

**Title**

P-Selectin Activated Platelet Monocyte Aggregates and Microvascular Dysfunction in patients with stable angina and non-ST elevation acute coronary syndrome undergoing PCI

**Authors**

Chrysostomos A Mavroudis MD, MRCP, Bikash Majumder MD, MRCP, Ray Sapsford BSc, Janet North MSc, Mark Lowdell PhD, Roby D Rakhit MD, FRCP

Corresponding author

Roby Rakhit MD, FRCP

Consultant Cardiologist & Honorary Senior Lecturer

Department of Cardiology

Royal Free London NHS Trust, London, United Kingdom NW3 2QG

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## **Abstract**

### **Background**

Microvascular dysfunction (MvD) is a predictor of outcomes in coronary heart disease (CHD). Activated platelets interact with leukocytes forming platelet monocyte aggregates (PMA), a marker of platelet activation.

### **Objectives**

To assess the relationship between MvD and PMA expression in CHD patients treated with percutaneous coronary intervention (PCI).

### **Methods**

Fourteen patients with NSTE-ACS and 11 patients with stable angina (SA) recruited. Blood samples aspirated sequentially from the right atrium (RA), ascending aorta (AO) and the coronary artery (CO) distal to the culprit lesion. MvD was assessed by measuring the index of microvascular resistance (IMR), coronary flow reserve (CFR) and the coronary wedge pressure (Pw). Flow cytometry was used for PMA estimation.

### **Results**

Total PMA and CD62P+PMA did not differ between CO, AO and RA in the two groups. CD62P+PMA expression in the CO and AO of the ACS group were higher compared to SA ( $p=0.005$  and  $p=0.03$  respectively). Pw (median) was higher in the ACS (52.5 mmHg) compared to the SA group (18 mmHg)  $p= 0.01$ . IMR and CFR did not differ between ACS and SA. Pw and IMR in both groups correlated positively with the total PMA ( $r^2=0.3$ ;  $p=0.009$  and  $r^2=0.4$ ;  $p=0.01$  for Pw and IMR respectively in ACS,  $r^2=0.2$ ;  $p=0.03$  and  $r^2=0.3$ ;  $p=0.01$  for Pw and IMR respectively in SA).

### **Conclusions**

Elevated CD62+PMA is a hallmark of ACS. PMA correlate with measured microvascular dysfunction in both stable angina and ACS patients. This study supports the hypothesis that

PMA may be important determinants of platelet activation, inflammation and microvascular dysfunction in CHD.

**Key words:** acute coronary syndrome, microvascular dysfunction, platelet activation and platelet-monocyte aggregates.

## **Background**

Microvascular dysfunction (MvD) is a powerful independent predictor of long term outcomes, left ventricular function and mortality, in patients presenting with acute coronary syndrome (ACS) and undergoing revascularization with percutaneous coronary intervention (PCI). **REFERENCE REQUIRED** Despite angiographic successful coronary artery stenting potential compromise of the coronary microcirculation during or after PCI is associated with “slow flow” or “no flow” with subsequent impaired myocardial tissue oxygenation.

**REFERENCE REQUIRED** A number of different non-invasive and invasive techniques have been developed to evaluate the coronary microvasculature. Among the invasive methods in the catheter laboratory the ‘Index of microvascular resistance’ (IMR) which is derived using a Doppler or a pressure-temperature sensing wire, has emerged as a novel index for the assessment of the coronary microcirculation (1-3).

Fearon et al demonstrated positive correlation of IMR measured immediately following primary PCI (pPCI) for STEMI patients with peak CK and left ventricular function assessed by echocardiography at 3 months follow up (4). It has also been shown that measured IMR during pPCI, in STEMI patients, predicts myocardial salvage, infarct characteristics, and left ventricular ejection fraction (5-7). Cuisset et al has demonstrated, in patients with stable angina who had elective PCI, that direct stenting was associated with significantly lower IMR compared to the patients who had pre-dilation with balloon. They have also demonstrated that patients with post-PCI troponin release had significantly higher IMR compared to the patients without post-PCI troponin release (8). In addition measurement of IMR in patients electively admitted for PCI may allow prospective identification of patients at risk of periprocedural myocardial infarction (9).

Activated endothelial cells and platelets express P-selectin on their surface which mediates the platelet-leukocyte interaction via the P-selectin-PSGL-1 bond leading to platelet

monocyte aggregate (PMA) formation with leukocyte activation, migration and recruitment at the site of inflammation (10;11). It has been shown that inflammatory mediators such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and LPS increase P-selectin production from the endothelial cells in addition to up-regulating P-selectin on the cell surface (12). Furthermore, activated platelets lose their surface P-selectin despite the fact they continue to circulate and function (13). Therefore PMA measurement may represent a more robust marker of platelet activation than detection of P-selectin on their surface. P-selectin on the surface of the activated platelets, within the PMA complex, induces monocyte TF expression which, in the ACS setting with a ruptured atheromatous plaque, may trigger or contribute to the cascade of athero-thrombosis (14). PMA levels, both at the site of the coronary lesion and systematically, has been described as a sensitive marker of platelet activation and inflammation. Increased PMA levels have been observed in both stable angina (15;16) and ACS patients (17). The extent of PMA formation depends mainly on platelet activation status rather than the monocyte activation (18). It has been shown that prevention of platelet adhesion to monocytes by interfering with the binding of platelet P-selectin to PSGL-1 reduces inflammation (19). Platelet binding to monocytes via the P-selectin dependant pathway increases the adhesive properties of monocytes by up-regulating the expression and functionality of beta1 and beta2 integrins (20;21). This process is facilitated by the presence of RANTES and CXCL10 chemokines produced by platelets on to the monocytes (22). A positive relationship between inflammatory markers (IL6 and CRP) and markers of platelet activation like P-selectin and PMA has been previously demonstrated (23). Therefore the possibility that PMA may contribute in the pathophysiology of microvascular dysfunction via exaggerating and sustaining the coagulation cascade in addition to perpetuating local coronary inflammation exist.

The role of PMA formation in the pathophysiology of ACS remains unknown. Whether PMA is just a bystander of the inflammatory and thrombotic process occurring in the setting of ACS or whether the PMA is an effector of the disease remains unclear. **EXPLAIN HERE EXACTLY WHAT A CD62+ PMA IS** Botto et al have demonstrated increased platelet-monocyte interaction and CD62P+PMA formation in blood samples aspirated from the infarct related artery (IRA) compared with the peripheral circulation in patients presented with myocardial infarction (24). The concept of an active involvement of PMA in ACS has been supported with the observations by Patel et al who demonstrated trans-coronary gradient of platelet-leukocyte formation in patients with ACS. They found increased CD62P+PMA level in the coronary sinus samples compared to the aortic samples in both patients with ACS and stable angina (25). In addition, recent data suggest that platelet monocyte interaction is a normal physiological process in the absence of overt platelet activation with little impact on the potential of monocytes to cause vascular injury (26). We identified this subpopulation of PMA as P-selectin positive PMAs (CD62+ PMAs). We hypothesized that 1) PMA is higher in the ACS group compared with the stable angina and 2) a positive correlation should exist between PMA expression and markers of microvascular dysfunction. The aim of our study was to assess the relationship between microvascular dysfunction and the level of PMA expression, intracoronary and systemic (aorta and right atrium) in patients presenting with NSTE-ACS or stable angina treated with PCI.

## **Methods**

### **Study population**

Twenty five patients (14 patients with NSTE-ACS and 11 patients with stable angina (SA) presenting to our hospital with symptomatic coronary heart disease and treated with PCI were recruited for the study. The diagnosis of NSTE-ACS was based on the history of chest pain with or without ischaemic ECG changes (other than ST elevation) and a troponin T value >

0.03 ng/l (27). SA patients were admitted electively for PCI. SA patients had been reviewed in the cardiology outpatient clinic with a diagnosis of angina pectoris based on typically ischaemic sounding chest pain and a positive non-invasive test such as exercise tolerance test, stress echocardiography or myocardial perfusion scan. The decision for diagnostic angiography was made by a cardiologist as part of routine clinical care. Informed consent was obtained prior to the procedure from all recruited patients outside the cardiac catheterisation laboratory. Ethical permission was granted by the local ethics committee of the Royal Free Hospital, and all participants provided fully informed written consent.

### **Exclusion criteria**

Patients with a history of chronic renal failure (defined as an estimated GFR<60ml/min), prior coronary artery bypass grafts and patients who had received Glycoprotein IIb/IIIa antagonists prior to sampling were excluded from the study.

### **Coronary angiography, coronary angioplasty and blood sampling**

Diagnostic coronary angiography followed by PCI was performed according to contemporary guidelines (27). Through a 6F venous sheath via the femoral vein a 5F multipurpose catheter (5F, Cordis ®, internal diameter 0.11cm) was placed in the right atrium. Left and right coronary artery angiography was performed with a 5F Judkin's Left 4 diagnostic catheter (Cordis ®, internal diameter 0.11 cm) and 5F Judkin's Right 4 diagnostic catheter (5F, Cordis ®, internal diameter 0.11 cm) respectively. Blood samples (10 ml from each site) were aspirated sequentially through the diagnostic 5F catheter from the right atrium and ascending aorta. After identification and wiring of the culprit lesion an aspiration catheter (Medtronic ® Export catheter, internal diameter 0.10 cm) was advanced just distal to the culprit lesion and 10 mls blood sample was aspirated. All blood samples were aspirated carefully through

catheters of similar internal diameter in order to maintain similar shear stress during aspiration and minimise shear stress related in vitro platelet activation. PCI was performed according to standard procedures following collection of the blood samples.

NSTE-ACS patients were treated with 300 mg of Aspirin, 600 mg of Clopidogrel and weight adjusted low molecular weight heparin (enoxaparin 1mg/kg twice daily) immediately after the diagnosis of NSTE-ACS. All patients received weight adjusted unfractionated heparin in the catheter laboratory during PCI to maintain the ACT between 200 and 250 sec. SA patients were treated with anti-anginal medications; aspirin 75mg od and clopidogrel 75mg od for 7 days prior to the PCI as per hospital protocol.

#### **Measuring IMR and CFR with pressure-temperature sensor wire**

Thermodilution technique using a 0.014 inch floppy, pressure wire (pressure wire 3, Radi Medical system) as described before (3) was used to measure IMR and CFR, in 7 patients with NSTE-ACS and 7 patients with SA. The shaft/microsensor of the wire can be used as an additional thermistor, providing the input signal at the coronary ostium of any fluid injection with a temperature difference from blood. In this manner the mean transit time of the injected 3 mls 0.9% saline at room temperature, in the coronary artery can be calculated from a coronary thermodilution curve. Measurements of the mean transit time (Tm) were calculated 3 times at baseline and 3 times at maximum hyperaemia. Maximum hyperaemia was achieved by adenosine infusion through the femoral vein at a rate 140 µg/kg/min. Distal coronary pressure (Pd) was recorded from the pressure wire. Mean aortic pressure (Pa) was measured from the coronary catheter and central venous pressure (Pv) was measured from the right heart catheter at maximum hyperaemia. Coronary wedge pressure (Pw) was measured at maximum hyperaemia by inflating a semi-compliant balloon (1mm smaller than the vessel diameter) in the coronary artery. IMR measurements were calculated after the completion of

the PCI. IMR was calculated using the equation  $IMR = Pa \times Tm \times [(Pd-Pw) \div (Pa-Pw)]$  as described before (1). All the above measurements were taken during maximum hyperaemia. CFR equals to the ratio of the baseline Tm/hyperaemic Tm provided that the time for analysis of the thermodilution equals at least one cardiac cycle, and variability between values of the 3 transit times < 20%.

### **Flow Cytometry and PMA methodology**

4mls of the sampled whole blood was poured into separate sterile vacutainer tubes containing a combination of 3.2% sodium citrate and EDTA for flow-cytometric estimation of PMA and CD62P+ PMA as described by our group before (28). Blood samples were immediately transferred to the haematology laboratory and prepared for flow cytometry and PMA estimation, within 2 hours of sample collection. Five  $\mu$ L of fluorescent monoclonal antibodies, anti CD61 FITC, anti CD14 PerCP and anti CD62P PE were added in each of the three (one tube for each compartment) round bottom polystyrene tubes (BD Falcon 12 x75 mm style). One hundred  $\mu$ L of whole blood was aliquoted into each tube. The samples were incubated in a dark place at room temperature for 20 minutes. After that, erythrocytes were lysed by addition of 2 ml of easy lyse<sup>TM</sup> solution (Dako) (1 in 10 dilution) for 15 minutes at room temperature in dark place. Red blood cells were washed with addition of 1ml of FACS flow and centrifuged at 300g for 5 minutes. The supernatant was discarded and the cells resuspended in 500  $\mu$ L of FACS flow for immediate flow cytometric acquisition and analysis (FACS Calibur equipped with Cell Quest<sup>®</sup> software – BD Biosciences, Oxford, UK). Events were acquired on a 2D dot plot arraying CD14 (logarithmic scale abscissa) and SSC height (linear scale ordinate). Monocytes were identified as CD14 positive events and distinctive intermediate side scatter height. A minimum of 5000 CD14 positive events were acquired from each sample. The monocyte population was gated and named as analysis region (R1).

Events within R1 plotted again on a 2D dot-plot arraying CD 61 signal width (linear scale abscissa) and CD14 signal height (logarithmic scale abscissa). Double positive CD14+CD61+ events were considered as total PMA and were expressed as percentage of the total monocytes. Triple positive CD14+CD61+CD62P+ events were identified as P-selectin positive PMA (CD62P+ PMA) and were expressed as percentage of the total PMA. The process was standardised in our laboratory with the percentage of PMCs in the peripheral circulation of normal healthy individuals was found to be  $2.57 \pm 0.31$  (CV  $13.96 \pm 8.30\%$ ).

### **Statistical analysis**

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

### **Results**

The demographics and baseline characteristics of the study population are shown in table 1. Briefly the majority of the study population was male (83%). The SA patients were more likely to have past medical history of hypertension compared to the NSTE-ACS group. Also the NSTE-ACS patients were more likely to have family history of CHD. The study population was well balanced with respect to other risk factors, diabetes mellitus, dyslipidaemia and smoking.

### **Total PMA expression in the CO, AO and RA sites in NSTE-ACS and SA groups**

Within NSTE-ACS and SA groups there were no difference seen in the expression of total PMA and CD62P+ PMA levels between the three sites (CO, AO and RA). Total PMA expression in the CO, AO and RA did not differ between NSTE-ACS and SA (Fig 1). However the expression of CD62P+ PMA in the CO and AO of the NSTE-ACS patients was higher compared to SA patients ( $p=0.005$  and  $p=0.03$  respectively) (Fig 1). There was no difference in the expression CD62P+ PMA in the RA between the two groups (Fig 1c).

### **IMR, CFR and Pw in the two groups**

Pw (median (IQR) was higher in the NSTE-ACS group [52.5 mmHg (34.2-65.5)] compared with the SA group [18 mmHg (17-32)]  $p=0.01$ ] Fig 2. IMR (median (IQR) did not differ between NSTE-ACS [19.6 mmHg\*sec (14.4-34.2)] and SA group [19.9 mmHg\*sec (17.6-26.8)] (Fig 2). CFR also did not differ between the two groups (data not shown).

### **Correlations between indices of microvascular dysfunction and PMA in the ACS and stable angina groups**

Pw and IMR in the NSTE-ACS correlated positively with the total PMA levels in the three compartments ( $r^2=0.35$ ;  $p=0.009$  and  $r^2=0.46$ ;  $p=0.01$  for Pw and IMR respectively). In addition there was a strong positive correlation between Pw and CD62P+ PMA ( $r^2=0.46$ ;  $p=0.007$ ). (Table 2)

In the SA group Pw and IMR correlated positively with total PMA ( $r^2=0.23$ ;  $p=0.03$  and  $r^2=0.28$ ;  $p=0.01$  for CwP and IMR respectively). CD62P+ PMA in the SA did not correlate with Pw or IMR. (Table 2)

## **Discussion**

The unique finding of our study is the positive correlation between invasive markers of microvascular dysfunction and PMA in patients with NSTE-ACS and stable angina. In line with previous studies, CD62P+ PMA in the coronary were higher in ACS patients compared with stable angina. The above findings support the knowledge that platelet activation is pivotal in ACS and as such CD62P+ PMA which is a sensitive marker of platelet activation is the hallmark of ACS. It also supports the notion that PMA formation is dependent on platelet activation and that PMA expression and platelet activation follow the same pattern of expression. Concomitant aortic and coronary blood sampling in this study demonstrated that CD62+PMA were significantly higher in acute coronary syndrome compared to stable angina indicating overspill of activated PMA generated at the site of plaque rupture from coronary to aorta. The fact that the NSTE-ACS patients were studied, blood sampling and IMR measurements, 2-4 days after the onset of symptoms whilst receiving treatment with anti-platelet and anti-coagulant medications may have altered the expression of total PMA and contributed to our observations that total PMA did not differ between the 2 groups. This may also explain the fact that only Pw was higher in the ACS group compared to the SA group whilst IMR and CFR did not differ between the 2 groups. The latter is probably due to sample size.

During ACS, PMA formation at the site of plaque rupture with subsequent monocyte activation and cytokine release may contribute to the impairment of microcirculatory flow. Ko et al have found increased levels of soluble CD40 ligand, IL-6, serotonin, TF and factor VII in the culprit coronary artery compared to those in peripheral blood (29). Also a positive relationship between inflammatory markers (IL6 and CRP) and markers of platelet activation like P-selectin and PMA has been demonstrated by Wang et al (30). Takahashi et al have

showed that neutrophilia on admission is associated with impaired microvascular reperfusion and poor functional recovery in patients with myocardial infarction treated with primary PCI. Similar finding were found by Sezer et al (31) who showed that neutrophilia and higher mean platelet volume were found to be associated with higher IMR, lower CFR and higher coronary wedge pressure in patients with ACS treated with PCI. The results of our study are in line with the above findings. Both total PMA and CD62P+ PMA correlated positively with the coronary wedge pressure in the ACS patients. Also IMR correlated positively with total PMA. Contrary to the above, in the SA group IMR and Pw correlated positively with total PMA only but not with CD62P+ PMA.

Clinical studies in animal models, with coronary artery occlusion and reperfusion, have shown that administration of recombinant monoclonal PSGL-1 antibodies targeting the P-selectin-PSGL interaction results in inhibition of leukocyte-platelet and leukocyte-endothelial binding with subsequent improvement of reperfusion in addition to the reduction of the infarct size (32-34). However in human beings administration of recombinant PSGL in patients with STEMI as an adjunct to thrombolysis have failed to improve clinical outcomes like TIMI flow, infarct size and mortality (35). This can be explained by the fact that in both studies the recombinant antibody was given intravenously and as such may have failed to reach the coronary circulation. This is because the recombinant antibody was human antibody and as such was probably absorbed by the antibody receptors expressed on the macrophages in the liver and spleen. Recently, Tardif et al have shown that intravenous administration of a P-selectin antagonist (inlacumab) in patients with ACS scheduled for PCI reduce myocardial damage (36). They found that peak Troponin I and creatine kinase reduced by 23%. Our findings may explain the mechanism behind the above positive findings. This raises the possibility that PMA may contribute to the pathophysiology of microvascular dysfunction via exaggerating and sustaining platelet activation and coagulation cascade in addition to

perpetuating local coronary inflammation. Therefore, targeting PMA formation in ACS may interrupt the platelet-monocyte interactions and limit intra-coronary inflammation and thrombus formation.

### **Conclusions**

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

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## **Figure legends**

Figure 1 Higher expression of CD62P+ PMA, but not total PMA, in the acute coronary syndrome (ACS) compared with the stable angina (SA) group in the coronary and aorta. (Total PMA expressed as % of monocytes and CD62P+ PMA as % of total PMA). PMA (platelet monocyte aggregates). Comparisons were performed using the Mann-Whitney U test.

Figure 2 Higher coronary wedge pressure (Pw) in the acute coronary (ACS) compared with the stable angina (SA) group. The index of microvascular resistance (IMR) did not differ in the two groups. Comparisons were performed using the Mann-Whitney U test.

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Table 1. Baseline characteristics, demographics and angiographic data of the study groups.

CHD (coronary heart disease), ACS (acute coronary syndrome)

	ACS		ACS		SA		SA	
	PMA vs Pw	CD62 + PMA vs Pw	PMA vs IMR	CD62 + PMA vs IMR	PMA vs Pw	CD62 + PMA vs Pw	PMA vs IMR	CD62 + PMA vs IMR
1/slope	4.865	1.438	3.053	-1.174	5.09	2.344	2.971	-2.569
R square	0.3503	0.4603	0.462	0.3183	0.2342	0.07371	0.2823	0.1726
P value	0.0097	0.0076	0.0106	0.0893	0.0306	0.3696	0.0192	0.1096

Table 2. The table above shows the linear regression analysis including p values of the PMA/CD62P+PMA, in the three sites, and indices of microvascular dysfunction. Platelet monocyte aggregates (PMA), Coronary wedge pressure (Pw), Index of microvascular resistance (IMR)

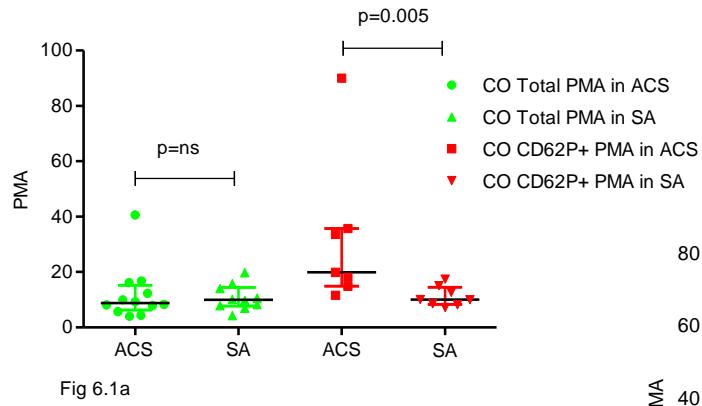


Fig 6.1a

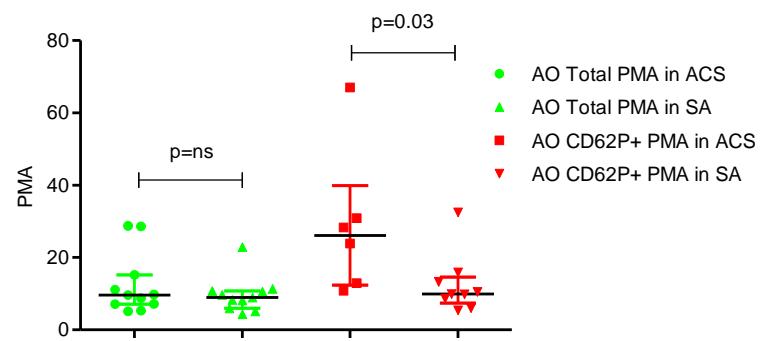


Fig 6.1b

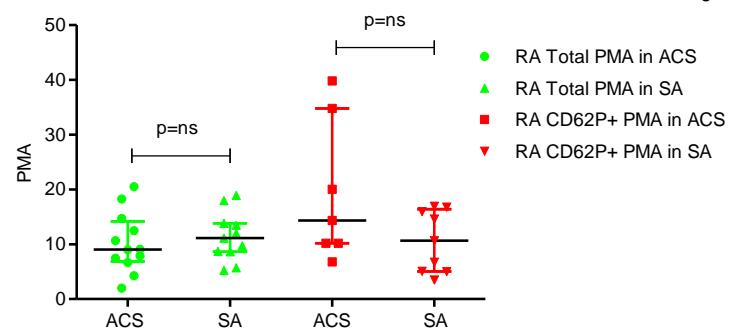


Fig 6.1c

Figure 1 Higher expression of CD62P+ PMA in the acute coronary syndrome compared with the stable angina.

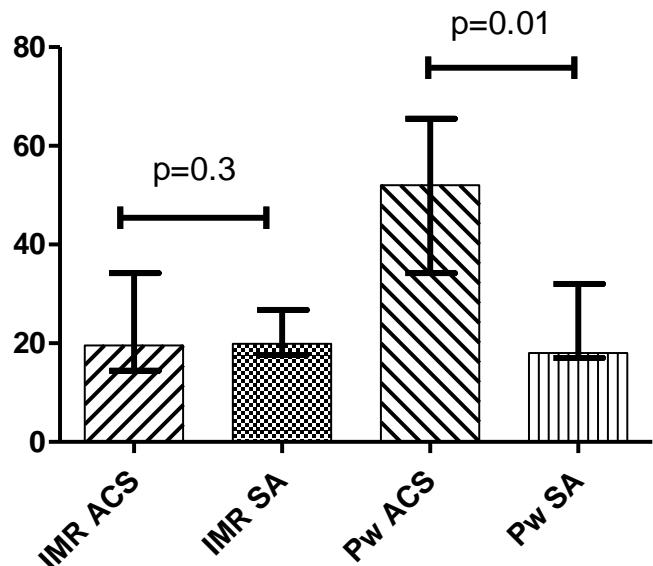


Figure 2 Higher coronary wedge pressure (Pw) in the acute coronary syndrome compared with stable angina.