Thrombosis Research

Persistent circulating platelet and endothelial derived microparticle signature may explain on-going pro-thrombogenicity after acute coronary syndrome.

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Abstract:	Aims: Microparticles (MPs) are submicron vesicles, released from activated, and apoptotic cells. MPs are shown to be elevated in the circulation of patients with coronary artery disease (CAD) and have pro-thrombotic potential. However, limited data exists on MP signature over time following an acute coronary event. Methods & Results: Circulating total annexin v+ (Anv+) MPs of endothelial (EMP), platelet (PMP), monocyte (MMP), neutrophil (NMP) and smooth muscle cell (SMMP) originwere quantified by flow cytometry. 13 patients with acute coronary syndrome (ACS) were prospectively enrolled and 12 patients with stable angina (SA) were included as a comparator group. A panel of MP was measured at baseline, after percutaneous coronary intervention (PCI) and at days 1, 7, 30 and 6 months. Intra & inter group comparison was made between various time points. MP mediated thrombin generation was measured by recording lag phase, velocity index, peak thrombin and endogenous thrombin potential at these time points and compared with healthy controls.The total AnV+ MP levels were similar in ACS and SA groups at baseline, peaked immediately after PCI and were at their lowest on day 1. PMP & EMP levels remained significantly elevated in ACS patients at 6 months when compared to SA. No such difference was noted with NMP, MMP and SMMP. Patients with coronary artery disease showed abnormal thrombograms when compared to controls. Peak thrombin (nano moles) was significantly higher in CAD when compared to controls (254 IQR [226, 239] in ACS, 255 IQR [219, 328] in SA and 132 IQR [57, 252] in controls; p = 0.006). Differences in thrombin generation between ACS and SA were not significant (p=1). Furthermore, thrombin parameters remained abnormal in ACS & SA patients at 6 months. Conclusions: Total MP and individual MP phenotypes were significantly elevated after PCI reflecting endothelial injury. Elevated PMP and EMP levels at 6 months in ACS patients is suggestive of on-going inflammation, endothelial injury and may explain on-going pro-thrombogenicity seen up to 6 months after ACS despite dual antiplatelet therapy.

Highlights:

- Microparticles (MPs) released in patients with coronary artery disease have prothrombotic potential. The fate of MPs released following acute coronary syndrome is not known.
- MPs and their prothrombotic potential measured by thrombin generation assay were assayed at various time points for up to 6 months following index presentation.
- Platelet derived & Endothelial derived MPs remained significantly elevated in patients presenting with acute coronary syndrome at 6 months.
- Patients with CAD showed abnormal thrombograms when compared to controls.

Persistent circulating platelet and endothelial derived microparticle signature may explain on-going pro-thrombogenicity after acute coronary syndrome.

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SK, DE, PAB, RDR – Design, execution, writing and revision of the study

SK, RG – Collection of samples and conducting flow cytometry

SK, YH, DE – Analysis on Flowjo

Essentials:

- Microparticles (MPs) released in patients with coronary artery disease have prothrombotic potential. The fate of MPs released following acute coronary syndrome is not known.
- MPs and their prothrombotic potential measured by thrombin generation assay were assayed at various time points for up to 6 months following index presentation.
- Platelet derived & Endothelial derived MPs remained significantly elevated in patients presenting with acute coronary syndrome at 6 months.
- Patients with CAD showed abnormal thrombograms when compared to controls.

Aims:

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

Methods & Results:

Circulating total annexin v+ (Anv+) MPs of endothelial (EMP), platelet (PMP), monocyte (MMP), neutrophil (NMP) and smooth muscle cell (SMMP) origin were quantified by flow cytometry. 13 patients with acute coronary syndrome (ACS) were prospectively enrolled and 12 patients with stable angina (SA) were included as a comparator group. A panel of MP was measured at baseline, after percutaneous coronary intervention (PCI) and at days 1, 7, 30 and 6 months. Intra & inter group comparison was made between various time points. MP mediated thrombin generation was measured by recording lag phase, velocity index, peak thrombin and endogenous thrombin potential at these time points and compared with healthy controls. The total AnV+ MP levels were similar in ACS and SA groups at baseline, peaked immediately after PCI and were at their lowest on day 1. PMP & EMP levels remained significantly elevated in ACS patients at 6 months when compared to SA. No such difference was noted with NMP, MMP and SMMP. Patients with coronary artery disease showed abnormal thrombograms when compared to controls. Peak thrombin (nano moles) was significantly higher in CAD when compared to controls (254 IQR [226, 239] in ACS, 255 IQR [219, 328] in SA and 132 IQR [57, 252] in controls; p = 0.006). Differences in thrombin generation between ACS and SA were not significant (p=1). Furthermore, thrombin parameters remained abnormal in ACS & SA patients at 6 months.

Conclusions:

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

Introduction:

Atherosclerotic coronary artery disease (CAD) is a pro-inflammatory and pro-coagulant condition (1, 2). CAD remains a major cause of mortality and morbidity across the world (3). Furthermore, recurrence of coronary events due to progression of de novo disease in patients with a history of CAD remains high (4, 5). Better risk stratification and aggressive secondary prevention tailored to the individual is required to combat this problem as we now know significant individual variation exists in the disease phenotype. Development of novel biomarkers may yield patient specific information reflecting the underlying pathophysiologic process of CAD. The concepts of "vulnerable patient" has been proposed in place of "vulnerable plaque" to better risk stratify individuals at a high risk of adverse cardiovascular events. The triad of vulnerable plaque, vulnerable blood and vulnerable myocardium can characterize the vulnerable patient; the vulnerability in blood is derived from a hypercoagulable state (6). Over the last two decades, emerging data has demonstrated the role of sub-micron vesicles called microparticles (MPs) in mediating inflammation (7) in atherosclerotic CAD (8). MPs are cell membrane-derived particles released from activated or apoptotic cells (9) and have been shown to be elevated in the circulation of patients with CAD (10) but no information exists regarding their long-term clearance following an acute event. The biological function of MPs depends upon the parent cell they originate from (11). Broadly they have a role in inflammation & coagulation (12, 13), endothelial dysfunction, angiogenesis(14) and microvascular dysfunction (15). We hypothesized that the levels of circulating endothelial derived (EMP), platelet derived (PMP), neutrophil derived (NMP), monocyte derived (MMP) and smooth muscle cell derived (SMMP) MPs in acute coronary syndrome (ACS) are dynamic. Furthermore we sought to assess MP mediated thrombin generation as a quantifiable measure of their pro-thrombotic potential. The evaluation of MP expression over time and their pro-thrombotic potential may have a role as a biomarker aiding in better patient risk stratification and represent a future therapeutic target.

Methods:

13 patients with ACS scheduled for invasive angiography and percutaneous coronary intervention (PCI) were included in the study. 12 patients with SA were recruited as a comparator group. PCI was done with contemporary drug eluting stents. Ethical approval was obtained from London – Stanmore research ethics committee. Informed consent was obtained from every patient prior to participation in the study. Blood was collected for MP analysis and thrombin generation assay (TGA) immediately pre and post PCI, on days 1,7,30 and 180. For comparative purposes the TGA was also analysed from an age-matched control group with no CAD.

MP assessment:

Dual antiplatelet therapy with Aspirin and Clopidogrel or Ticagrelor was given according to global risk for cardiovascular events (GRACE) score (16). Pre-PCI samples were collected before the administration of unfractionated heparin or GP IIb/IIIa antagonists. In addition, ACS patients received treatment with Low molecular weight heparin (LMWH) up until PCI. However, where clinically possible we ensured that there was a delay of 12 hours between the last dose of LMWH and blood collection to minimise any potential effect on TGA levels.

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

Microparticle mediated thrombin generation assay (TGA):

The procedure for blood collection, storage and preparation for assay was similar to MP assessment. MPs were sedimented from PPP and resuspended in 200 **µl** of control microparticle-free plasma (MPFP) containing 30 mg/mL of corn trypsin inhibitor (Sigma) to inhibit contact activation. The MPFP was prepared from approximately 50 **µl** plasma obtained from healthy volunteers. Subsequently, 40 **µl** of MPs resuspended in control MPFP was added to the plate well, followed by 50 **µl** of calcium-fluorogenic substrate (0.5 mmol/L of Z-G-G-R-AMC and 7.5 mmol/L of calcium final concentrations (Pathway Diagnostics). No exogenous TF or phospholipids were added. The thrombin generated was measured by fluorogenic excitation/emission at 360/460 nm at 1-minute time intervals for 60 minutes in an Optima fluorescence plate reader (BMG). Measures of peak thrombin, lag time, velocity index, and endogenous thrombin potential were recorded. As patients with ACS were treated with low molecular weight heparin a time delay of at least 12 hours was maintained after the last dose of LMWH before aspirating blood so that there are no errors in interpreting thrombin generation assay. TGA was not interpretable on immediate post PCI samples due to administration of systemic Heparin and hence excluded from analysis.

Statistics:

Normally distributed data were presented as mean ± SD. Non-parametric data were reported as median and interquartile range (IQR). Categorical variables were compared with chi-square or Fisher's exact test, as appropriate. All numeric parameters of interest were measured on continuous scales. As the same patients were assessed at the different time points, the analysis was performed using multilevel linear regression. Two-level models were used with individual measurements contained within patients. Variables with positively skewed distributions were log transformed before analysis. Analyses were performed for all patients combined, and then separately for ACS and SA patients.

Additional analyses compared between the thrombin parameters at the first time point between the patient groups and a control group. The analyses were performed using Analysis of Variance (ANOVA). Variables with skewed

distributions were analysed on a log scale. In addition to an overall comparison of the three groups, post-hoc tests were used to compare between each pair of groups. The p-values from these post-hoc comparisons were given a Bonferroni adjustment to allow for an increased risk of finding a statistically significant result due to multiple comparisons.

The strength of association between the microparticle measurements and the thrombin variables was performed using Spearman's rank correlation (due to the skewed distribution of the microparticle measurements (and some of the thrombin variables). Analyses were performed for the values at each timepoint.

Results:

Patient characteristics:

The majority of the patients across both groups were male. More numbers of patients in the ACS group were on statins. Both groups were evenly matched for other variables (Table-1).

Total Microparticles:

At all time points except immediately post PCI total MPs were numerically higher in the ACS group (Figure 2). The time points at which MPs were at their peak and nadir were immediately post PCI and on day 1 post PCI respectively. The results are as follows with values of microparticles in 100,000s and presented in median and interquartile range (IQR). In the ACS group, the levels of total AnV+MPs at baseline, post PCI, days 1, 7, 30 & 180 post PCI were 306 (270, 360), 728 (226, 1043), 114 (55, 264), 343 (192, 609), 117 (60, 206) and 245 (109, 354) respectively; p =0.007. In SA group the levels of total AnV+MPs at baseline, post PCI, days 1, 7, 30 & 180 post PCI were 202 (63, 502), 962 (422, 1323), 88 (40, 251), 294 (172, 951), 92 (44, 157) and 165 (77, 490) respectively; p<0.001.

Individual MP Phenotypes:

PMPs:

A trend similar to total AnV+ MPs was also noted with PMPs (Figure 3). In the ACS group the levels of activated PMPs at six months were higher than the SA group; 3.6 (1.2, 6.7) vs. 0.7 (0.4, 2.7); p=0.03.

EMPs:

Similar results to PMPs were also noted with CD62E+ and CD54+ EMPs (Figure 4 and 5, respectively). Levels of CD62E+ EMPs were higher in the ACS group when compared to the SA group at six months – 11.9 (4.7, 29.0) vs. 3.4 (1.8, 6.7); p=0.02. Levels of CD54+ EMPs were also higher in the ACS cohort when compared to SA cohort – 9.9 (3.8, 18.7) vs. 2.6 (0.7, 5.2); p=0.008.

NMPs, MMPs, & SMMPs:

The levels of NMPs, MMPs and SMMPs were similar between groups with no statistical difference. Full results are given in supplementary index.

MP mediated TGA:

MP mediated TGA at various time points revealed abnormal lag phase, peak thrombin, velocity index and endogenous thrombin potential as reflected by area under curve (AUC) in both ACS and SA groups compared to healthy controls. A separate comparative analysis of TGA with healthy controls at baseline showed significantly abnormal thrombograms in the ACS and SA cohort. Comparison with healthy controls was only carried out at baseline and not at any other time points. AUC, a measure of endogenous thrombin generation potential, at baseline for ACS, SA and control groups was 3411 (685), 3726 (751) and 2334 (1319) respectively (p=0.006).

Correlation between MPs and TGA:

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

Discussion:

MPs were previously shown to be elevated in CAD; more so in ACS than SA and were associated with adverse outcomes (10, 17, 18). MPs were also shown to correlate with inflammatory markers such as Interleukin - 6 and C-reactive protein in previous studies (18-20). This, in addition to lab based studies demonstrating the capability of MPs to act as transporters for micro

RNAs, lends further credence to their role in CAD (21-23). Although MPs are known to be elevated at the time of the index event in ACS patients (24), no information exists regarding the balance on production and clearance post ACS. Studies in the past quantified the levels of MPs post PCI but not beyond 48 hours (24, 25). By carrying out serial quantification of MPs to 6 months we have demonstrated that production and clearance of MPs is dynamic in patients with CAD. MPs were higher following index event in ACS patients though statistically not significant when compared to the SA cohort. This finding perhaps reflects the chronic inflammatory state seen in CAD, with an elevated baseline level of MPs secondary to on-going apoptosis or cellular/platelet activation. Following the index event in ACS, a spike in MPs occurs due to activation of various cells. This is evident by the fact that in the SA cohort total and individual phenotypes of various MPs returned to near baseline levels in the longer term. Furthermore, we observed the levels of MPs to be dynamic at various time points. Our study has shown higher individual phenotypes of MPs in the ACS cohort at 6 months when compared to the SA cohort despite antiplatelet drugs and high dose statins. The individual phenotypes of MPs that were elevated at 6 months in the ACS cohort when compared to the SA cohort were CD54+ EMPs, CD62E+ EMPs and CD62P+PMPs. These findings suggest on-going endothelial injury across both cohorts of patients, though significantly more in the ACS cohort. Previous studies demonstrated that constitutively expressed EMPs (CD31+, CD105) were elevated in relation to apoptosis where as inducible EMPs (CD62E, CD54 & CD106) were elevated in activation (26). Thus CD62E EMPs expressed on activated endothelium (27) is likely to be reflective of on-going endothelial injury in ACS patients, either directly related to the index lesion causing the ACS, and/or from areas of activated endothelium remote from the index lesion. Similarly, CD54 or intercellular adhesion cell molecule (ICAM-1), expressed on activated endothelial cells and activated monocytes, is reflective of underlying inflammation. CD31 or platelet endothelial adhesion cell molecule (PECAM) is expressed on platelets, monocytes and on endothelial cells particularly in apoptosis, has a role in angiogenesis and in neutrophil recruitment (28 - 31). We have also characterised SMMPs and found no difference between two groups at any of the time points. The clinical

importance of these elevated MPs at 6 months need to be explored further in a large cohort of patients as data relating to prognostic importance of activated platelets and certain phenotypes of MPs following acute coronary event is emerging (32-34)

Another observation we made was higher total and individual phenotypes of MPs following PCI. Boos et al, also noted elevated markers of endothelial damage/ dysfunction in their study following PCI but not coronary angiography (35). In the present study the levels were particularly high in the SA cohort, decreased levels of MPs and thrombin parameters were seen on day 1 and higher MPs along with thrombin parameters were seen at 6 months suggesting the role of underlying inflammation. In their respective studies Biasucci et al & Inoue et al also noted similar response(24, 25).

The abnormal TGA was also worthy of note: thrombin generation remained abnormal at 6 months with shorter lag time and higher thrombin in the ACS cohort but did not discriminate between ACS and SA. Previous studies have shown enhanced thrombin generation in patients with CAD, though correlation with MP was not carried out. Borissoff et al showed positive independent association between severe coronary atherosclerosis and in vivo thrombin generation (36). Another relatively old study from the era when Aspirin was still the mainstay of therapy following AMI showed enhanced thrombin generation for up to 2 years following myocardial infarction (37). Our study has shown that this is still the case despite patients being treated with potent secondary prevention regimes. Figueras et al demonstrated greater thrombin generation at 10 days in patients who develop angina following AMI or UA when compared to other who attain early stability (38). The explanation for the findings in our study and observed correlations is uncertain. It may be secondary to activated PMPs that are rich in procoagulant phosphatidylserine (39). Furthermore, TF present on activated endothelium as reflected by higher number of CD54 and CD62E+ EMPs may also be contributing. Larger studies with longer follow up may also help us to understand the mechanism behind

stent thrombosis and if enhanced thrombogenecity by MPs has any potential role.

Limitations:

Limitations associated with a prospective observational study involving small sample size at a single centre are worthy of note. However, the study design allowed us to carry out robust analysis of MPs and TGA at various time points giving valuable information. The study is underpowered with a limited follow up to observe any hard end points. Thus, elevated MPs and abnormal TGA at 6 months remains an observation and if they have any association with recurrent cardiovascular events is not established. Furthermore, the pattern of MP clearance in our study also remains an observation and whether underlying inflammation and treatment modulates MP release and clearance is not proven. Another limitation is use of platelet poor plasma for TGA. Platelet poor plasma is a pool of all MP phenotypes thus it was not possible to pinpoint which MP fraction contributed most to the thrombogenesis. Finally limitations related to technological issues in measuring MPs such as sizing, and counting is worthy of note. **Another limitation of note is potential bias due to usage of using 1 and 3 um beads, as that would have counted more events when compared to using smaller beads. Finally data is not normalised for platelet MPs as usually they constitute a large percentage of total MPs.**

Conclusions:

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

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

Table 1 – Baseline characteristics for cohort of 25 patients with coronary artery disease. M – Male, CAD – Coronary artery disease, PCI – Percutaneous coronary intervention, CRP - C-reactive protein, LVEF – left

ventricular ejection fraction, ACE – I – Angiotensin converting enzyme inhibitors, ARB – Angiotensin receptor blockers, LAD – Left anterior descending artery, LCX – Left circumflex artery and RCA – Right coronary artery.

Figures

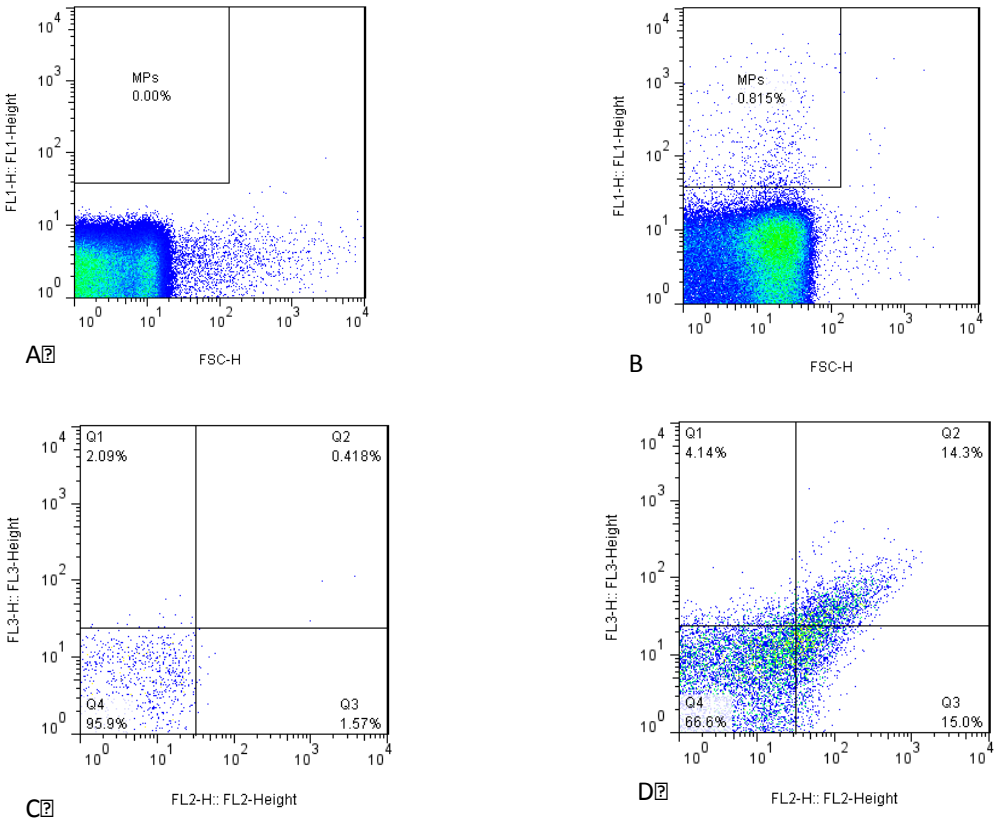

Figure 1:

Flow cytometric analysis of annexin V+ MPs from platelet poor plasma (PPP) of a patient with Non ST elevation myocardial infarction (NSTEMI). These are post intervention samples from a peripheral vein.
A) Events shown here are not stained with annexin V and serve as control. This, in combination with 1.1µm latex beads is used to determine the gating of annexin V+ MPs.
B) Annexin V+ MPs acquired from PPP of a patient with NSTEMI.
C) The IgG1k isotype control.
D) Figure depicting CD31+MPs.

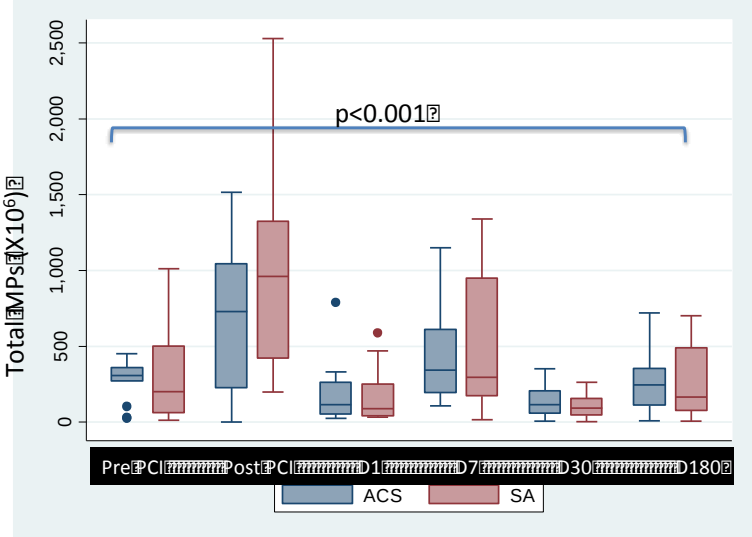

Figure 2: Box plots comparing total ANV+ MPs between ACS and SA across all time points. Mps are measured /ml

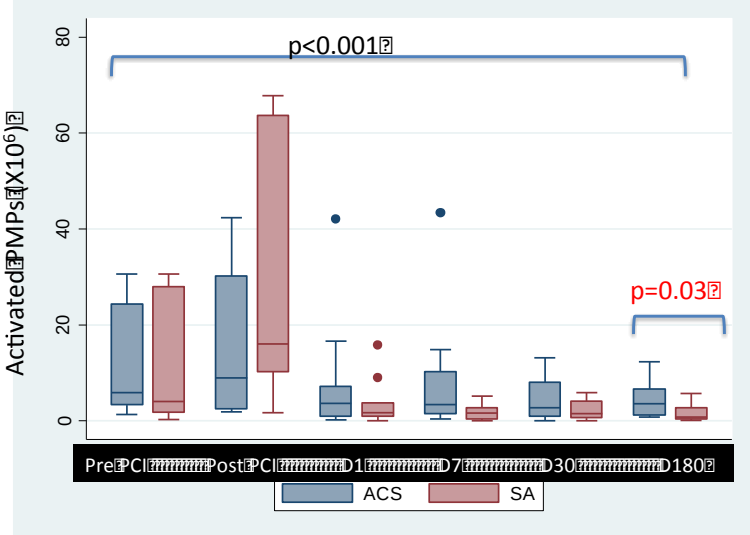

Figure 3: AnV+CD42-62P+PMP. As shown in the figure there was significant difference in the levels of MPs with in groups. PMP levels were high in ACS patients at 6 months when compared to SA. The difference between levels of PMPs between ACS and SA at other time points was not significant.

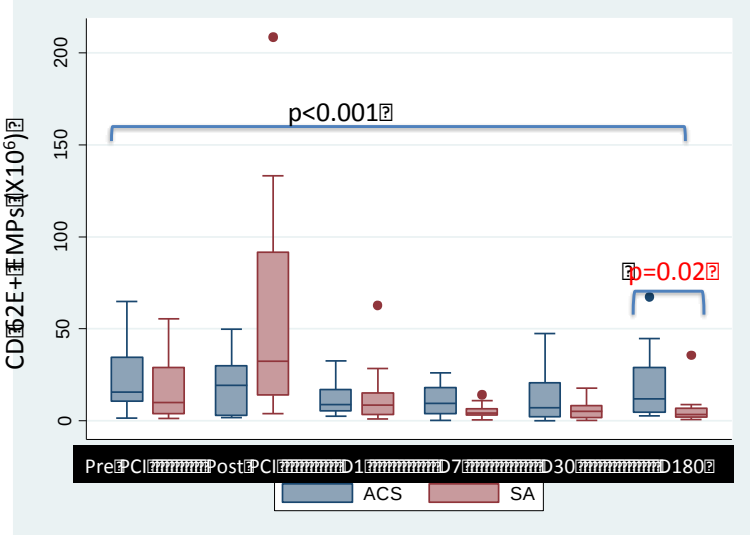

Figure 4: AnV+CD42-62E+EMP. As shown in the figure there was a significant difference in the levels of MPs within groups. EMP levels were high in ACS patients at 6 months when compared to SA. The difference between levels of EMPs between ACS and SA at other time points was not significant.

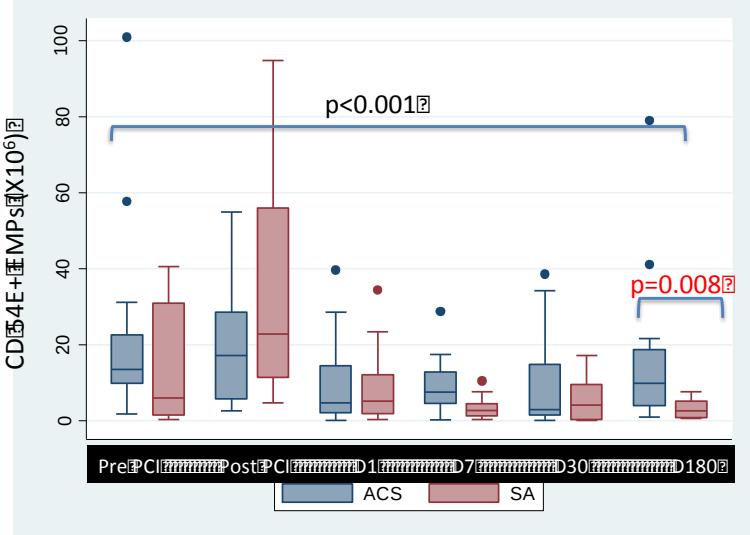

Figure 5: AnV+CD42-54+EMP. As shown in the figure there was significant difference in the levels of MPs with in groups. EMP levels were high in ACS patients at 6 months when compared to SA. The difference between levels of EMPs between ACS and SA at other time points was not significant.

References:

1. Libby P, Ridker PM, Hansson GK, Leducq Transatlantic Network on A. Inflammation in atherosclerosis: from pathophysiology to practice. Journal of the American College of Cardiology. 2009;54(23):2129-38.
2. Virmani R, Burke AP, Farb A, Kolodgie FD. Pathology of the vulnerable plaque. Journal of the American College of Cardiology. 2006;47(8 Suppl):C13-8.
3. WHO. The leading cause of death in the world, 2000 and 2012. fact sheet N310. Updated 2014. http://wwwwhoint/topics/martality/en/. 2014.
4. Perk J, De Backer G, Gohlke H, Graham I, Reiner Z, Verschuren WM, et al. European guidelines on cardiovascular disease prevention in clinical practice (version 2012) : the fifth joint task force of the European society of cardiology and other societies on cardiovascular disease prevention in clinical practice (constituted by representatives of nine societies and by invited experts). Int J Behav Med. 2012;19(4):403-88.
5. Stone GW, Maehara A, Lansky AJ, de Bruyne B, Cristea E, Mintz GS, et al. A prospective natural-history study of coronary atherosclerosis. The New England journal of medicine. 2011;364(3):226-35.
6. Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, et al. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part II. Circulation. 2003;108(15):1772-8.
7. Rautou PE, Leroyer AS, Ramkhelawon B, Devue C, Duflaut D, Vion AC, et al. Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration. Circulation research. 2011;108(3):335-43.
8. Rautou PE, Vion AC, Amabile N, Chironi G, Simon A, Tedgui A, et al. Microparticles, vascular function, and atherothrombosis. Circulation research. 2011;109(5):593-606.
9. Shai E, Varon D. Development, cell differentiation, angiogenesis--microparticles and their roles in angiogenesis. Arteriosclerosis, thrombosis, and vascular biology. 2011;31(1):10-4.
10. Bernal-Mizrachi L, Jy W, Jimenez JJ, Pastor J, Mauro LM, Horstman LL, et al. High levels of circulating endothelial microparticles in patients with acute coronary syndromes. American heart journal. 2003;145(6):962-70.
11. Mackman N. On the trail of microparticles. Circulation research. 2009;104(8):925-7.
12. Habersberger J, Strang F, Scheichl A, Htun N, Bassler N, Merivirta RM, et al. Circulating microparticles generate and transport monomeric C-reactive protein in patients with myocardial infarction. Cardiovascular research. 2012;96(1):64-72.
13. Ueba T, Nomura S, Inami N, Nishikawa T, Kajiwara M, Iwata R, et al. Correlation and association of plasma interleukin-6 and plasma platelet-derived microparticles, markers of activated platelets, in healthy individuals. Thrombosis research. 2010;125(6):e329-34.
14. Leroyer AS, Rautou PE, Silvestre JS, Castier Y, Leseche G, Devue C, et al. CD40 ligand+ microparticles from human atherosclerotic plaques stimulate endothelial proliferation and angiogenesis a potential mechanism for

intraplaque neovascularization. Journal of the American College of Cardiology. 2008;52(16):1302-11.
15. Porto I, Biasucci LM, De Maria GL, Leone AM, Niccoli G, Burzotta F, et al. Intracoronary microparticles and microvascular obstruction in patients with ST elevation myocardial infarction undergoing primary percutaneous intervention. European heart journal. 2012;33(23):2928-38.
16. Fox KA, Dabbous OH, Goldberg RJ, Pieper KS, Eagle KA, Van de Werf F, et al. Prediction of risk of death and myocardial infarction in the six months after presentation with acute coronary syndrome: prospective multinational observational study (GRACE). BMJ. 2006;333(7578):1091.
17. Sinning JM, Losch J, Walenta K, Bohm M, Nickenig G, Werner N. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. European heart journal. 2011;32(16):2034-41.
18. Mavroudis CA, Eleftheriou D, Hong Y, Majumder B, Koganti S, Sapsford R, et al. Microparticles in acute coronary syndrome. Thrombosis research. 2017;156:109-16.
19. Cui Y, Zheng L, Jiang M, Jia R, Zhang X, Quan Q, et al. Circulating microparticles in patients with coronary heart disease and its correlation with interleukin-6 and C-reactive protein. Molecular biology reports. 2013;40(11):6437-42.
20. Chirinos JA, Zambrano JP, Virani SS, Jimenez JJ, Jy W, Ahn E, et al. Correlation between apoptotic endothelial microparticles and serum interleukin-6 and C-reactive protein in healthy men. The American journal of cardiology. 2005;95(10):1258-60.
21. Diehl P, Fricke A, Sander L, Stamm J, Bassler N, Htun N, et al. Microparticles: major transport vehicles for distinct microRNAs in circulation. Cardiovascular research. 2012;93(4):633-44.
22. Koganti S, Eleftheriou D, Brogan PA, Kotecha T, Hong Y, Rakhit RD. Microparticles and their role in coronary artery disease. International journal of cardiology. 2017;230:339-45.
23. Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. Circulation research. 2010;107(9):1047-57.
24. Biasucci LM, Porto I, Di Vito L, De Maria GL, Leone AM, Tinelli G, et al. Differences in Microparticle Release in Patients With Acute Coronary Syndrome and Stable Angina. Circulation Journal. 2012;76(9):2174-82.
25. Inoue T, Komoda H, Kotooka N, Morooka T, Fujimatsu D, Hikichi Y, et al. Increased circulating platelet-derived microparticles are associated with stent-induced vascular inflammation. Atherosclerosis. 2008;196(1):469-76.
26. Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, Ahn YS. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. Thrombosis research. 2003;109(4):175-80.
27. Tomchuck SL, Zwezdaryk KJ, Coffelt SB, Waterman RS, Danka ES, Scandurro AB. Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. Stem Cells. 2008;26(1):99-107.
28. Vaporciyan AA, DeLisser HM, Yan HC, Mendiguren, II, Thom SR, Jones ML, et al. Involvement of platelet-endothelial cell adhesion molecule-1 in neutrophil recruitment in vivo. Science. 1993;262(5139):1580-2.

29. Koganti S, Karanasos A, Tu S, Rakhit RD, Regar E. Visualization of extensive intraplaque neovascularization by optical coherence tomography. Hellenic J Cardiol. 2017.
30. Lenglet S, Mach F, Montecucco F. Role of matrix metalloproteinase-8 in atherosclerosis. Mediators of inflammation. 2013;2013:659282.
31. Ren J, Zhang J, Xu N, Han G, Geng Q, Song J, et al. Signature of circulating microRNAs as potential biomarkers in vulnerable coronary artery disease. PloS one. 2013;8(12):e80738.
32. Chiva-Blanch G, Bratseth V, Ritschel V, Andersen GO, Halvorsen S, Eritsland J, et al. Monocyte-derived circulating microparticles (CD14+, CD14+/CD11b+ and CD14+/CD142+) are related to long-term prognosis for cardiovascular mortality in STEMI patients. International journal of cardiology. 2017;227:876-81.
33. Aurigemma C, Scalone G, Tomai F, Altamura L, De Persio G, Stazi A, et al. Persistent enhanced platelet activation in patients with acute myocardial infarction and coronary microvascular obstruction: clinical implications. Thrombosis and haemostasis. 2014;111(1):122-30.
34. Empana JP, Boulanger CM, Tafflet M, Renard JM, Leroyer AS, Varenne O, et al. Microparticles and sudden cardiac death due to coronary occlusion. The TIDE (Thrombus and Inflammation in sudden DEath) study. Eur Heart J Acute Cardiovasc Care. 2015;4(1):28-36.
35. Boos CJ, Balakrishnan B, Blann AD, Lip GY**.** Effects of Percutaneoud Coronary Intervention on Peripheral Venous Blood Circulating Endothelial cells and Plasma Indices of Endothelial Damage/Dysfunntion. Chest, ISSN: 0012-3692, Vol: 132, Issue: 6, Page: 1920-1926
36. Borissoff JI, Joosen IA, Versteylen MO, Spronk HM, ten Cate H, Hofstra L. Accelerated in vivo thrombin formation independently predicts the presence and severity of CT angiographic coronary atherosclerosis. JACC Cardiovascular imaging. 2012;5(12):1201-10.
37. Martinez-Sales V, Vila V, Reganon E, Goberna MA, Ferrando F, Palencia MA, et al. Elevated thrombotic activity after myocardial infarction: A 2-year follow-up study. Haemostasis. 1998;28(6):301-6.
38. Figueras J, Monasterio Y, Lidon RM, Nieto E, Soler-Soler J. Thrombin formation and fibrinolytic activity in patients with acute myocardial infarction or unstable angina: in-hospital course and relationship with recurrent angina at rest. Journal of the American College of Cardiology. 2000;36(7):2036-43.
39. Bidot L, Jy W, Bidot C, Jr., Jimenez JJ, Fontana V, Horstman LL, et al. Microparticle-mediated thrombin generation assay: increased activity in patients with recurrent thrombosis. Journal of thrombosis and haemostasis : JTH. 2008;6(6):913-9.

Persistent circulating platelet and endothelial derived microparticle signature may explain on-going pro-thrombogenicity after acute coronary syndrome.

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SK, YH, DE – Analysis on Flowjo

Essentials:

- Microparticles (MPs) released in patients with coronary artery disease (CAD) have prothrombotic potential. The fate of MPs released following acute coronary syndrome (ACS) is not known.
- MPs and their prothormbotic potential measured by thrombin generation assay were assayed at various time points for up to 6 months following index presentation.
- Platelet derived & Endothelial derived MPs remained significantly elevated in patients presenting with acute coronary syndrome at 6 months.
- Patients with CAD showed abnormal thrombograms when compared to controls.

Aims:

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

Methods & Results:

Circulating total annexin v+ (Anv+) MPs of endothelial (EMP), platelet (PMP), monocyte (MMP), neutrophil (NMP) and smooth muscle cell (SMMP) origin were quantified by flow cytometry. 13 patients with acute coronary syndrome (ACS) were prospectively enrolled and 12 patients with stable angina (SA) were included as a comparator group. A panel of MP was measured at baseline, after percutaneous coronary intervention (PCI) and at days 1, 7, 30 and 6 months. Intra & inter group comparison was made between various time points. MP mediated thrombin generation was measured by recording lag phase, velocity index, peak thrombin and endogenous thrombin potential at these time points and compared with healthy controls. The total AnV+ MP levels were similar in ACS and SA groups at baseline, peaked immediately after PCI and were at their lowest on day 1. PMP & EMP levels remained significantly elevated in ACS patients at 6 months when compared to SA. No such difference was noted with NMP, MMP and SMMP. Patients with coronary artery disease showed abnormal thrombograms when compared to controls. Peak thrombin (nano moles) was significantly higher in CAD when compared to controls (254 IQR [226, 239] in ACS, 255 IQR [219, 328] in SA and 132 IQR [57, 252] in controls; p = 0.006). Differences in thrombin generation between ACS and SA were not significant (p=1). Furthermore, thrombin parameters remained abnormal in ACS & SA patients at 6 months.

Conclusions:

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

Introduction:

Atherosclerotic coronary artery disease (CAD) is a pro-inflammatory and pro-coagulant condition (1, 2). CAD remains a major cause of global mortality and morbidity (3). Furthermore, recurrence of coronary events due to progression of de novo disease in patients with a history of CAD remains high (4, 5). Better risk stratification and aggressive secondary prevention tailored to the individual is required to combat this problem as we now know significant individual variation exists in disease phenotype. Development of novel biomarkers may yield patient specific information reflecting the underlying pathophysiologic process of CAD. The concept of “vulnerable patient” has been proposed in place of “vulnerable plaque” to better risk stratify individuals at risk of adverse cardiovascular events. The vulnerable patient can be characterized by a triad of vulnerable plaque, vulnerable blood and vulnerable myocardium; the vulnerability in blood is derived from a hypercoagulable state (6). Over the last two decades, emerging data has demonstrated the role of sub-micron vesicles called microparticles (MPs) in mediating inflammation (7) in atherosclerotic CAD (8). MPs are cell membrane-derived particles released from activated or apoptotic cells (9) and have been shown to be elevated in the circulation of patients with CAD (10) but no information exists regarding their long-term clearance following an acute event. The biological function of MPs depends upon the parent cell they originate from (11). Broadly they have a role in inflammation & coagulation (12, 13), endothelial dysfunction, angiogenesis(14) and microvascular dysfunction (15). We hypothesized that the levels of circulating endothelial derived (EMP), platelet derived (PMP), neutrophil derived (NMP), monocyte derived (MMP) and smooth muscle cell derived (SMMP) MPs in acute coronary syndrome (ACS) are dynamic. Furthermore we sought to assess MP mediated thrombin generation as a quantifiable measure of their pro-thrombotic potential. The evaluation of MP expression over time and their pro-thrombotic potential may have a role as a biomarker aiding in better patient risk stratification and represent a future therapeutic target.

Methods:

13 patients with ACS scheduled for invasive angiography and percutaneous coronary intervention (PCI) were included in the study. 12 patients with SA

were recruited as a comparator group. Ethical approval was obtained from London – Stanmore research ethics committee. Informed consent was obtained from every patient prior to participation in the study. Blood was collected for MP analysis and thrombin generation assay (TGA) immediately pre and post PCI, on days 1,7,30 and 180. For comparative purposes the TGA was also analysed from an age-matched control group with no CAD.

MP assessment:

Dual antiplatelet therapy with Aspirin and Clopidogrel or Ticagrelor was given according to global risk for cardiovascular events (GRACE) score (16). Pre-PCI samples were collected before the administration of unfractionated heparin or GP IIb/IIIa antagonists. In addition, ACS patients received treatment with Low molecular weight heparin (LMWH) up until PCI. However, where clinically possible we ensured that there was a delay of 12 hours between the last dose of LMWH and blood collection to minimise any potential effect on TGA levels.

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

Microparticle mediated thrombin generation assay (TGA):

The procedure for blood collection, storage and preparation for assay was similar to MP assessment. MPs were sedimented from PPP and resuspended in 200 **µl** of control microparticle-free plasma (MPFP) containing 30 mg/mL of corn trypsin inhibitor (Sigma) to inhibit contact activation. The MPFP was

prepared from approximately 50 **µl** plasma obtained from healthy volunteers. Subsequently, 40 **µl** of MPs resuspended in control MPFP was added to the plate well, followed by 50 **µl** of calcium-fluorogenic substrate (0.5 mmol/L of Z-G-G-R-AMC and 7.5 mmol/L of calcium final concentrations (Pathway Diagnostics). No exogenous TF or phospholipids were added. The thrombin generated was measured by fluorogenic excitation/emission at 360/460 nm at 1-minute time intervals for 60 minutes in an Optima fluorescence plate reader (BMG). Measures of peak thrombin, lag time, velocity index, and endogenous thrombin potential were recorded. As patients with ACS were treated with low molecular weight heparin a time delay of at least 12 hours was maintained after the last dose of LMWH before aspirating blood so that there are no errors in interpreting thrombin generation assay. TGA was not interpretable on immediate post PCI samples due to administration of systemic Heparin and hence excluded from analysis.

Statistics:

Normally distributed data were presented as mean ± SD. Non-parametric data were reported as median and interquartile range (IQR). Categorical variables were compared with chi-square or Fisher's exact test, as appropriate. All numeric parameters of interest were measured on continuous scales. As the same patients were assessed at the different time points, the analysis was performed using multilevel linear regression. Two-level models were used with individual measurements contained within patients. Variables with positively skewed distributions were log transformed before analysis. Analyses were performed for all patients combined, and then separately for ACS and SA patients.

Additional analyses compared between the thrombin parameters at the first time point between the patient groups and a control group. The analyses were performed using Analysis of Variance (ANOVA). Variables with skewed distributions were analysed on a log scale. In addition to an overall comparison of the three groups, post-hoc tests were used to compare between each pair of groups. The p-values from these post-hoc comparisons were given a Bonferroni adjustment to allow for an increased risk of finding a statistically significant result due to multiple comparisons.

The strength of association between the microparticle measurements and the thrombin variables was performed using Spearman's rank correlation (due to the skewed distribution of the microparticle measurements (and some of the thrombin variables). Analyses were performed for the values at each timepoint.

Results:

Patient characteristics:

The majority of the patients across both groups were of male sex. More numbers of patients in the ACS group were on statins. Both groups were evenly matched for other variables (Table-1).

Total Microparticles:

In all patients, total MPs increased considerably following PCI and rapidly decreased at day 1, though these changes were not significant (Figure 3). At all time points except immediately post PCI total MPs were numerically higher in the ACS group, though not statistically significant (Figure 4). The time points at which MPs were at their peak and nadir were immediately post PCI and on day 1 post PCI respectively. The results are as follows with values of microparticles in 100,000s and presented in median and interquartile range (IQR).

In the ACS group, the levels of total AnV+MPs at baseline, post PCI, days 1, 7, 30 & 180 post PCI were 306 (270, 360), 728 (226, 1043), 114 (55, 264), 343 (192, 609), 117 (60, 206) and 245 (109, 354) respectively; p =0.007. In SA group the levels of total AnV+MPs at baseline, post PCI, days 1, 7, 30 & 180 post PCI were 202 (63, 502), 962 (422, 1323), 88 (40, 251), 294 (172, 951), 92 (44, 157) and 165 (77, 490) respectively; p<0.001.

At six months ACS patients had numerically higher MPs although statistically not significant [245 (109, 354 vs. 165 (77, 490); p=0.89] (Figure 4). The difference between levels of MPs varied significantly between various time points with in the groups

Individual MP Phenotypes:

PMPs:

A trend similar to total AnV+ MPs was also noted with PMPs (Figure 5). In the ACS group the levels of activated PMPs at six months were higher than the SA group; 3.6 (1.2, 6.7) vs. 0.7 (0.4, 2.7); p=0.03.

EMPs:

Similar results to PMPs were also noted with CD62E+ and CD54+ EMPs (Figure 6 and 7, respectively). Levels of CD62E+ EMPs were higher in the ACS group when compared to the SA group at six months – 11.9 (4.7, 29.0) vs. 3.4 (1.8, 6.7); p=0.02. Levels of CD54+ EMPs were also higher in the ACS cohort when compared to SA cohort – 9.9 (3.8, 18.7) vs. 2.6 (0.7, 5.2); p=0.008.

NMPs, MMPs, & SMMPs:

The levels of NMPs, MMPs and SMMPs were similar between groups with no statistical difference. Full results are given in supplementary index.

MP mediated TGA:

MP mediated TGA at various time points revealed abnormal lag phase, peak thrombin, velocity index and endogenous thrombin potential as reflected by area under curve (AUC) in both ACS and SA groups compared to healthy controls. The healthy controls consisted of 10 patients (5 male and 5 female subjects). The healthy controls were age matched with ACS and SA groups but did not have any documented CAD, risk factors for CAD and were not on any medications. A separate comparative analysis of TGA with healthy controls at baseline showed significantly abnormal thrombograms in the ACS and SA cohort. Comparison with healthy controls was only carried out at baseline and not at any other time points.

Individual TGA parameters:

Lag phase-

Lag phase at baseline for ACS, SA and controls were; median16.8 (range 7.1), 15.7 (6.5) and 20.5 (7.1) seconds respectively (p=0.31). Lag phase was

shortest at 1 month across both groups (9.8 (7.0) for ACS vs. 6.8 (4.5) seconds for SA; p=0.45) before reaching baseline levels by 6 months (15.8 (4.0) for ACS vs. 18.0 (3.8) seconds for SA, p= 0.31).

Thrombin-

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

Area under curve (AUC)-

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

Correlation between MPs and TGA-

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

Discussion:

MPs were previously shown to be elevated in CAD; more so in acute coronary syndromes than stable angina and are associated with adverse outcomes (10, 17, 18). MPs were also shown to correlate with inflammatory markers such as IL-6 and CRP in previous studies (18-20). This, in addition to lab based studies demonstrating the capability of MPs to act as transporters for micro RNAs, lends further credence to their role in CAD (21-23). Although MPs are known to be elevated at the time of the index event in ACS patients (24), no information exists regarding the balance on production and clearance post ACS. Studies in the past quantified the levels of MPs post PCI but not beyond

48 hours (24, 25). By carrying out serial quantification of MPs to 6 months we have demonstrated that production and clearance of MPs is dynamic in patients with CAD. MPs were higher following index event in ACS patients though statistically not significant when compared to the SA cohort. This finding perhaps reflects the chronic inflammatory state seen in CAD, with an elevated baseline level of MPs secondary to on-going apoptosis or cellular/platelet activation. Following the index event in ACS, a spike in MPs occurs due to activation of various cells. This is evident by the fact that in the SA cohort total and individual phenotypes of various MPs returned to near baseline levels in the longer term. It is not possible to assume a similar situation in the ACS cohort as the baseline was not known given the event had already occurred a few days prior to obtaining blood samples for MP measurement. Furthermore, the attenuating effects of potent antiplatelet drugs and low molecular weight heparin / direct thrombin inhibitors may have had resulted in the reduction of MP levels in ACS cohort. However, we observed the levels of MPs to be dynamic at various time points. The most striking finding and to the best of our knowledge demonstrated for the first time, was higher individual phenotypes of MPs in the ACS cohort at 6 months when compared to the SA cohort despite antiplatelet drugs and high dose statins. The individual phenotypes of MPs that were elevated at 6 months in the ACS cohort when compared to the SA cohort were CD54+ EMPs, CD62E+ EMPs and CD62P+PMPs. These findings suggest on-going endothelial injury across both cohorts of patients, though significantly more in the ACS cohort. Previous studies demonstrated that constitutively expressed EMPs (CD31+, CD105) were elevated in relation to apoptosis where as inducible EMPs (CD62E, CD54 & CD106) were elevated in activation (26). Thus CD62E EMPs expressed on activated endothelium (27) is likely to be reflective of on-going endothelial injury in ACS patients, either directly related to the index lesion causing the ACS, and/or from areas of activated endothelium remote from the index lesion. Similarly, CD54 or intercellular adhesion cell molecule (ICAM-1), expressed on activated endothelial cells and activated monocytes, is reflective of underlying inflammation. CD31 or platelet endothelial adhesion cell molecule (PECAM) is expressed on platelets, monocytes and on endothelial cells particularly in apoptosis, has a

role in angiogenesis and in neutrophil recruitment (28). The role of angiogenesis and neutrophil recruitment in plaque instability is well established; angiogenesis and resultant formation of friable neovessels can lead to intraplaque haemorrhage (29) and matrix metalloproteinases released by neutrophils in atherosclerotic plaque can lead to degradation of collagen / extracellular matrix(30). Furthermore, the role of CD 31+ MPs in amplifying the expression of the selected miRNAs in patients with vulnerable CAD was also noted (31). Another novelty in our study was characterisation of SMMPs. To the best of our knowledge this was not carried out before though no significant difference in levels was found between groups at any of the time points. The clinical importance of these elevated MPs at 6 months need to explored further in a large cohort of patients as data relating to prognostic importance of activated platelets and certain phenotypes of MPs following acute coronary event is emerging (32-34)

Another interesting observation of note was the massive spike in total and individual phenotypes of MPs following PCI. The spike was particularly large in the SA cohort reflecting the fact the iatrogenic endothelial injury by balloon angioplasty in a non-primed environment whereas in the ACS cohort there was preceding smouldering endothelial injury and inflammation. By stabilising the culprit plaque with mechanistic PCI therapy, the resultant passivation most likely led to decreased levels of MPs and thrombin parameters as seen on day 1 in our study. However, a rather curiously rebound phenomenon of MPs and thrombin parameters were seen at 6 months suggesting the role of underlying inflammation. In their respective studies Biasucci et al & Inoue et al also noted similar response(24, 25).

The abnormal TGA was also worthy of note: thrombin generation remained abnormal at 6 months with shorter lag time and higher thrombin in the ACS cohort but did not discriminate between ACS and SA. Previous studies have shown enhanced thrombin generation in patients with CAD, though correlation with MP was not carried out. Borissoff et al showed positive independent association between severe coronary atherosclerosis and in vivo thrombin generation (35). In our study TGA was abnormal in both groups at index event

and at 6 months, though intuitively one would expect abnormal thrombin generation assay in ACS cohort. This could be explained by the fact that multi-vessel disease was seen in a similar percentage of patients from both groups; and by the observation that optical coherence tomography studies demonstrate incidental healed ruptured plaques in the SA cohort (albeit less frequently; data from our optical coherence tomography sub study – manuscript in preparation). Another relatively old study from the era when Aspirin was still the mainstay of therapy following AMI showed enhanced thrombin generation for up to 2 years following myocardial infarction (36). Our study has shown that this is still the case despite patients being treated with potent secondary prevention regimes. Figueras et al demonstrated greater thrombin generation at 10 days in patients who develop angina following AMI or UA when compared to other who attain early stability (37). The explanation for the findings in our study and observed correlations is uncertain. It may be secondary to activated PMPs that are rich in procoagulant phosphatidylserine (38). Furthermore, TF present on activated endothelium as reflected by higher number of CD54 and CD62E+ EMPs may also be contributing. Whilst the comparable thrombograms between ACS and SA at the time of index event could be attributed to the attenuating effects of low molecular weight heparin treatment given to ACS patients, the comparable thrombograms at 6 months remain unexplained. This may well be explained by the fact that silent atherothrombotic events are common in stable CAD when compared to annual incidence of myocardial infarction (39).

Limitations:

Limitations associated with a prospective observational study involving small sample size at a single centre are worthy of note. However, the study design allowed us to carry out robust analysis of MPs and TGA at various time points giving valuable information. The study is underpowered with a limited follow up to observe any hard end points. Thus, elevated MPs and abnormal TGA at 6 months remains an observation and if they have any association with recurrent cardiovascular events is not established. Furthermore, the pattern of MP clearance in our study also remains an observation and whether

underlying inflammation and treatment modulates MP release and clearance is not proven. Another limitation is use of platelet poor plasma for TGA. Platelet poor plasma is a pool of all MP phenotypes thus it was not possible to pinpoint which MP fraction contributed most to the thrombogenesis.

Conclusions:

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

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

Table 1 – Baseline characteristics for cohort of 25 patients with coronary artery disease. M – Male, CAD – Coronary artery disease, PCI – Percutaneous coronary intervention, CRP - C-reactive protein, LVEF – left ventricular ejection fraction, ACE – I – Angiotensin converting enzyme inhibitors, ARB – Angiotensin receptor blockers, LAD – Left anterior descending artery, LCX – Left circumflex artery and RCA – Right coronary artery.

Figures

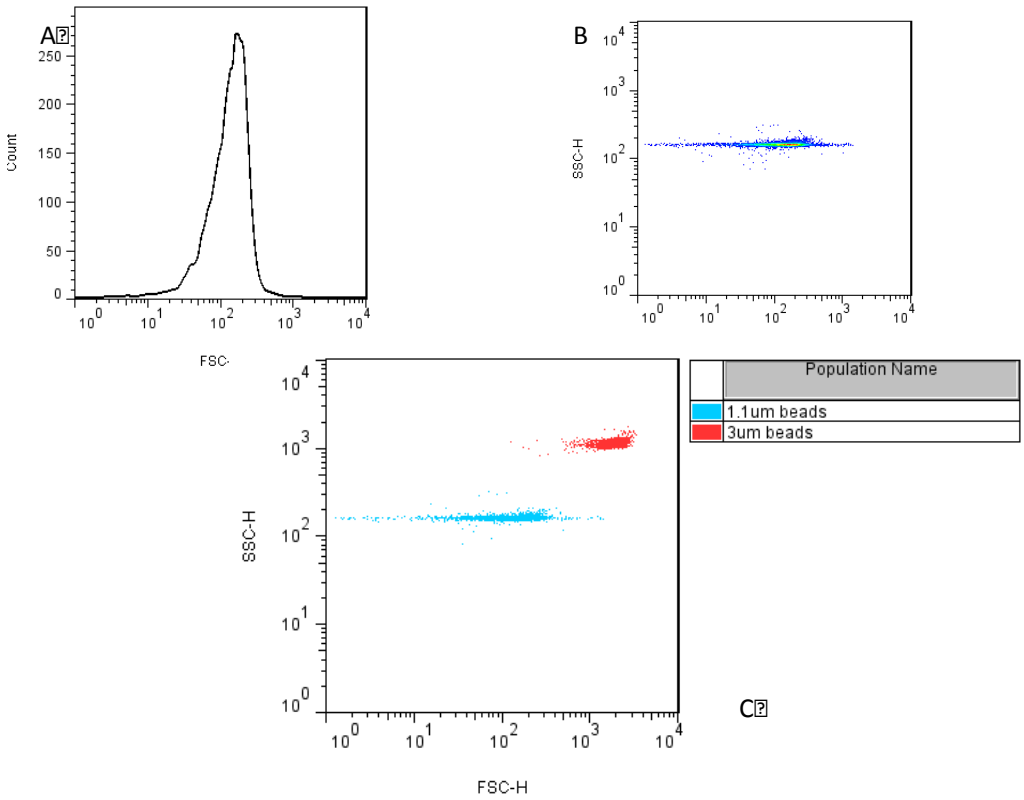

Figure 1: MP gating strategy using latex beads
A) Histogram of 1.1µm beads on the forward scatter scale.
B) 1.1µm beads on flow cytometry. Data is presented as side scatter (particle granularity) vs. forward scatter (particle size).
C) 1.1µm and 3 µm beads on flow cytometry. Data is presented as side scatter (particle granularity) vs. forward scatter (particle size) and nicely shows two different bead populations. 1.1µm beads are used for gating, 3 µm beads are also concurrently run at the time of flow cytometry to determine absolute number of MPs/ml of plasma.

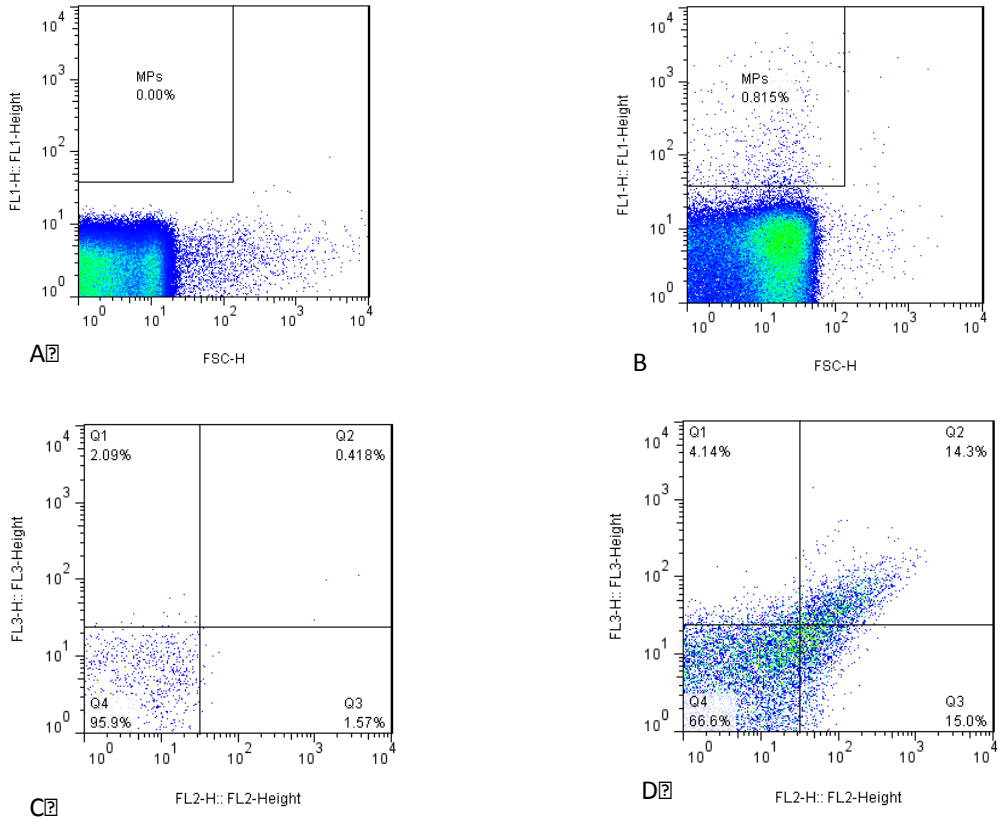

Figure 2:

Flow cytometric analysis of annexin V+ MPs from platelet poor plasma (PPP) of a patient with Non ST elevation myocardial infarction (NSTEMI). These are post intervention samples from a peripheral vein.
A) Events shown here are not stained with annexin V and serve as control. This, in combination with 1.1µm latex beads is used to determine the gating of annexin V+ MPs.
B) Annexin V+ MPs acquired from PPP of a patient with NSTEMI.
C) The IgG1k isotype control.
D) Figure depicting CD31+MPs.

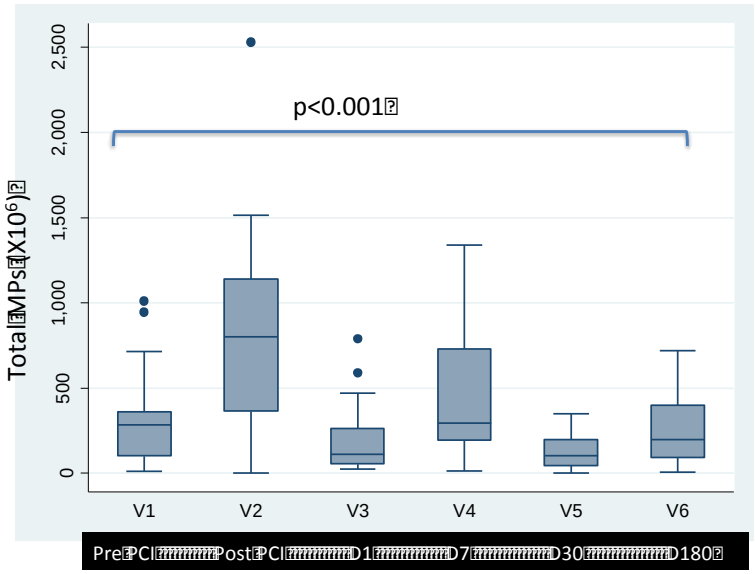

Figure 3: Total AnV+ MPs across all time points.

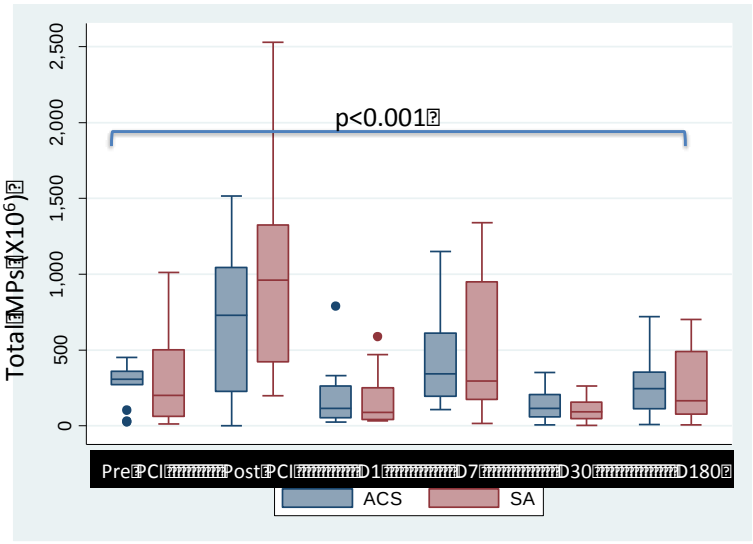

Figure 4: Box plots comparing total ANV+ MPs between ACS and SA across all time points.

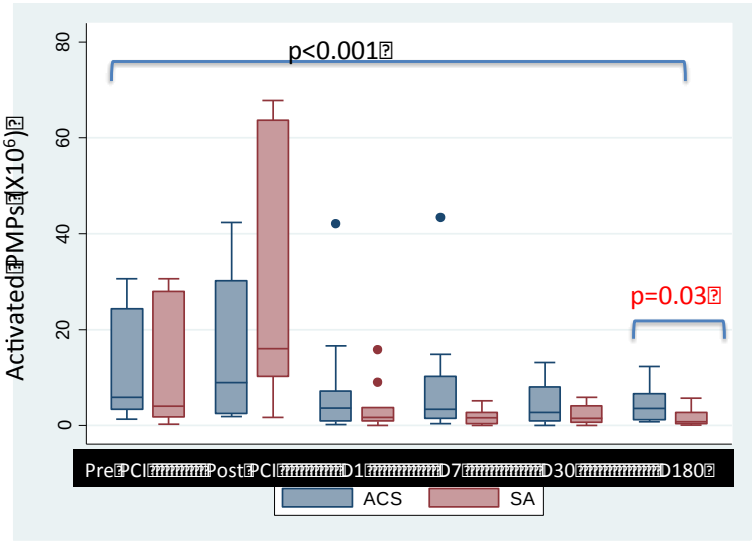

Figure 5: AnV+CD42-62P+PMP. As shown in the figure there was significant difference in the levels of MPs with in groups. PMP levels were high in ACS patients at 6 months when compared to SA. The difference between levels of PMPs between ACS and SA at other time points was not significant.

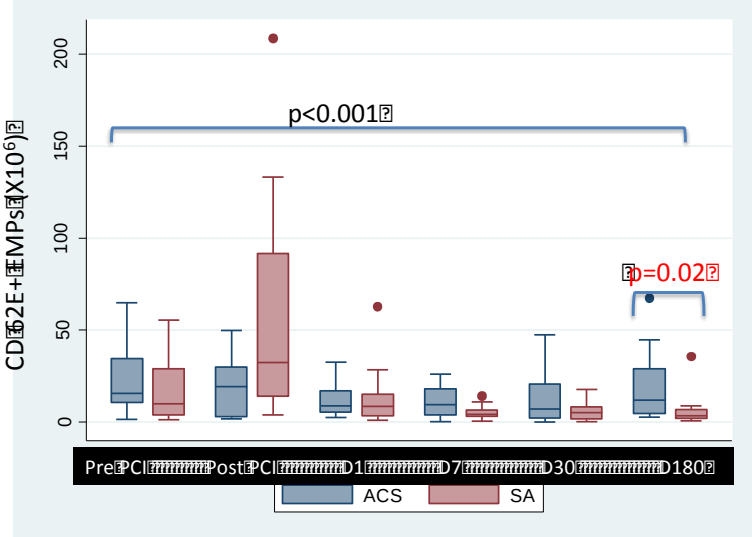

Figure 6: AnV+CD42-62E+EMP. As shown in the figure there was a significant difference in the levels of MPs within groups. EMP levels were high in ACS patients at 6 months when compared to SA. The difference between levels of EMPs between ACS and SA at other time points was not significant.

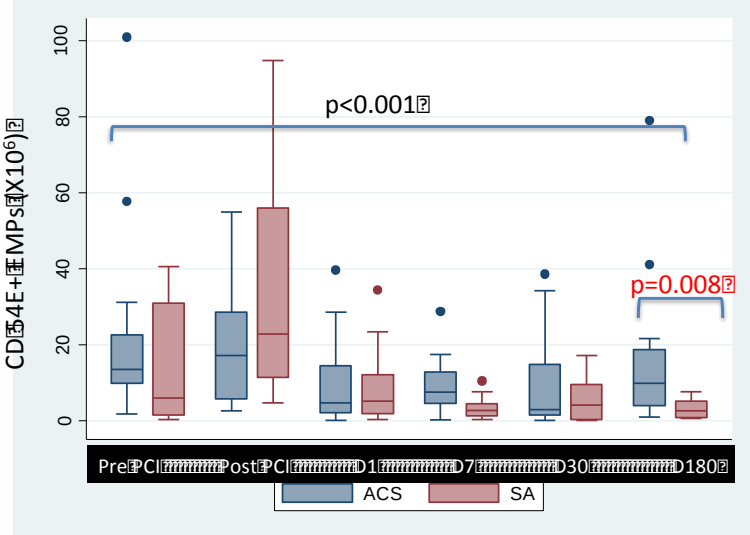

Figure 7: AnV+CD42-54+EMP. As shown in the figure there was significant difference in the levels of MPs with in groups. EMP levels were high in ACS patients at 6 months when compared to SA. The difference between levels of EMPs between ACS and SA at other time points was not significant.

References:

1. Libby P, Ridker PM, Hansson GK, Leducq Transatlantic Network on A. Inflammation in atherosclerosis: from pathophysiology to practice. Journal of the American College of Cardiology. 2009;54(23):2129-38.
2. Virmani R, Burke AP, Farb A, Kolodgie FD. Pathology of the vulnerable plaque. Journal of the American College of Cardiology. 2006;47(8 Suppl):C13-8.
3. WHO. The leading cause of death in the world, 2000 and 2012. fact sheet N310. Updated 2014. http://wwwwhoint/topics/martality/en/. 2014.
4. Perk J, De Backer G, Gohlke H, Graham I, Reiner Z, Verschuren WM, et al. European guidelines on cardiovascular disease prevention in clinical practice (version 2012) : the fifth joint task force of the European society of cardiology and other societies on cardiovascular disease prevention in clinical practice (constituted by representatives of nine societies and by invited experts). Int J Behav Med. 2012;19(4):403-88.
5. Stone GW, Maehara A, Lansky AJ, de Bruyne B, Cristea E, Mintz GS, et al. A prospective natural-history study of coronary atherosclerosis. The New England journal of medicine. 2011;364(3):226-35.
6. Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, et al. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part II. Circulation. 2003;108(15):1772-8.
7. Rautou PE, Leroyer AS, Ramkhelawon B, Devue C, Duflaut D, Vion AC, et al. Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-dependent monocyte adhesion and transendothelial migration. Circulation research. 2011;108(3):335-43.
8. Rautou PE, Vion AC, Amabile N, Chironi G, Simon A, Tedgui A, et al. Microparticles, vascular function, and atherothrombosis. Circulation research. 2011;109(5):593-606.
9. Shai E, Varon D. Development, cell differentiation, angiogenesis--microparticles and their roles in angiogenesis. Arteriosclerosis, thrombosis, and vascular biology. 2011;31(1):10-4.
10. Bernal-Mizrachi L, Jy W, Jimenez JJ, Pastor J, Mauro LM, Horstman LL, et al. High levels of circulating endothelial microparticles in patients with acute coronary syndromes. American heart journal. 2003;145(6):962-70.
11. Mackman N. On the trail of microparticles. Circulation research. 2009;104(8):925-7.
12. Habersberger J, Strang F, Scheichl A, Htun N, Bassler N, Merivirta RM, et al. Circulating microparticles generate and transport monomeric C-reactive protein in patients with myocardial infarction. Cardiovascular research. 2012;96(1):64-72.
13. Ueba T, Nomura S, Inami N, Nishikawa T, Kajiwara M, Iwata R, et al. Correlation and association of plasma interleukin-6 and plasma platelet-derived microparticles, markers of activated platelets, in healthy individuals. Thrombosis research. 2010;125(6):e329-34.
14. Leroyer AS, Rautou PE, Silvestre JS, Castier Y, Leseche G, Devue C, et al. CD40 ligand+ microparticles from human atherosclerotic plaques stimulate endothelial proliferation and angiogenesis a potential mechanism for

intraplaque neovascularization. Journal of the American College of Cardiology. 2008;52(16):1302-11.
15. Porto I, Biasucci LM, De Maria GL, Leone AM, Niccoli G, Burzotta F, et al. Intracoronary microparticles and microvascular obstruction in patients with ST elevation myocardial infarction undergoing primary percutaneous intervention. European heart journal. 2012;33(23):2928-38.
16. Fox KA, Dabbous OH, Goldberg RJ, Pieper KS, Eagle KA, Van de Werf F, et al. Prediction of risk of death and myocardial infarction in the six months after presentation with acute coronary syndrome: prospective multinational observational study (GRACE). BMJ. 2006;333(7578):1091.
17. Sinning JM, Losch J, Walenta K, Bohm M, Nickenig G, Werner N. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. European heart journal. 2011;32(16):2034-41.
18. Mavroudis CA, Eleftheriou D, Hong Y, Majumder B, Koganti S, Sapsford R, et al. Microparticles in acute coronary syndrome. Thrombosis research. 2017;156:109-16.
19. Cui Y, Zheng L, Jiang M, Jia R, Zhang X, Quan Q, et al. Circulating microparticles in patients with coronary heart disease and its correlation with interleukin-6 and C-reactive protein. Molecular biology reports. 2013;40(11):6437-42.
20. Chirinos JA, Zambrano JP, Virani SS, Jimenez JJ, Jy W, Ahn E, et al. Correlation between apoptotic endothelial microparticles and serum interleukin-6 and C-reactive protein in healthy men. The American journal of cardiology. 2005;95(10):1258-60.
21. Diehl P, Fricke A, Sander L, Stamm J, Bassler N, Htun N, et al. Microparticles: major transport vehicles for distinct microRNAs in circulation. Cardiovascular research. 2012;93(4):633-44.
22. Koganti S, Eleftheriou D, Brogan PA, Kotecha T, Hong Y, Rakhit RD. Microparticles and their role in coronary artery disease. International journal of cardiology. 2017;230:339-45.
23. Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. Circulation research. 2010;107(9):1047-57.
24. Biasucci LM, Porto I, Di Vito L, De Maria GL, Leone AM, Tinelli G, et al. Differences in Microparticle Release in Patients With Acute Coronary Syndrome and Stable Angina. Circulation Journal. 2012;76(9):2174-82.
25. Inoue T, Komoda H, Kotooka N, Morooka T, Fujimatsu D, Hikichi Y, et al. Increased circulating platelet-derived microparticles are associated with stent-induced vascular inflammation. Atherosclerosis. 2008;196(1):469-76.
26. Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, Ahn YS. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. Thrombosis research. 2003;109(4):175-80.
27. Tomchuck SL, Zwezdaryk KJ, Coffelt SB, Waterman RS, Danka ES, Scandurro AB. Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. Stem Cells. 2008;26(1):99-107.
28. Vaporciyan AA, DeLisser HM, Yan HC, Mendiguren, II, Thom SR, Jones ML, et al. Involvement of platelet-endothelial cell adhesion molecule-1 in neutrophil recruitment in vivo. Science. 1993;262(5139):1580-2.

29. Koganti S, Karanasos A, Tu S, Rakhit RD, Regar E. Visualization of extensive intraplaque neovascularization by optical coherence tomography. Hellenic J Cardiol. 2017.
30. Lenglet S, Mach F, Montecucco F. Role of matrix metalloproteinase-8 in atherosclerosis. Mediators of inflammation. 2013;2013:659282.
31. Ren J, Zhang J, Xu N, Han G, Geng Q, Song J, et al. Signature of circulating microRNAs as potential biomarkers in vulnerable coronary artery disease. PloS one. 2013;8(12):e80738.
32. Chiva-Blanch G, Bratseth V, Ritschel V, Andersen GO, Halvorsen S, Eritsland J, et al. Monocyte-derived circulating microparticles (CD14+, CD14+/CD11b+ and CD14+/CD142+) are related to long-term prognosis for cardiovascular mortality in STEMI patients. International journal of cardiology. 2017;227:876-81.
33. Aurigemma C, Scalone G, Tomai F, Altamura L, De Persio G, Stazi A, et al. Persistent enhanced platelet activation in patients with acute myocardial infarction and coronary microvascular obstruction: clinical implications. Thrombosis and haemostasis. 2014;111(1):122-30.
34. Empana JP, Boulanger CM, Tafflet M, Renard JM, Leroyer AS, Varenne O, et al. Microparticles and sudden cardiac death due to coronary occlusion. The TIDE (Thrombus and Inflammation in sudden DEath) study. Eur Heart J Acute Cardiovasc Care. 2015;4(1):28-36.
35. Borissoff JI, Joosen IA, Versteylen MO, Spronk HM, ten Cate H, Hofstra L. Accelerated in vivo thrombin formation independently predicts the presence and severity of CT angiographic coronary atherosclerosis. JACC Cardiovascular imaging. 2012;5(12):1201-10.
36. Martinez-Sales V, Vila V, Reganon E, Goberna MA, Ferrando F, Palencia MA, et al. Elevated thrombotic activity after myocardial infarction: A 2-year follow-up study. Haemostasis. 1998;28(6):301-6.
37. Figueras J, Monasterio Y, Lidon RM, Nieto E, Soler-Soler J. Thrombin formation and fibrinolytic activity in patients with acute myocardial infarction or unstable angina: in-hospital course and relationship with recurrent angina at rest. Journal of the American College of Cardiology. 2000;36(7):2036-43.
38. Bidot L, Jy W, Bidot C, Jr., Jimenez JJ, Fontana V, Horstman LL, et al. Microparticle-mediated thrombin generation assay: increased activity in patients with recurrent thrombosis. Journal of thrombosis and haemostasis : JTH. 2008;6(6):913-9.
39. Newby DE. Triggering of acute myocardial infarction: beyond the vulnerable plaque. Heart. 2010;96(15):1247-51.