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## Differences in responses of platelets to fluid shear stress in patients with peripheral artery disease (PAD) and coronary artery disease (CAD)

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### Abstract

Information on differences in platelet function between patients with peripheral arterial disease (PAD) and patients with coronary artery disease (CAD) is limited. We sought to examine the differences in the platelets response to shear stress in patients with PAD compared to those with CAD. Men with symptomatic PAD (ankle brachial index [ABI] <0.9; n=29) were compared with similarly aged men with CAD (post coronary artery bypass grafting; n=40) but without PAD. All participants were on aspirin, and none were on clopidogrel. We measured changes in shear-induced platelet aggregation (SIPA) and shear-induced P-selectin expression (SIPE) under fluid shear rates of 5,000 and 10,000 sec<sup>-1</sup> which are typically found in arterioles and stenosed arteries, respectively. Aggregation was also induced by a combined stimulation of collagen, fluid shear stress, and adenosine diphosphate (ADP) or epinephrine using a platelet function analyzer (PFA-100) as well as optical aggregometry (arachidonic acid, collagen and epinephrine). Analyses of covariance adjusted for age, aspirin dose, and statin use were used to estimate differences between the groups. Values of SIPA at fluid shear rates of 5,000 and 10,000 sec<sup>-1</sup> were significantly higher in the PAD group, while there were no differences between the PAD and CAD groups in SIPE at both fluid shear rates. However, baseline shear-induced P-selectin expression was higher in patients with PAD than CAD (mean fluorescence intensity [MFI]=2.93 ±1.37 vs.1.94 ±0.67; p=0.01), while the percentage increases in SIPA and SIPE at fluid shear rates of 5,000 and 10,000 sec<sup>-1</sup> were significantly higher in patients with CAD when compared to PAD (p <0.001 for all comparisons). Although there were several similarities in platelet function between men with PAD and men with CAD, significant

differences in platelet responses to shear stress were observed in men with PAD when compared to those with CAD. Although the mechanism for these observed differences are not clear, we hypothesize that *in vivo* platelet activation in PAD patients may contribute to the differences and will need to be further investigated.

## Keywords

PAD; CAD; Platelet function; shear

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## Introduction

Peripheral arterial disease (PAD) or lower extremity arterial disease is among the most severe manifestations of atherosclerosis. Patients with PAD have worse outcomes when compared to those with coronary artery disease (CAD) [1-3], and the 5-year mortality reported in some studies has been worse than the 5-year mortality for some malignancies [4]. Almost 2 out of every 3 patients with PAD have concomitant atherosclerotic involvement of other arterial beds [5,6]. The majority of the PAD-associated poor outcomes are mediated through adverse events related to the atherothrombotic events involving other arterial beds, namely coronary and cerebral. However, given the worse outcomes associated with PAD, it would be important to identify whether there are differences in the various pathways associated with atherogenesis and atherothrombosis when compared to those with atherosclerosis of other arterial beds. Inflammatory markers such as C-reactive protein (CRP) have been already shown to be increased in patients with concomitant PAD and CAD when compared to those with CAD alone [7,8]. Given the importance of platelets in mediating arterial thrombosis, we hypothesized that platelets in patients with PAD will be more active and have greater response to shear stress when compared to platelets in patients with CAD. To test this, we examined whether there were significant differences in platelet function as measured by platelet responsiveness to fluid shear stress in the presence and absence of platelet agonists in PAD patients, whose platelets are likely to be exposed to greater shear stress as a result of an increased burden of atherosclerosis, local stenosis and the resultant turbulence of blood flow, when compared to those with CAD. Data already suggests that such differences in platelet function may exist: PAD patients benefit from dual antiplatelet therapy with clopidogrel and aspirin [9,10], while CAD patients seem to benefit from the addition of clopidogrel only in specific circumstances such as in acute coronary syndrome and post percutaneous interventions. The benefit of clopidogrel in patients with stable CAD is not clear [9-13]. Hence, identifying differences in platelet function between patients with CAD and PAD may be important in understanding why patients with PAD tend to have the worst outcomes among those individuals with atherosclerotic vascular disease.

## Methods

### Subjects

Men with PAD were selected from those being recruited to an ongoing study, Effect of Lipid Modification on Peripheral Arterial Disease after Intervention Trial (ELIMIT) (enrolling patients from 2004 to date) [14], a NIH-sponsored, randomized, double-blinded, placebo-controlled trial, which has approval from our Institutional Review Board. ELIMIT is evaluating whether additional reductions in atherogenic lipoproteins and increases in high-density lipoprotein cholesterol (HDL-C) with a triple combination therapy of lipid-altering drugs (statin, ezetimibe, and niacin) will halt/regress atherosclerosis as compared with statin monotherapy. Pre-randomization samples from ELIMIT were used for this post-hoc exploratory analysis. Samples from men with CAD were obtained from the Platelet and Genes

Physiology-2 (PGAP-2) study [15], which recruited CAD patients approximately 3–6 months post coronary artery bypass grafting from 2001-2003. None of these patients were reported to have concomitant PAD. All patients from the PAD and CAD groups were on aspirin, and none were on clopidogrel. Furthermore, due to the very small number of women enrolled in ELIMIT, only men were included for our current analysis.

### Blood collection

All 29 PAD patients enrolled in ELIMIT and all 40 CAD patients enrolled in PGAP-2 had blood samples collected using similar phlebotomy techniques. Patients were required to fast overnight (8 hours minimum) and refrain from smoking for at least 4 hours prior to an early morning phlebotomy. At resting state, after discarding the first 2 mL, blood was collected with either a 19-gauge or 21-gauge needle, into a syringe containing 3.8% sodium citrate (final concentration, 0.38%). To obtain platelet-rich plasma (PRP), blood was centrifuged at  $150\times g$  for 15 minutes at 24 °C. The platelet counts in PRP were standardized to be in between 200,000–250,000/mm<sup>3</sup> with autologous platelet-poor plasma (obtained by centrifuging PRP at  $1500\times g$  for 10 minutes at 24 °C). PRP was used for shear-induced platelet aggregation (SIPA) and shear-induced P-selectin expression (SIPE). The same phlebotomist collected the blood samples in >95% of the individuals included in this analysis.

### Assays

The assays were performed within 2 hours of sample collection. All equipment had quality control procedures performed every time they were started.

For SIPA, 0.5 mL of PRP was loaded onto a cone and plate viscometer (HAAKE-RS1, Thermo Fisher Scientific, Newton, NH) and exposed to a shear rate of either 5,000 or 10,000 sec<sup>-1</sup> for 1 minute at room temperature. An aliquot of 10 µL of sheared sample was mixed with 10 mL of isotonic fluid (Beckman Coulter, Miami, FL) containing 1.0% glutaraldehyde (Sigma, St Louis, MO), and the number of single platelets was then counted on a Z2 particle counter (Beckman Coulter). SIPA was defined as percent of reduction in single platelets after exposure to fluid shear stress, as previously described [16].

In addition, 10 µL of the sample was incubated with a phycoerythrin (PE)-conjugated monoclonal P-selectin antibody (1 µg/mL, BD Pharmingen, San Diego, CA) for 20 minutes at room temperature for detection of P-selectin expression (SIPE) before and after shear exposure by an EPICS XL-MCL flow cytometer (Beckman Coulter). P-selectin expression was presented as geometrical mean fluorescence detected on 10,000 platelets.

In addition, SIPA was also measured in whole blood by a combined stimulation of shear stress with the platelet agonists, collagen, and ADP or epinephrine, using the Platelet Function Analyzer (PFA-100, Dade Behring, Deerfield, IL). Briefly, 0.8 mL of citrated whole blood was loaded into two types of cartridges: one containing collagen and ADP; and the other, collagen and epinephrine. The blood samples were injected through an aperture at a shear rate of 5000 sec<sup>-1</sup>. The time required to occlude the aperture was measured and correlated with shear-induced platelet aggregation.

Finally, we also measured spontaneous platelet aggregation and aggregation induced by arachidonic acid (0.5 mg/mL, BioData Corp., Horsham, PA) or epinephrine (10 µM, Sigma) on an optical PAP-4 aggregometer (BioData). The platelet aggregation was monitored for 10 minutes at 37 °C with constant stirring (1,000× rpm). The same 2 technicians performed all the tests.

There were several considerations in choosing these tests. Spontaneous platelet aggregation, which does not occur in healthy individuals, served as a baseline detector of platelet

hyperactivity associated with systemic inflammation. The arachidonic acid-induced aggregation tested for aspirin intake, while the epinephrine-induced aggregation was used as a standard for identifying platelet hyper-reactivity [17]. SIPA and SIPE examined platelet activation and aggregation which was specifically induced by the glycoprotein Ib-von Willebrand factor (GP Ib-VWF) interaction, a process which may be enhanced due to oxidative shear stress. We tested two shear rates ( $5,000 \text{ sec}^{-1}$  and  $10,000 \text{ sec}^{-1}$ ) which are normally encountered in physiological and pathophysiological (stenosis) arterial flow. The PFA-100 complements SIPA to test how platelets are activated and aggregated not only by fluid shear stress, but also by other platelet agonists in combination with shear stress. These platelet agonists are pathophysiologicaly relevant because they are found in the ruptured atherosclerotic plaque (collagen) or released by erythrocytes (ADP) when ruptured by turbulent high shear and rough atherosclerotic surface. Together, these tests will examine platelet function in a more physiologically relevant condition which involves several ligand-receptor interactions.

### Statistical methods

Our null hypotheses were that platelets from PAD patients will have the same responses to shear as platelets in CAD patients. All patients were males and used aspirin. CAD patients were selected to be similar in age to the PAD patients. Comparisons between the two groups were tested using analysis of covariance (ANCOVA) adjusted for age, diabetes history, aspirin dose and statin use. We also conducted additional analysis by adding the CAD status of PAD patients to the above model in order to determine the effect of CAD status on the results. When the variances of the two groups were inhomogeneous and were not correctable by transformations, a regression model with complex variance terms was fitted by maximum likelihood methods. A Tobit transformation was used to account for the 22 right-censored observations for the outcome PFA-epinephrine. Because this study was exploratory, no adjustments were made for multiple tests. P values  $<0.05$  were interpreted as significant. Data is presented as mean  $\pm$  standard deviation (SD).

### Results

Baseline characteristics of the patients are presented in Table I. The 29 patients in the PAD group and 40 patients in the CAD group were of similar age and had similar prevalence of hypertension and tobacco use. All patients were on aspirin. The number of statin users was significantly higher in the CAD patients ( $p=0.004$ ), while there was a trend towards a significantly higher prevalence of diabetes in the PAD patients ( $p=0.05$ ). None of the individuals with CAD reported having PAD, while 52% of the individuals with PAD also had CAD.

We first measured whole blood platelet activation and aggregation induced by a combination of agonists including fluid shear stress ( $5000 \text{ sec}^{-1}$ ), collagen, and either ADP or epinephrine. There was no difference between the two groups with respect to platelet aggregation induced by epinephrine ( $p=0.59$ ) or ADP cartridges ( $p=0.25$ ) (PFA-100: Table II). One concern regarding the experimental approach was that PFA-100 tests a platelet phenotype induced by a combined stimulation of three different agonists which target three different sets of receptors. As a result, any effect related to one ligand-receptor pathway (such as GP Ib-VWF interaction induced by shear stress) may not be sufficiently detected. To address this concern, we separately measured platelet activation and aggregation induced by fluid shear stress and platelet activation and aggregation induced by other agonists.

When we measured the effects of fluid shear stress, analyses of covariance (ANCOVA), after adjusting for age, diabetes history, statin use and aspirin dose, were performed to compare the PAD and CAD groups. As shown in Table III, percentage increases in SIPA under shear rates

of  $5,000 \text{ sec}^{-1}$  and  $10,000 \text{ sec}^{-1}$  were significantly greater in the CAD group ( $66.0 \pm 17.4\%$  and  $91.39 \pm 4.68\%$ , respectively) as compared to patients in the PAD group ( $44.39 \pm 14.0\%$  and  $83.2 \pm 9.24\%$ , respectively;  $p < 0.0001$ ).

Parallel to the measurements of shear-induced platelet aggregation, we also tested shear-induced platelet expression of P-selectin (after adjusting for age, diabetes history, statin use and aspirin dose), which is commonly used as a marker for platelet activation, before and after shear exposure. As shown in Table III, the levels of baseline shear-induced P-selectin expression were significantly higher in PAD patients as compared to baseline levels from CAD patients ( $p = 0.01$ ). However, a difference in P-selectin expression was not detected after exposure to shear stress at shear rates of  $5,000 \text{ sec}^{-1}$  ( $p = 0.11$ ) and  $10,000 \text{ sec}^{-1}$  ( $p = 0.96$ ), indicating that the percent increases of SIPE from baseline levels were significantly less in the PAD group as compared to the CAD group at both shear rates ( $25.4 \pm 21.8\%$  vs.  $107 \pm 56.3\%$  at  $5,000 \text{ sec}^{-1}$ ,  $p < 0.0001$ ; and  $176 \pm 112.3\%$  vs.  $292.9 \pm 171.6\%$  at  $10,000 \text{ sec}^{-1}$ ,  $p = 0.03$ ). These results suggest that platelets in patients with PAD are at least partially activated *in vivo* (as suggested by higher baseline SIPE levels) before exposure to the shear stress *ex vivo*, and as a result of this baseline, *in vivo* activation has less responsiveness to *ex vivo* shear stress (as suggested by lesser percent increases in SIPE).

We then tested the response of the platelets to various agonists (Table IV). Spontaneous aggregation, which can serve as an indicator of platelet hyper-reactivity; arachidonic acid-induced aggregation, which serves as a measure of response to aspirin; and epinephrine-induced aggregation were all found to be similar between the two groups ( $p = 0.68$ ;  $p = 0.17$ ; and  $p = 0.09$ , respectively). When CAD status was added to the model, the significance of the results remained essentially the same except for a percentage increase in SIPE at shear rates of  $10,000 \text{ sec}^{-1}$  which was no longer significant ( $p = 0.13$ ). However, we believe that these results must be interpreted cautiously given that unrecognized CAD may have existed in several individuals with PAD.

## Discussion

In this exploratory analysis, we have described differences in platelet function in response to shear stress between patients with PAD and CAD. Although patients with CAD had a greater increase in platelet aggregation and activation (as measured by percent increases in shear-induced P-selectin expression) in response to *ex-vivo* shear at rates of  $5,000 \text{ sec}^{-1}$  and  $10,000 \text{ sec}^{-1}$ , platelets in PAD patients had an increased expression of baseline P-selectin, a marker of platelet activation. Previous studies have suggested that patients with PAD have worse outcomes when compared to those with coronary artery disease (CAD) [1-3]. The cause for these poor outcomes is largely related to the morbidity and mortality associated with the concomitant atherosclerotic involvement of other vascular beds (namely, from myocardial infarction and stroke).

Several biomarkers associated with inflammation such as CRP [7,18-21], interleukin-6 (IL-6) [18,20], monocyte chemoattractant protein-1 (MCP-1), [22] adhesion molecules [18,23], fibrinogen [20,24], and D-dimer [21,24] have been associated with worse outcomes in individuals with PAD; and in addition, levels of markers such as CRP have been reported to be further increased in patients with concomitant PAD and CAD when compared to those with CAD alone [7,8]. Although it is not clear whether the increased activity of pro-atherogenic pathways leads to increased atherosclerosis, consequently leading to PAD (diffuse atherosclerosis), or whether the increased activity of these pathways is secondary to the increased burden of atherosclerosis, it does seem that these pro-atherogenic pathways are more active in patients with PAD.

Enhanced platelet aggregation and activation have also been reported in patients with PAD [25-28]. Reininger et al described that patients with PAD have an increased spontaneous aggregation of platelets when compared to normal controls [25]. Now, we measured several parameters that globally tests platelet function, but focused primarily on platelet response to fluid shear stress (SIPA, SIPE, and PFA) and identified differences between patients with PAD when compared to those with CAD. The rationale for focusing on shear-induced platelet aggregation and activation is that increased shear stress, which is well known to affect platelet function [29], also occurs *in vivo*, in response to the alteration of the vessel wall and lumen, secondary to atherosclerosis. Furthermore, shear-induced platelet aggregation has previously been shown to predict acute coronary events and mortality [30]. As noted in our results, patients with CAD had increased platelet aggregation and P-selectin expression in response to *ex vivo* shear, as compared to those with PAD. However, baseline P-selectin expression was greater in PAD patients as compared to CAD patients. Together, the results suggest that platelets from patients with PAD may have already been partially activated *in vivo*, secondary to the constant exposure to the atherosclerosis-induced changes in profiles of shear stress and systemic inflammation.

However, the modest increase in P-selectin expression suggests that only a small percentage of the platelets were activated in the PAD patients, and therefore, platelet aggregation was not significantly affected in response to higher doses of agonists. On the other hand, even a small percentage of activated platelets could affect the von Willebrand factor cross-linking with platelets in response to fluid shear stress because the glycoprotein Ib receptor density is significantly less on activated platelets. A low receptor density, even if only in a smaller percentage of the platelets, is known to affect the multivalent receptor cross-linking induced by *ex vivo* fluid shear stress.

The activated platelets, in turn, release multiple cyto- and chemokines that can propagate atherosclerosis and alter the stability of atherosclerotic plaques. Supporting this hypothesis that the increased burden of atherosclerosis is associated with more platelet activation is a recent paper that suggested that the severity of PAD is associated with platelet activation. Rajagopalan et al [31] examined 182 patients with intermittent claudication or subcritical limb ischemia and measured platelet p-selectin expression and bound fibrinogen by flow cytometric analysis and platelet aggregation using rapid platelet function assay with arachidonic acid and thrombin receptor activation peptide as agonists and reported that as the severity of PAD increased (as estimated by the ABI), the platelet activation increased as well.

This possibility is consistent with the clinical observation that additional anti-platelet therapy with clopidogrel confers benefit for PAD patients using aspirin [9,10] but not for patients with stable CAD [10]. One explanation is that ADP, which activates platelets and is released from erythrocytes in response to systemic atherosclerosis in PAD patients, could play a greater role in PAD, and clopidogrel blocks platelet ADP receptors [32], thereby possibly conferring additional benefit in these patients.

It is important that further studies confirm the findings from this exploratory analysis in order to better understand the effect of atherosclerosis on platelets.

## Limitations

Several limitations must be acknowledged: Patients with CAD enrolled in the PGAP-2 study did not have symptomatic PAD. However, they did not have any ABI testing done and hence there may have been some undiagnosed, asymptomatic PAD in these patients. Similarly, although almost 50% of the patients with PAD had concomitant CAD, reflective of the higher burden of atherosclerosis in PAD patients, several patients with PAD may have had undiagnosed CAD. Studies[33] have suggested that almost 90% of patients with symptomatic



PAD (as enrolled in ELIMIT) have had coronary atherosclerosis on a pre-operative coronary angiogram. It is likely that more of our patients in the PAD group had concomitant CAD which had not yet become overtly manifested. However, it is highly likely that patients in our PAD group had more diffuse atherosclerosis and an increased burden of atherosclerosis when compared to those in our CAD group. The 2 groups differed with respect to statin use and prevalence of diabetes. Although these were adjusted for statistically, given the potential effect of these factors on platelet function these differences must be acknowledged. Further, we did not have information on other factors that may alter platelet activation such as exercise. The testing for PGAP-2 and ELIMIT while similar were not performed contemporaneously. Although P-selectin is the most widely used marker of platelet activation, there are other markers which may be more sensitive. Several quality control measures were however undertaken to ensure reproducibility of the measurements. It is also important to note that the platelet function testing was completed with a background of aspirin therapy as this is the standard of care in patients with PAD and CAD. It is of interest that differences in platelet function and activation were still present. Finally, we performed multiple statistical tests but did not adjust for the multiple testing as this was an exploratory analysis.

## Conclusion

In conclusion, although there were several similarities in platelet function between men with symptomatic PAD when compared to those with CAD, significant differences in platelet response to shear stress were also noted. Additional studies will help us better characterize these important differences with potential clinical consequences.

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**Table I**

## Baseline characteristics

	<b>ELIMIT (PAD) (n=29)</b>	<b>PGAP 2 (CAD) (n=40)</b>	<b>P value</b>
Age (years)	62.5	61.8	0.72
Hypertension	83%	68%	0.18
Diabetes	41%	18%	0.05
Tobacco	52%	60%	0.62
Statins	79%	100%	0.004
Aspirin ( <b>mean daily dose, mg</b> ) *	100% ( <b>260.5</b> )	100% ( <b>270.1</b> )	
CAD	52%	100%	<0.001

\* The mean daily dose for aspirin was 270.1 mg in the CAD group, and 260.5 mg in the PAD group. In the CAD group, 77.5% of the individuals were on 325 mg, and 22.5% of the individuals were on 81 mg. In the PAD group, 72.4%, 24.1%, 3.5% were on, respectively, 325 mg, 81 mg, and 162 mg of aspirin daily.

**Table II**

Platelet aggregation with PFA-100 and optical aggregometer

	<b>PAD</b>	<b>CAD</b>	<b>P value</b>
PFA-100 Epinephrine-induced (closure time: seconds) ( $\pm$ SD) (*)	219.31 $\pm$ 86.20	210.74 $\pm$ 74.61	0.59
PFA-100 ADP (closure time: seconds) ( $\pm$ SD) (*)	98.55 $\pm$ 38.9	85.56 $\pm$ 12.8	0.25

PAD: Peripheral arterial disease; CAD: coronary artery disease; SD: standard deviation; PFA: platelet function analyzer

(\*) Models adjusted for age, diabetes history, aspirin dose and statin use

**Table III**

Shear-induced platelet aggregation (SIPA) and shear-induced P-selectin expression (SIPE) and percentage increases from baseline in SIPA and SIPE measured at shear rates of 5,000 sec<sup>-1</sup> and 10,000 sec<sup>-1</sup>

	<b>PAD</b>	<b>CAD</b>	<b>P-value</b>
SIPA 5,000 sec <sup>-1</sup> (10 <sup>1</sup> /μL) (± SD) (*)	12,083 ± 2920	7,706 ± 3,876	<0.0001
SIPA % aggregation 5,000 sec <sup>-1</sup> (%) (± SD) (*)	44.39 ± 14.0%	66.0 ± 17.4%	<0.0001
SIPA 10,000 sec <sup>-1</sup> (10 <sup>1</sup> /μL) (± SD) (*)	3,638 ± 1,870	1,961 ± 1,064	<0.0001
SIPA % aggregation 10,000 sec <sup>-1</sup> (%) (± SD) (*)	83.2 ± 9.24%	91.39 ± 4.68%	<0.0001
SIPE 0 (MFI) (± SD) (*)	2.93 ± 1.37	1.94 ± 0.67	0.01
SIPE 5,000 sec <sup>-1</sup> (MFI) (± SD) (*)	3.60 ± 1.63	3.97 ± 1.58	0.11
SIPE P-S 5,000 sec <sup>-1</sup> (%) (± SD) (*)	25.4 ± 21.8%	107.1 ± 56.3%	<0.0001
SIPE 10,000 sec <sup>-1</sup> (MFI) (± SD) (*)	7.07 ± 1.79	7.29 ± 3.41	0.96
SIPE P-S (± SD) 10,000 sec <sup>-1</sup> (%) (*)	176 ± 112.3%	292.9 ± 171.6%	0.03

PAD: peripheral arterial disease; CAD: coronary artery disease; SD: standard deviation; SIPA: shear-induced platelet aggregation; SIPE: shear-induced P-selectin expression; MFI: mean fluorescence intensity

(\*) Models adjusted for age, diabetes history, aspirin dose and statin use

**Table IV**

Platelet aggregation mediated by epinephrine and arachidonic acid

	<b>PAD</b>	<b>CAD</b>	<b>P-value</b>
Spontaneous aggregation ( $\pm$ SD) (*)	8.72 $\pm$ 5.21	7.92 $\pm$ 4.75	0.68
Arachidonic acid-induced aggregation ( $\pm$ SD) (*)	14.72 $\pm$ 23.5	6.72 $\pm$ 11.1	0.17
Epinephrine-induced aggregation ( $\pm$ SD) (*)	59.07 $\pm$ 24.1	50.42 $\pm$ 12.9	0.09

PAD: Peripheral arterial disease; CAD: coronary artery disease; SD: standard deviation;

(\*) Models adjusted for age, diabetes history, aspirin dose and statin use