changes and produce different inflammatory cytokines and TF.

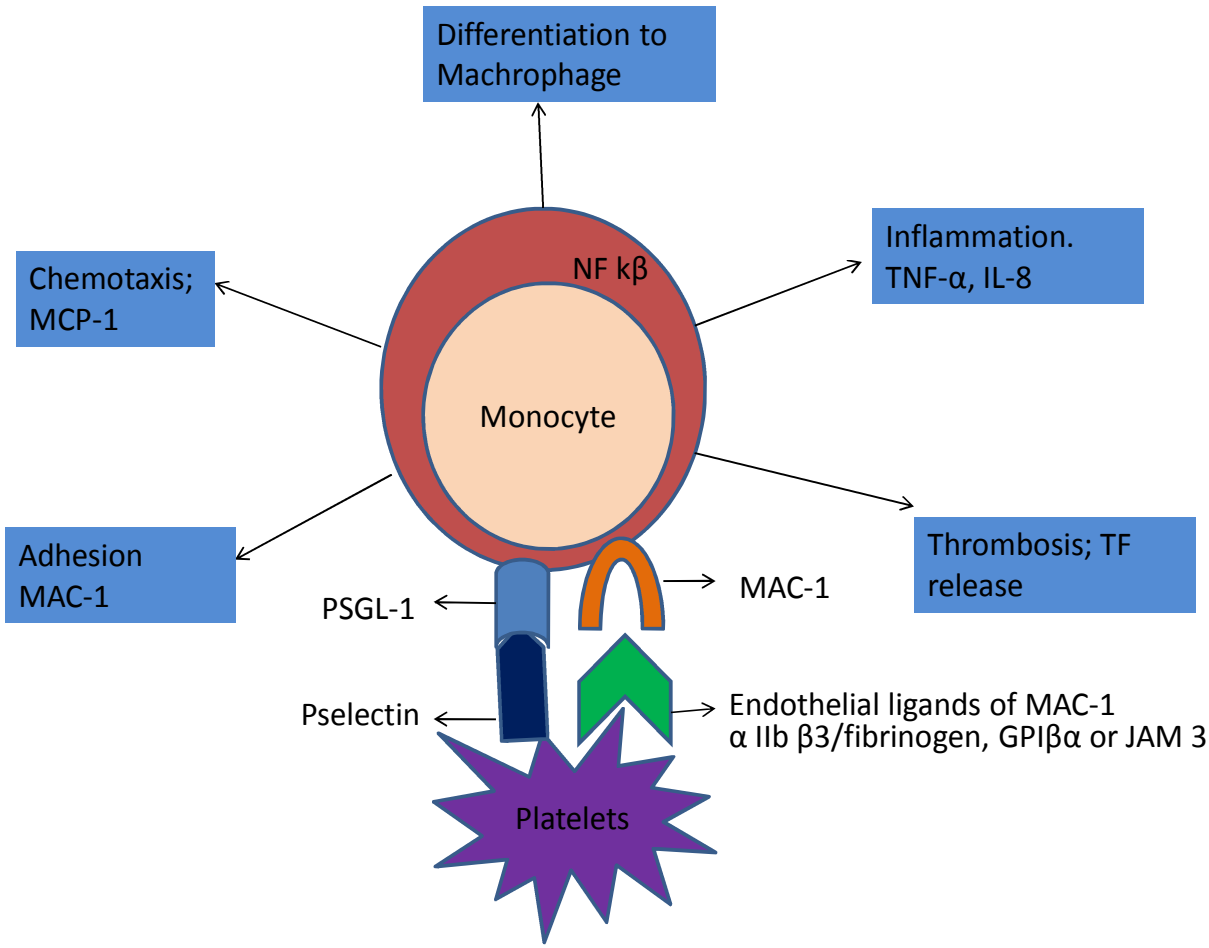


Figure 1.2:- Platelet and monocyte interaction induced thrombotic and inflammatory changes in the monocyte. Monocytes undergo certain changes following interaction with platelets becoming more thrombotic, inflammatory and more adhesive.

1.5.2 INFLAMMATION - SYSTEMIC OR INTRACORONARY EVENT?

It remains unclear whether inflammatory markers originate from the rupture of plaque or represent a systemic process. Inflammatory activity has been assessed mainly by measuring the levels of CRP, SAA or pro-inflammatory cytokines in the peripheral blood. Increased local levels of pro-inflammatory cytokines are known to promote hepatic synthesis of CRP and SAA. Measurement of inflammatory markers in the peripheral circulation may not be appropriate for evaluation of local inflammatory activity. Approaches to the evaluation of local inflammatory markers include use of local perfusate or by measuring the difference in concentration across the coronary circulation. It has been reported that the concentration of IL-6, a major inducer of CRP and SAA synthesis, increases immediately in the coronary sinus after PTCA (94). An elevated coronary sinus-arterial difference in the level of IL-6 has been reported in patients with acute coronary syndrome (95). Cusack et al have demonstrated rise in the levels of IL-6 and TNF alpha between coronary sinus and aortic root suggestive of intra-myocardial synthesis of these inflammatory markers in unstable angina. When dividing these patients according to the troponin T levels the trans-myocardial gradient is mostly associated with the evidence of myocardial injury (96).Ko et al.(25) have shown increased levels of IL-6, serotonin, TF, CD40 ligand, factor VII in culprit coronary artery in comparison to peripheral blood during percutaneous coronary interventions in 18 patients with acute myocardial infarction. These findings also suggest local intracoronary production of inflammatory and thrombotic markers in acute myocardial infarction. Some studies have demonstrated the existence of SAA and CRP in

focal atherosclerotic lesions, e.g CRP has been found to co localize with the terminal complement complex in the intima of early atherosclerotic coronary arteries (97) and mRNA of CRP has also been detected in both smooth muscle like cell and thickened intima of atherosclerotic plaques (98). Therefore CRP may be synthesized in the intima of atherosclerotic coronary arteries for certain period and may interact directly with atherosclerotic coronary arteries.

Maier et al. (53) have demonstrated increase in levels of IL-6 by > 70% and SAA by around 10 % in the culprit coronary artery relative to those in the aorta in the STEMI patients. According to their conclusion this differential expression of increased inflammatory markers must be related to the arterial wall within the ruptured plaque or by the blood cells trapped in the occluded coronary artery. With immunohistochemistry they have also demonstrated co localization of IL-6 with monocytes in the thrombus occluding the culprit coronary artery. Though SAA is produced predominantly in the liver (99) the increase in local SAA blood levels in the culprit coronary artery is suggestive of local production of SAA at the site of occlusion either by cells of the atherosclerotic arterial wall or by the white blood cells trapped in the thrombus. In line with this interpretation SAA mRNA and protein have been detected in human atherosclerotic lesions and cultured arterial smooth muscle cells. (100).

The principal source of CRP and complement components has always been assumed to be the liver. Up-regulation of CRP after tissue injuries such as acute myocardial infarction has been attributed to induction of CRP in hepatocytes by inflammatory cytokines such as interleukin-6 (101). Apart from systemic response to inflammation it has now been shown that cells within the atherosclerotic plaque also synthesise CRP (98, 102). Macrophage and smooth

muscle like cells in atherosclerotic plaques produce seven times more CRP mRNA than the liver (98).

Many types of cells including lymphocytes, monocytes, vascular smooth muscle cells and endothelial cells produce interleukin-6 (IL-6) (103) which is a major determinant of CRP production in liver (104). Deliargyris et al found that IL-6 is produced in the coronary circulation of patients who have unstable angina but not in the patients who have stable angina (95).

Several lines of evidence suggest SAA may play a pathophysiological role in atherosclerosis. SAA is present in human atherosclerotic lesions (100) and SAA proteins can be produced by cells of the artery wall.

Increased local levels of pro-inflammatory cytokines are known to promote hepatic CRP and SAA, but concentrations of these markers in the peripheral circulation have been found to have no correlation with angiographic severity of coronary artery disease in patients with stable angina (105, 106). This fact indicates that measurement of inflammatory markers only in the peripheral blood samples may not be appropriate for evaluation of local inflammatory activity in coronary artery. Efficient evaluation of such local inflammatory markers can be done by the use of local perfusate or by difference in concentrations across coronary circulation.

1.6 INFLAMMATORY MARKERS IN CORONARY ARTERY DISEASE:-

1.6.1 ROLE OF CRP IN CORONARY ARTERY DISEASE:-

CRP stimulates production of TF by mononuclear cells, (107) the main initiator for blood coagulation. CRP interacts with low density lipoprotein and with damaged cell membranes (108) and can also activate the complement system (109). CRP induces PAI -1 expression from the endothelial cells and suppresses NO release from the endothelial cells (110).

Numerous studies have demonstrated that an elevated CRP in the setting of unstable angina and non Q wave myocardial infarction is associated with a worse prognosis (83, 111) (112) (11). Elevated levels of CRP in patients with Q wave myocardial infarction is reported to be associated with cardiac rupture, left ventricular aneurysm and cardiac death at one year (113). Tommasi et al have also reported prognostic value of CRP levels in patients with first acute myocardial infarction. Only increased CRP levels are independently associated to the incidence of patients who develop cardiac events (cardiac deaths, new onset angina, and recurrent myocardial infarction). Importantly this group has not reported any correlation with CRP levels and extent of rise in cardiac enzymes (114, 115). Biasscci et al have reported on the prognostic significance of CRP elevation in patients with unstable angina without myocardial injury (116). In the setting of percutaneous coronary revascularisation a hyper responsive reaction of the inflammatory system defined by elevation of CRP, IL-6 and SAA after angioplasty is considered to be a worse prognostic factor (117). Gaspardone et al have confirmed this by showing that persistent elevation in CRP 72 hours after

coronary artery stenting identified all patients who later suffered adverse outcome. In contrast no cardiac events occurred in those with normal levels at one year follow up (118).

CRP levels are a strong predictor of future cardiac events in apparently healthy men as suggested by MONICA (Monitoring Trends and Determinants in Cardiovascular Disease Study) study (119). Patients with highest quintile of CRP levels are shown to have 2.6 fold increased risk of suffering a fatal or nonfatal myocardial infarction or sudden cardiac death. Similarly in Women's Health Study patients with higher baseline hsCRP levels than control subjects are found to be associated with cardiovascular events and patients with highest baseline levels are associated with five and seven fold increase in any vascular events and combined stroke or myocardial infarction respectively (120).

1.6.2 ROLE OF SAA IN CORONARY ARTERY DISEASE:-

Several lines of evidence suggest SAA may play a pathophysiological role in atherosclerosis. First SAA is found as an apolipoprotein on HDL particles and may play a role in acute modification of cholesterol transport during physiological stress. Secondly SAA have been shown to be chemotactic for monocytes (121). During the acute phase of inflammation, SAA is associated with HDL, during which it rapidly becomes a predominant apolipoprotein, and acute-phase HDL may mediate delivery of phospholipids, cholesterol, and cholesterol esters to regenerating tissue at sites of inflammation, where neutrophil, monocyte and macrophages are present. Thus SAA may play an important role in the inflammatory process and reversal of cholesterol transport in the atherosclerotic

coronary circulation (122).Transition from chronic stable atherosclerotic coronary artery disease into an ACS is associated with an increase in inflammatory activity within the plaque, (123) reflected in an increase in CRP and SAA levels (83). SAA induces IL-1 and IL-8 in neutrophils and interferon gamma in lymphocytes, promotes monocyte chemotaxis and adhesion (124),(125, 126), induces matrix-metalloproteinases and activates NF-kB in monocytoid THP-1 cells (127). SAA deposits are seen in the atherosclerosis lesions (53) and activated macrophages express SAA genes (128). SAA also induces TNF in normal neutrophils and monocytes (129). SAA is also known to be a potent and rapid inducer of TF on normal monocytes via the NF-Kb, ERK ½ and p38 MAPK pathways (130).

Matsubara et al (131) have reported severity of coronary atherosclerosis is positively related to the increment of SAA across the coronary circulation. The TIMI 11A sub-study suggested SAA may provide important prognostic information with respect to short term mortality among patients presenting with an acute coronary syndrome without ST elevation. Markedly elevated SAA is predictive of increased mortality at 14 days even among those with a negative troponin (132). Liuzzo et al in a study of 31 patients presenting with unstable angina without evidence of myocardial necrosis have reported more frequent recurrent angina, myocardial infarction, higher rates of revascularization, death in those patients who presented with elevated SAA (83) .

Song et al have demonstrated peripheral blood mononuclear cells from patients with ACS, but not stable angina, express higher basal TF than controls suggesting a hypercoagulable state in ACS (133). They have also demonstrated SAA induced higher TF activity in patients with ACS, but not stable angina, indicating that SAA may contribute to the hypercoagulable state in patients with

ACS. In a different study Cai et al have shown excessive SAA synthesis under inflammatory conditions leads to an imbalance of SAA/HDL plasma levels(130).The deposition/production of SAA in plaque (53) may represent a powerful and rapid pro-thrombotic amplification mechanism. Their findings suggest TF response to SAA differs in ACS patients compared to controls, and the difference in induced TF between ACS and stable angina is of borderline significance. SAA, that strongly induces both TF and TNF, may have the capacity to influence acute events in CAD. Song et al have also demonstrated upregulation of TNF mRNA in SAA-stimulated peripheral blood mononuclear cells within 30 min and peaked at 1 h, indicating that TNF is an early response gene product of SAA. They have reported unlike TF, basal peripheral blood mononuclear cell-derived TNF levels are similar in patients and controls, and low dose SAA (1 ng/mL) only induces TNF in cells from ACS patients. Their major findings is that across the range of doses, SAA induces more TNF in cells from ACS patients than in stable angina or controls, suggesting SAA may be a pro-inflammatory amplifier in ACS (133).

1.6.3 ROLE OF TNF- ALPHA IN CORONARY ARTERY DISEASE:-

TNF contributes to plaque instability by promoting inflammatory processes and inducing matrix metalloproteinases in atherosclerotic lesions (134). Elevated serum levels of TNF in CAD predict coronary events in population studies (135).Reperfusion induces TNF-alpha expression in the coronary microcirculation and TNF-alpha can impair endothelium- dependent coronary flow reserve (136) which may adversely affect outcome. TNF-alpha can cause depression of cardiac contractility either directly (137) or by induction of inducible NO synthase (i NOS) in cardiocytes and other cellular constituents within the heart (138, 139). Upregulation of TNF- alpha is known to mediate and influence a multitude of interactions resulting in progressive inflammation and plaque destabilization and prothrombotic tendencies. Administration of TNF alpha antibody has been demonstrated to rapidly downregulate a spectrum of cytokines (IL-6) and acute phase proteins e.g amyloid A, haptoglobin and fibrinogen (140). It is not entirely known whether TNF- alpha is generated locally in the coronary bed or it is a systemic response. A significant transmyocardial gradient is expected if TNF- alpha is generated in the coronary circulation of patients with ACS. In some experimental (141) and clinical (142, 143) studies, the TNFα concentrations do not differ in paired arterial and coronary sinus blood samples, suggesting that TNF- alpha is not of cardiac, but of systemic origin. Aged rats have increased circulating levels of TNFα which are associated with endothelial dysfunction in coronary arteries (144, 145) and chronic TNFα inhibition improves flow-mediated dilatation in mesenteric arteries of aged rats (146).

Increased TNFα concentration contributes to vascular dysfunction in peripheral as well as in the coronary arteries. TNFα induces the rapid expression of cell adhesion molecules (CAM) such as E-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1) at the endothelial surface. These molecules mediate the attachment and transmigration of leukocytes from the blood stream into the vascular wall (147). In the cellular level following contact with platelets, activated monocytes produce TNFα which impacts primarily on the extrinsic pathway of the coagulation cascade with its central component, i.e. TF (148). TF mediates thrombin formation, leading to fibrin clot formation and intravascular fibrin deposition. Under pathophysiological conditions, TF is expressed on the surface of macrophages, neutrophils, and endothelial cells (149) (150) (81, 151).TNFα also contributes to increased uptake of LDL into monocytes/macrophages; uptake of oxidized LDL in turn increases TNFα release, thus initiating a vicious cycle (152).

1.6.4 ROLE OF IL-6 IN CORONARY ARTERY DISEASE:-

IL-6 is one of the most widely studied biomarkers and has been accepted as a valuable inflammatory marker to identify those at high risk of cardiac events. IL-6 is one of the main triggers of CRP release from the liver. In patients with unstable angina IL-6 is a strong predictor of risk of serious coronary events (153). Ohtsuka et al. have reported that circulating IL-6 levels correlate closely with left ventricular geometric changes during the remodelling process in patients with reperfused MI (154). Tan et al have demonstrated IL-6 significantly and independently correlates with the onset of STEMI and cardiac mortality during 24

month follow-up suggesting that IL-6 may play a key role in the development of CAD (155). IL- 6 has also been shown to be elevated in ACS and is associated with increased risk of in-hospital events (156).IL- 6 is produced by variety of inflammatory cell types and has been shown to remain elevated up to 4 weeks after a myocardial infarction. It increases fibrinogen and PAI-1 and promotes adhesion of neutrophils and monocytes during myocardial reperfusion and produce a negative inotropic effect on myocardium (157) (137, 158, 159).

Cusack et al have shown during the first 48 h after admission, the levels of IL-6, CRP and TnT are high in those who subsequently had a major adverse cardiac events. One week after admission, the prognostic value of IL-6 had been lost (96). Date et al have studied 35 patients (11 patients with angiographically proven coronary artery disease and 24 patients with angiographically normal coronary arteries) and they have demonstrated the amount of IL-6 produced in the coronary circulation is significantly higher in patients with coronary atherosclerosis compared with who had normal coronary artery. They have measured transcoronary IL-6 by measuring the concentration difference between coronary artery and great cardiac vein. They have also demonstrated a significant positive correlation between IL-6 and CRP in coronary circulation and have established a relationship between coronary microvascular resistance and transcoronary IL-6 production(160). IL-6 is one of the main triggers of C reactive protein release. In apparently healthy male 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 (161).

From the above discussion it is clear that there is a wealth of data to support the hypothesis that PMC formation, subsequent monocyte activation and inflammation play an important role in the entire process of coronary artery disease. PMC formation and inflammation are interlinked. As PMC formation can initiate an inflammatory response, similarly inflammatory cytokines can also initiate platelet activation and subsequent PMC formation. Formation of PMC is P- selectin dependent. It is not clear whether PMC formation is a systemic phenomenon or it is mainly a local intracoronary event which drives the local intracoronary inflammation and play its part in the pathogenesis of acute coronary syndrome. It is also not clear whether the platelets and monocytes within the complex are equally active or they demonstrate differential increased site specific activation in the coronary circulation of the patients with ACS. It is also unclear how this activation status can be responsible for driving systemic or local intracoronary inflammation in patients with CAD and specifically in patients with ACS. Similarly it is yet to be established what relationship soluble P-selectin has with activated PMCs and whether there is any relationship with soluble P- selectin and inflammation in patients with coronary artery disease.

Jurk et al. have investigated the role of leukocyte and platelet activation in patients with severe asymptomatic and symptomatic carotid artery disease. They have demonstrated monocytes from symptomatic (acute stroke aetiology) and asymptomatic patients are highly activated, shown by significantly enhanced presentation of inflammatory markers CD11b and TSP-1 on the surface. Both are found to have positive correlation with monocyte-platelet association rate. Circulating single as well as monocyte-bound platelets from symptomatic

patients have shown significantly enhanced surface expression of P-selectin and TSP-1, whereas platelets from asymptomatic patients are not found to be significantly activated(162). These results emphasise the importance of monocyte and platelet activation status during the pathogenesis of atherothrombotic cerebral ischaemia.

1.7 OBJECTIVES:-

1 To develop a flow cytometric method for the accurate estimation of PMC by effectively reducing the influence of coincidence events.

2 To compare systemic and site specific (coronary circulation, aorta and systemic venous) PMC, P-selectin and TF expression on the PMCs between ACS and stable angina patients.

3 To examine the presence of site specific differential activation status of platelet and monocytes within these complexes.

4 To evaluate the relationship of inflammatory parameters with PMCs and P-selectin positive PMCs in systemic as well as coronary circulation in ACS patients.

5 To examine the role of sP- selectin and its correlation with inflammation in patients with coronary artery disease.

CHAPTER 2

MATERIAL & METHODS

2.1 General Methods

2.1.1 Patients

2.1.1.1 STEMI patients.

2.1.1.2 NSTEMI patients.

2.1.1.3 Stable patients.

2.1.2 Coronary Intervention.

2.2 Flow Cytometry.

2.2.1 Collection of blood sample for flow cytometry.

2.2.2 Parameters measured by flow cytometry.

2.2.3 Preparation of samples for flow cytometry.

2.2.4 Flow cytometry technique.

2.3 Estimation of Inflammatory Parameters and Soluble P Selectin.

2.3.1 Collectin and preparation of blood samples for estimation of CRP, SAA, TNF- alpha, IL-6 and soluble P-selectin.

2.3.2 ELISA

2.3.2.1 Estimation of TNF- alpha and IL-6.

2.3.2.2 Estimation of soluble P -Selectin.

2.3.3 Estimation of high sensitive CRP and SAA.

2.1 GENERAL METHODS:-

2.1.1 PATIENTS

Fifteen patients with acute STEMI, 8 patients with NSTEMI and 7 patients with stable angina were recruited for the study.

2.1.1.1 STEMI PATIENTS: -

Fifteen consecutive STEMI patients were recruited following diagnosis based on the chest pain and characteristic ECG changes of ST elevation in the community by ambulance paramedics or by medical staff in the emergency department of a nearby district general hospital. Acute ST elevation myocardial infarction patients were brought straight to our hospital catheter laboratory for PPCI as a preferred method of treatment for acute myocardial infarction. These STEMI patients were consented and recruited before PPCI outside the cardiac catheterisation laboratory. Ethical permission was granted by the local ethics committee of the Royal Free Hospital. All the 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. All the patients were given weight adjusted unfractionated heparin to keep the ACT between 200- 250 sec before the PPCI.

2.1.1.2 NSTEMI PATIENTS: -

Eight NSTEMI patients who were treated with PCI were recruited. NSTEMI was diagnosed on the basis of a history of cardiac sounding chest pain at rest with or without acute ECG changes and a 12 hour troponin T value > 0.03 ng/l. On arrival in our hospital all 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) prior to PCI. All NSTEMI patients were consented and recruited for the study before scheduled coronary angiography. Angiography was performed in all cases within 72 hours of onset of chest pain. All the NSTEMI patients also received weight adjusted unfractionated heparin in the catheter laboratory before PCI to maintain the ACT between 200 and 250 sec.

2.1.1.3 STABLE ANGINA PATIENTS:-

Elective patients with stable angina pectoris scheduled for day case coronary angiography and PCI were recruited for the study. All patients had a prior diagnosis of angina via the chest pain clinic or in the community on the basis of cardiac sounding chest pain and a positive exercise tolerance test or other non-invasive test such as stress echocardiography or myocardial scintigraphy. All the patients had been reviewed by a cardiologists and decision for diagnostic angiography had been made by the cardiology team. Seven consecutive stable angina patients who were treated with percutaneous intervention were recruited. These patients had already received anti - anginal medication. Apart from anti-anginal medications stable angina patients had also been treated with 75mg of

Aspirin a day. Seventy five milligram of Clopidogrel per day was added 7 days prior to the angiographic procedure as per hospital protocol. All patients were treated with weight adjusted unfractionated heparin before the intervention to achieve an ACT between 200 and 250 sec.

2.1.2 CORONARY INTERVENTION:-

Diagnostic coronary angiography was performed via the right femoral artery route through a 6F sheath following 1% lignocaine local anaesthesia. A 6 F 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) and 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 5 F pigtail catheter (5F, Cordis ®, internal diameter 0.11cm) was placed in the right atrium. Following diagnostic angiography the culprit lesion was identified and weight adjusted unfractionated heparin was given. Blood samples (10 ml from each site) were aspirated through the diagnostic 5F catheter with the help of a 10ml syringe from the ascending aorta and 5F pigtail catheter from right atrium. After identification of the culprit lesion an appropriate guiding catheter was inserted and lesion crossed with an angioplasty wire. Once wired 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 the help of a syringe. In NSTEMI and stable angina patients if due to technical reasons passage of export catheter was difficult initially then

predilation of the lesion with a balloon was performed in order to pass the export catheter distal to the lesion. All the samples were collected before patients were treated with GP IIb/IIIa antagonists. Following collection of samples PCI was performed according to standard procedure.

Coronary samples were collected to measure the expression of local intracoronary inflammatory parameters,(CRP, SAA, TNF alpha, IL-6) and markers of platelet activation e.g s P- selectin, PMCs, P- selectin positive PMC and TF positive PMC at the site of the culprit lesion. Similar parameters were measured in aortic and right atrial samples to ascertain the systemic arterial and systemic venous concentrations of inflammatory markers, soluble and cell surface platelet activation markers. To evaluate the true systemic values ; overall median values were calculated by calculating the median values from all the 3 sites rather than relying only on values from one sample from the venous circulation. Trans-culprit lesion gradient of the above parameters was calculated by the difference between the values from coronary artery and aorta. Samples from all the sites were carefully aspirated through catheters of similar internal diameter in order to maintain similar shear stress during aspiration of samples to prevent shear stress related differential in vitro platelet activation from different sites.

2.2 FLOW CYTOMETRY:-

2.2.1 COLLECTION OF BLOOD SAMPLE FOR FLOW CYTOMETRY:-

Ten milliliter blood was collected from each of the 3 sites. Out of this 10 milliliter 4 milliliter was collected into a separate sterile vacutainer tubes containing a

combination of 3.2% sodium citrate and EDTA for flow cytometric analysis of PMC, p-selectin and TF expression on PMC . EDTA was added to sodium citrate to stop in vitro calcium dependent PMC formation. Samples were immediately put onto ice and transported to the haematology laboratory for immediate preparation for flow cytometry.

2.2.2 PARAMETERS MEASURED BY FLOW CYTOMETRY:-

From the samples collected from the coronary artery, aorta and right atrium flow cytometric analysis was performed to estimate PMCs, p-selectin expression on these PMCs (p-selectin positive PMCs) and TF expression on these PMCs (TF positive PMCs).

Overall median PMC, overall median P-selectin positive PMC and overall median TF positive PMCs were estimated by calculating the median values from all these 3 sites. P- selectin positive PMC indicates presence of activated platelet within the complex and TF positive PMC indicates presence of activated monocytes within the complex. Differential expression of P-selectin and TF positive PMCs in these 3 sites indicated differential platelet and monocyte activation status within the complexes in different sites. Difference between aorta and coronary artery values were also calculated to obtain a transculprit lesion gradient of the above mentioned parameters.

2.2.3 PREPARATION OF SAMPLES FOR FLOW CYTOMETRY:-

Collected blood samples were immediately transferred to the laboratory and prepared for flow cytometry. Analysis of all the samples were complete within 2

hours of collection. Two round bottom polystyrene tubes (BD Falcon 12 x75 mm style) were taken. Five microlitre of anti CD61 FITC, anti CD14 PerCp and anti CD 142 PE were taken in the first tube (tube 1) and in the second tube anti CD142 PE was replaced by anti CD62P PE (tube-2). One hundred microlitres of whole blood were aliquoted into each of the tubes. The samples were incubated at room temperature for 20 minutes. After that erythrocytes were lysed by addition of 2 ml of easy lyse™ solution (Dako) (1 in 20 dilution) for 12 minutes at room temperature. Cells were washed with addition of 1000 microlitres of FACS flow and centrifuged at 300g for 5 minutes. After that the supernatant was discarded and the cells resuspended in 500 microlitres of FACS flow for immediate flow cytometric analysis (FACS Calibur equipped with Cell Quest ® soft ware – BD Biosciences, Oxford, UK).

2.2.4 FLOW CYTOMETRY TECHNIQUE:-

Cells were acquired on a 2D dot plot arraying CD14 (logarithmic scale abscissa) and SSC height (linear scale ordinate). The monocyte population was identified by CD14 expression and distinctive intermediate side scatter height. A minimum of 5000 monocytes were acquired from each sample. An analysis region (R1 in Fig 2.1a) was drawn around the monocyte population and cells within R1 plotted again on a 2D dotplot arraying CD 61 signal width (linear scale abscissa) and CD14 signal height (logarithmic scale ordinate). To exclude false-positive PMC arising from co-incident analysis of free platelets and monocytes, a region (R2 in Fig 2.1b) was drawn around the cells with narrow CD61 width and events falling within both R1 and R2 subsequently plotted onto another 2D dotplot arraying

CD61 FITC signal height (logarithmic scale abscissa) and CD 142 PE or CD 62P PE (logarithmic scale ordinate) (Fig 2.1 c & d) depending on the presence of antibody in tube 1 or tube 2. 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. CD14 & CD61 double positive true PMCs were expressed as the percentage of total monocyte population. P-selectin expression on the complexes was identified by co-expression of CD62P and TF expression on the bound monocytes identified by CD 142 PE positivity (figure- 2.1c). The P-selectin and TF expression on the complex were expressed as percentage of the total monocyte population as well as a percentage of PMC. The process was standardised in our laboratory with the percentage of PMCs in the peripheral circulation of normal healthy individuals was found to be 2.57± 0.31 (CV 13.96± 8.30 %).

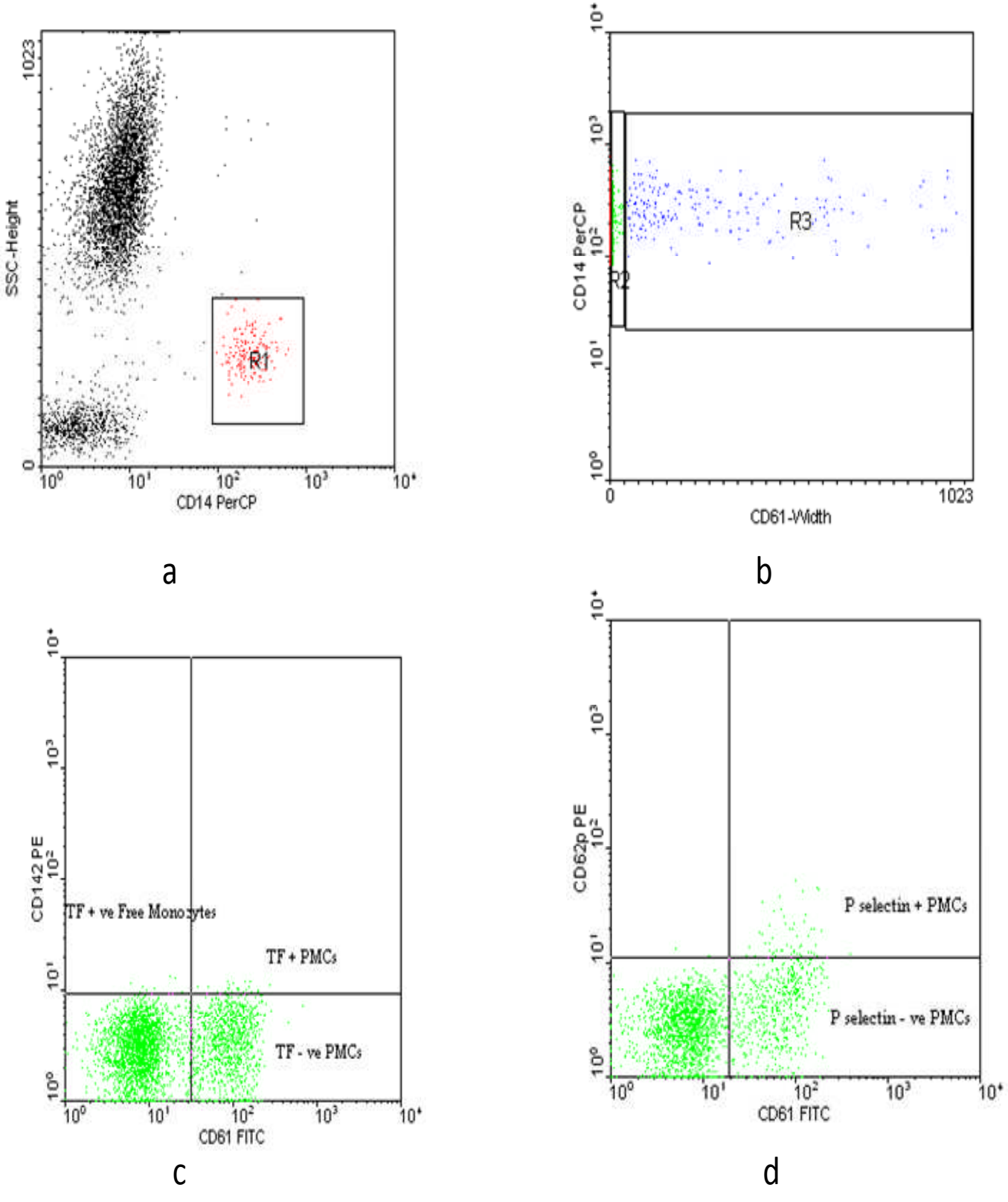


Figure 2.1- Flow Cytometry Dotplots

Figure 2.1 Flowcytometry

a. Monocyte population was identified by CD14 positivity and distinctive intermediate side scatter. R1 analysis region was drawn around the monocyte population.
b. Cells within R1 were plotted again on a 2D dotplot arraying CD 61 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 CD61 FITC signal height (logarithmic scale abscissa) and CD 142 PE or CD62 P PE (logarithmic scale ordinate) (Fig c & d) depending on the presence of antibody in tube 1 or tube 2. Cells dual positive for CD14 and CD61 were identified as PMCs. PMCs positive for CD142 were considered as TF positive complex (right upper quadrant, figure c). Whereas CD14 positive cells which only expressed CD142 (Left upper quadrant, figure c) are identified as tissue factor expressing free monocytes. Co expression ofCD62P was identified as P-selectin positive complexes (right upper quadrant figure- d).

2.3 ESTIMATION OF INFLAMMATORY PARAMETERS AND SOLUBLE P SELECTIN:-

2.3.1 COLLECTION AND PREPARATION OF BLOOD SAMPLES FOR ESTIMATION OF CRP, SAA, TNF- ALPHA, IL-6 AND SOLUBLE P SELECTIN.

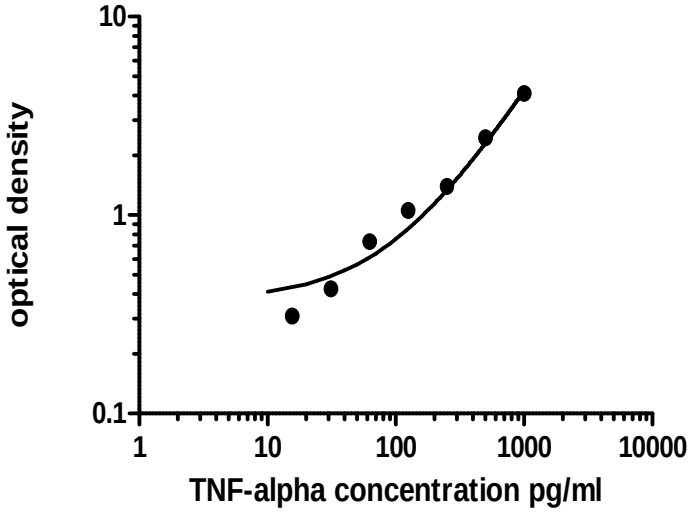
Two vacutainer tubes with 3.2 % sodium citrate were filled with 2.7 ml blood collected from each site; these samples were used to prepare double spun platelet poor plasma by centrifuging the samples initially at 800g for 10 minutes at room temperatur. The supernatant was collected into Eppendorfs and recentrifuged at 2500g for 5 minutes and 90% of the supernatant plasma was aspirated and immediately stored at -80^0 C for later analysis of IL-6 and TNF-alpha, 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).

2.3.2 ELISA-

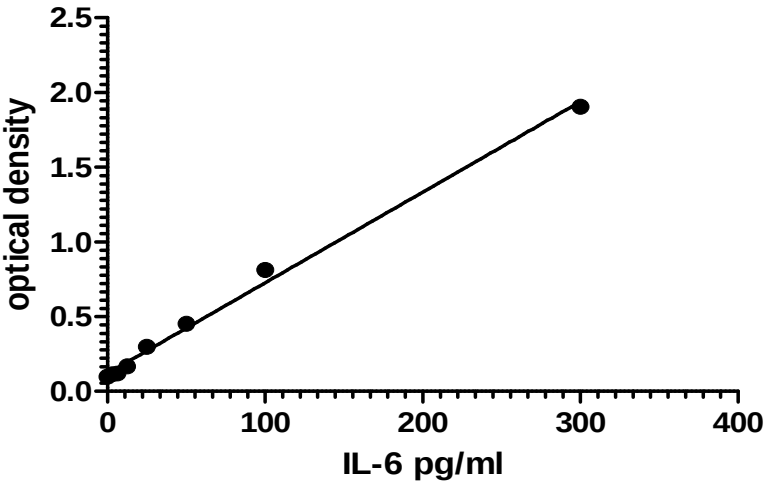
2.3.2.1 ESTIMATION OF TNF- ALPHA AND IL-6:-

TNF-alpha and IL-6 were measured using a quantitative enzyme immunoassay technique (R&D Systems). Respective monoclonal antibobies specific for TNF-alpha or IL-6 were precoated onto a microplate. Standards and samples were pipetted into the wells and any TNF-alpha or IL-6 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-alpha 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-alpha 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-alpha or IL-6 concentrations versus the log of optical density and the best fit line was determined by regression analysis. The CV of intraassay precision of this particular TNF alpha kit varied between 4.6% and 5.2% and the CV of interassay precision varied between 5.4% and 7.4%. The mean minimal detectable dose of TNF-alpha was 1.6 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 0.70 pg/ml.



a



b

Figure 2.2- Standard curve for TNF alpha (a) and IL-6 (b). Standard curve was produced by plotting the log of TNF– alpha (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.

2.3.2.2 ESTIMATION OF SOLUBLE P SELECTIN:-

Soluble P- Selectin was estimated as a soluble marker of platelet activation. Soluble P- selectin was assayed by the quatitative sandwich immunoassay technique using the R& D Systems kit. In this kit a monoclonal antibody specific for sP-selectin was precoated into the wells together with a polyclonal antibody specific for sPselectin 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.

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 sP- selectin was less than 0.5 ng/ml. Mean value from healthy individual was 29 ng/ml (± 1sd range 18-40 ng/ml).

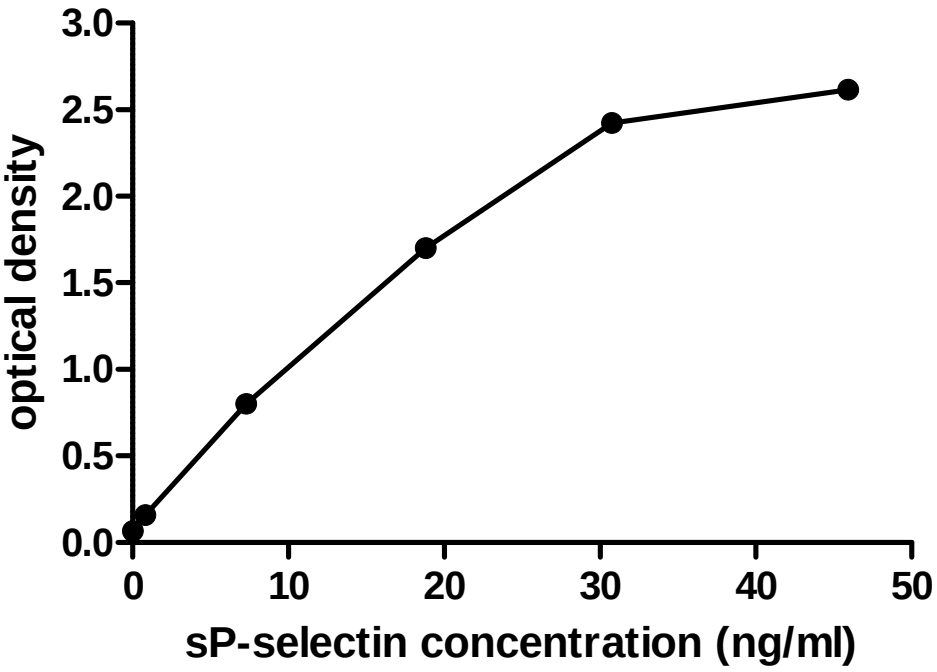


Figure 2.3- Standard curve for s P- Selectin. Standard curve for s P-selectin was drawn by plotting the mean absorbance on the y axis against the concentration on the x axis. Values of the samples obtained from the standard curve was multiplied by the dilution factor of 20 to get the true sP- selectin concentration.

2.3.3 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) (163). 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 (164),(165).

CHAPTER 3: PMC METHODOLOGY

IMPROVED ACCURACY AND REPRODUCIBILITY OF ENUMERATION OF PLATELET-MONOCYTE COMPLEXES THROUGH USE OF DOUBLET DISCRIMINATOR STRATEGY

3.1

Introduction

3.2 Doublet Discriminator Strategy

3.3 Patient Material and Methods

3.3.1 Subjects and blood collection

3.3.2 Sample preparation

3.4 Flow Cytometry

3.5 Statistics

3.6 Results

3.6.1 The doublet discriminator gating strategy

3.6.2 Normal peripheral blood

3.6.3 Diluted samples

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3.6.3.2 Comparison of effectiveness of serial dilution technique with doublet discriminator strategy in reducing coincidence.

3.6.4 Reproducibility of doublet discriminator technique in pathological samples of patients with coronary artery disease

3.6.5 Stability of samples

3.7 Discussion

3.1 INTRODUCTION:-

The importance of PMC formation as a sensitive marker of platelet activation has been demonstrated in several clinical conditions including ACS (22, 31, 166-169) however estimation of PMCs by flow cytometry suffers from several methodological shortcomings and in vitro artefacts. Modifications of flow cytometric methods of platelet analysis have been reported including the use of either washed platelets (170) or whole blood (171). Some investigators have used red cell lysis for better delineation of platelet and white cell populations (14).

One of the challenges of platelet monocyte complex estimation is in vitro activation of the platelets. Mody et al (172) and Kim et al (173) demonstrated that use of citrate, theophylline, adenosine, dipyridamole (CTAD) anticoagulant minimises the ex vivo activation and Pearson et al found that CTAD samples are stable for up to 2 hours post venesection (37). Some investigators have used whole blood methods to avoid red cell lysis, and centrifugation (24, 171) but the relative abundance of red cells makes the analysis both time consuming and insensitive (174). Removal of erythrocytes by cell separation or red cell lysis facilitates measurements (175, 176) but these procedures might also lead to significant in vitro artefacts (34).

A consistent problem of overestimation of the platelet-monocyte complexes remains due to coincident events (177). Two colour flow cytometry using monoclonal antibodies specific for monocytes and platelet has been widely used for PMC estimation; platelet-monocyte complexes appearing as double positive events. However double positive events can originate not only from true

complexes but also from non-interacting coinciding platelets and monocytes. As the concentration of platelets is considerably higher than that of monocytes the chances of having one or multiple platelets close to but not attached to a monocyte are high and when a non-conjugated monocyte transits through the laser adjacent to (but not conjugated to) one or more platelets the cells can be detected as one event and appear as a PMC (39, 40). To reduce the artefact we have investigated the use of doublet discriminator (DDM) strategy in estimation of PMCs in normal peripheral blood, in arterial and femoral venous samples of patients with CAD and also studied the effect of this strategy on serially diluted samples and vice versa.

3.2 DOUBLET DISCRIMINATOR STRATEGY:-

The doublet discrimination technique was designed to overcome the problem of co-incident analysis of doublets of bare nuclei in DNA ploidy experiments (178). In the analysis of PMC, co-incident platelets and monocytes had a longer time of flight through the focal point of the laser than true PMC so, using pulse processed analysis, they elicit signals with a greater fluorescent width than the single PMC event and a concomitant greater signal area. We hypothesized that co-incident events may be differentiated from true PMC events by plotting the cells in a dot plot of signal width and area or signal height and width.

3.3 PATIENTS, MATERIALS AND METHODS :-

3.3.1 SUBJECT AND BLOOD COLLECTION

Fourteen normal healthy volunteers aged between 25 and 60 years were included. Samples were collected from the peripheral vein with 19 G needle and syringe. Twenty patients with coronary artery disease aged between 56 and 75 years were also included. Arterial samples from different sites (coronary artery ostium, aorta, femoral artery) were aspirated through the diagnostic coronary angiogram catheter (5FJudkin's right or left diagnostic catheter with internal diameter of 0.11 cm) and venous samples through the femoral venous sheath (5F) with the help of 5 ml syringe during the angiogram procedures (Table 3.1).

Serial No	Sex	Age	Individual	Site of Sample	Dilution (µl)	Time of analysis since sample collection (minutes)	Total number of measured observations
Normal peripheral venous sample							
N001	M	29	Normal healthy	Peripheral vein	500	80	3
N002	M	25	Normal healthy	Peripheral vein	500	90	3
N003	M	60	Normal healthy	Peripheral vein	500	70	3
N004	F	53	Normal healthy	Peripheral vein	500	80	3
N005	F	45	Normal healthy	Peripheral vein	500	90	3
Samples used to evaluate dilution effect							
N006	M	35	Normal healthy	Peripheral vein	500	90	1
	M	35	Normal healthy	Peripheral vein	750	90	1
	M	35	Normal healthy	Peripheral vein	1000	90	1
N007	M	33	Normal healthy	Peripheral vein	500	80	1

	M	33	Normal healthy	Peripheral vein	750	80	1
	M	33	Normal healthy	Peripheral vein	1000	80	1
N008	F	45	Normal healthy	Peripheral vein	500	70	1
	F	45	Normal healthy	Peripheral vein	750	70	1
	F	45	Normal healthy	Peripheral vein	1000	70	1
N009	F	38	Normal healthy	Peripheral vein	500	80	1
	F	38	Normal healthy	Peripheral vein	750	80	1
	F	38	Normal healthy	Peripheral vein	1000	80	1
N010	F	35	Normal healthy	Peripheral vein	500	110	1
	F	35	Normal healthy	Peripheral vein	750	110	1
	F	35	Normal healthy	Peripheral vein	1000	110	1
N011	M	45	Normal healthy	Peripheral vein	500	120	1
	M	45	Normal healthy	Peripheral vein	750	120	1

	M	45	Normal healthy	Peripheral vein	1000	120	1
N012	M	40	Normal healthy	Peripheral vein	500	110	1
	M	40	Normal healthy	Peripheral vein	750	110	1
	M	40	Normal healthy	Peripheral vein	1000	110	1
N013	M	30	Normal healthy	Peripheral vein	500	120	1
	M	30	Normal healthy	Peripheral vein	750	120	1
	M	30	Normal healthy	Peripheral vein	1000	120	1
N014	F	35	Normal healthy	Peripheral vein	500	110	1
	F	35	Normal healthy	Peripheral vein	750	110	1
	F	35	Normal healthy	Peripheral vein	1000	110	1
Patients' sample							
N015	F	60	Stable angina	Femoral artery	500	90	3
	F	60	Stable Angina	Aorta	500	90	3
	F	60	Stable angina	Coronary artery	500	90	3

N016	M	65	NSTEMI	Femoral artery	500	100	3
	M	65	NSTEMI	Aorta	500	100	3
	M	65	NSTEMI	Coronary artery	500	100	3
N017	M	70	Stable angina	Femoral artery	500	120	3
	M	70	Stable angina	Aorta	500	120	3
	M	70	Stable angina	Coronary artery	500	120	3
N018	M	66	Stable angina	Femoral artery	500	100	3
	M	66	Stable angina	Aorta	500	100	3
	M	66	Stable angina	Coronary artery	500	100	3
N019	M	75	NSTEMI	Femoral artery	500	90	3
	M	75	NSTEMI	Aorta	500	90	3
	M	75	NSTEMI	Coronary artery	500	90	3
N020	M	70	NSTEMI	Femoral artery	500	80	3
	M	70	NSTEMI	Aorta	500	80	3

	M	70	NSTEMI	Coronary artery	500	80	3
N021	M	56	Stable angina	Femoral artery	500	90	3
	M	56	Stable angina	Aorta	500	90	3
	M	56	Stable angina	Coronary artery	500	90	3
N022	F	68	Stable angina	Femoral artery	500	80	3
	F	68	Stable angina	Aorta	500	80	3
	F	68	Stable angina	Coronary artery	500	80	3
N023	F	56	NSTEMI	Femoral artery	500	90	3
	F	56	NSTEMI	Aorta	500	90	3
	F	56	NSTEMI	Coronary artery	500	90	3
N024	M	69	Stable angina	Femoral artery	500	110	3
	M	69	Stable angina	Aorta	500	110	3
	M	69	Stable angina	Coronary artery	500	110	3
N025	M	55	Stable angina	Femoral artery	500	90	3

	M	55	Stable angina	Aorta	500	90	3
	M	55	Stable angina	Coronary artery	500	90	3
N026	M	74	NSTEMI	Femoral artery	500	80	3
	M	74	NSTEMI	Aorta	500	80	3
	M	74	NSTEMI	Coronary artery	500	80	3
N027	M	70	STEMI	Femoral vein	500	90	3
	M	70	STEMI	Aorta	500	90	3
	M	70	STEMI	Coronary	500	90	3
N028	F	72	NSTEMI	Femoral vein	500	80	3
	F	72	NSTEMI	Aorta	500	80	3
	F	72	NSTEMI	Coronary artery	500	80	3
N029	M	65	STEMI	Femoral vein	500	100	3
	M	65	STEMI	Aorta	500	100	3
	M	65	STEMI	Coronary artery	500	100	3
N030	M	72	NSTEMI	Femoral vein	500	90	3
	M	72	NSTEMI	Aorta	500	90	3

	M	72	NSTEMI	Coronary artery	500	90	3
N031	F	70	NSTEMI	Femoral vein	500	120	3
	F	70	NSTEMI	Aorta	500	120	3
	F	70	NSTEMI	Coronary	500	120	3
N032	M	71	NSTEMI	Femoral vein	500	110	3
	M	71	NSTEMI	Aorta	500	110	3
	M	71	NSTEMI	Coronary artery	500	110	3
N033	F	65	Stable angina	Femoral vein	500	120	3
	F	65	Stable angina	Aorta	500	120	3
	F	65	Stable angina	Coronary artery	500	120	3
N034	M	66	NSTEMI	Femoral vein	500	110	3
	M	66	NSTEMI	Aorta	500	110	3
	M	66	NSTEMI	Coronary artery	500	110	3

Table 3.1 – General descriptions of the collected samples.

3.3.2 SAMPLE PREPARATION:-

Protocol 1 - *Normal peripheral blood* - Peripheral blood samples from 5 healthy individuals aged between 25 and 60 years were collected from a peripheral vein with 19 G needle and 5 ml syringe. Blood (2.7 ml) was collected into sodium citrate (0.109 M/3.2%) anticoagulant (BD vacutainer systems, USA). The samples were transported to the laboratory within 15 minutes on ice for analysis. Five microlitres each of anti-CD61 FITC (BD Biosciences, Oxford, UK) and anti-CD14 PerCP (BD Biosciences, Oxford, UK) monoclonal antibodies were added to labelled 12mm x 75 mm polystyrene tubes. One hundred microlitres of whole blood was added to each tube, mixed with the antibodies and incubated for 20 minutes on ice. After labelling, erythrocytes were lysed with the addition of 2ml BD FACS lysing solution (BD Biosciences, Oxford, UK) (1in 10 dilution) for 10 minutes at room temperature. The samples were washed with 1ml of FACS flow (BD Biosciences) and then centrifuged at 300g for 5 minutes and the supernatant was discarded. The cells were resuspended in 0.5ml FACS flow for immediate flow cytometric analysis. Flow cytometry was performed at low flow rate (not more than 1000 cells/sec) using a FACS Calibur (BD Biosciences, Oxford, UK) with Cell Quest Pro ® software. Three thousand gated monocyte events were acquired and PMCs were expressed as a percentage of the total monocyte population. All the samples were analysed by flow cytometry within 2 hours of sample collection.

Protocol 2 - *Effect of serial dilution of the samples* – Peripheral venous samples from another 9 healthy individuals were prepared as Protocol 1. After acquisition the samples were serially diluted to 150% (750µl final volume) and 200% (1ml

final volume) in order to physically reduce co-incidence. The diluted samples were reanalysed by flow cytometry with and without electronic doublet discrimination (figure 3. 1).

Protocol 3 - *Reproducibility in pathological samples* - To see the reproducibility and effectiveness of doublet discriminator strategy in reducing coincident events in the pathological samples from different sites we included 20 patients (10 NSTEMI, 2 STEMI and 8 Stable angina patients). In STEMI patients samples were collected during the acute stage while the patients were having PPCI. In NSTEMI patients samples were collected during their coronary angiogram procedure within 72 hours of onset of chest pain. In stable angina patients samples were collected during their elective coronary angiography. Twelve patients had samples taken from the femoral artery, aorta and coronary artery ostium and 8 patients had samples from the femoral vein, aorta and coronary artery ostium. Arterial Samples (2.7 ml from each site) were collected by aspiration through the Judkin's 5F right or left diagnostic coronary angiogram catheter (Cordis) (Internal diameter – 0.11 cm) with the help of 5 ml syringe. Femoral venous samples were collected from the 5F femoral venous sheath with the help of 5 ml syringe. Samples were prepared in the same way as peripheral blood samples from normal donors above and each sample was analysed in triplicate with the flow cytometry method with DDM on and DDM off (described below). PMC frequencies were estimated from the average of the triplicate values.

Protocol 4 - Stability of the samples post labelling - To determine the stability of the complexes in the samples prepared in the above described method we prepared 9 samples from 3 patients [2 stable angina patients and 1 NSTEMI

patient.] Citrated samples (2.7 ml) were collected from the coronary artery ostium, aorta and femoral vein. Samples were analysed in triplicate and following initial flow cytometry analysis the prepared samples were kept in the fridge at $4^{o}C$ for 24 hours without addition of any further preservatives or fixatives and were reanalysed in the same manner.

3.4 FLOW CYTOMETRY:-

Cells were acquired on a 2D analysis dot plot arraying CD14 (logarithmic scale abscissa) and side scatter (SSC) height (linear scale ordinate) (Figure 3. 1 A) and the region was drawn (R1) around the monocyte population identified by their distinctive CD14 positivity and intermediate SSC height. Cells in the R1 were gated (G1) and plotted on a 2D dotplot arraying CD61 FITC (linear abscissa) vs SSC height (Figure 3.1B). Cells with the narrow CD61-width were magnified on the dot plot C to select events with the narrowest fluorescence width as putative “singlet” events. All CD14+ events with higher CD61 width were gated together as putative “multiplets”. Cells both positive for CD61 and CD14 with narrowest CD61 width were considered as true PMCs (subsequently described as “DDM on”). Cells both positive for CD61 and CD14 with wider CD61 width were hypothesised to be coincident events. PMC frequency data with ‘DDM off’ were determined for all CD14 positive cells.

After validation of the “DDM on” and “DDM off” criteria future experiments estimated the frequencies of PMCs with and without doublet discriminator use. Values were measured in triplicate and the averages were taken to estimate the

PMC frequency. Co efficient of variation (CV) was calculated to ascertain the reproducibility.

Experimental design: Using the above flow cytometry technique we conducted series of experiments to ascertain the effectiveness and reproducibility of doublet discriminator strategy in different situations. Cells with wider FL1 width were considered as coincidents and excluded from the analysis. Cells double positive for CD14 and CD61 with narrow FL1 width were considered as true PMCs.

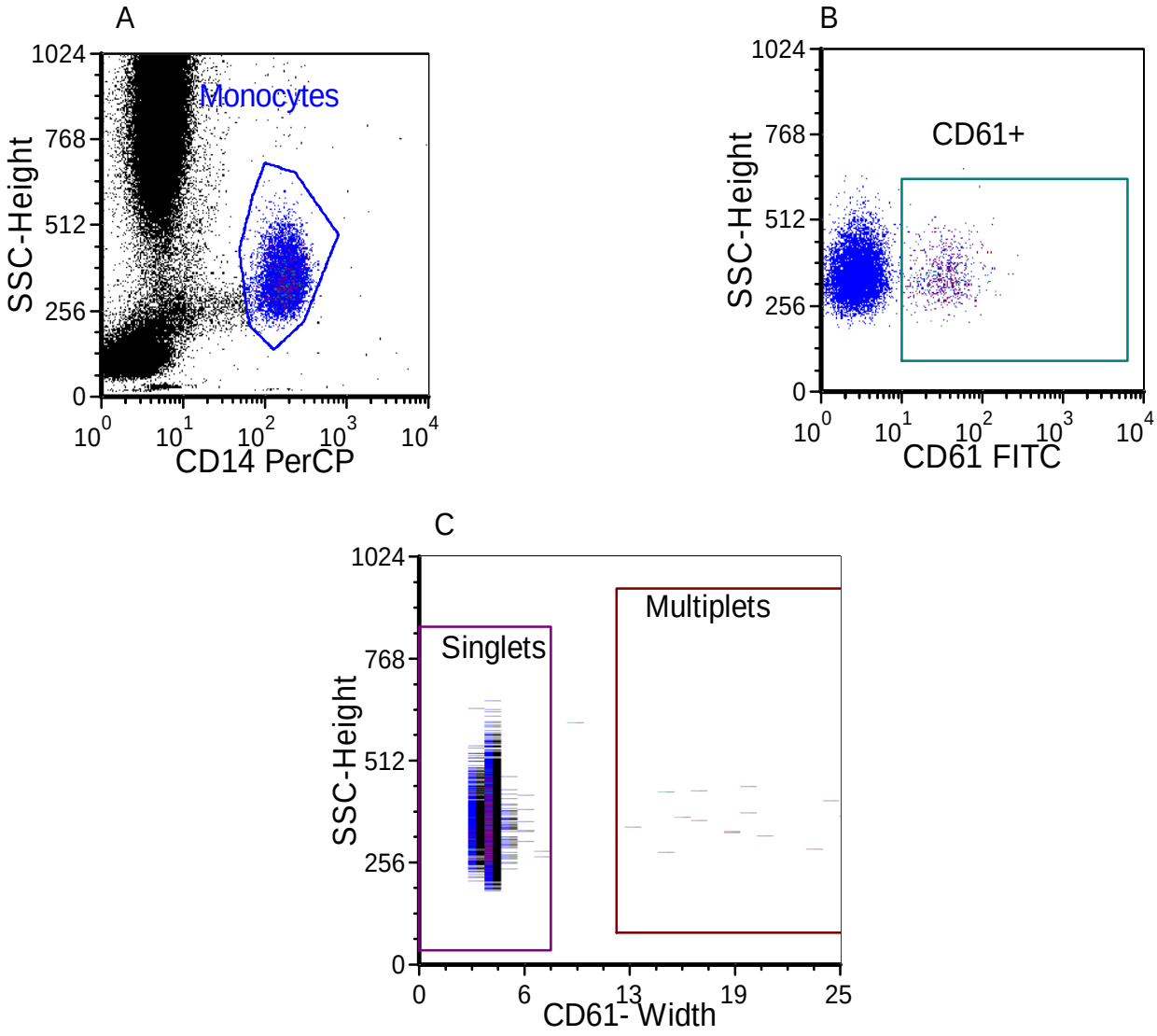

Figure.3. 1. Flow cytometry:- Monocytes were identified with CD14 positivity and distinctive intermediate sidescatter height. Region (R1) was drawn around the monocyte populations (A). Monocytes were plotted on a 2D dotplot arraying CD61-height (abscissa) vs SSC (ordinate) (B) and the CD61+ve events plotted on a CD61-width vs SSC height array. The plot was zoomed to expand the CD61 width data array (C). Regions were set around the “singlet” population and the “multiplets”. PMC were calculated as the percentage of CD61+ cells within the CD14+ population either with inclusion of the multiplets (described as DDM ”off” results) or after exclusion of the multiplets (described as DDM “on” results).

3.5 STATISTICS:-

Data were represented as mean and standard deviation.The paired t test was used to compare the PMC frequencies with DDM on and DDM off and also to compare the baseline PMC values with 24 hour PMC values. One way ANOVA with Bonferroni's multiple comparison was used to compare the PMC frequencies between different dilutions. To evaluate the relationship of PMC values with DDM on and DDM off we used linear regression analysis. A two tailed p value <0.05 was considered to be significant .Statistical analysis was performed using the Graphpad prism® software (version 5).

3.6 RESULTS:-

3.6.1 THE DOUBLET DISCRIMINATOR GATING STRATEGY:-

Support for the hypothesis that analysis of the CD61 fluoresence width signal could discriminate between true PMC and co-incident events of free monocytes and free platelets was provided by the exemplar gating strategy in figure 3. 1. The singlet events as determined by CD61 width could be clearly distinguished from signals with a wider distribution of CD61 fluorescence.

3.6.2 NORMAL PERIPHERAL BLOOD:-

The frequency of platelet-monocyte complexes within the circulating monocytes in the peripheral blood of 5 healthy normal individuals was found to be 6.27% ± 1.77 (mean ± sd) without the use of doublet discrimination strategy. This frequency fell significantly to 2.57% ± 0.99 using the doublet discriminator strategy (p=0.02). (CV 13.96 ± 8.30 %) (Fig:3. 2).

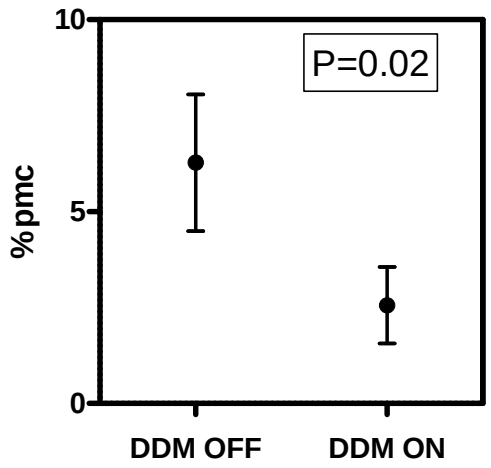

Figure. 3.2-Comparison of frequency of PMCs in the peripheral blood of normal healthy individuals (n=5) with (DDM on) and without (DDM off) the use of doublet discriminator (DDM) technique. DDM use effectively reduced the co incident events hence lower values compared to DDM off.

3.6.3 DILUTED SAMPLE:-

3.6.3.1 EFFECT OF DOUBLET DISCRIMINATOR STRAGEY ON DILUTED SAMPLE:-

Peripheral venous samples from 9 different individuals were prepared as described above and the cells were resuspended in FACS flow with serial dilution of 500, 750 and 1000 microlitres before analysis. The PMC frequencies (%) without the doublet discriminator use (DDM off) were calculated as 16.44 ± 4.09, 12.21± 3.44, and 9.67± 2.91 respectively confirming the ability of dilution to reduce coincident acquisition of free platelets and monocytes in the PMC gate. However, the additional use of doublet discrimination (DDM on) reduced the PMC values in the same samples to 6.46± 1.65, 4.52± 1.27 and 4.05± 1.19 respectively; all of which were significantly lower ($p<0.05$) than the matched "DDM Off" results. PMC values were consistently lower using the doublet discriminator strategy (Figure: 3.3,3.4). The diluted samples tested without DDM showed significant differences between dilutions and the co-efficient of variance remained constant despite dilution. In contrast, the 750µl and 1000µl samples when analysed with doublet discrimination showed no significant difference, suggesting that the "true" value of PMC had been determined and that no co-incidence was occurring but it is unlikely that doublet discrimination alone is able to remove the artefact of co-incidence (Figure 3.5).

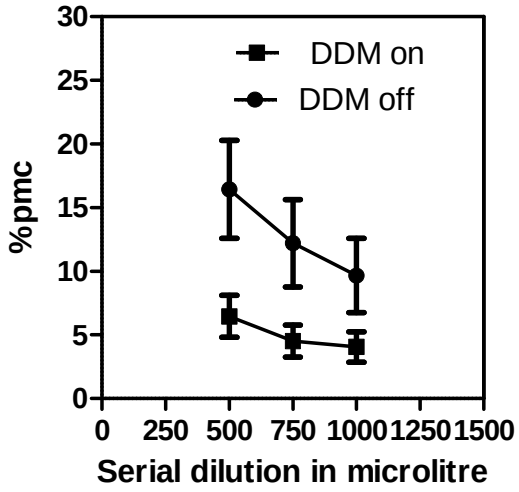

Figure 3. 3- Effect of serial dilution in reducing coincident events with and without doublet discriminator use Serial dilution reduced the frequency of PMCs as dilution reduced coincident acquisition of free platelets and monocytes in the PMC gate. However, the additional use of doublet discrimination reduced the PMC values in the same samples even further.

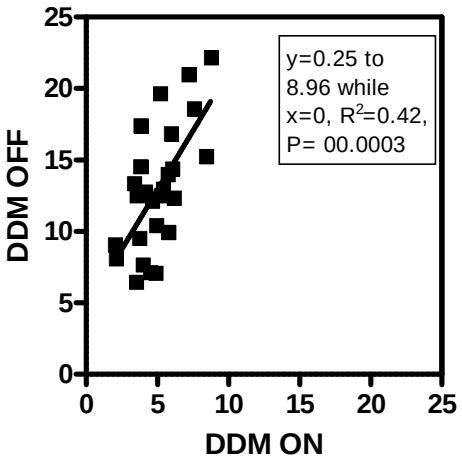

Figure 3. 4. - Effect of doublet discriminator mode use in diluted samples. Without the use of doublet discriminator (DDM off) the PMC frequency was significantly overestimated even in the samples with serial dilution. Serial dilution alone was not sufficient to reduce the coincident events effectively. Use of doublet discriminator (DDM on) reduced the coincident events in the diluted samples even further.

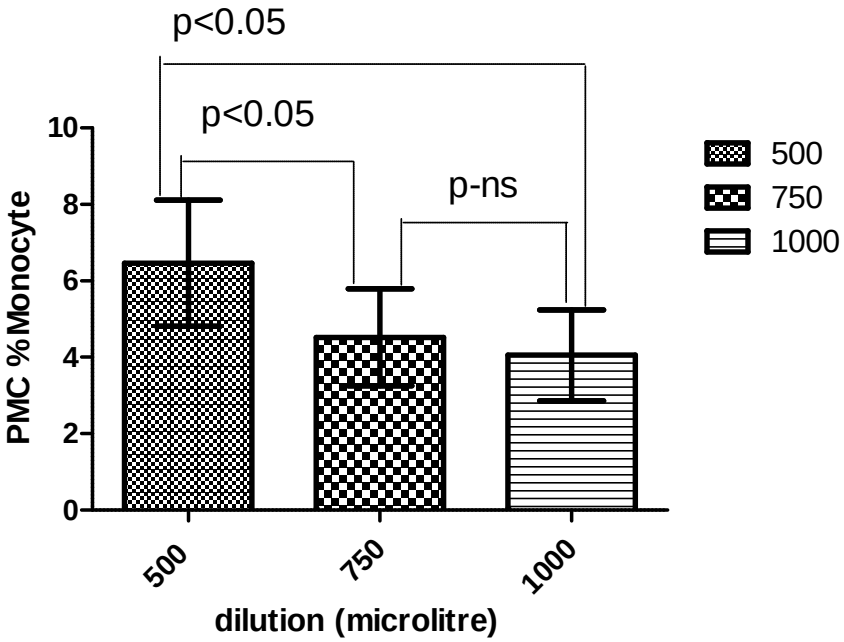

Figure 3. 5. Effectiveness of doublet discriminator use for estimation of true PMC value. Doublet discriminator use did not fully abolish the effect of serial dilution but no significant difference in PMC values between 750 µl and 1000 µl dilution suggests with the use of DDM true value of PMC has been achieved (n=9).

3.6.3.2 COMPARISON OF EFFECTIVENESS OF SERIAL DILUTION TECHNIQUE WITH DOUBLET DISCRIMINATOR STRATEGY IN REDUCING COINCIDENCE.

To determine the efficiency of doublet discrimination in reducing the coincidence events compared to the sample dilution strategy we estimated the difference of average percentage of PMCs in the 500µl dilution with DDM off and DDM on and compared with the result of estimated difference in PMC frequency between the 500 and 750 microlitre and 500 and 1000 microlitre dilutions. The average reduction in percentage of PMC frequency by dilution alone was 4.23% (± 2.19) and 6.78% (± 2.68) in the 750 and 1000 microlitre samples respectively. In contrast, the use of doublet discrimination on the samples with 500µl dilution reduced the PMC frequency by an average of 9.98% (± 3.54). These results suggested that serial dilution does lower co-incidence but it is a less efficient strategy than doublet discrimination strategy (Figure 3.6).

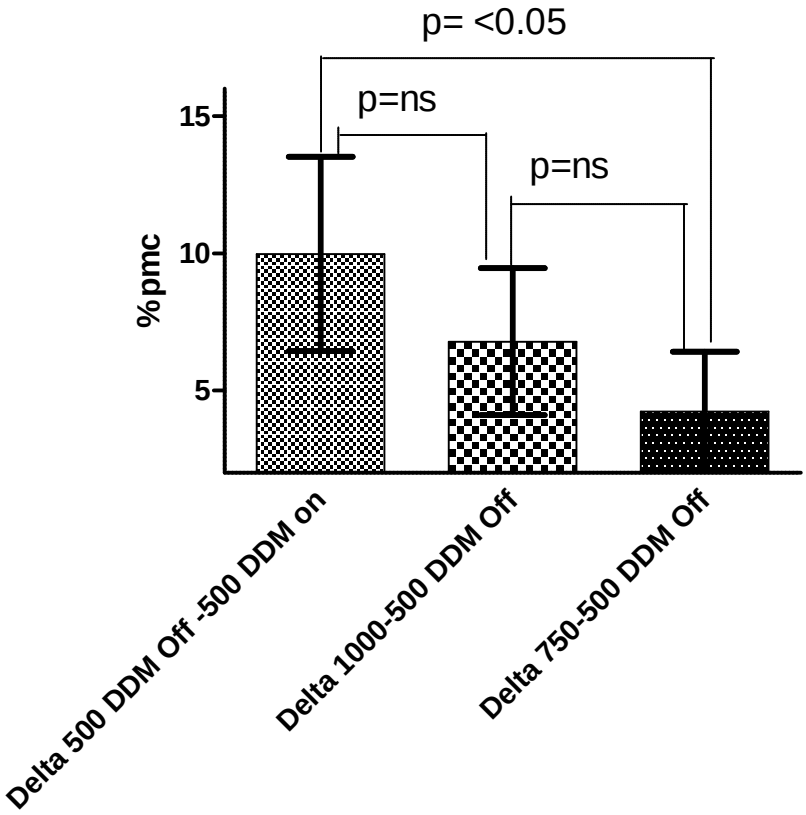

Figure 3.6- Comparison of effectiveness of doublet discriminator use and serial dilution in reducing the coincident events. Average reduction in coincident events were higher in 1000 microlitre dilution compared to the 750 microlitre dilution as higher dilution reduced the chance of detection of coincident events but use of doublet discriminator strategy alone without any further dilution of samples reduced the coincident events more effectively. This proved the doublet discriminator strategy was more effective than serial dilution in reducing the coincident events.

3.6.4 REPRODUCIBILITY OF DOUBLET DISCRIMINATOR TECHNIQUE IN PATHOLOGICAL SAMPLES OF PATIENTS WITH CORONARY ARTERY DISEASE.

PMC analysis using the doublet discriminator strategy was performed with 60 samples collected from the 20 patients with coronary artery disease during coronary angiography. This confirmed its value in significant reduction in coincidence events. PMC frequency within the monocyte population was 16.04% (± 11.26) without the use of doublet discriminator strategy which reduced to 7.66% (± 5.18) using doublet discrimination ($p<0.01$).

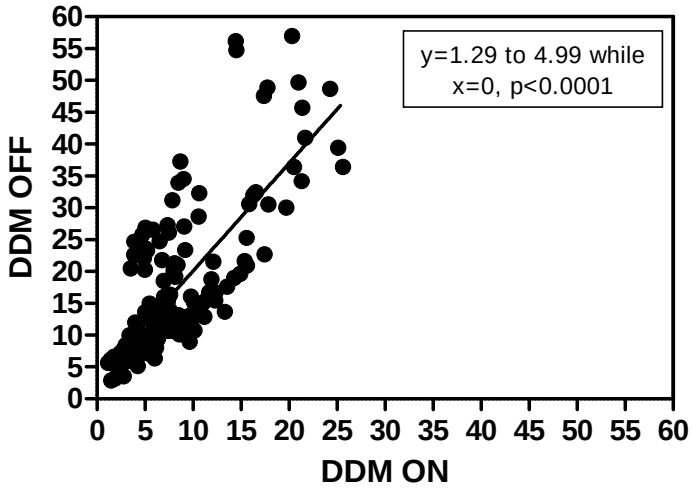

Figure 3. 7. Effect of doublet discriminator use in pathological samples. Linear regression analysis showed doublet discriminator use (DDM on) effectively reduced the co incident events in samples from patients with coronary artery disease in the same manner as it did in normal individuals (n= 20).

3.6.5 STABILITY OF SAMPLES:-

Prepared samples were stable even after 24 hrs while kept at 4^0C. In these 3 patients the baseline and 24 hrs average PMC frequency (%) were 15.11± 9.83 and 14.03± 9.44 (p=0.22) respectively (DDM off). Those values with DDM on were 8.84 ± 6.04 and 9.40 ± 6.13. (p=0.36) (Figures 3. 8a & 3. 8b).

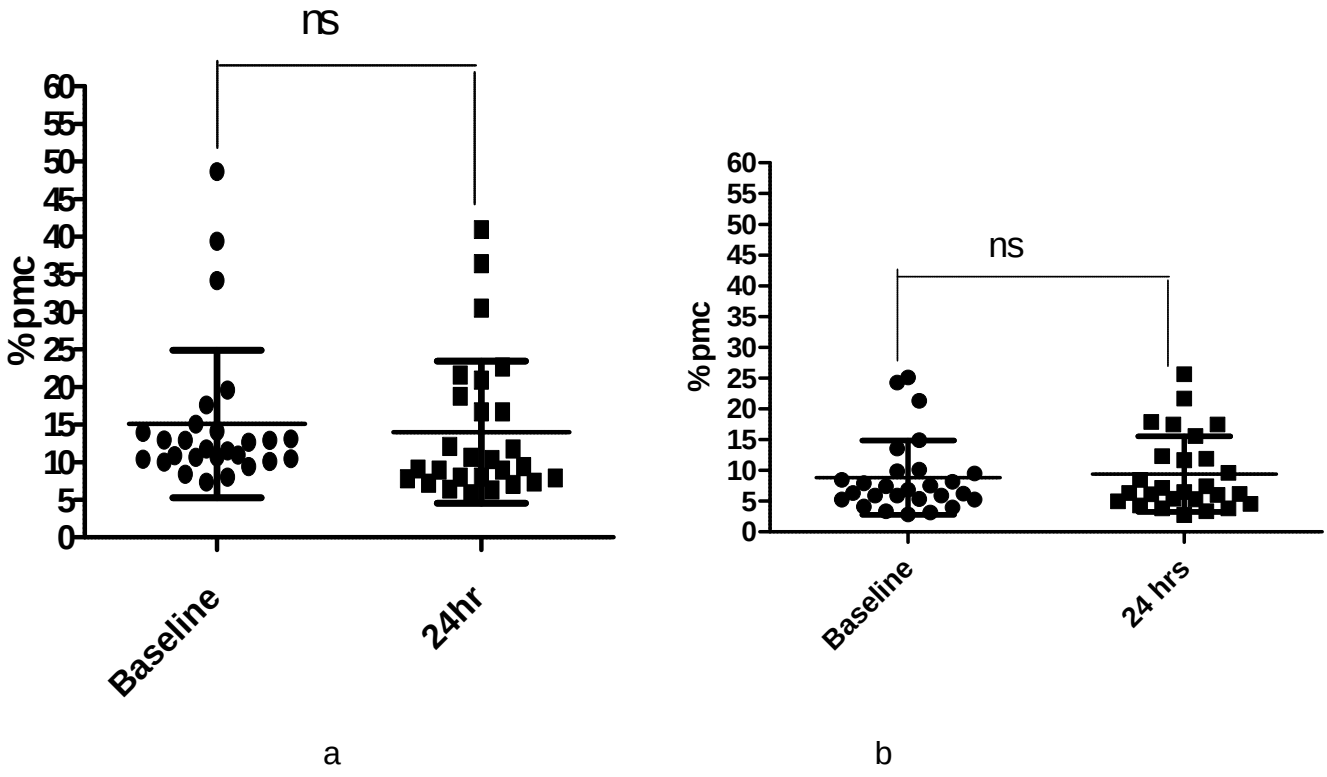

Figure 3.8 a &b. Stability of samples over 24 hour period. Comparison of the PMC values at baseline and reanalysis of the same samples after 24 hours with DDM off and DDM on respectively. Following preparation and initial flow cytometry assessment samples were kept in the fridge at 4 ^{0} C without addition of any further preservatives or fixatives. Samples were reanalyzed after 24 hours in the similar manner. No significant difference was noted between the baseline and 24 hour mean PMC values with DDM off , p= 0.22 and with DDM on,

p=0 .36 (n=3).

3.7 DISCUSSION:-

In peripheral circulation platelets are found both as free entities and in complexes with monocytes, granulocytes and even erythrocytes and it has been presumed that this affected their activation and function in vivo. The frequency of platelet-monocyte complexes appears to have a role in coronary disease and stroke (22, 31, 168) (179). Erythrocyte-platelet complexes have recently been found at the centre of atherosclerotic plaques by scanning electron microscopy in an experimental murine model (180). The potential importance of complexed platelets in normal and pathological thrombosis is clear but flow cytometric assays for these complexes are poorly standardised.

Different methods of platelet monocyte complex estimation are described in the literature. Many concerns have been raised regarding in vitro platelet activation and artefactual platelet-monocyte complex formation resulting in overestimation of platelet-monocyte complexes. It has been reported that the choice of anticoagulant, sample preparation e.g. fixation, erythrocyte lysis, centrifugation, washing technique etc might influence the estimated frequency of platelet-monocyte complexes. So whole blood techniques are now preferred, most of which exclude fixation, erythrocyte lysis and centrifugation steps in order to minimise the in vitro platelet activation and artefactual platelet-monocyte complex formation. Sample dilution and flow rate also influence PMC estimation. So in different studies the frequency (%) of PMC in the normal peripheral venous sample varies widely from 3.72± 1.39 (13) to 12.3± 3.3 (24, 32, 36).This wide variation could also be due to the fact that coincident events were probably not considered during estimation of PMCs.

The potential influence of coincident events has remained one of the major concerns that needed to be addressed. In order to exclude the coincident events Bihari et al used a subtraction method where they divided the blood samples into two parts one part was stained for platelets and granulocytes, the other part was stained only for platelets and fluorescent beads were mixed with it. Then measuring the platelet-granulocyte and platelet-bead double positivity at the same dilution (platelet concentrations needed to be the same) the exact amount of platelet-granulocyte complexes could be calculated by subtraction method (177). Another way of reducing coincidence was to use higher dilution (200 or 400 fold). Coincidence was found to be negligible resulting in very low (1-5%) frequencies of complexes. It should be taken into account that dilution might result in the dissociation of the complexes, therefore, analysis of highly diluted samples did not allow the authors to draw conclusions with respect to the undiluted samples in terms of its complex content (166, 169). A method independent of dilution for exclusion of coincidence was thus needed.

In this study we confirmed the importance of coincidence events in the overestimation of the frequency platelet:monocyte complexes in clinical samples. The use of doublet discrimination to overcome co-incidence was not new but did not appear to have been used in studies of platelet:leukocyte complexes previously. Many publications in this field have ignored the problem of co-incidence entirely whist others have used extremely diluted samples and/or low sample flow rate to reduce its impact on precision (179).Whilst effective this very significantly extended sample acquisition time which severely hindered its use in busy routine or research laboratories or settings where a high throughput is required.

This strategy was reproducible and similarly effective in reducing the co-incident events in the peripheral venous samples of normal individuals, in the diluted samples and in the arterial, femoral venous, aortic and coronary artery samples of the patients with coronary artery disease.

In our study for the first time we demonstrated the doublet discriminator strategy could be used to exclude the coincident events to a great extent thus improving the accuracy of estimation and reducing intra-assay variability. As predicted, we found that the percentage of PMCs within the monocyte population in the peripheral venous circulation of normal individual was 2.57± 0.31 which was much lower than some of the previous published data (32, 36) but comparable to the data published by Ashman et al.(24). In samples from other 9 individuals in which we looked at the use of dilution to exclude co-incidence the PMC frequencies were found to be higher as they were taken at different times of the day and they were not age matched. Dilution of the samples reduced the PMC frequency. This could be due to the fact that dilution further reduced the chance of co-incidence as the probability of presence of cells in the detection volume was lower with serial dilution. Alternatively, the additional dilution could have led to spontaneous dissociation of cells. We did demonstrate that sample dilution alone could not exclude the co-incident events completely. Using the doublet discriminator strategy we managed to reduce the co-incident events much more effectively. Doublet discriminator strategy proved effective but not completely independent of the dilution effect. The effect of dilution, still present with the doublet discriminator strategy was probably due to the fact that with increasing dilution there was a possibility of spontaneous dissociation of platelet-monocyte complexes resulting in lowering of true complex estimation.

We have demonstrated that use of a doublet discriminator strategy effectively reduces the co-incident events in the samples of the patients with coronary artery disease taken from different sites (femoral vein, femoral artery, aorta and coronary artery) and can eliminate co-incidence if used with moderate sample dilution. This study also demonstrated that prepared samples remain stable with respect to PMC complex content even after 24 hrs at 4^{o} C; an important consideration for analysis of samples in busy laboratories where flow cytometer access is at a premium.

CHAPTER 4

COMPARISON OF PLATELET-MONOCYTE COMPLEX (PMC) EXPRESSION AND DIFFERENTIAL PLATELET AND MONOCYTE ACTIVATION STATUS WITHIN THE COMPLEXES IN THE ACUTE CORONARY SYNDROME AND STABLE ANGINA PATIENTS

4.1Introduction.

4.2 Objectives.

4.3 Patients and Methods

4.3.1 Group 1- Acute coronary syndrome patients (ACS).

4.3.2 Group 2- Stable angina patients.

4.3.3 Exclusion criteria.

4.3.4 Sample collection and preparation.

4.3.5 Flow cytometry.

4.4 Statistical analysis.

4.5 Results.

4.6 Discussion.

4.7 Conclusion.

4.1 INTRODUCTION:-

Formation of PMCs has already been demonstrated in the ACS. The role of PMCs in the pathogenesis of ACS remains unclear. It is not clear whether platelet activation and PMC formation is a systemic response or whether there is a direct role for intracoronary PMC formation in the pathophysiology of acute myocardial infarction via monocyte activation, TF expression and inflammation. Therefore, PMCs may be a link between platelet mediated thrombogenesis and monocyte mediated inflammation in ACS. Following phosphorylation of the membrane protein p-selectin in the platelet alpha granule (86) the granule moves to the platelet surface exposing P-selectin and tethering the platelet with the monocyte via PSGL-1 to form PMCs. In addition, other integrin dependant mechanisms may be involved in the formation of PMCs (104) . Binding of platelets to monocytes has been shown to regulate various monocyte actions. Platelets play a significant role in supplying cholesterol to monocytes assisting monocytes to mature into lipid laden macrophages (181),(182). Furthermore, the binding of thrombin stimulated platelets induces monocyte cytokine expression (2) and monocyte chemokine synthesis may have been regulated by platelet surface p selectin in conjunction with the platelet chemokine RANTES (8). It is shown that activated platelets express CD40 ligand (CD40L) on their surface (9). Binding of CD40L to CD40 on monocytes results in monocyte activation and production of cytokines including IL-6 (10) which often parallels elevation of CRP (12) seen in ACS (183). A further hypothesis is that binding of activated platelets might alter monocyte activation status resulting in an increase in TF expression from the monocyte component acting as a trigger for the initiation of a cascade of

thrombogenesis and the release of proinflammatory cytokines, including IL-6, IL-1β and IL-12. Therefore, it is likely that, following platelet activation, a cascade of different processes occur in parallel to augment local inflammation and thrombogenesis. However, binding with unstimulated platelets might not be associated with a similar cellular response (13). Therefore, in the absence of platelet activation PMC formation might just represent a physiological phenomenon. Depending upon the level of activation, the platelets within PMCs might actually be responsible for triggering a pro-inflammatory response in monocytes. P-selectin expression on the PMCs is indicative of activated platelets on these complexes and TF expression on these complexes demonstrates the presence of activated monocytes within the complexes.

In the context of ACS it is unclear whether PMC formation is a systemic phenomenon or a local intracoronary event. Presuming PMCs are mainly formed at the site of plaque disruption at the culprit lesion site a significant transculprit lesion gradient (gradient between distal coronary and aorta) might be expected and PMC expression would be highest in the coronary circulation compared to the other systemic vascular compartments (aorta and right atrium).

4.2 OBJECTIVES:-

1. To compare overall median PMC (calculated by taking the median PMC expression from coronary, aorta and right atrium) and overall median P selectin and TF expression between the ACS and stable angina group.

2. To identify any possible site specific difference in PMC expression between these two groups.

3. To examine whether a trans-culprit lesion gradient exists in PMC expression, P- selectin and TF expression in ACS patients.

4. To examine whether platelet and monocyte activation status (evidenced by P-selectin and TF expression respectively) influences site-specific expression of PMC.

4.3 PATIENTS AND METHODS:-

4.3.1 Group 1:-ACS PATIENTS

Twenty three patients with acute coronary syndrome who underwent PCI were recruited for the study. Among the 23 ACS patients 15 patients were admitted with STEMI and treated with PPCI as immediately.Eight patients had NSTEMI and treated with PCI within 72 hours of symptom onset. Informed

consent was obtained before the procedure. Ethical approval for the study was granted from the local research and ethics committee. All patients were given 600mg of Clopidogrel and 300mg of Aspirin before the procedure as per protocol for performing PCI in our institution. Prior to PCI patients also received weight adjusted unfractionated heparin to maintain ACT between 200-250 sec.

4.3.2 GROUP 2 – STABLE ANGINA PATIENTS:-

Seven patients with stable angina were also recruited prior to elective PCI. Patients were already taking 75 mg of Aspirin and 75mg of Clopidogrel for 7 days prior to the procedure according to the protocol for performing elective PCI in our hospital. Prior to PCI they also received weight adjusted unfractionated heparin to maintain the ACT between 200-250 sec.

4.3.3 EXCLUSION CRITERIA:-

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

4.3.4 SAMPLE COLLECTION AND PREPARATION:-

Samples were aspirated from the coronary artery distal to the culprit lesion (using a coronary aspiration, Medtronic ® Export catheter , internal diameter 0.10 cm), aorta using a Judkin's diagnostic catheter (5F, Cordis ®, internal diameter 0.11 cm) and from the right atrium (using a pigtail cather (5F, Cordis ®, internal diameter 0.11 cm). It has been demonstrated before that aspiration of blood through the Export catheter does not induce artefactual platelet activation (184). Four milliliters of blood from each site was collected into 3 separate sterile vacutainer tubes containing a combination of 3.2% sodium citrate and EDTA for flow cytometric analysis of PMC, p-selectin and TF expression . EDTA was added to sodium citrate to stop in vitro calcium dependent PMC formation .

Samples were prepared for flow cytometry analysis as described in chapter 2.

4.3.5 FLOW CYTOMETRY:-

PMC, P -Selectin positive PMC and TF positive PMCs from all the 3 sites were estimated with flow cytometry as described in chapter 2. Overall median values were calculated by taking median values from the three sample sites and site specific values were (aorta, right atrium and coronary) calculated separately.

4.4 STATISTICAL ANALYSIS:-

*D*ata were presented as median and interquartile range. To compare variables between the two groups non-parametric Mann-Whitney test was used. To compare variables within the same group for calculation of transculprit lesion gradients paired t test was used. A Two-tailed p value < 0.05 was considered statistically significant. All calculations were performed using graphpad prism® (version 5) software.

4.5 RESULTS:-

Twenty three ACS patients (15 STEMI and 8 NSTEMI) and 7 stable angina patients were recruited for the study. The baseline demoraphic and clinical charcteristics of the patients are described in table 4.1.

	ACS (n= 23)	Stable (n= 7)
Age median (IQR), years	60 (48 - 69)	70 (58-75)
Male	18	6
Female	5	1
Risk Factors		
History of hypertension	8	4
History of smoking	11	5
Diabetes	3	0
Dyslipidaemia	9	5
Angiographic characteristics		
1 vessel disease	12	4
2 vessel disease	8	1
3 vessel disease	3	2
Culprit vessel		
Left Anterior Descending	6	4
Left Circumflex	8	0
Right Coronary Artery	9	3
Time of coronary sampling since the onset of pain		
STEMI [Median (IQR)] hours	4 hours and 50 minutes (1 hour and 45 minutes-6 hours)	
NSTEMI [Median (IQR)] hours	66 (46-72)	-

Table 4.1 -Baseline demographic and clinical characteristics of the patients recruited.

There was no significant difference (median ; IQR) in overall median PMC expression (% monocyte) or overall median TF positive PMCs (%PMC) between ACS and stable angina groups. However, overall median P- selectin positive PMC (% PMC) was significantly higher in the ACS group compared to the stable angina group (p= 0.006). See Table 4.2.

	ACS median (IQR)	Stable Angina median (IQR)	p
Overall median PMC (%monocyte)	9.98 (7.17- 18.02)	10.18 (6.22- 15.28)	0.43
Overall median P-selectin + PMC (%PMC)	19.34 (14.05-32.20)	13.98 (7.93- 16.85)	0.006
Overall median TF +PMC (%PMC)	4.79 (3.09-6.14)	5.25 (3.16-8.69)	0.48

Table 4.2- Comparison of Overall Median PMC, P-Selectin Positive PMC and TF Positive PMC between ACS and Stable Angina Group. There was no significant difference in overall median PMC and overall median TF positive PMCs between the ACS and stable angina group but the overall median P- selectin positive PMC expression was significantly higher in the ACS group compared to the stable angina group. This finding suggested a higher percentage of activated platelets within the PMCs in the ACS patients compared with stable angina patients.

There was no significant site specific difference in PMC expression [median (IQR)] between the ACS and the stable angina group. However, P-selectin positive PMC expression (%PMC) [median (IQR)] was significantly higher in the coronary circulation of ACS patients compared with the coronary circulation of stable angina patients (p= 0.003). In the peripheral circulation (aorta and right atrium) this difference was not significant. Also, no site specific difference was seen in TF positive PMC expression between the two groups (Table 4.3).

	ACS (n = 23)	STABLE (n=7)	p
PMC(%Monocytes)			
CO	12.4 (7.3-17.3)	8.8 (6.3-16.75)	0.41
AO	9.6 (6.4-17.9)	8.2 (5.13-1.34	0.45
RA	12.4(8.7-18.26)	12.0 (8.7-17.9)	0.29
P-Selectin positive PMCs (%PMC)			
CO	29.05 (21.27-55.03)	9.16 (7.4-15.6)	0.003
AO	15.99(11.97-22.11)	13.3 (7.97-24.18)	0.54
RA	15.57(10.89-23.00)	15.98(10.76-16.89)	0.71
TF Positive PMC (%PMC)			
CO	4.9 (3.3-8.6)	4.5 (4.0-7.7)	0.69
AO	3.6 (2.5- 4.9)	6.3 (3.4-9.8)	0.09
RA	5.2 (2.5- 6.6)	3.2 (2.1- 8.5)	0.90

Table-4.3 Site specific comparison between the ACS and stable angina group**:-**

PMC expression was non-significantly higher in all the compartments in the ACS group. Intracoronary P- selectin positive PMC expression was significantly higher in the ACS group compared with the stable angina patients suggesting increased intracoronary expression of activated platelets within the complexes in ACS patients.

In ACS patients although there was no significant transculprit lesion gradient (p=0.35) seen in total PMC expression (Figure 4.1) a significant transculprit lesion gradient in P-selectin positive (p= 0.01) (Figue 4. 2) and TF positive PMC expression (p=0.04) (Figure 4. 3) was seen. In the stable angina patients there were no significant transculprit lesion gradient in total PMC, P-selectin positive and TF positive PMC expressions (data not shown).

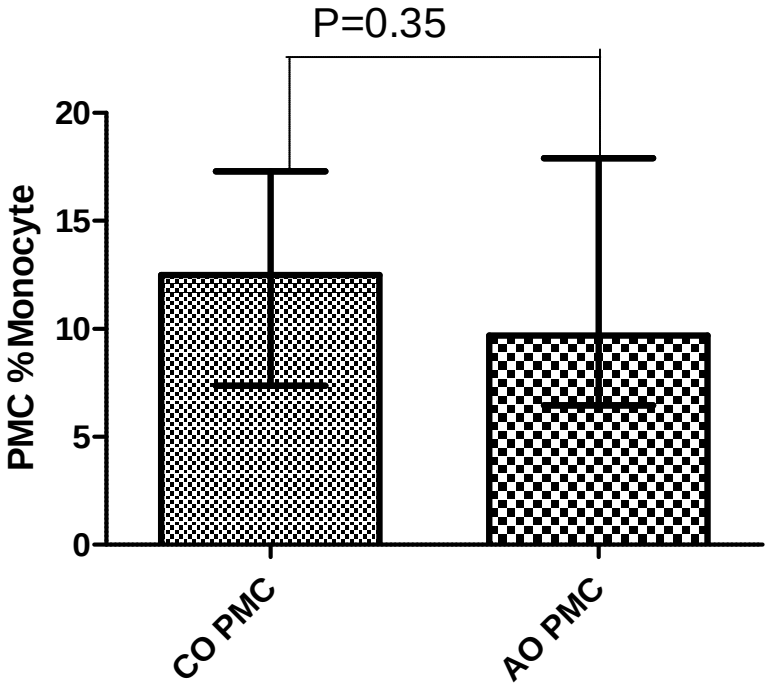

Figure 4. 1-Trans culprit lesion PMC gradient in ACS. There was no significant trans culprit lesion gradient in PMC expression in the ACS patients. (n= 22)

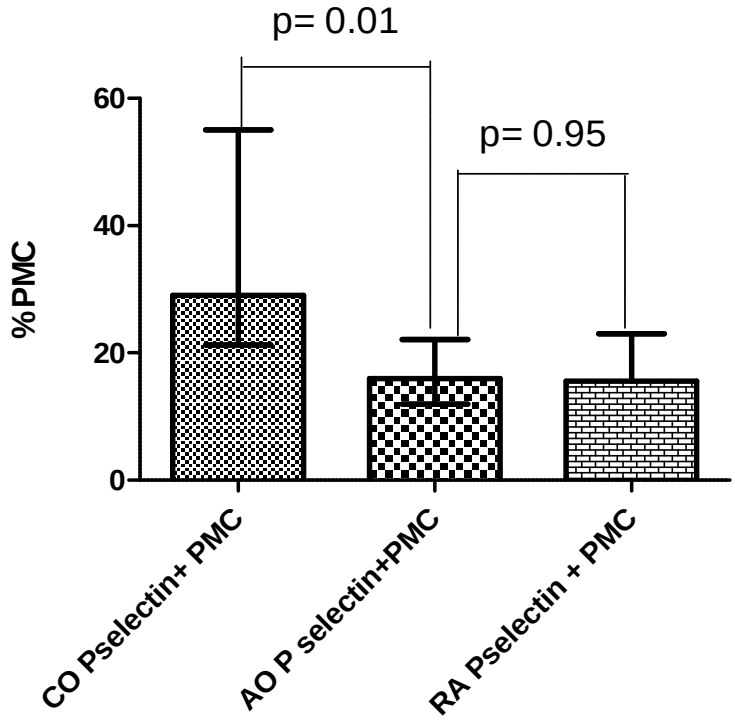

Figure 4. 2- Site specific comparison of P- selectin positive PMC expression in the ACS patients showing significant transculprit lesion gradient.(n= 14)

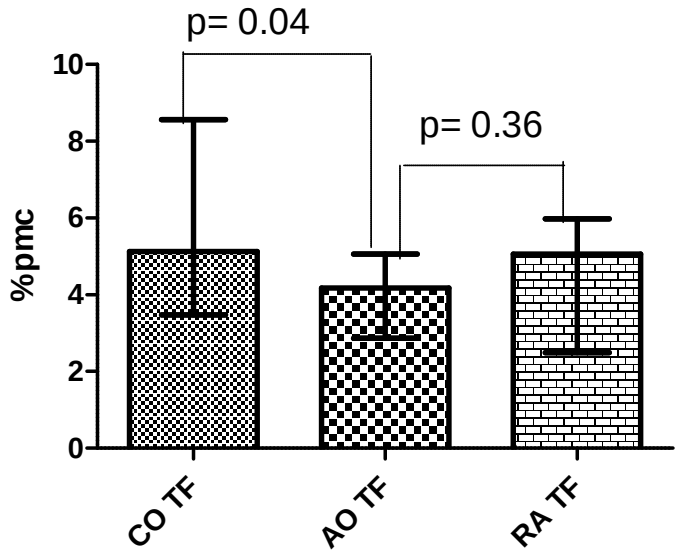

Figure- 4.3 Site specific comparison of TF positive PMCs in ACS.TF positive PMC expression had significant trans culprit lesion gradient in the ACS patients. This finding suggested heightened intracoronary monocyte activation within the PMC in the ACS patients.

4.6 DISCUSSION:-

Monocyte and macrophages are known to produce cytokines, extracellular matrix molecules, enzymes and other mediators which might play a central role in inflammatory disease including atherosclerosis (185). Antibody inhibition studies indicate that monocytes bind platelets primarily via PSGL-1 and P-selectin. The extent of monocyte-platelet interaction via PSGL-1 could lead to the development of a proatherogenic monocyte phenotype (22). Furman et al (21) first showed that circulating PMCs might be an early marker of acute myocardial infarction. As expected, our data showed increased site specific PMC expresson in all the 3 sites in ACS patients compared with stable angina patients but the difference was not statistically significant. This is either due to the limited number of patients recruited or the acknowledgement that overall PMC formation per se is most likely a non-specific systemic phenomenon in the context of coronary artery (vascular) disease. These findings did not implicate a significant pathophysiological role for overall PMC formation per se in acute coronary syndrome. Bournazos et al suggested that, in the absence of platelet activation, PSGL-1 mediated platelet adhesion to circulating monocytes represents a physiological process with little impact on cell physiology. In contrast high levels of P- selectin on the surface of activated platelets or binding of multiple platelets per monocyte were required to trigger monocyte activation via PSGL-1 (13). Our in vivo findings in human subjects showed that the percentage of PMCs which expressed P-selectin on their surface (P-selectin positive PMC) was significantly higher in the ACS group. Our findings suggested that although PMC formation appeared to be more of a general phenomenon it was platelet activation status within the complex (expressed by P- selectin positivity) which distinguished ACS

and stable angina patients. Therefore, we concluded that not all the complexes were equally active. In ACS patients activated platelets expressing P-selectin were higher compared with the stable angina group. This difference was most evident in the coronary circulation of ACS patients. These findings support the findings of Bournazos et al. However, unexpectedly monocyte activation status within the complexes (TF positive PMCs) did not significantly differ between the ACS and stable angina groups. In the ACS group a significant transculprit lesion gradient of both P-selectin and TF positive PMC suggested heightened intracoronary platelet and monocyte activation status within the complex at the site of plaque disruption. This provided clear support to the hypothesis that only activated PMC are of pathophysiological importance in ACS whereas overall PMC is merely a marker of generalised vascular inflammation. The role of heightened intracoronary platelet and monocyte activated PMC in contributing to microvascular injury requires further investigation.

4.7 CONCLUSION:-

Increased expression of PMCs was seen in ACS and stable angina patients compared with normal healthy individuals. No significant difference existed in total PMC expression between ACS and stable angina patients suggesting that PMC formation more likely a physiological marker of generalised vascular inflammation in coronary artery disease but not specifically implicated in the pathogenesis of ACS. However, activated PMC (with either activation of the platelet or monocyte portions) are increased in ACS patients and are more likely to be implicated in the pathogenesis of ACS. This hypothesis is supported by the

observation of a clear transculprit gradient for activated PMC in ACS patients. This study provides key insights into the role of platelet and monocyte interactions in coronary artery disease.

CHAPTER 5

COMPARISON OF SYSTEMIC AND SITE SPECIFIC INFLAMMATORY BURDEN BETWEEN ACS AND STABLE ANGINA PATIENTS.

5.1 Introduction.

5.2 Patients and Methods.

5.2.1 Sample collection and preparation.

5.3 Objectives.

5.4 Statistical methods.

5.5 Results.

5.6 Discussion.

5.7 Conclusion.

5.1 INTRODUCTION:-

In ACS inflammation has been found to play a significant role in the disease process. Mulvihill and colleagues have reported that a CRP concentration of < 3mg/l is associated with a negative predictive value of 97% for major adverse cardiovascular events within six months. Conversely a CRP concentration of > 3 mg/l has shown to have a sensitivity of 96% for predicting adverse cardiovascular events, with a specificity of 52 % (82). It is not clear whether increased levels of these inflammatory markers have any causal relationship with the pathogenesis of ACS (85). Using the principle of Mendelian randomisation CRP genetic variants as proxies a recent study by C Reactive Protein Coronary Heart Disease Genetics Collaboration (CCGC) group has shown that genetically raised concentrations of CRP are unrelated to conventional risk factors and risk of coronary heart disease. This group have also suggested that CRP concentration is unlikely to have even a modest causal role in coronary heart disease. Furthermore, this group did not find any material associations between CRP genetic variants and fibrinogen, interleukin 6, and leukocyte count. These results suggest that C reactive protein concentration is unlikely to be causally relevant (186). However other inflammatory markers like SAA, TNF alpha, IL-6 may have some relationship with the pathogenesis of acute coronary syndrome.

It remains unclear whether inflammatory markers originate from the site of plaque rupture or represent a systemic process. Measurement of inflammatory markers in the peripheral circulation may not be appropriate for evaluation of local inflammatory activity. Accurate evaluation of local inflammatory markers can be done by use of either local perfusate or by measuring the difference in concentration across the coronary circulation. An elevated coronary sinus-arterial

difference in the levels of IL-6 has been reported in patients with ACS (95). Deliargyris et al have described that IL-6 is produced in the coronary circulation of patients who have unstable angina but not in the patients who have stable angina (95).

Maier et al. (53) have demonstrated increase in levels of IL-6 by > 70% and SAA by around 10 % in the culprit coronary artery relative to those in the aorta in the STEMI patients. According to their conclusion this differential expression of increased inflammatory markers must be related to the arterial wall within the ruptured plaque or by the blood cells trapped in the occluded coronary artery.

Though SAA is known to be produced predominantly in the liver (99) the increase in local SAA blood levels in the culprit coronary artery can suggest local production of SAA at the site of occlusion either by cells of the atherosclerotic arterial wall or by white blood cells trapped in the thrombus. In line with this interpretation SAA mRNA and protein have been detected in human atherosclerotic lesions and cultured arterial smooth muscle cells (100).

Although In general it is believed that inflammation is a systemic effect of acute coronary syndrome and inflammatory markers are mostly synthesised in the liver, nevertheless the evidence above supports the idea of a local intracoronary synthesis of inflammatory markers. In accordance with these findings it is expected there should be a significant transculprit lesion gradient of inflammatory marker expression in the ACS patient and inflammatory burden will be highest at the coronary circulation distal to the culprit lesion. We also expect inflammatory burden will be higher in the ACS patients compared with the stable patients.

5.2 PATIENTS AND METHODS:-

In order to prove the above hypothesis blood samples were collected from 23 ACS patients (15 STEMI, 8 NSTEMI) and 7 stable angina patients. STEMI patients were recruited immediately after their admission in hospital just before the PPCI. NSTEMI patients were recruited within 72 hours of onset of chest pain before their percutaneous interventions. Stable angina patients were recruited before their elective percutaneous coronary intervention. Patients' baseline characteristics and drug treatments have already been described in the previous chapter. Samples were collected from aorta, coronary artery (distal to the culprit lesion) and right atrium. Rather than having a single venous sample we chose to measure overall median values by taking the median concentrations from all the 3 sites to ascertain the systemic inflammatory burden.

5.2.1 SAMPLE COLLECTION AND PREPARATION:-

Samples were aspirated from the coronary artery distal to the culprit lesion (using a coronary aspiration, Medtronic ® Export catheter , internal diameter 0.10 cm), aorta (using a Judkin's diagnostic catheter (5F, Cordis ®, internal diameter 0.11 cm) and from the right atrium (using a pigtail cather (5F, Cordis ®, internal diameter 0.11 cm). Blood samples (2.7ml) collected from each site were placed into 3.2% sodium citrate vacutainer tubes and the samples were used to prepare double spun platelet poor plasma by centrifuging the samples initially at 800g for 10 minutes at room temperature then supernatant was collected into eppendorfs and was recentrifuged at 2500g for 5 minutes and 90% of the supernatant

plasma was aspirated and immediately stored at -80^0 C for later analysis of IL-6 and TNF-alpha with ELISA (R&D systems) ; hs CRP and SAA protein using a high sensitivity automated microparticle enhanced latex turbidimetric immunoassay (COBAS MIRA; Roche Diagnostics GmbH).

5.3 OBJECTIVES:-

To compare the overall median values and site specific values of different inflammatory markers to ascertain the difference of inflammatory burden between the ACS and stable angina patients.

To examine the presence of differential expression of inflammatory parameters e.g CRP, SAA, TNF-alpha and IL-6 between the culprit coronary artery and peripheral circulation in the ACS group.

5.4 STATISTICAL METHODS:-

Data were presented as median (interquartile range). For comparison of continuous variables within a group p values were calculated by using a non parametric (Kruskal-Wallis) test. To compare variables between the two groups the nonparametric Mann-Whitney test was used. Correlation analysis was performed with Spearman's correlation analysis. A two-tailed p values < 0.05 was considered statistically significant. All calculations were performed using graphpad prism® (version 5) software.

5.5 RESULTS:-

The overall median CRP and SAA and IL- 6 concentrations were significantly higher in the ACS group compared with the stable angina group [CRP median (IQR) mg/l 2.9 (0.8- 6.4) vs 0.80 (0.70-1.8); p= 0.001. SAA median (IQR) mg/l 4.5 (2.4-12.8) vs 2.5 (1.4-2.7); p= 0.0007 and IL-6 median (IQR) pg/ml 5.9 (2.9-10.9) vs 1.7 (1.1-5.6) p= 0.03]. However, the difference was not significant in overall median TNF- alpha concentrations between these two groups [TNF- alpha median (IQR) pg/ml 25.4 (16.6- 39.2) vs 31.9 (21.8- 37.9) p= 0.29]. IL-6 concentration was below lower detection level of 0.7 pg/ml in the coronary artery and aorta of 3 patients and in right atrium of 5 patients with stable angina .

In the ACS group intracoronary CRP and SAA concentrations [median (IQR)] were found to be lower [2.4 (0.8-5.9) mg/l and 3.7 (1.8-12.7) mg/l] compared with the aorta [3.0 (1.07-6.5) mg/l and 5.3 (2.6-14.0) mg/l] and right atrium [3.0 (1.07-6.5) mg/l and 5.5 (2.5-13.6) mg/l] although the differences were not statistically significant (p= 0.76 and p= 0.81 respectively).No significant site specific difference in the TNF-alpha and IL-6 concentrations were noted in the ACS patients (p= 0.59 and p= 0.18 respectively).In the stable angina group there were no significant site specific difference in the inflammatory parameters either.

SAA (mg/ l)	ACS	Stable	p
CO	3.7 (1.8-12.7)	2.4 (1.1-2.7)	0.07
AO	5.3 (2.6-14.0)	2.6 (1.4-3.0)	0.04
RA	5.5 (2.5-13.6	2.5 (1.6-2.8)	0.04
CRP (mg/ l)			
CO	2.4 (0.8-5.9)	0.8 (0.5-1.5)	0.09
AO	3.0 (1.07-6.5)	0.8 (0.4-2.3)	0.07
RA	3.0 (1.07-6.5)	0.8 (0.7-2.1)	0.03
TNF-alpha (pg/ml)			
CO	24.24 (14.61- 33.42)	25.95 (13.84-36.88)	0.79
AO	26.36 (13.98-45.14)	31.85 (21.58- 39.40)	0.42
RA	29.89 (19.38- 52.30)	35.52 (26.33- 46.10)	0.81

Table -5.1 Site specific comparison of inflammatory parameters between the ACS and Stable angina group - CRP and SAA concentrations were higher in the ACS group compared to the stable angina group in all the sites.

5.6 DISCUSSION:-

In our study we have demonstrated the concentrations of CRP and SAA are higher in the ACS patients compared to the stable angina patients. These findings suggest the inflammatory burden in the ACS patients is higher than the stable angina patients. Findings of below lower detection level of IL-6 in different sites of the stable angina patients also emphasise the fact of lower inflammatory burden in the stable disease. Deliargyris et al have demonstrated that IL-6 is produced in the coronary circulation of patients of unstable angina but not in the patients who have stable angina (95). Though traditionally CRP and SAA productions by the liver are regarded as a systemic response to inflammation it has now been shown that cells within the atherosclerotic plaque also synthesise CRP (95, 99-101). Macrophage and smooth muscle like cells in atherosclerotic plaques have been shown to produce seven times more CRP mRNA than the liver (100). SAA is shown to be chemotactic for monocytes (121) and SAA proteins are shown to be produced by cells of the artery wall. Transition from chronic stable atherosclerotic CAD into an acute coronary syndrome has been found to be associated with an increase in inflammatory activity within the plaque, reflected in an increase in C- reactive protein and SAA levels. (83, 123).

These findings raise the question whether these pro inflammatory cytokines play any pathogenic local role within the coronary artery of the ACS patients. If that is the case we expect concentrations of inflammatory markers will be highest in the coronary artery compared to the aorta and right atrium. In contrary to the expectation the levels of CRP and SAA are found to be slightly low in the coronary circulation of the ACS patients compared to the peripheral circulation.

In absence of any increased coronary artery specific differential expression in the different inflammatory cytokine markers we conclude inflammation is a systemic response. Maier et al (53) also reported low intracoronary CRP values in acute myocardial infarction patients. This could be due to the fact CRP must have undergone partial catabolism in the vessel wall while it is trapped. Previous studies have demonstrated CRP protein in the coronary plaques (97, 187).

5.7 CONCLUSSION:-

We conclude inflammatory burden is higher in the ACS patients compared to the stable angina patients. It has been suggested in the previous studies that apart from being a systemic response inflammation can be a local intracoronary phenomenon in the pathogenesis of acute coronary syndrome.Increased levels of intracoronary inflammatory parameters compared to the peripheral circulation has also supported this hypothesis in the past. In contrary to the expectation our findings have not shown any significant transcoronary gradient of CRP, SAA, TNF- alpha and IL-6 levels. This suggests inflammation is more of a systemic phenomenon rather than a local intracoronary event. Surprisingly enough our findings of slightly lower CRP and SAA concentrations in the coronary circulation compared to the aorta and right atrium of the ACS patient can be due to the fact CRP and SAA may have undergone partial catabolism in the vessel wall as postulated by Maier et al (53).

CHAPTER 6

THE RELATIONSHIP BETWEEN TOTAL INTRACORONARY PMCs, P- SELECTIN, TISSUE FACTOR POSITIVE PMCs AND INTRACORONARY INFLAMMATION IN ACUTE CORONARY SYNDROME

6.1

Introduction

6.2 Objective.

6.3 Patients and Methods.

6.4 Statistical methods.

6.5 Results.

6.6 Discussion.

6.7Conclusion

6.1 INTRODUCTION:-

In ACS inflammation plays a significant role in the disease process and increased levels of inflammatory parameters e.g CRP and SAA have been associated with adverse outcomes (82-84). There is evidence that an increased state of inflammation is paralleled by activation of platelets with phosphorylation of the membrane protein P-selectin in the platelet alpha granule membrane (86). The granule moves to the platelet surface exposing P-selectin and tethering the platelet with the monocyte via P-selectin glycoprotein ligand-1 (PSGL-1) to form platelet-monocyte complexes. In addition, other integrin dependant mechanisms may be involved in the formation of PMCs (188).

Another hypothesis is that binding of activated platelets may alter monocyte activation status resulting in an increase in TF expression from the monocyte component acting as a trigger for the initiation of a cascade of thrombogenesis and the release of proinflammatory cytokines, including IL-6, IL-1β and IL-12. It has been shown activated platelets express CD 40 ligand on their surface (9). Binding of CD40L to CD 40 on monocytes leads to monocyte activation and production of cytokines including IL-6 which is associated with unstable angina (10, 11) and is often associated with increased CRP.

CRP also stimulates production of TF by mononuclear cells, (107) the main initiator for blood coagulation. CRP interacts with low density lipoprotein and with damaged cell membranes (108) and can also activate the complement system (109). CRP induces PAI -1 expression from the endothelial cells and suppresses NO release from the endothelial cells (110).

SAA (81) promotes monocyte chemotaxis and adhesion. SAA also induces TNF alpha in normal neutrophils and monocytes (124, 129). SAA was also shown to be a potent and rapid inducer of TF on normal monocytes (130).

In summary platelet interaction with monocytes results in monocyte activation which subsequently renders the monocyte more adhesive, more procoagulant through TF expression and more inflammatory. In addition increased inflammatory activities may also be directly related to platelet activation and PMC formation. Therefore, as platelet activation and inflammation appear to be inter-related there should be a correlation between PMC formation, platelet activation and inflammation in ACS. However, it is not clear whether this interrelationship is coronary artery specific or systemic.

In the previous chapters we have demonstrated that PMC formation is a generalised systemic phenomenon but P- selectin expression and TF expression on the PMCs is highest within the coronary circulation of ACS patients. These findings suggest that in ACS it is the activation status of the PMC which is most important at a local intracoronary level. We have also established that inflammation is a systemic phenomenon in CAD with no site specific difference seen in measured inflammatory parameters in ACS patients.

6.2 OBJECTIVE:-

To examine intracoronary correlation between PMC, P-selectin positive PMC,TF positive PMC and inflammation in ACS patients.

6.3 PATIENTS AND METHODS:-

Twenty three patients with acute ACS (15 STEMI and 8 NSTEMI) who had undergone percutaneous coronary intervention (PCI) were recruited for the study. Patients‘ details, recruitment process and characteristics have already been described in the previous chapters. Sample collection, flow cytometry and ELISA methods have also been described in the previous chapter.

6.4 STATISTICAL METHODS:-

Data were presented as median (Interquartile range). Correlation analysis was performed with Spearman’s correlation analysis. Two-tailed p values < 0.05 was considered statistically significant. All calculations were performed using graphpad prism® (version 5) software.

6.5 RESULTS:-

CORRELATION OF INTRACORONARY PMC, P- SELECTIN POSITIVE PMC AND INFLAMMATION IN ACS PATIENTS:-

A significant positive correlation was found between intracoronary PMC and TNF-alpha (r = 0.54, p= 0.02) and a non-significant positive correlation with intracoronary CRP (r= 0.32, p=0.14), SAA (r= 0.32, p=0.14) and IL-6 (r= 0.47, p= 0.06).

Intracoronary P-selectin positive PMCs also demonstrated a non significant positive correlation with TNF –alpha (p= 0.53, r= 0.09), CRP (r= 0.16, p= 0.56) and SAA (r=0.12, p=0.66) similarly TF positive PMCs have shown a non significant positive correlation with IL-6 (r= 0.21, p= 0.53) and TNF- alpha (r=0.23, p= 0.48).

Ten patients in the ACS group were found to have intracoronary SAA concentrations ≥ 4.5 mg/l (≥ overall median value). In this subgroup of patients with relatively higher intracoronary inflammatory burden there was a significant correlation between intracoronary SAA and intracoronary PMC expression (r= 0.75, p= 0.01) (figure -6.1). This correlation was not observed in the peripheral circulation of the ACS patients. (data not shown).

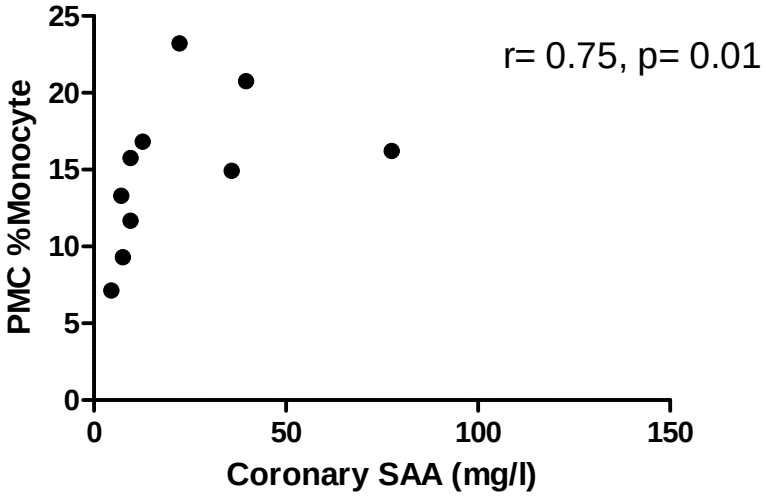

Figure –6. 1. Correlation of intracoronary PMC and SAA in ACS. In ACS patients with higher intracoronary inflammatory burden SAA (≥ 4.5 mg/l) there was significant correlation of intracoronary PMC and SAA.

CORRELATION OF INTRACORONARY PMCs AND INFLAMMATORY MARKERS IN THE STEMI SUBGROUP.

In the STEMI subgroup intracoronary PMC expression demonstrated a significant positive correlation with intracoronary TNF-alpha (r= 0.68, p= 0.03) (figure-6.2). TF positive PMCs (% monocytes) also showed a positive correlation with intracoronary TNF-alpha and IL-6 (r=0.66, p=0.05 & r=0.71, p= 0.05 respectively) (Figure-6.3). In the peripheral circulation no significant correlations were observed between PMC expression, P-selectin and TF factor expression and inflammatory markers (data not shown).

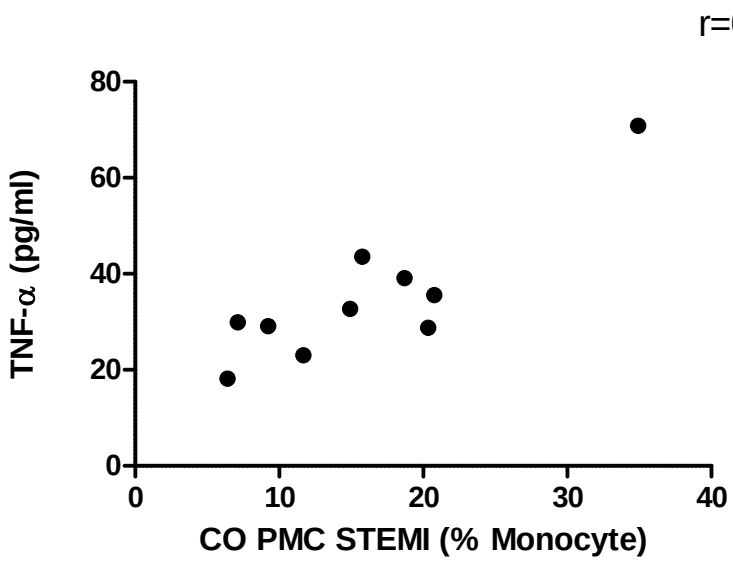

Figure –6.2.Correlation of intracoronary PMC and TNF- alpha in STEMI. In STEMI patients intracoronary PMC has significant positive correlation with intracoronary TNF- alpha.

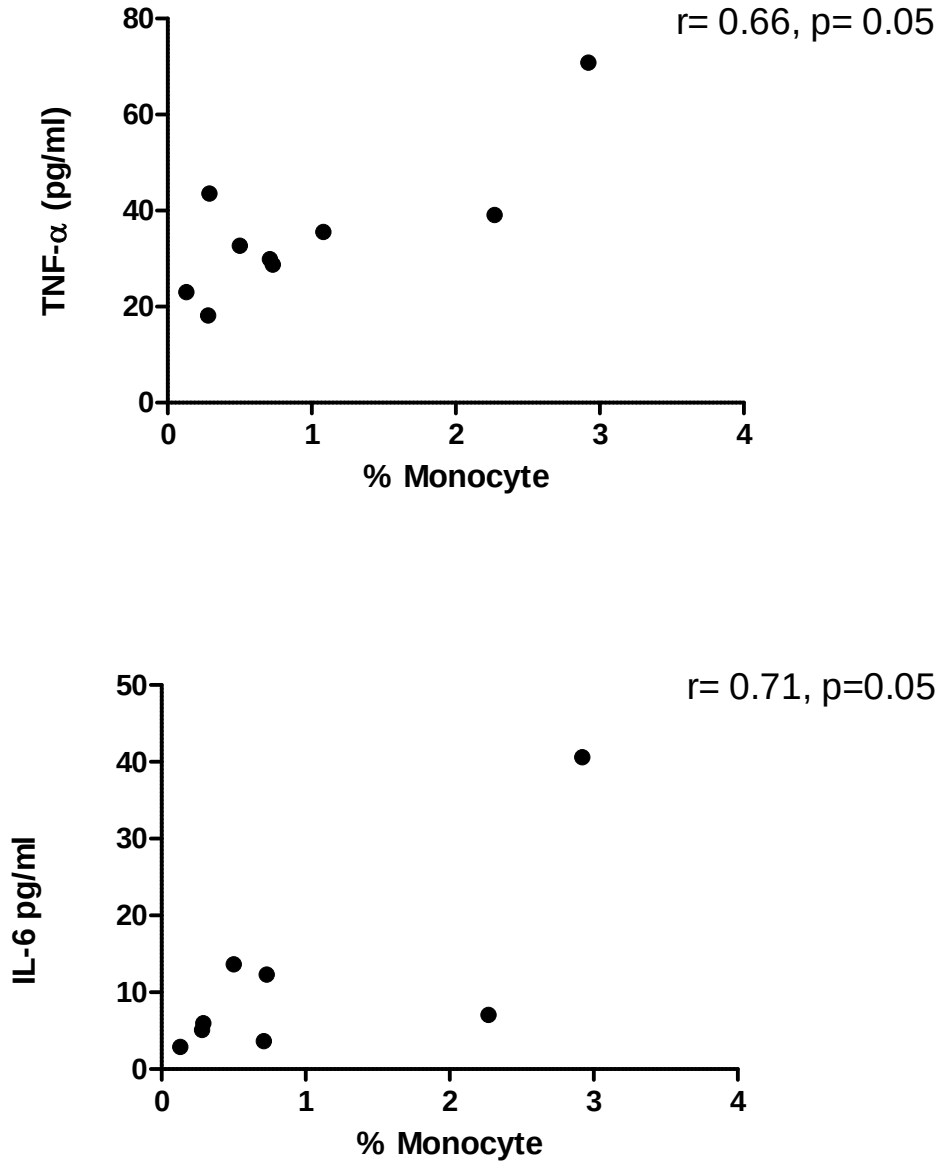

Figure-6.3 Correlation of intracoronary TF positive PMC with TNF- alpha and IL-6 in STEMI.Intracoronary TF positive PMC showed positive correlations with intracoronary IL-6 (n= 8) and TNF- alpha (n=9).

6.6 DISCUSSION:-

The finding of a significant positive correlation with intracoronary PMC and TNF alpha overall in the ACS patients and in the STEMI subgroup emphasises the local relationship between PMC formation and inflammation in the acute stage of the disease. Similarly, positive correlation of intracoronary TF positive PMC with TNF alpha and IL-6 in the STEMI patients provides a further possible mechanistic insight into the role of activated monocytes within the PMC in the pathogenesis of intracoronary inflammation and thrombosis. Unstimulated monocytes are not proinflammatory but within activated monocytes there is significant upregulation in the expression of inflammatory parameters. TF positive complexes indicate presence of monocyte activation within these complexes. It has previously been shown binding of CD40L on platelets to CD 40 on monocytes leads to monocyte activation and production of cytokines including IL-6 which is associated with unstable angina (10),(11). Monocyte expression of chemokines is also shown to be induced by thrombin activated platelets and is regulated by NF-kB activity. Ligation of monocyte PSGL-1 together with RANTES induces NF-kB activity and subsequent secretion of MCP-1 , TNF-alpha and IL-8 (8, 20). Therefore, from the summary of in vitro studies it is clear that upon activation monocytes secrete different inflammatory cytokines including TNF alpha and IL-6. Our in vivo study in patients with acute STEMI supports these in vitro study findings.

In the ACS patients intracoronary PMC has shown positive correlation with CRP, SAA, IL-6 andTNF- alpha suggesting a local intracoronary relationship between PMC and inflammatory mediators. The correlation is statistically significant only with TNF- alpha. This finding suggest TNF alpha may be a more coronary artery

specific marker dependent on PMC formation. It has been shown before that TNF- alpha is a central mediator of inflammation and is localised in atherosclerotic lesions. And may contribute to triggering, progression and rupture of plaque (189-191). It has been reported previously that TNF- alpha can cause depression of cardiac contractility either directly (137) or by induction of inducible NO synthase (Inos) in cardiocytes and other cellular constituents within the heart (138).

Correlation of intracoronary SAA with PMC in the subgroup of patients with higher inflammatory burden (SAA≥ 4.5 mg/l) further emphasises the local intracoronary inter-relationship between PMC formation and inflammation. Dotsenko et al. in a previous study demonstrated an association between elevated CRP and increased PMC in patients with atherosclerotic patients suggesting a relationship between inflammation and PMC formation (192). Positive correlation of PMC and TF expression with intracoronary TNF alpha suggests PMC may play a contributory role through heightened TF expression in the pathogenesis of intracoronary inflammation in the STEMI patients. Celi et al demonstrated platelet surface P-selectin induces the expression of TF on monocytes and promotes fibrin deposition within the growing thrombus at the site of the vascular injury (6),(193).

6.7 CONCLUSION:-

This study demonstrates a local intracoronary interrelationship between PMC formation and inflammation in ACS patients. TNF- alpha is the most significant intracoronary inflammatory marker related to PMC formation and monocyte

activation within the PMC; particularly in STEMI patients in the hyperacute stages of intracoronary inflammation. In STEMI patients PMC may play a significant local role through TF expression in determining TNF-alpha and IL-6 production.

CH APTER 7

SOLUBLE P SELECTIN AND P SELECTIN POSITIVE PLATELET MONOCYTE COMPLEXES (PMCs) AS DETERMINANTS OF INFLAMMATION IN PATIENTS WITH CORONARY ARTERY DISEASE.

7.1

Introduction.

7.2 Objectives.

7.3 Patients and Methods.

7.4 Exclusion criteria.

7.5 Statistical Method.

7.6 Results.

7.7Discussion.

7.1 INTRODUCTION:-

Heightened level of inflammation is a well recognised phenomenon in patients with CAD. Increased CRP has been implicated as an adverse prognostic marker in patients with coronary artery disease (82-84). Formation of PMCs can be a link between platelet mediated thrombogenesis and monocyte mediated inflammation but the mechanism by which PMCs contribute to intracoronary inflammation is not clearly established. One hypothesis is that binding of activated platelets may alter monocyte activation status resulting in an increase in TF expression from the monocyte component acting as a trigger for the initiation of a cascade of thrombogenesis and the release of proinflammatory cytokines, including IL-6, IL-1β and IL-12. However, binding with unstimulated platelets may not be associated with a similar cellular response (13). Therefore, in the absence of platelet activation PMC formation may just represent a physiological phenomenon. Depending upon the level of activation, the platelets within PMCs may actually be responsible for triggering a pro-inflammatory response in monocytes. On the other hand there is evidence that an increased inflammatory state is paralleled by activation of platelets with phosphorylation of the membrane protein p-selectin in the platelet alpha granule membrane (86).Then the granule moves to the platelet surface exposing P-selectin and tethering the platelet with the monocyte via PSGL-1 to form PMC. Apart from cell surface expression a variant of P-selectin can also be detected in the plasma (s p- selectin) leading to presumption that they are secreted, shed or cleaved from the cell surface however, the changes in soluble levels in the plasma may not reflect levels at the cell membrane (194). Mere PMC formation may not have any

significant relationship with inflammation but platelet activation status within this complexes (P-selectin positive PMC) and sP-selectin may have some relationship with inflammation in coronary artery disease. P-selectin has been implicated at the cellular level as a causative factor for inflammation as it contributes to leukocyte rolling and triggers TF release from the acitvated monocytes (195). So far no direct correlation with inflammatory biomarkers such as CRP, SAA and TNF alpha have been demonstrated in vivo in human subjects with CAD.

There have been some suggestions that CRP and SAA may play a local intracoronary role in the pathogenesis of ACS (53, 98, 102, 196). If these inflammatory markers are responsible for local intracoronary inflammation we expect their concentrations will be higher in the coronary circulation compared to the peripheral circulation. However, data largely implicates these biomarkers are a systemic response synthesised in response to coronary artery disease (117, 197, 198).

7.2 OBJECTIVES:-

To investigate whether a relationship exists between P-selectin expression on the PMCs and s P-selectin levels.

To examine whether any differential expression exists between expression of inflammatory biomarkers in the coronary circulation compared with the peripheral circulation.

To investigate the relationship between different inflammatory markers e.g CRP, SAA and TNF- alpha and P-selectin expression on the PMCs and soluble plasma P-selectin.

7.3 PATIENTS AND METHODS:-

Thirty patients with coronary artery disease were recruited for the study. Consent was obtained from 15 patients with STEMI immediately after their admission to hospital for PPCI, 8 patients with NSTEMI within 72 hours of their symptom onset and 7 patients with stable angina just before their elective percutaneous coronary intervention (PCI). Prior ethical approval was obtained from the local research and ethics committee. Patients with STEMI and NSTEMI were loaded with 600 mg of clopidogrel and 300 mg of Aspirin prior to the percutaneous procedure. Stable angina patients had already received 75 mg of Aspirin and 75mg of Clopidogrel for 7 days prior to the procedure according to the protocol for performing elective PCI in our hospital. Prior to PCI all the patients were treated with weight adjusted unfractionated heparin to maintain the ACT between 200-250 seconds.

Samples were collected from the coronary artery distal to the lesion, aorta and right atrium as previously described in chapter 2. PMC estimation and P-selectin expression on the PMCs were estimated by flow cytometry as described in chapter 2. PMCs were expressed as % monocyte and P- selectin positive PMCs were expressed as % monocyte as well as % of PMC.

High sensitive CRP, SAA,TNF-alpha and s-Pselectin were estimated from double spun platelet poor plasma prepared from collected blood samples from all the 3 sites as described in chapter 2.

Coronary samples distal to the lesion were taken to estimate the local intracoronary concentrations of soluble P-selectin, inflammatory markers, total PMC and P-selectin positive PMC expression. Aortic and right atrial samples were taken to estimate similar parameters in the arterial and venous circulation respectively. Overall median values were measured to estimate the systemic expression of PMC, P-selectin positive PMC, CRP, SAA and TNF- alpha. Overall median values were calculated by calculating the median concentrations from all the 3 sites rather than having a single value from a venous sample.

7.4 EXCLUSION CRITERIA:--

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

7.5 STATISTICAL METHODS:-

Data were presented as median and inter-quartile range. Inflammatory parameters, PMC, P-selectin positive PMC in the coronary circulation, aorta and right atrium were compared using the non parametric Kruskal- Wallis one way analysis of variance test. Correlation analysis was performed by using spearman's correlation analysis. Two tailed p value < 0.05 was considered significant.

7.6 RESULTS:-

Patient demographics and coronary characteristics are described in table 7.1. No significant site specific difference in PMC expression was seen. Similarly there was no significant site specific difference in CRP, TNF alpha, SAA and s P-selectin concentration (table-7.2). However, percentage of PMC expressing P-selectin on their surface (P-selectin positive PMC) was significantly higher in the coronary circulation compared with the aorta and right atrium (p= 0.02) (Table-7.2)

Age median (IQR), years	61(51-72)
Male	24
Female	6
Risk Factors	
History of hypertension	12
History of smoking	16
Diabetes	3
Dyslipidaemia	14
Angiographic characteristics	
1 vessel disease	16
2 vessel disease	9
3 vessel disease	5
Culprit vessel	
Left Anterior Descending	10
Left Circumflex	8
Right Coronary Artery	12

Table-7.1 Baseline patient and coronary characteristics**.**

	Aorta Median (IQR)	Right Atrium Median (IQR)	CoronaryArtery Median (IQR)	p
CRP mg/l	1.7 (0.8- 5.5)	1.9 (0.8- 5.8)	1.6(0.72- 4.85)	0.77
SAA mg/l	3.8(2.05- 9.35)	3.6 (2.4-8.8)	2.8 (1.5- 9.0)	0.77
TNF–alpha pg/ml	27.30 (14.25-43.11)	34.86 (19.38-45.81)	23.06 (13.24-35.57)	0.27
sP -selectin ng/ml	21.90 (14.90-29.80)	23.40 (14.20-39.00)	16.80 (13.00-25.20)	0.49
P-Selectin positive PMC (% monocyte)	1.98 (0.98- 3.06)	1.62 (0.89-3.34)	3.08 (1.77- 7.85)	0.12
P-selectin positive PMC (%PMC)	15.37 (9.88-19.18)	15.98 (10.96-19.76)	24.17 (17.95-43.37)	0.02

Table 7. 2:- Site specific concentrations of inflammatory markers, s P- selectin, PMC and P-selectin positive PMC in patients with coronary artery disease (n=30). Percentage of P- selectin positive PMC (%PMC) was significantly higher in the coronary circulation compared with aorta and right atrium.

Overall median PMC [% monocyte ; median (IQR)] was 9.98 (7.13- 17.46)% and overall median P selectin positive PMC [% PMC median (IQR)] was 16.89 (11.14 – 29.76) %; % monocyte median (IQR) was 2.07 (1.15- 3.77)%.]

Overall median P selectin positive PMC (% monocyte) showed a significant positive correlation with overall median CRP (r= 0.37, p=0.005), overall median SAA (r=0.38, p=0.003) and a non significant positive correlation with overall median TNF- alpha (r= 0.26, p= 0.09) (Figure- 7.1)

Similarly, overall median s P-selectin showed a significant positive correlation with overall median CRP, SAA and TNF-alpha (Figure-7. 2)

In addition there was a significant positive correlation between overall median P-selectin positive PMC (%monocyte) and s P-Selectin. (Figure-7. 3)

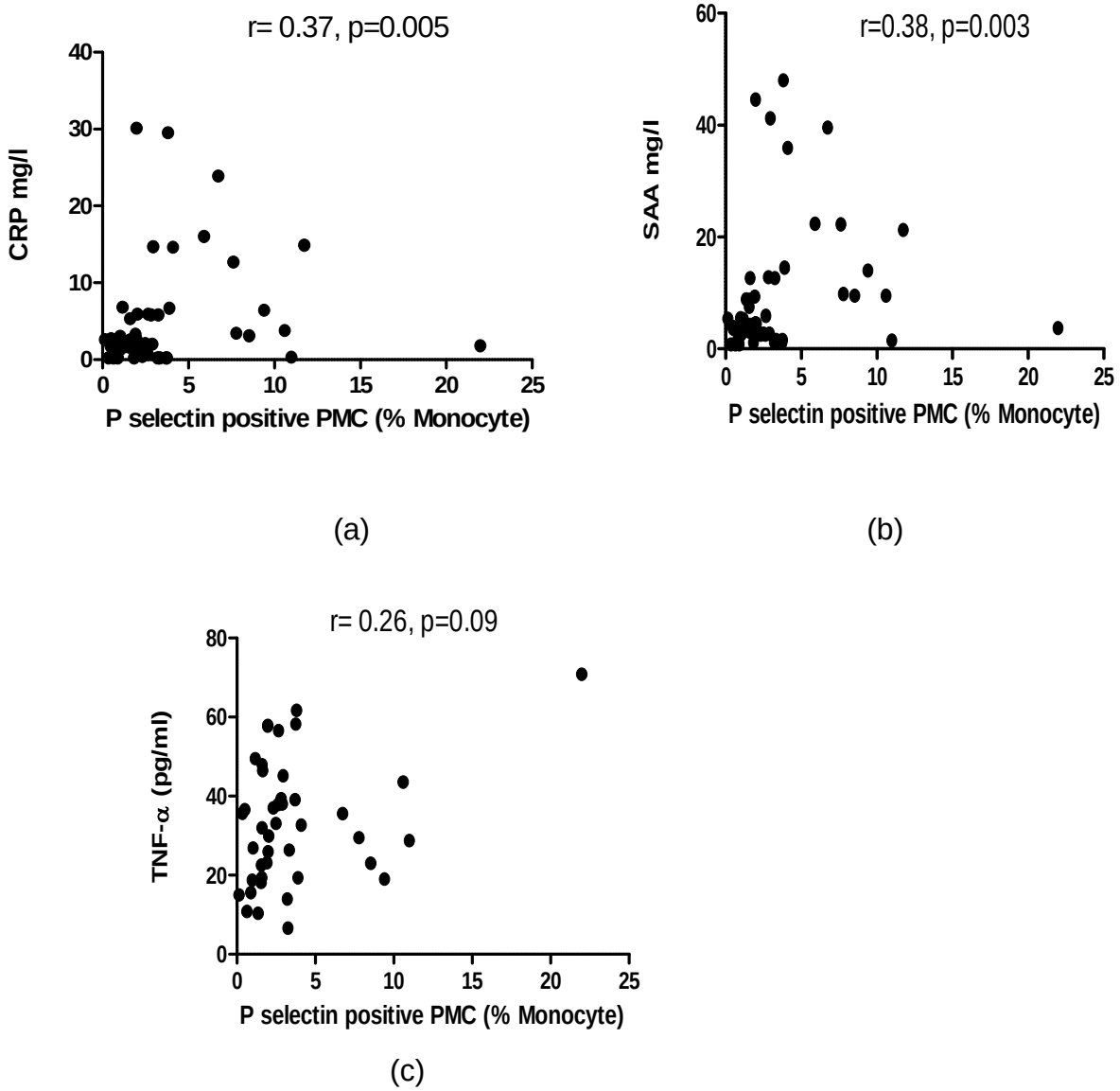

Figure 7.1- Correlation of overall median P-Selectin positive PMC with inflammatory parameters in CAD Patients Overall median P- selectin positive PMC (%monocyte) has significant positive correlation with overall median CRP (a), SAA (b) and non-significant positive correlation with overall median TNF-alpha (c).

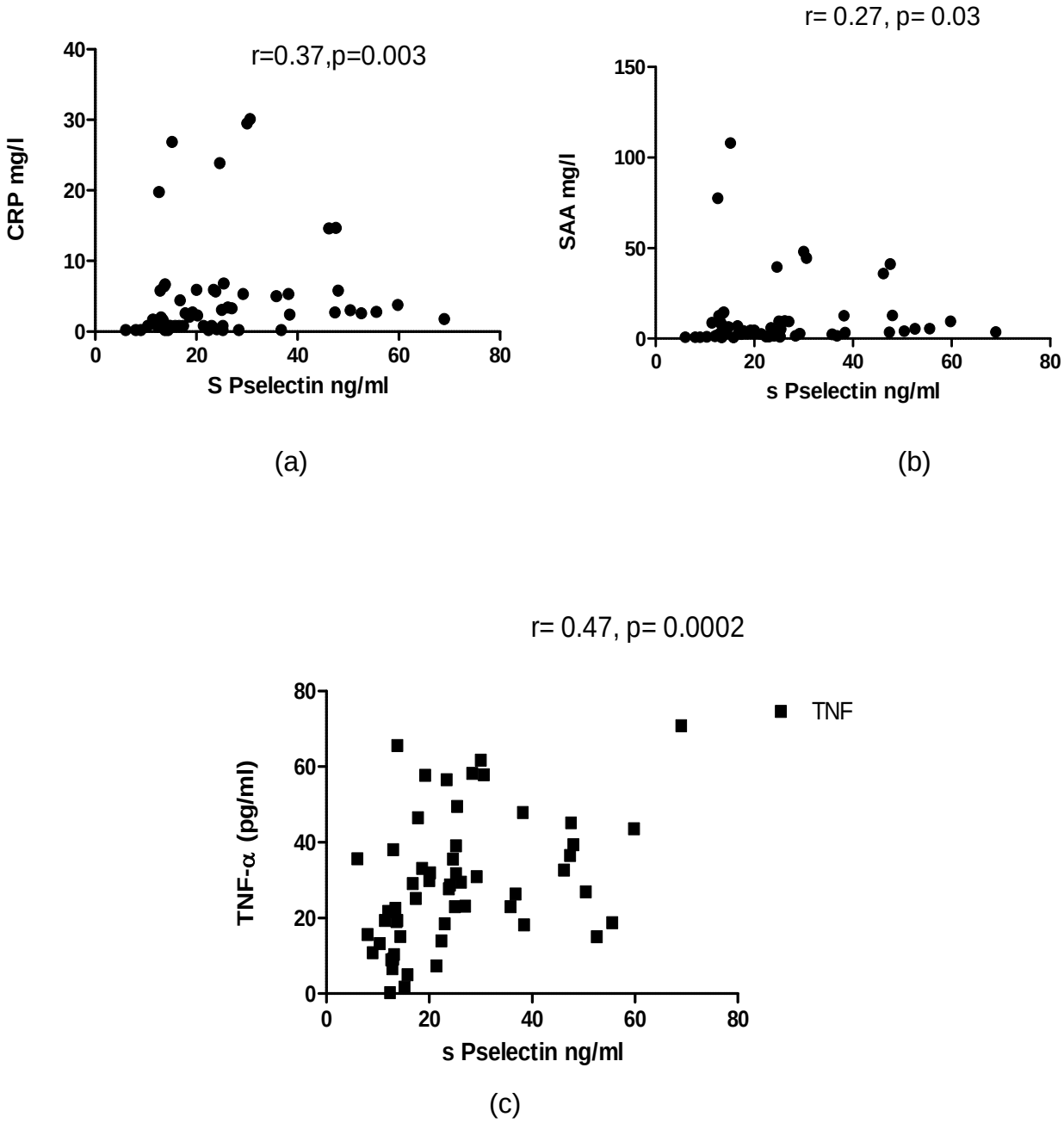

Figure 7.2- Correlation of overall median sP-selectin with inflammatory parameters in CAD Patients. Overall median sP-selectin demonstrated significant positive correlation with overall median CRP (a), overall median SAA (b) and overall median TNF- alpha (c).

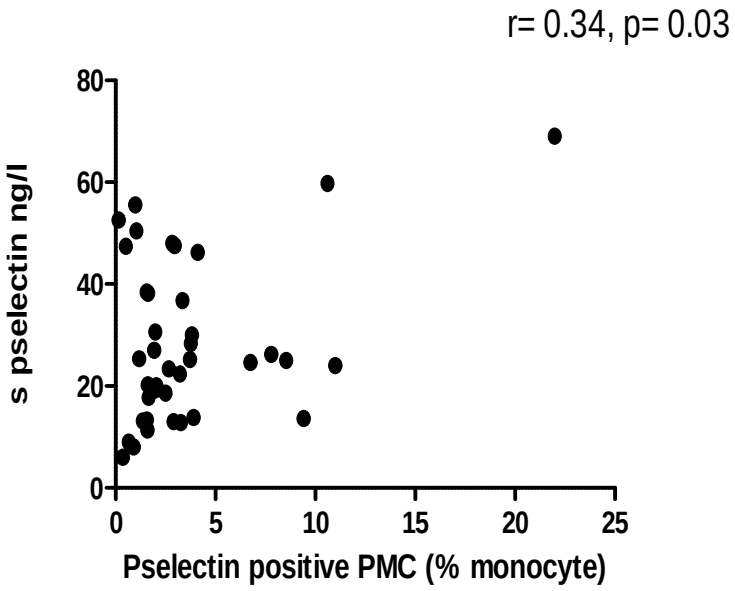

Figure - 7.3 Correlation of P- selectin positive PMC and s P-selectin. P-selectin positive PMC (% monocyte) and sP-selectin showed a significant positive correlation.

7.7 DISCUSSION:-

Although traditionally CRP and SAA production by the liver are regarded as a systemic response to inflammation it has now been shown that cells within the atherosclerotic plaque also synthesise CRP (98, 102, 196). Macrophage and smooth muscle - like cells in atherosclerotic plaques are shown to produce seven times more CRP mRNA than the liver (98). SAA has also been shown to be chemotactic for monocytes (121). It has also been shown that SAA proteins are produced by cells of the artery wall. The transition from chronic stable atherosclerotic coronary artery disease into an acute coronary syndrome has been found to be associated with an increase in inflammatory activity within the plaque, reflected in an increase in C- reactive protein and SAA levels (83) (123). These findings raise the question whether these pro inflammatory cytokines play any pathogenic local role within the coronary artery of the ACS patients. Therefore, if inflammation is a local intracoronary event one would expect local intracoronary levels of inflammatory markers to be higher in the coronary circulation compared to the peripheral circulation. Maier et al. (53) demonstrated increase in levels of IL-6 by > 70% and SAA by around 10 % in the culprit coronary artery relative to those in the aorta in the STEMI patients. Our study does not support the hypothesis that inflammation is primarily an intracoronary event as there is no differential increased expression of intracoronary inflammatory parameters in the coronary compared with systemic circulation. Interestingly, contrary to what one might have expected, CRP, SAA and TNF alpha concentrations are non-significantly lower in the coronary circulation compared to the peripheral circulation. Decreased intracoronary CRP values in

acute myocardial infarction patients have been reported by Maier et al (53) as described in the chapter 5. This could be due to the fact CRP must have undergone partial catabolism in the vessel wall while it is trapped. CRP protein within coronary plaques have been reported in previous studies (97), (187).

Upon activation from inflammatory cytokines, mainly TNF- alpha, cell surface expression of P-selectin is enhanced. This process is vital in the early phase of inflammation mediating leukocyte recruitment and transient leukocyte to endothelial interaction (tethering and rolling phase) (199, 200) (201). P- selectin is expressed in platelets , stored in membrane alpha granules. Upon activation, it is translocated to the surface of the platelets (202, 203).

P- selectin sheds from the surface of platelets and circulate in the soluble form as well. P –selectin, either on the surface of activated platelets or as a soluble form in plasma, may play a significant role in thrombosis, inflammation and thrombosis- mediated inflammation events. One hypothesis is that binding of activated platelets alters monocyte activation status resulting in an increase in TF expression from the monocyte component acting as a trigger for the initiation of a cascade of thrombogenesis and the release of proinflammatory cytokines promotes fibrin deposition within the growing thrombus at the site of the vascular injury (193),(204, 205). It is not clear how P-selectin, either on the cell surface or in the soluble form, contributes to inflammation as previous data have not established a correlation between P- selectin and inflammatory parameters. Schmacher et al. measured IL-6, CRP, TNF alpha alongside the slouble adhesion molecules but they did not find any significant correlations between s P- selectin and CRP, TNF- alpha or IL-6 (79),(80). To our knowledge our study is the first of its kind where we have clearly demonstrated significant positive

correlations of sP- selectin with CRP, SAA and TNF- alpha. We have also demonstrated a significant positive correlation of P-selectin positive PMCs (% monocyte) with the CRP and SAA. These findings establish the relationship of s P- selectin with inflammation in CAD. Similarly relationship of P-selectin positive PMC (% monocyte) with inflammatory parameters emphasises the fact that monocytes bound within the complex with P selectin positive platelets may play a significant role in the systemic inflammatory response associated with ACS. Also in this study we have demonstrated a significant correlation between P-selectin positive PMC and sP- selectin. We conclude P -selectin plays a significant role in the inflammatory process in coronary artery disease both in the soluble form and by influencing monocytes within the platelet monocyte complexes. P-selectin has been linked with an enhanced inflammatory response, restenosis and increased risk of cardiovascular events and may be a predictor of future thrombotic events (74). Though this study does not definitely establish the causal relationship of P-selectin and inflammation nevertheless it emphasises the importance of platelet activation, P- selectin expression and inflammation in coronary artery disease. P-selectin may be a future therapeutic target to modulate the inflammatory response in coronary artery disease.

CHAPTER 8

DISCUSSION

Over the last decade PMCs have generated considerable interest in understanding the pathophysiology of ACS. PMCs have been implicated as a link between platelet mediated thrombogenesis and monocyte mediated inflammation. The exact mechanism through which PMCs contribute to the pathogenesis of ACS is yet to be understood. Similarly it is not clear whether PMC formation is a systemic phenomenon or a local intracoronary phenomenon which augments the intracoronary inflammatory response in patients with ACS. There are some suggestions that PMC formation may be merely a physiological phenomenon without much pathological significance (13). On the other hand existing flow cytometry methods have not taken into consideration the possibility of coincident events which may result in over estimation of PMCs. Similarly, the exact role of inflammation in the pathogenesis of ACS is also controversial. Data suggests that inflammatory markers can be generated locally in the coronary artery of the patients with acute coronary syndrome (25). With this background in mind the first aim of this thesis was to establish a flow cytometric method which can exclude the coincident events carefully so that the true PMCs can be estimated accurately. The aim of further investigations in this study was to first evaluate whether PMC formation and inflammation represented systemic or local phenomena. Also, to establish the relationship between PMC, and inflammation and to find out whether any local intracoronary relationship existed between PMC and inflammation in the ACS patients.

In chapter 3 we have described the flow cytometric methodology using a double discriminator technique previously designed to overcome the problem of co-incident analysis of doublets of bare nuclei in DNA ploidy experiments. With this technique we have successfully excluded coincident platelets and monocytes as

they have a longer time of flight through the focal point of the laser. Using pulse processed analysis, they elicit signals with a greater fluorescent width than the single cell and a concomitant greater signal area. Using this technique we have successfully excluded the coincident events with wider fluorescent width and managed to accurately evaluate true PMC expression.

In the chapter 4 we have demonstrated that overall PMC is higher in all vascular compartments of ACS patients compared with stable angina patients. However, no differential expression of heightened PMC expression was obvious within the intracoronary compartment compared with venous or aortic, suggestin a general systemic phenomenon.We have demonstrated that activated PMC (complexes expressing P-selectin or TF, suggestive of activated platelets and monocytes within the complexes respectively) have been found to be significantly increased with a significant trans - culprit lesion gradient demonstrated. We have also demonstrated P selectin positive PMC expression in the coronary circulation is significantly higher in ACS compared with stable angina patients. These findings suggest differential site specific platelet and monocyte activation status within the complex may be more important in the pathogenesis of ACS rather than overall PMC formation.

In chapter 5 we have demonstrated that inflammatory burden is higher in ACS patients compared with stable angina patients but we have not found any significant trans-coronary gradient in inflammatory parameters to suggest that inflammation is a local intracoronary event. We conclude inflammation is a systemic phenomenon in coronary artery disease.

In Chapter 6 we have described the local intracoronary relationship of PMC and inflammation in ACS patients. We have found positive correlation with

intracoronary TNF alpha with PMC and TF positive PMC .This findings suggest local monocyte activation on the PMC may be the trigger for local intracoronary inflammation.

In chapter 7 we have emphasised the role of P selectin and systemic inflammation in patients with coronary artery disease in general both in the soluble form and on the cell surface of PMC. In this chapter we have also demonstrated the significant positive correlation between soluble and PMC surface P-selectin.

Our study is an observational study and although it does not establish a causal relationship between PMC formation and inflammation in ACS patients. Nevertheless our data importantly establishes the fact mere PMC formation may not have much pathological significance but instead represents a general systemic marker of inflammation. However, it is the platelet and monocyte activation status within the complex which appears directly related to inflammation in patients with ACS. This provides an important pathophysiological insight into patelet-monocyte interaction in coronary disease. Our data also support the hypothesis that PMC may contribute to systemic inflammation through P-selectin expression and local intracoronary inflammation through increased P-selectin and TF expression.

Future Work-

This study provides some mechanistic insight about the role of P-selectin and TF expression on the PMC in relation to systemic as well as local intracoronary inflammation in ACS patients. The activated PMC (expressing P- selectin or TF) may represent a novel therapeutic targets in abrogating the intracoronary inflammatory burden in ACS. This in turn by reducing intracoronary

thrombogenesis and cytokine-mediated microcellular damage may represent a strategy to reduce microvascular injury and aid myocardial salvage.

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