Accepted Manuscript

Microparticles in acute coronary syndrome

Chrysostomos A. Mavroudis, Despina Eleftheriou, Ying Hong, Bikash Majumder, Sudheer Koganti, Ray Sapsford, Janet North, Mark Lowdell, Nigel Klein, Paul Brogan, Roby D. Rakhit

Title

Microparticles in Acute Coronary Syndrome

Authors

Chrysostomos A Mavroudis MD, MRCP, *Despina Eleftheriou MRCPCH, PhD, *Ying Hong PhD, Bikash Majumder MD, MRCP, Sudheer Koganti MRCP, Ray Sapsford MSc, §Janet North MSc, §Mark Lowdell PhD, *Nigel Klein FRCPCH, PhD, *Paul Brogan FRCPCH, PhD, Roby D Rakhit MD, FRCP

Corresponding author

Roby Rakhit MD FRCP

Department of Cardiology

Royal Free London Foundation Trust, Pond Street, London NW3 2QG

Tel: 020 7794 0500 extension: 38017

E-mail: roby.rakhit@nhs.net

*Infection, Inflammation and Rheumatology section, UCL Institute of Child Health London WC1N 1EH

§Haematology department, Royal Free Foundation Trust, London NW3 2QG

Word count: 4882

Abstract

Background

Emerging evidence supports the role of cell-derived microparticles (MPs) in the pathophysiology of acute coronary syndrome (ACS).

Objectives

To explore the relationship between coronary and systemic MP levels, investigate the correlation between MPs, inflammatory markers and Troponin T in patients with ACS.

Methods

Thirty seven patients with ACS scheduled for percutaneous coronary interventions (PCI) were studied. Eleven patients with stable angina (SA) were included as a control group. AnnexinV+MPs (AnV+MPs) and activated platelet-monocyte aggregates (PMA) from right atrium (RA) and culprit coronary artery (CO) distal to culprit lesion were measured using flow cytometry. High sensitivity C- reactive protein (CRP), Interleukin - 6 (IL-6), tumour necrosis factor – α (TNF-α), serum amyloid A (SAA) and Troponin T were assayed.

Results

Total and cell specific AnV+MP expression were higher in the ACS group in both the CO and RA, with greater levels detected in the CO. Platelet activation showed positive correlation with Troponin-T and platelet MP in both CO and RA of the ACS group (r=0.4 for both; p=0.04 & p=0.03 respectively). Inflammatory markers levels did not differ between the ACS and SA patients.

Conclusions

Elevated coronary and systemic MP levels and positive correlation of platelet activation with Troponin-T and platelet MPs suggest a pathogenic role for MPs in ACS.

Key words: acute coronary syndrome, microparticles, inflammation, platelet activation and myocardial necrosis

Background

Inflammation plays a pivotal role in the pathogenesis of coronary artery disease (CAD) (1)controlling not only the evolution of atheromatous plaque but also its propensity for rupture or erosion, by altering the matrix composition (2). In addition, inflammation, through the activation and differentiation of the inflammatory cells involved, increases the thrombogenicity of the plaque's interior by promoting tissue factor bearing microparticle (MP) release with subsequent thrombus formation over the ruptured or eroded plaque (3-5). Patients with diseases associated with chronic inflammation such as diabetes mellitus, metabolic syndrome and chronic inflammatory autoimmune diseases are at increased risk of developing CAD (6). In vivo inflammatory activity can be assessed by measuring C-reactive protein (CRP), serum amyloid antigen (SAA) tumour necrosis factor α (TNF-α) and Interleukin-6 (IL-6). High levels of inflammatory markers in ACS patients were shown to be associated with adverse outcomes (7-10). However, whether or not increased levels of these inflammatory markers is merely an association or have any causal relationship with the pathogenesis of ACS remains to be established (9,11,12). Furthermore, the source of inflammatory markers in ACS is still a matter of debate. It has also been shown that higher levels of IL-6 are found in the coronary artery when compared to the systemic arterial circulation in patients with ACS (13,14). While elevated inflammatory markers such as CRP and SAA, which are synthesised in the liver, indicate systemic inflammation, the above evidence supports the concept that intracoronary inflammation may also occur in close to proximity to the site of injury. This indicates that assessment of inflammatory markers in the peripheral circulation may not truly reflect the true burden of the intracoronary inflammatory process.

Emerging evidence indicates that MPs released from activated cells are potent pro-coagulant and pro-inflammatory molecules that may contribute to thrombus formation (3) and

3

endothelial dysfunction (15). It is possible that locally produced MPs may therefore contribute to the local coronary inflammatory processes in ACS (16-19), although the exact mechanisms are currently poorly understood. Several clinical studies have shown correlation between circulating MP levels and clinical outcomes, markers of myocardial damage, inflammation and indices of microvascular dysfunction (20-25).

The aim of this study was therefore to explore the relationship between coronary and systemic MP levels, circulating markers of inflammation, and extent of myocardial necrosis in patients with ACS.

Methods

Study population

Thirty seven ACS patients [23 ST segment elevation myocardial infarction (STEMI), 14 non-ST segment elevation (NSTEMI)] and 11 stable angina (SA) patients treated with percutaneous coronary intervention (PCI) were recruited. The diagnosis of STEMI was based on the history of chest pain and ST segment elevation on ECG. NSTEMI was diagnosed based on history, ECG changes and Troponin T value > 0.03 ng/l (26). Patients with STEMI underwent primary PCI and patients with NSTEMI had PCI in line with recommended guidelines. SA patients were admitted electively for PCI after having undergone out patient review and investigations. The local ethics committee (LREC London) granted permission to undertake the study. Patients with history of prior coronary artery bypass graft surgery and those who received Glycoprotein IIb/IIIa antagonists prior to blood sampling were excluded from the study.

Coronary angiography, PCI and blood sampling

STEMI cohort was loaded with 300 mg of Aspirin and 600 mg of Clopidogrel prior to PCI. NSTEMI patients received 300mg Aspirin followed by 75mg once daily (OD); 300mg Clopidogrel followed by 75mg OD and low molecular weight heparin prior to PCI. SA patients were treated with aspirin 75mg OD and clopidogrel 75mg OD for at least 7 days prior to PCI.

Coronary angiography was carried out through trans femoral access. Right atrial blood samples were obtained using a 5F multipurpose catheter (5F, Cordis ®, internal diameter 0.11cm) via the femoral vein. Left and right coronary artery angiography was performed with a 5F Judkin's left 4 and right 4 diagnostic catheters (Cordis ®, internal diameter 0.11cm). Following diagnostic angiography the culprit lesion was identified and weight adjusted unfractionated heparin was given prior to advancing the guide wire to achieve an activated clotting time (ACT) of 200 and 250 sec. After wiring of the culprit lesion an aspiration catheter (Medtronic ® Export catheter, internal diameter 0.10 cm) was advanced distal to the culprit lesion and 10 millilitres (mls) of blood was aspirated. Catheters with similar internal diameter were used for blood sample collection to minimise shear stress related in vitro platelet activation. PCI was performed according to standard procedures following blood sampling.

MP identification and characterization using flow cytometry

Blood was collected in 3.2% buffered citrate bottle and transferred immediately from cardiac catheterization laboratory to the haematology laboratory. The samples were centrifuged twice at 5,000 g for 5 minutes to obtain platelet-poor plasma (PPP) which was stored at $-80o$ Celsius for later analyses of microparticle (MP) estimation and enzyme-linked immunosorbent assay (ELISA) studies. MPs were sedimented from 200μL of PPP after

5

centrifugation at 17,000 *g* for 60 minutes and resuspended in AnV binding buffer (BD

PharMingen, Oxford, UK). The MP pellets were labelled and characterised with fluorescent

monoclonal antibodies: fluorescein isothiocyanate (FITC) or Phycoerythrin (PE) labelled

annexin V, CD62E, CD54, CD105, CD31, CD66b, CD14, TF (clone VD8, American

Diagnostica), P-selectin (CD62P) CD42a. Using this methodology we defined the following

types of microparticles: total AnV+ MP, platelet derived MP (PMP; AnV+CD42a+),

endothelial derived MP (EMP; dual positive for AnV+ and CD62E or CD105; or

AnV+CD42a-CD31+), neutrophil derived MP (NMP; AnV+CD42a-CD66b+) and tissue

factor positive monocyte-derived MP (MMP; AnV+CD14+TF+). The samples were

incubated in the dark for 15 minutes at room temperature prior to analysis by flow cytometry

using FACS caliber machine (BD Pharmingen) (27). The 1μm beads are used for gating the

MPs and 3μm beads are used for enumeration. To obtain optimal forward and side scatter

instrument settings for MP, 1μm and 3μm latex beads (Sigma) were run concurrently with the

microparticle samples. Logarithmic forward and side scatter plots were obtained. Gates were

then set to include particles less than approximately 1.5μm, but to exclude the first forward

scatter channel containing maximal noise. Particles less than 1.5μm in size and binding

annexin V were then gated, and histograms obtained for this gated population for binding to

individual monoclonal antibodies to determine the cell of origin of the MP. To convert flow

cytometer counts to an estimate of the number of MP per ml of plasma, a predetermined

number (always 200000, calculated as per manufacturer recommendations) of 3μm latex

beads (Sigma) was divided by the number of 3μm beads counted. The absolute number of

annexin V binding microparticles per ml of plasma was then calculated by multiplying the

above ratio (200000/number of 3μm beads counted) with the number of MP flow cytometer

counts, the number of wells into which the plasma sample was divided (7 wells) and divided

6

by the exact volume of plasma (200μl) from which the microparticles were analysed, as described by Combes et al(28).

Preparation of whole blood for platelet-monocyte aggregate (PMA) flow cytometry

PMA were identified as previously described (29). In brief blood was collected in 3.2% sodium citrate bottles with addition of EDTA to block the in vitro calcium dependent PMA formation. Following staining with fluorescent monoclonal antibodies, anti-CD61 FITC, anti-CD62P PE and anti-CD14 PerCP, and red blood cell lysis with erythrocytes with easy lyse™ solution (Dako) samples were washed with 1mL of FACS flow and centrifuged at 300g for 5 minutes. The supernatant was discarded and the cells resuspended in 500μL of FACS flow for immediate flow cytometric acquisition and analysis (FACS Calibur equipped with Cell Quest ® software − BD Biosciences, Oxford, UK). Events were acquired on a 2D dot plot of CD14 (logarithmic scale abscissa) and SSC height (linear scale ordinate). Monocytes were identified as CD14 positive events and distinctive intermediate side scatter height. A minimum of 5000 CD14 positive events were acquired from each sample. The monocyte population was gated and named as analysis region (R1). To exclude false-positive PMA arising from co-incident analysis of free platelets adjacent to, but not directly interacting with monocytes, the events with a narrow CD61 width was drawn (R2). Events falling within both R1 and R2 regions were subsequently plotted onto another 2D dot-plot arraying CD61 FITC signal height (logarithmic scale abscissa) and CD62P PE (logarithmic scale ordinate). Double positive CD14+ and CD61+ events were consider as total PMA and expressed as percentage of the total monocytes. P-selectin positive PMA (CD62P+PMA) were identified as triple positive CD14+CD61+CD62P+ events and were expressed as a percentage of the total PMA.

Measurement of plasma TNF-α, IL-6, hs-CRP, SAA and Troponin T

Plasma TNF-α and IL-6 levels were measured using a quantitive sandwich enzyme immunoassay technique as per manufacturer instructions (R&D systems, Europe). CRP was measured in plasma using a high sensitivity automated microparticle enhanced latex turbidimetric immunoassay (COBAS MIRA; Roche Diagnostics GmbH). SAA was measured in plasma using latex nephelometry (BNII autoanalyser; Dade Behring, Marburg, Germany). Troponin T was measured from plasma using the Elecsys troponin T high-sensitive assay (Roche Diagnostics).

Statistical analyses

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

Results

The demographics and baseline characteristics of the study population are shown in table 1. Briefly, the majority of the study population was male (79%). The SA patients were more likely to have a medical history of hypertension compared with the ACS group (p=0.01).

Levels of MP are higher in the CO than in the RA.

Table 2 summarises the CO MP levels between the two patient groups. Total AnV+ MP were higher in the CO than RA in all patients. The ACS population had higher MPs when compared to those with SA (p=0.04). All MP sub-populations, endothelial, monocyte and platelet, were consistently higher in the ACS group (Figure 1). Blood from the RA also showed similar patterns (Figure 2).

Levels of MPs reflect the clinical presentation

Total MPs from the CO were higher in the STEMI population compared with NSTEMI (p=0.02). PMP, EMP and NMP sub-populations were higher in the STEMI group compared to the NSTEMI and SA (Table 3). TF+MMP levels did not differ between the STEMI and NSTEMI groups. TF+MMP were lower in the SA group compared with the STEMI and NSTEMI groups. Total AnV+MP and the other MP sub-populations (except from TF+MMP) did not differ between the NSTEMI and SA groups. Similarly all MP were higher in the RA in the STEMI population compared with NSTEMI and SA patients (p=0.02) (Table 4). There was no difference in TF+MMP levels between STEMI and NSTEMI. All MP were higher in the STEMI group compared with the NSTEMI group.

MP expression during ACS is time dependent

The median (IQR) ischemic times (defined as time from onset of symptoms to PCI, expressed in minutes) in the STEMI and NSTEMI groups were 285 (116-360) and 4320 (2100-6060) respectively (p<0.0001). Total and cell specific AnV+MPs, isolated from the CO of the NSTEMI group, were inversely correlated with ischaemic time (Figure 3). Although there was a trend for negative correlation between the ischaemic time and all MP groups, the

9

correlation was statistically significant only for the total AnV+MP, PMP and EMP. There was no correlation between AnV+MP and ischemic time in the STEMI group.

Platelet activation is a feature of ACS

Having observed elevated levels of platelet microparticles, (Figures 1-2), further assessment of the role of platelets in ACS was undertaken. Levels of activated platelet MPs were measured in patients with ACS and SA. Figures 1 and 2 show that AnV+CD42a+CD62P+ PMP were much higher in the ACS group compared with the SA group in both the CO and RA (p=0.0008 and p=0.001 respectively). We have previously shown that activated platelets bind to leukocytes and can be detected as platelet monocyte aggregates (PMA) (30). Levels of activated PMA were higher in the ACS group than in the SA group in both the CO (p=0.0003) and RA (p=0.04) Figure 5. Soluble P-Selectin was also higher in the ACS group compared to the SA (Figure 4), and did not differ between CO and RA.

Inflammatory indices are not good indicators of myocardial injury

There was a non-significant increase in levels of inflammatory markers in ACS patients as compared to those with SA (Table 5). Interestingly, with the exception of IL6, inflammatory indices were higher in the RA as compared to the CO. This contrasts with MPs, which were always higher in the CO. There was a broad correlation between levels of MPs, inflammatory indices and troponin T in ACS but not in SA. IL-6 in particular, correlated with levels of AnV+CD31+EMP (r=0.5; p=0.03) and AnV+TF+MMP (r=0.6; p=0.01). (Figure 5)

Discussion

Our study has shown elevated levels of total MPs and MP subpopulations in CAD. Levels of MPs varied across the spectrum of CAD with STEMI cohort demonstrating higher levels

followed by NSTEMI and SA. Furthermore, levels were highest in the coronary artery, thus indicating culprit lesion as the possible source. Levels of MPs were better at identifying patients with severe ACS, than inflammatory markers taken from either the CO or RA.

Our results are in agreement with previous studies demonstrating elevated platelet and endothelial MPs in ACS (31-33). We have also now demonstrated that the levels of leukocyte (neutrophil and monocyte) MPs are elevated in ACS.

We further noted negative correlation between the ischaemic time and total MP, PMP, EMP AnV+ MP release in the CO in the NSTEMI group but not in the STEMI group. This could be because the PPCI in the STEMI group occurred within hours after the onset of symptoms (compared to the PCI in the NSTEMI which occurred days after the onset of symptoms) which is probably the peak time of MP expression during ACS. This suggests that, in line with previous studies (20,34), there is a peak in MP release during the first 24-48 hours and thereafter MP levels fall (35). Arguably, earlier sampling (on admission) of the NSTEMI patients would have yielded an expression of MP levels similar to that of the STEMI patients.

Our observation of high levels of AnV+CD62P+ MP in the CO raises the possibility that these could amplify vascular inflammation and thus contribute to lesion progression by further recruitment of inflammatory cells expressing P-selectin glycoprotein ligand (PSGL), which binds CD62P (P-selectin) (36,37). Since we also detected high CO AnV+CD62E+ MP, almost certainly derived from activated coronary endothelium, it is likely that E-selectin-PSGL binding could further mediate the interaction between inflammatory cells and activated endothelial cells and contribute to lesion progression (38). In addition, the high coronary artery neutrophil derived microparticles we observed could further amplify vascular inflammation by binding to activated endothelium and resultant endothelial activation (39).

11

Further evidence of the importance of platelets in ACS came from our finding that patients with ACS had higher CD62P+PMA and soluble P-selectin compared to patients with SA, in both CO and the RA. The role of PMA formation in the pathophysiology of CHD remains unknown. Activated platelets express P-selectin on their surface, which mediates the platelet-leukocyte interaction via the P-selectin-PSGL-1 bond leading to PMA formation with leukocyte activation, migration and recruitment at the site of inflammation (40,41). The platelet-monocyte interaction via the P-selectin-PSGL-1 pathway further increases the adhesive and pro-thrombotic properties of monocytes by up-regulating the expression and functionality of beta1 and beta2 integrins (42,43) and by TF expression on the monocyte surface respectively (44,45). It has been shown that activated platelets lose their surface P-selectin despite the fact they continue to circulate and function (46). Therefore PMA measurement may represent a more robust marker of platelet activation than detection of P-selectin on their surface (47,48). Increased PMA levels have been observed in patients with stable angina (49,50), ACS patients (51) and also after coronary interventions (52). Studies in patients with ACS who underwent PCI after administration of glycoprotein IIb/IIIa inhibitors have shown that inhibition of platelet adhesion and activation via glycoprotein IIb/IIIa inhibitors leads to PMA formation reduction in addition to the reduction of platelet activation and degranulation (52-54). It has also been shown that prevention of platelet adhesion to monocyte by blocking the P-selectin-PSGL-1 interaction reduces inflammation (55). As well as amplifying inflammation, MP are also prothrombotic (56). Our finding of high CO TF+ MMP could implicate their role in clot propagation, since higher levels were observed in STEMI and NSTEMI patients than those with SA. A potentially important finding is the positive correlation between total AnV+ microparticles and MP subpopulations with markers of inflammation. Intracoronary inflammatory markers correlated with total AnV+ microparticles and MP subpopulations (including platelets, endothelial and leukocytes

12

derived MP) whilst in the RA markers of inflammation correlated only with endothelial and monocyte derived TF+MMP. The fact that in SA the markers of inflammation did not correlate with MP formation could suggest a pivotal role of intracoronary inflammation as a driver of MP release from the culprit plaque in the pathogenesis of ACS. The observation of diverse correlation of MPs and inflammatory markers in the CO and RA could be due to the fact different inflammatory markers relate to different activation status of the endothelium. Variable EMP profile may indicate variable endothelial activation status (57). In addition, due to the small number of patients conclusions cannot be made and further studies needed.

Limitations of the study

Our study has a number of limitations. Firstly, the study sample was relatively small, making the interpretation of correlations less reliable in addition to statistical error (i.e. the observation that there was no statistical difference of the inflammatory markers expression between ACS and SA patients). Secondly, the effects of antiplatelet and anticoagulant therapy on MP production, even though standardised for all patient groups, and their prothrombotic activity (i.e. measuring TF activity which was not done in this study) was not fully analysed and needs further study. In addition sampling of the infarct related artery proximally to the culprit lesion or sampling of a bystander coronary artery could have strengthened our hypothesis that the culprit atheromatous coronary plaque is the predominant source of microparticle release. We also acknowledge the fact that our flow cytometry protocol is not according to international society of thrombosis and haemostasis recommended protcols.

Conclusions

Future improvements in outcomes for patients with ACS will come from earlier recognition of the disease and a better understanding of its pathogenesis. While there has been considerable focus on the role of inflammation in ACS, we and others have failed to find that inflammatory markers alone identify patients with ACS as compared to SA. MPs appear to be a sensitive indicator of disease activity, both within the coronary artery and within the systemic circulation. This may reflect the local genesis of the MPs but may also indicate an important role for MPs in the pathogenic processes operating in ACS. Future studies should now address the potential for using MPs to characterise the nature and severity of ACS and explore the potential for modulating MP mediated pathogenic mechanisms to improve the outcome of patients with coronary vascular disease.

14

Conflict of Interest: None

References

1.   Libby P. Inflammation in atherosclerosis. Nature 2002;420:868-74.
2.   Libby P. The molecular mechanisms of the thrombotic complications of atherosclerosis. J Intern Med 2008;263:517-27.
3.   Morel O, Morel N, Freyssinet JM, Toti F. Platelet microparticles and vascular cells interactions: a checkpoint between the haemostatic and thrombotic responses. Platelets 2008;19:9-23.
4.   Morel O, Jesel L, Freyssinet JM, Toti F. Cellular mechanisms underlying the formation of circulating microparticles. Arteriosclerosis, thrombosis, and vascular biology 2011;31:15-26.
5.   Suades R, Padro T, Badimon L. The Role of Blood-Borne Microparticles in Inflammation and Hemostasis. Seminars in thrombosis and hemostasis 2015;41:590-606.
6.   Peters MJ, Symmons DP, McCarey D et al. EULAR evidence-based recommendations for cardiovascular risk management in patients with rheumatoid arthritis and other forms of inflammatory arthritis. Ann Rheum Dis 2010;69:325-31.
7.   Monaco C, Rossi E, Milazzo D et al. Persistent systemic inflammation in unstable angina is largely unrelated to the atherothrombotic burden. Journal of the American College of Cardiology 2005;45:238-43.
8.   Mulvihill NT, Foley JB, Murphy RT, Curtin R, Crean PA, Walsh M. Risk stratification in unstable angina and non-Q wave myocardial infarction using soluble cell adhesion molecules. Heart 2001;85:623-7.
9.   Liuzzo G, Biasucci LM, Gallimore JR et al. The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. The New England journal of medicine 1994;331:417-24.
10.   Saadi S, Holzknecht RA, Patte CP, Stern DM, Platt JL. Complement-mediated regulation of tissue factor activity in endothelium. J Exp Med 1995;182:1807-14.
11.   Lagrand WK, Visser CA, Hermens WT et al. C-reactive protein as a cardiovascular risk factor: more than an epiphenomenon? Circulation 1999;100:96-102.
12.   Horne BD, Muhlestein JB, Carlquist JF et al. Statin therapy, lipid levels, C-reactive protein and the survival of patients with angiographically severe coronary artery disease. Journal of the American College of Cardiology 2000;36:1774-80.
13.   Deliargyris EN, Raymond RJ, Theoharides TC, Boucher WS, Tate DA, Dehmer GJ. Sites of interleukin-6 release in patients with acute coronary syndromes and in patients with congestive heart failure. The American journal of cardiology 2000;86:913-8.
14.   Maier W, Altwegg LA, Corti R et al. Inflammatory markers at the site of ruptured plaque in acute myocardial infarction: locally increased interleukin-6 and serum amyloid A but decreased C-reactive protein. Circulation 2005;111:1355-61.
15.   Hugel B, Martinez MC, Kunzelmann C, Freyssinet JM. Membrane microparticles: two sides of the coin. Physiology (Bethesda) 2005;20:22-7.
16.   Distler JH, Pisetsky DS, Huber LC, Kalden JR, Gay S, Distler O. Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases. Arthritis Rheum 2005;52:3337-48.
17.   Mallat Z, Hugel B, Ohan J, Leseche G, Freyssinet JM, Tedgui A. Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. Circulation 1999;99:348-53.
18.   Morel O, Toti F, Hugel B et al. Procoagulant microparticles: disrupting the vascular homeostasis equation? Arteriosclerosis, thrombosis, and vascular biology 2006;26:2594-604.
19.   Hartopo AB, Puspitawati I, Gharini PP, Setianto BY. Platelet microparticle number is associated with the extent of myocardial damage in acute myocardial infarction. Arch Med Sci 2016;12:529-37.

20. Biasucci LM, Porto I, Di Vito L et al. Differences in Microparticle Release in Patients With Acute Coronary Syndrome and Stable Angina. Circulation Journal 2012;76:2174-2182.

21. Montoro-Garcia S, Shantsila E, Tapp LD et al. Small-size circulating microparticles in acute coronary syndromes: relevance to fibrinolytic status, reparative markers and outcomes. Atherosclerosis 2013;227:313-22.

22. Porto I, Biasucci LM, De Maria GL et al. Intracoronary microparticles and microvascular obstruction in patients with ST elevation myocardial infarction undergoing primary percutaneous intervention. European heart journal 2012;33:2928-38.

23. 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;32:2034-41.

24. Ueba T, Nomura S, Inami N 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:e329-34.

25. Chiva-Blanch G, Laake K, Myhre P et al. Platelet-, monocyte-derived and tissue factor-carrying circulating microparticles are related to acute myocardial infarction severity. PloS one 2017;12:e0172558.

26. Updated ESC Guidelines for managing patients with suspected non-ST-elevation acute coronary syndromes. European heart journal 2011;32:2909-10.

27. Brogan PA, Shah V, Brachet C et al. Endothelial and platelet microparticles in vasculitis of the young. Arthritis Rheum 2004;50:927-36.

28. Combes V, Dignat-George F, Mutin M, Sampol J. A new flow cytometry method of platelet-derived microvesicle quantitation in plasma. Thrombosis and haemostasis 1997;77:220.

29. Majumder B, North J, Mavroudis C, Rakhit R, Lowdell MW. Improved accuracy and reproducibility of enumeration of platelet-monocyte complexes through use of doublet-discriminator strategy. Cytometry B Clin Cytom 2012;82:353-9.

30. Inwald DP, McDowall A, Peters MJ, Callard RE, Klein NJ. CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. Circulation research 2003;92:1041-8.

31. Bernal-Mizrachi L, Jy W, Jimenez JJ et al. High levels of circulating endothelial microparticles in patients with acute coronary syndromes. American heart journal 2003;145:962-70.

32. Singh N, Gemmell CH, Daly PA, Yeo EL. Elevated platelet-derived microparticle levels during unstable angina. The Canadian journal of cardiology 1995;11:1015-21.

33. Stepien E, Stankiewicz E, Zalewski J, Godlewski J, Zmudka K, Wybranska I. Number of microparticles generated during acute myocardial infarction and stable angina correlates with platelet activation. Archives of medical research 2012;43:31-5.

34. Skeppholm M, Mobarrez F, Malmqvist K, Wallen H. Platelet-derived microparticles during and after acute coronary syndrome. Thrombosis and haemostasis 2012;107:1122-9.

35. Suades R, Padro T, Crespo J et al. Circulating microparticle signature in coronary and peripheral blood of ST elevation myocardial infarction patients in relation to pain-to-PCI elapsed time. International journal of cardiology 2016;202:378-87.

36. Bournazos S, Rennie J, Hart SP, Fox KA, Dransfield I. Monocyte functional responsiveness after PSGL-1-mediated platelet adhesion is dependent on platelet activation status. Arteriosclerosis, thrombosis, and vascular biology 2008;28:1491-8.

37. Li N, Hu H, Lindqvist M, Wikstrom-Jonsson E, Goodall AH, Hjemdahl P. Platelet-leukocyte cross talk in whole blood. Arteriosclerosis, thrombosis, and vascular biology 2000;20:2702-8.

38. Huo Y, Xia L. P-selectin glycoprotein ligand-1 plays a crucial role in the selective recruitment of leukocytes into the atherosclerotic arterial wall. Trends Cardiovasc Med 2009;19:140-5.

39. Hong Y, Eleftheriou D, Hussain AA et al. Anti-neutrophil cytoplasmic antibodies stimulate release of neutrophil microparticles. Journal of the American Society of Nephrology : JASN 2012;23:49-62.

40. Neumann FJ, Marx N, Gawaz M et al. Induction of cytokine expression in leukocytes by binding of thrombin-stimulated platelets. Circulation 1997;95:2387-94.

41. Galkina E, Ley K. Double jeopardy: how soluble P-selectin activates leukocytes in peripheral arterial occlusive disease. Circulation research 2006;98:12-4.

42. da Costa Martins PA, van Gils JM, Mol A, Hordijk PL, Zwaginga JJ. Platelet binding to monocytes increases the adhesive properties of monocytes by up-regulating the expression and functionality of beta1 and beta2 integrins. J Leukoc Biol 2006;79:499-507.

43. Yago T, Tsukuda M, Minami M. P-selectin binding promotes the adhesion of monocytes to VCAM-1 under flow conditions. J Immunol 1999;163:367-73.

44. Lindmark E, Tenno T, Siegbahn A. Role of platelet P-selectin and CD40 ligand in the induction of monocytic tissue factor expression. Arteriosclerosis, thrombosis, and vascular biology 2000;20:2322-8.

45. Celi A, Pellegrini G, Lorenzet R et al. P-selectin induces the expression of tissue factor on monocytes. Proc Natl Acad Sci U S A 1994;91:8767-71.

46. Michelson AD, Barnard MR, Hechtman HB et al. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. Proc Natl Acad Sci U S A 1996;93:11877-82.

47. Patel PB, Pfau SE, Cleman MW et al. Comparison of coronary artery specific leukocyte-platelet conjugate formation in unstable versus stable angina pectoris. The American journal of cardiology 2004;93:410-3.

48. Gawaz M, Reininger A, Neumann FJ. Platelet function and platelet-leukocyte adhesion in symptomatic coronary heart disease. Effects of intravenous magnesium. Thrombosis research 1996;83:341-9.

49. Furman MI, Benoit SE, Barnard MR et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. Journal of the American College of Cardiology 1998;31:352-8.

50. Ott I, Neumann FJ, Gawaz M, Schmitt M, Schomig A. Increased neutrophil-platelet adhesion in patients with unstable angina. Circulation 1996;94:1239-46.

51. Furman MI, Barnard MR, Krueger LA et al. Circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction. Journal of the American College of Cardiology 2001;38:1002-6.

52. Ray MJ, Walters DL, Bett JN, Cameron J, Wood P, Aroney CN. Platelet-monocyte aggregates predict troponin rise after percutaneous coronary intervention and are inhibited by Abciximab. International journal of cardiology 2005;101:249-55.

53. Wang J, Zhang S, Jin Y, Qin G, Yu L, Zhang J. Elevated levels of platelet-monocyte aggregates and related circulating biomarkers in patients with acute coronary syndrome. International journal of cardiology 2007;115:361-5.

54. Furman MI, Kereiakes DJ, Krueger LA et al. Leukocyte-platelet aggregation, platelet surface P-selectin, and platelet surface glycoprotein IIIa after percutaneous coronary intervention: Effects of dalteparin or unfractionated heparin in combination with abciximab. American heart journal 2001;142:790-8.

55. Sprague DL, Sowa JM, Elzey BD, Ratliff TL. The role of platelet CD154 in the modulation in adaptive immunity. Immunol Res 2007;39:185-93.

56. Sinauridze EI, Kireev DA, Popenko NY et al. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. Thrombosis and haemostasis 2007;97:425-34.

57. Jimenez JJ, Jy W, Mauro LM, Horstman LL, Soderland C, Ahn YS. Endothelial microparticles released in thrombotic thrombocytopenic purpura express von Willebrand factor and markers of endothelial activation. British journal of haematology 2003;123:896-902.
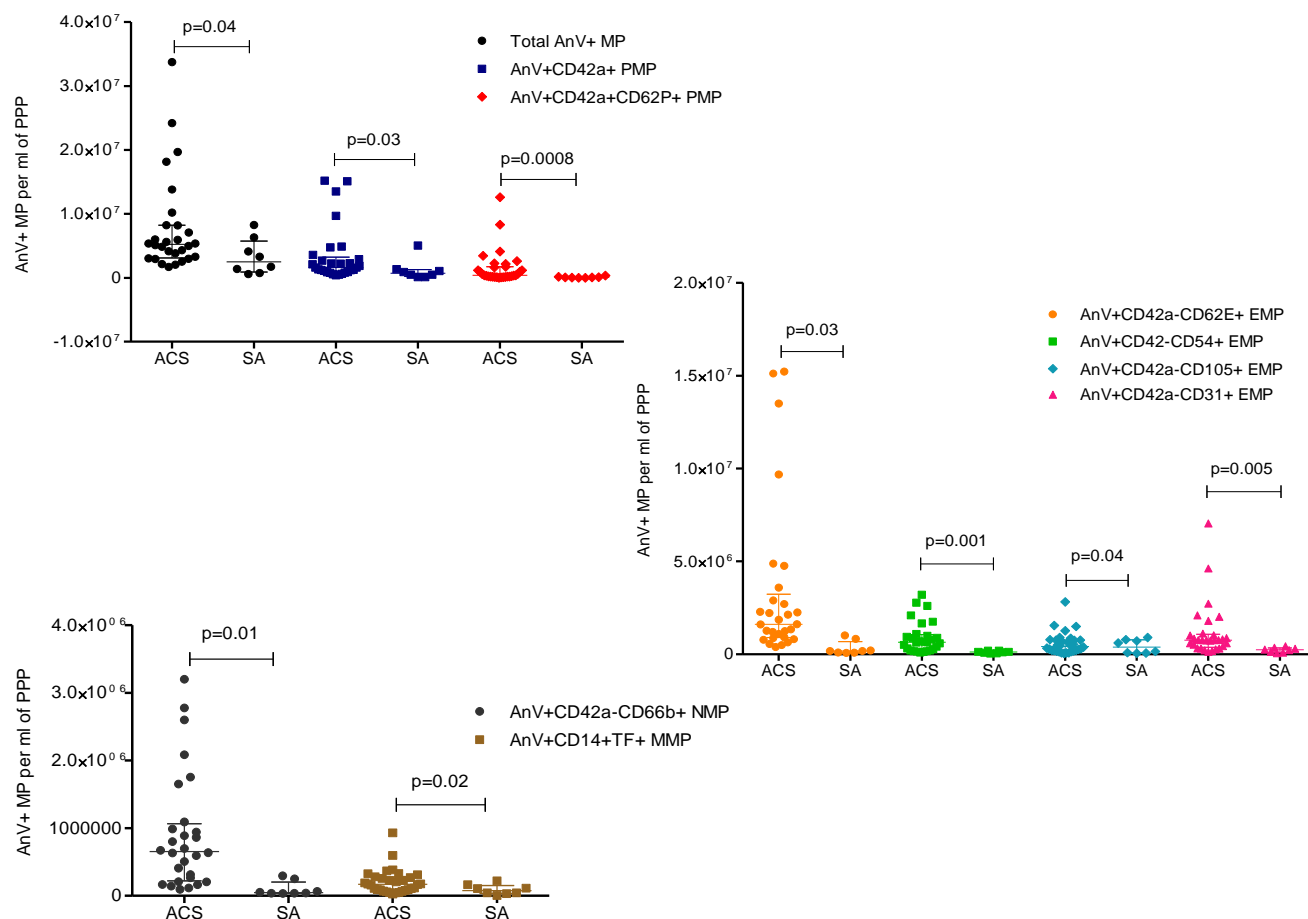
Figure 1. Differences of the total AnnexinV+ Microparticles (AnV+MP) and MP subpopulations in the coronary artery (CO) between the two groups, acute coronary syndrome (ACS) and stable angina (SA). Statistical analysis performed using the Mann-Whitney U test. MP (microparticles), PMP (platelet derived microparticles), EMP (endothelial derived microparticle), NMP (neutrophil derived microparticle), MMP (monocyte derived microparticle).
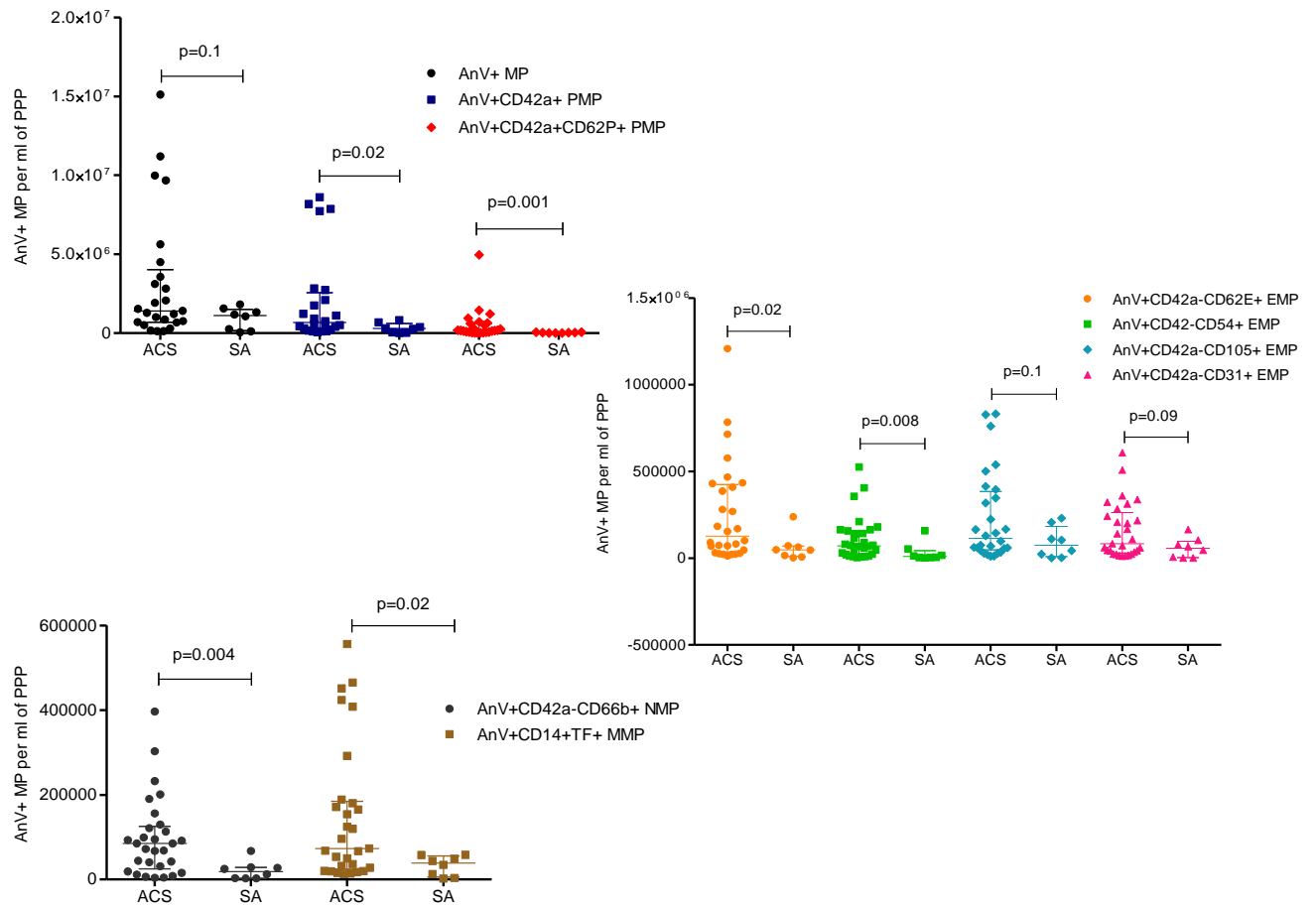
Figure 2. Differences of the total AnnexinV+ Microparticles (AnV+MP) and MP subpopulations in right atrium (RA) in the acute coronary syndrome (ACS) and stable angina (SA) groups. Statistical analysis performed using the Mann-Whitney U test. MP (microparticles), PMP (platelet derived microparticles), EMP (endothelial derived microparticle), NMP (neutrophil derived microparticle), MMP (monocyte derived microparticle).
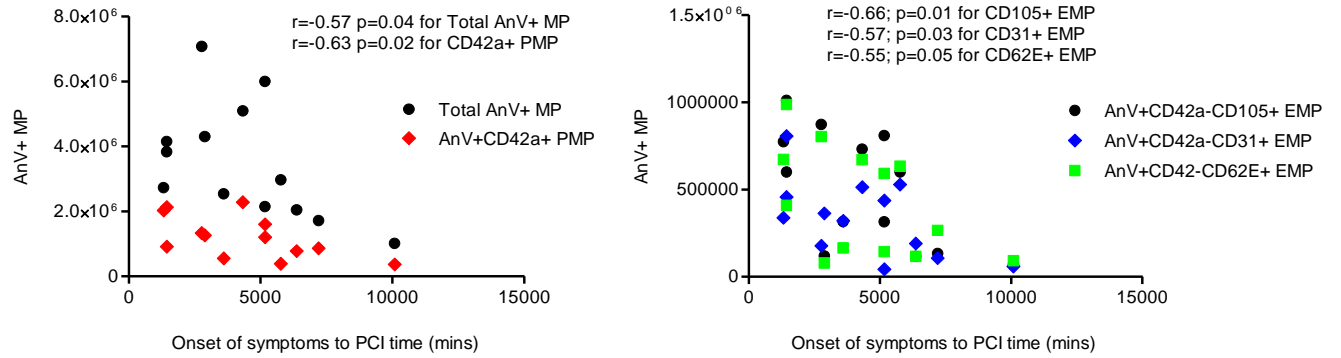
21

Figure 3. Negative correlation between the ischemic time and AnnexinV+ Microparticles (AnV+ MPs) in the coronary artery in the non-ST-segment elevation myocardial infarction group suggesting a time dependant mode of MP expression during acute coronary syndrome. MP (microparticles), PMP (platelet derived microparticles), EMP (endothelial derived microparticle).

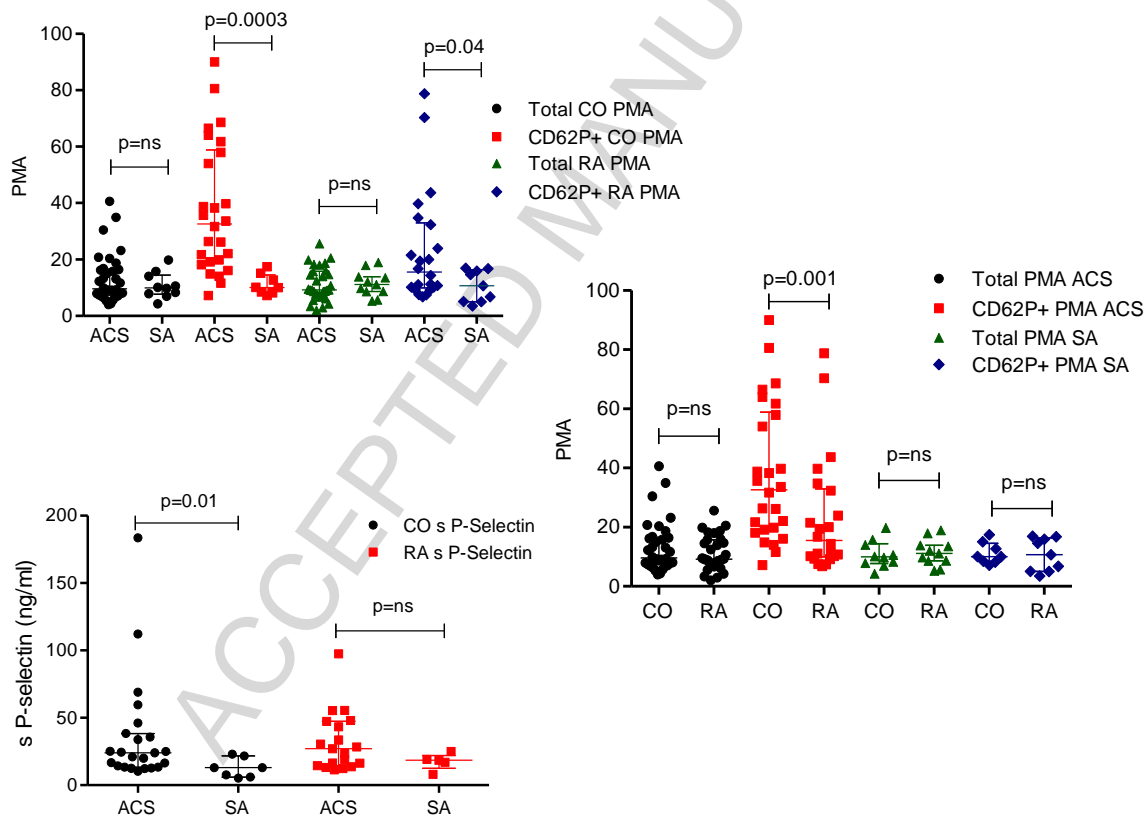Figure 4. Differences in total platelet monocyte aggregates (PMA), CD62P+PMA and soluble p-selectin in the acute coronary syndrome (ACS) and stable angina (SA) groups. Total PMA were expressed as percentage of the total monocytes. P-selectin positive PMA (CD62P+PMA) were expressed as percentage of the total PMA. Statistical analysis performed using the Mann-Whitney U test. PMA (platelet monocyte aggregates)
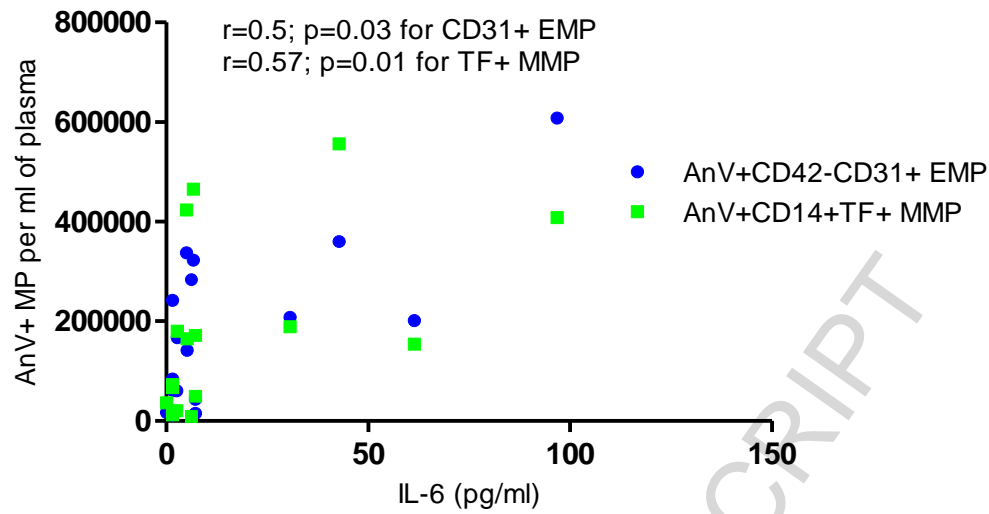
22

Figure 5. Positive correlation between AnnexinV+ Microparticles (AnV+MP) and markers of inflammation Interleukin (IL-6) in the right atrium (RA) in the acute coronary syndrome (ACS) group. EMP (endothelial derived microparticle), MMP (monocyte derived microparticle), IL-6.

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

Table 1. Baseline characteristics, demographics and angiographic data of the acute coronary syndrome (ACS) and stable angina (SA) groups. Fisher exact test was used for comparison of proportions between the groups. Coronary heart disease (CHD).

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

Table 2. Differences of the total AnV+MP and MP subpopulations ($*10^6$/ml of plasma) between the acute coronary syndrome (ACS) and stable angina (SA) groups in the coronary (CO) and right atrium (RA). Statistical analysis performed using the Mann-Whitney U test.

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

Table 3. Differences of microparticles expression ($*10^6$/ml of plasma) in the three groups in

the coronary artery (CO). MP levels expressed as medians with interquartile range (IQR).

Statistical analysis performed using Kruskal-Wallis test.

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

Table 4. Differences of microparticles expression ($*10^6$/ml of plasma) in the three groups in

the right atrium (RA). MP levels expressed as medians with interquartile range (IQR).

Statistical analysis performed using Kruskal-Wallis test.

27

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

Table 5. Differences of the inflammatory markers (medians (IQR)) between the acute coronary syndrome (ACS) and stable angina (SA) patients in the coronary (CO) and right atrium (RA). Statistical analysis performed using the Wilcoxon test. IL-6 (interleukin 6), SAA (serum amyloid A), TNF-α (tumour necrosis alpha) and hs-CRP (high sensitivity C-reactive protein).