THE EFFECTS OF PHYSIOLOGICALLY RELEVANT SHEAR STRESS AND PLATELET-ENDOTHELIAL CELL INTERACTION ON PLATELET ACTIVATION AND PLATELET MICROPARTICLE GENERATION

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THE EFFECTS OF PHYSIOLOGICALLY RELEVANT

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.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

1.1 Objective 2

II. BACKGROUND 4

2.1 Platelets 4
2.2 Shear induced platelet activation 9
2.2.1 P-selectin expression 12
2.2.2 GPIbα expression 13
2.3 Platelet Microparticles (PMP) 16
2.3.1 PMP physiology 16
2.3.2 Effect of shear stresses on PMP generation 16
2.3.3 Role of PMP in disease progression 18
2.4 Endothelial cell (EC) 19
2.5 Platelet and Endothelial cell (EC) interaction under shear stress 20
2.6 Coronary circulation 22

III. MATERIALS AND METHODS 25

3.1 Shear stress 25
3.1.1 Cone-Plate shearing device 25
3.1.2 Platelet Shear stress 28
3.1.2.1 Constant shear stress 28
3.1.2.2 Dynamic shear stress 28
3.2 Materials 30
3.2.1 Buffers used 30
3.2.2 Washed Platelet 31
3.2.3 Endothelial Cells (EC) 31
3.3 Platelet activation 32
3.3.1 P-selectin expression 32
3.3.2 GPIbα expression 33
3.3.3 Mathematical modeling 33

Chapter Page

3.4 Platelet microparticle (PMP) generation....34
3.5 Endothelial cell (EC) and platelet interaction....35
3.5.1 Platelet activation....36
3.5.2 PMP generation....36
3.6 Statistical Analysis....36

IV. RESULTS....38

4.1 Platelet activation in absence of EC....38
4.1.1 P-selectin expression....38
4.1.1.1 Constant shear stress....38
4.1.1.2 Dynamic shear stress....45
4.1.1.3 Mathematical model of P-selectin expression....47
4.1.1.4 Mathematical model validation....49
4.1.2 GPIbα expression....51
4.1.2.1 Constant shear stress....51
4.1.2.2 Dynamic shear stress....56
4.1.2.3 Mathematical model of GPIbα expression....59
4.1.2.4 Mathematical model validation....61
4.2 Platelet microparticle (PMP) generation in absence of EC....63
4.2.1 Constant shear stress....63
4.2.2 Dynamic shear stress....66
4.3 Platelet activation in presence of EC....67
4.3.1 P-selectin expression under dynamic shear stress....67
4.3.2 GPIbα expression under dynamic shear stress....72
4.4 PMP generation in the presence of untreated EC....77
4.5 PMP generation in the presence of TNF-α treated EC....78

V. DISCUSSION....79

VI. CONCLUSION....87

REFERENCES....89

APPENDIX....101

LIST OF TABLES

Table Page

4.1 Summary of P value (two-way ANOVA) for P-slectin. 40

4.2 Combined effect of shear stress magnitude and shear exposure time on P-selectin expression 41

4.3 Rate of change in P-selectin expression...43

4.4 Mathematical model validation (P-selectin expression)....................................50

4.5 Summary of the statistically significant P values (two-way ANOVA) for GPIbα expression 52

4.6 Combined effect of shear stress magnitude and shear exposure time on GPIbα expression 54

4.7 Mathematical model validation (GPIbα expression) 62

4.8 Effect of cell interaction on platelet activation (CD62P expression). 71

4.9 Effect of shear stress on P-selectin expression. 71

4.10 Effect of shear exposure time on surface P-selectin expression. 71

4.11 Effect of cell interaction on GPIbα expression. 76

4.12 Effect of shear stress on surface GPIbα expression. 76

4.13 Effect of shear exposure time on GPIbα expression. 76

LIST OF FIGURES

Figure Page

2.1 Platelet dependent thrombus formation 6

2.2 Coagulation cascade representating intrinsic and extrinsic pathway....................8

2.3 Effect of shear stress and shear exposure time on red blood cells (hemolysis) and platelet activation 12

2.4 Structure of GPIb-IX-V complex...15

2.5 Human heart with coronary arteries...24

3.1 Schematic diagram of the cone-plate shearing device.......................................26

3.2 Hemodynamic single cone-plate shearing device..27

3.3 Shear stress history of platelets passing through the left coronary artery...........29

3.4 Pseudocolor scatter plots from flow cytometry showing different gates............35

4.1 P-selectin expression by activated platelets under constant shear stress 39

4.2 Flow cytometry plots (P-selectin) under constant shear stress 44

4.3 P-selectin expression by activated platelets under dynamic shear stress............46

4.4 Flow cytometry plot (P-selectin) under dynamic shear stress 47

4.5 Mathematical model of surface P-selectin expression.......................................48

4.6 Representation of P-selectin expression at different shear stress- exposure time 49

Figure Page

4.7 GPIbα expression by activated platelets under constant shear stress 52

4.8 Flow cytometry plots (GPIbα) under constant shear stress 55

4.9 GPIbα expression by activated platelets under dynamic shear stress 57

4.10 Flow cytometry plot (GPIbα) under dynamic shear stress 58

4.11 Mathematical model of GPIbα expression...60

4.12 Representation of GPIbα expression at different shear stress- exposure time 61

4.13 Fluorescence histogram of PMP population 64

4.14 PMP generation under constant shear stress 65

4.15 PMP generation under dynamic shear stress 66

4.16 Comparison of P-selectin expression of platelet, platelet with untreated EC, and platelet with treated EC under normal shear stress 68

4.17 Comparison of P-selectin expression of platelet, platelet with untreated EC, and platelet with treated EC under recirculation shear stress 69

4.18 Comparison of P-selectin expression of platelet, platelet with untreated EC, and platelet with treated EC under stenosis shear stress......................................70

4.19 Comparison of GPIbα expression of platelet, platelet with untreated EC, and platelet with treated EC under normal shear stress 73

4.20 Comparison of GPIbα expression of platelet, platelet with untreated EC, and platelet with treated EC under recirculation shear stress 74

4.21 Comparison of GPIbα expression of platelet, platelet with untreated EC, and platelet with treated EC under stenosis shear stress......................................75

4.22 PMP generation by platelets interacting with untreated EC under dynamic shear stresses 77

4.23 PMP generation by platelets interacting with treated EC under dynamic shear stresses 78

CHAPTER I

INTRODUCTION

Cardiovascular diseases (CVD) cause the most death all over the world. In 2006, approximately 81.1 million people in the USA suffered from different forms of CVD and around 51% of total death (by CVD) was due to coronary heart diseases (CHD)[1].

CHD is characterized by the narrowing of coronary blood vessels that hinders supply of oxygen rich blood to the cardiac muscle mass. Insufficient or complete blockage of blood supply may cause death of the cardiac tissues in the downstream regions of the blockage and lead to myocardial infarction (MI). One of the main reasons behind MI and most CVD is the localization of atherosclerosis, which can be detrimental to the vasculature and can promote formation of acute thrombus. Atherosclerosis is a common vascular disease, resulting in the formation of lesions/occlusions in the blood vessels, and improper functioning of the endothelial cells (EC). Formation and localization of atherosclerotic plaque in coronary arteries can reduce the lumen diameter, and disturb the local flow conditions, which may further induce EC activation, increase the sub-endothelial migration of lipids and other plaque substances, and lead to the growth and rupture of the atheromatous plaque.

Numerous studies have been conducted in the past to analyze the effects of disturbed shear stress on platelets and EC. However, most of those studies were limited to applying constant shear stresses, which did not represent physiological conditions very well. Moreover, those studies investigated the effects of shear stress on platelets and EC separately. In the current study, physiologically relevant dynamic shear stress was used to study platelet activation and platelet microparticle (PMP) generation, as well as the effects of platelet-EC interactions on these platelet responses. It would help us to better understand the role of disturbed shear stress in platelet activation and PMP generation (as increased number of PMP are observed in the blood circulation under different CVD diseases), and their role in the pathogenesis of cardiovascular diseases.

1.1 Objective

The goal of this study was to **investigate the effects of physiologically relevant shear stresses and platelet-endothelial cell interactions on platelet activation, and platelet microparticle (PMP) generation.**

Global Hypothesis: Altered shear stress induced by disturbed blood flow can activate platelets and EC and promote their interactions, leading to the pathogenesis of various cardiovascular diseases.

The specific aims of this study are as follows:

Specific aim 1: To expose platelets to constant and physiologically relevant dynamic shear stress *in vitro* in a cone-plate shearing device to investigate the effects of altered shear stress on platelet activation and PMP generation. Post shear exposure, platelet activation (based on P-selectin

and GPIbα expression) and platelet microparticle (PMP) generation were quantified.

Specific aim 2: To study the effects of physiologically relevant dynamic shear stress on platelet activation and PMP generation in the presence of normal or damaged EC (activated by cytokine). Platelets were sheared in the presence of normal or activated EC (TNF-α treated) in a cone-plate shearing device. After shearing, platelet activation (based on P-selectin and GPIbα expression) and PMP generation were measured.

CHAPTER II

BACKGROUND

2.1 Platelets

Platelets are small anucleate cells that circulate in blood. They are discoid in shape with a dimension of approximately 4 μm in diameter and 0.5 μm in thickness. Platelets are produced by megakaryocytes in bone marrow. Their average lifespan ranges from 10 to 12 days and the physiological concentration is between 150,000/μl to 400,000/μl in human plasma. Platelets play important roles in coagulation and hemostasis.

Resting platelets have a sturdy internal tri-laminate cytoskeletal membrane composed of a bilayer of phospholipids embedded with cholesterol, glycolipids, and a number of glycoproteins[2]. This cytoskeleton gives platelets a discoid shape, which helps platelets to adapt to various shear forces generated by the blood flow. In their resting state, platelets have negatively charged phospholipids inside their membrane. Translocation of these phospholipids to their outer surface is one of the major platelet responses during activation, resulting in a negatively charged surface, which promotes

coagulation. Two important organelles of platelets are the dense bodies and α-granules. Dense bodies have high concentration of calcium (Ca^{2+}), serotonin, ADP/ATP that are released upon activation. Release of dense granules acts as an important positive feedback mechanism for platelet activation and aggregation. α-granules are the most abundant granules in platelets. There are about 50 to 80 α-granules per platelet[3]. They contain von Willebrand Factors (vWF), fibronectin, platelet derived growth factor (PDGF), Factor V, and P-selectin. Upon activation, platelets release α-granules, increasing the expression of P-selectin (a glycoprotein) on their surface. Hence, P-selectin is one of the most commonly used activation marker. Platelet membrane also contains a number of glycoproteins including GPIb-IX-V, GPIIb/IIIa, GPV, and GPVI. GPIb-IX-V plays an important role in the initial phase of platelet-vessel wall interaction, which eventually leads to platelet activation. After activation, platelets tend to form aggregates by interacting with other circulating platelets through a surface glycoprotein GPIIb/IIIa.

Platelets can be activated by both biochemical and mechanical agonists. Activation can also occur when platelets come in contact with any foreign surface such as mechanical heart valves or artificial pacemakers. The major biochemical agonists include ADP, thrombin, thromboxane A_2 (TxA_2), serotonin, and collagen. Blood flow induced mechanical shear stress, especially the disturbed shear stress commonly found near the sites of arterial narrowing due to atherosclerosis, can be regarded as a mechanical agonist to activate platelets[4].

Activated platelets play major roles in hemostasis and thrombosis. Hemostasis deals with prevention of blood loss during a vascular injury by forming a platelet plug.

Hemostasis not only means blood-clotting process (coagulation), but also involves the clot dissolution and damaged tissue repairing. Thrombosis is an unregulated hemostatic reaction resulting in the formation of blood clot inside a diseased blood vessel (Figure 2.1). In both hemostasis and thrombosis, the three major platelet responses involved are activation, adhesion, and aggregation.

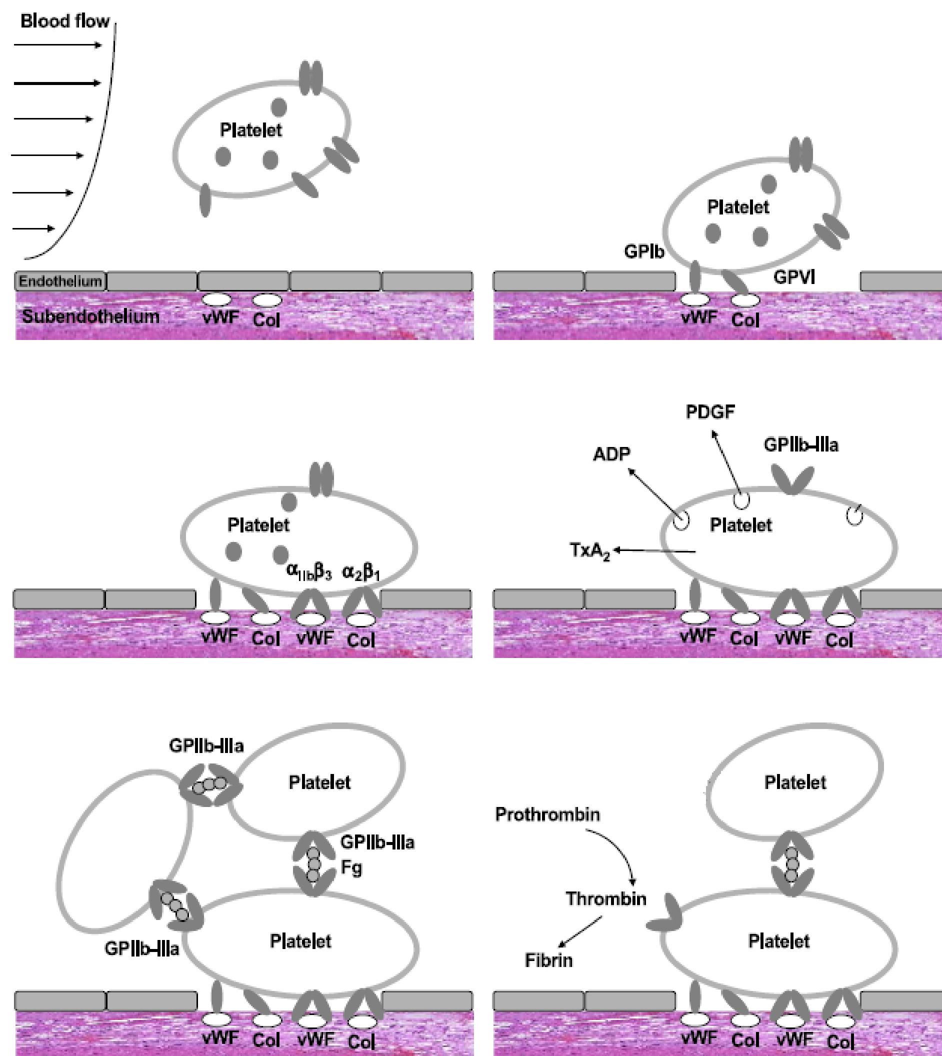

Figure 2.1: Platelet dependent thrombus formation[5].

Platelets arrive at the injured vascular site and initiate adhesion. Blood flow induced shear stress can govern platelet adhesion. Under high shear conditions, plasma vWF associates with collagen of the exposed sub-endothelium surface to act as a substrate for platelet adhesion. vWF multimers first interact with the GPIb-IX-V receptors of platelets to capture them[6]. GPIb-IX-V enables activated platelets to roll and tether on damaged endothelium surface via endothelial bound P-selectin[7]. At low shear stresses, circulating platelets adhere to collagen through GPIa/IIa receptor[8]. This initial adhesion to the vessel wall leads to platelet activation.

Upon activation platelets undergo shape change, phospholipid translocation, calcium (Ca^{2+}) and α-granule secretion. Platelet shape change includes formation of pseudopods that increases the surface contact area. In addition, platelet activation promotes translocation of phospholipids, especially phosphatidylserine, to the outer membrane. These negatively charged phospholipids help to bind Ca^{2+} and some specific proteins of the clotting system. ADP further accelerates platelet aggregation process; vWF and fibrinogen help in adhesion and aggregation. Release of both α-granules and dense granules, induce a positive feedback mechanism to activate more platelets.

Initial adhesion also results in integrin GPIIb/IIIa (fibrinogen receptor) and $\alpha_2\beta_1$ (collagen receptor) receptors activation. Interaction of these two receptors with extracellular matrix proteins stabilizes adhesion and initiates aggregation. Activated GPIIb/IIIa enables activated platelets to bind to fibrinogen[9]. Fibrinogen is a dimeric molecule, which has two GPIIb/IIIa binding sites. Through GPIIb/IIIa, activated platelets recruit more platelets, adhere to each other, and form a platelet plug.

Coagulation cascade (Figure 2.2), triggered during vascular damage, involves the interaction of a number of coagulation factors and results in the formation of insoluble fibrin to prevent blood loss.

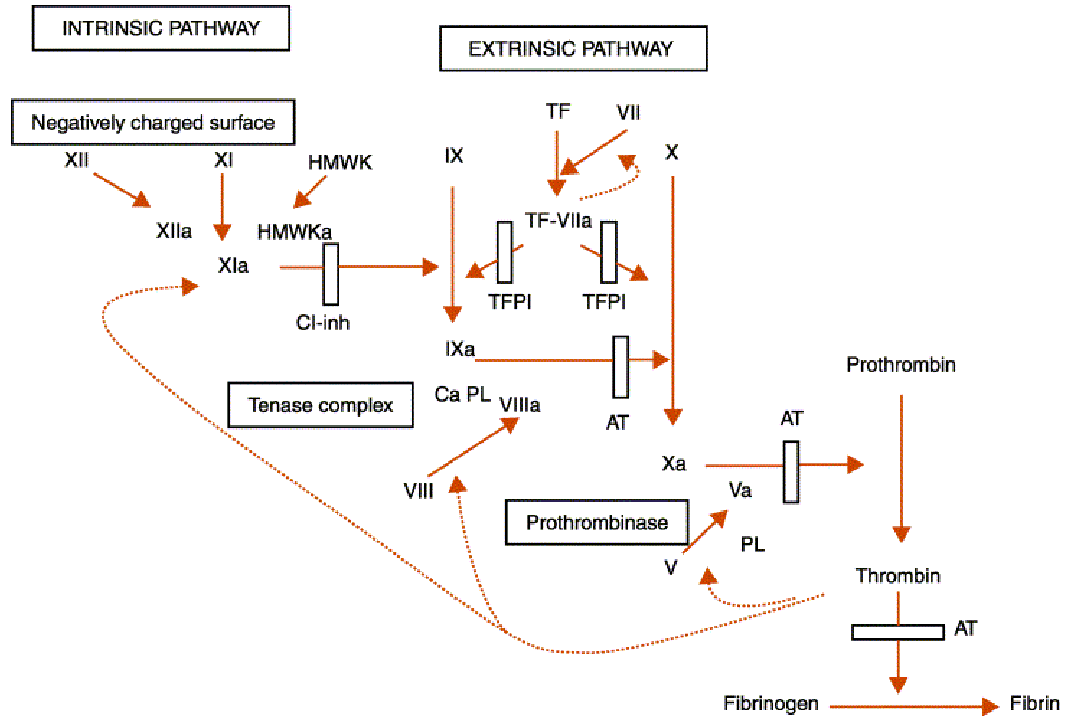

Figure 2.2: Coagulation cascade representating intrinsic and extrinsic pathway.

Coagulation phase consists of two different pathways: the extrinsic (tissue factor pathway) and the intrinsic (contact activation pathway). A damaged vascular wall triggers the extrinsic pathway by exposing sub-endothelial tissue factor (TF), which combines with Factor VII in plasma (F-VII) and forms the TF-VIIa complex. The TF-VIIa complex converts FX (clotting factor) into FXa. On the other hand, the intrinsic pathway is triggered by the formation of a complex on collagen by high molecular weight kininogen (HMWK) and FXII. FXIIa can convert FXI into FXIa which further activates FIX. This (FIX) leads to the activation of FX into FXa. FXa, from both intrinsic and extrinsic

pathway, can convert prothrombin (F-II) into thrombin. Thrombin is responsible for the conversion of soluble fibrinogen into insoluble fibrin. It is also a strong positive agonist for platelet activation. As the insoluble fibrin network forms, it can trap passing blood cells and additional platelets preventing blood loss at the site of injury thereby maintaining hemostasis.

Coagulation is a highly regulated process. However, when the activated cascade overwhelms normal regulatory controls, thrombus formation is no more contained and localized to the site of injury, which can lead to the pathogenic thrombosis condition. Thrombosis initiates with platelet activation (by biochemical agonists or mechanical agonists). Platelets are highly susceptible to hemodynamic shear forces. A variation in shear stress can act as a potent agonist for platelet activation, which can lead to thrombus formation. Unstable thrombi can take off from the blood vessel wall, forming a free emboli. Circulating emboli can get trapped and completely block narrowing small blood vessel leading to other clinical complications including stroke, myocardial infarction, pulmonary embolism, and deep vein thrombosis.

2.2 Shear induced platelet activation

Mechanical forces induced by blood flow (physiological and pathological) can affect platelet function. The most relevant mechanical force is hemodynamic shear stress, especially altered shear stress induced by cardiovascular diseases. In the human circulatory system, blood flow induces shear stress on both the blood vessel wall and the circulating blood cells. Shear stress distribution is majorly governed by the geometrical complexities of the vasculature and the arterial lumen diameter. This results in a varying

shear stress distribution at different arterial sections, especially under pathological conditions.

Shear stress level usually varies in between 10 to 30 dyne/cm^2 in a healthy normal coronary artery, whereas shear stress below 10 dyne/cm^2 usually characterizes low shear stress commonly found inside a recirculation zone and flow stagnation points[10]. Shear level can reach as high as 350 dyne/cm^2 in stenosed coronary arteries[11]. Numerous studies have been conducted to investigate the effect of these altered shear stresses on platelet activation[12;13]. To study the effect of constant high shear, Brown *et al*. exposed platelets to shear stress of 50, 100, and 250 dyne/cm^2 in a cone-plate shearing device. Their results demonstrated an elevated level of platelet activation along with aggregate formation when exposed to a shear stress greater than 50 dyne/cm^2. In addition, they also reported that platelets were lysed when exposed to 100 dyne/cm^2, and a shear of 250 dyne/cm^2 induced platelet fragmentation[14]. Hellums *et al*. have shown an increment in platelet activation by measuring intracellular Ca^{2+} concentration at shear stress level of 30, 60, 90, and 120 dyne/cm^2 for an exposure time of 60 s[4]. Sakariassen's group used a higher shear stress (315 dyne/cm^2 for 0.075 s) to show significant increase in activation of platelets[15]. Low shear stress level can also induce platelet activation. A recent study conducted by Yin *et al*. shows enhanced platelet activation by exposing platelets to low shear stress of 2.4 and 9 dyne/cm^2 for 30 min. This study summarized that shear stress at low level can induce platelet activation with elongated exposure time.

Recent studies have focused on investigation of platelet activation when they are exposed to dynamic shear stress conditions. Bluestein *et al*. exposed platelets initially to a series of high shear stress with magnitudes of 50, 60, 70 dyne/cm^2 for 40 s followed by

an exposure to a low shear of 1 dyne/cm^2 for around 14 min. Interestingly, platelets exposed to a combination of high and low shear were more activated than those that were exposed only to low shear. High shear stress exposed platelets, though only for a very short duration, were more sensitized. That is why, a subsequent exposure to low shear triggered 20 times faster platelet activation[16]. This may also be due to the fact that activated platelets interact with other quiescent platelets in the vicinity.

In addition to the magnitude of shear stress, shear exposure time also plays an important role in platelet activation. Ramstack *et al.* established that platelet stimulation is a nonlinear phenomenon. They exposed platelets to 300, 750, 1000 dyne/cm^2 for an average time of 25-1650 msec and have shown that larger shear stress induced significant platelet activation at shorter time duration[17]. Hellums *et al*. have shown that at any particular shear stress magnitude, a minimum time of exposure was required to induce activation. They termed that minimum exposure time as activation threshold[4]. Wurzinger *et al*. have shown in their study that at least 7 msec is necessary at a shear stress magnitude of 170 dyne/cm^2 to instigate platelet activation[18]. To give a time estimate of initiation of platelet activation, Hellums *et al*. summarized the results of previous researchers in a shear stress vs. exposure time plot that depicts the combined effect of constant shear stress and shear exposure time on platelet activation (shown in Fig 2.3)[4].

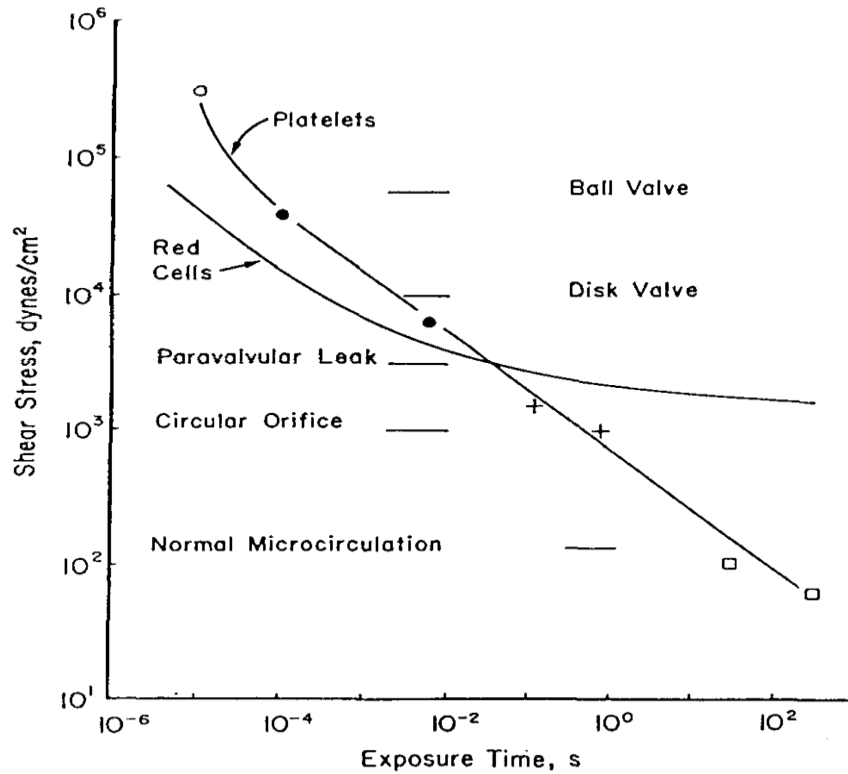

Fig 2.3: Effect of shear stress and shear exposure time on red cells (hemolysis) and platelet activation (measured as a function of shear-related serotonin release). This is a summary of several researchers work using various devices.

All these studies summarized that high shear or low shear or a combination of both can lead to platelet activation. However, not many investigations have been carried out to examine the combined effect of shear stress magnitude and shear exposure duration on platelet activation. Recently, Rubenstein *et al.* quantified the combined effect of shear stress and exposure time on platelet activation and defined a new parameter - "shear stress-exposure time"[19]. The results from that study indicated that platelets that were exposed to waveforms with similar shear-exposure time, were activated to the same level. This indicated that shear stress-exposure time may be a good parameter to predict platelet activation.

Shear induced activation results in the expression of a number of glycoproteins such as P-selectin, GPIbα, GPIIb on the platelet membrane. Quantification of these proteins is very important as increased number of activated platelets in the circulation system can perpetuate many cardiovascular diseases like myocardial infarction and stroke[20;21]. Most cardiovascular diseases, act as a positive feedback mechanism, can further enhance platelet activation. Platelet activation can be measured *in vitro* using different methods including platelet activated state (PAS) assay, enzyme linked immunosorbent assay (ELISA), and flow cytometry[22-24]. PAS assay enables probing the activated state of platelet by measuring the amount of thrombin generation, while ELISA and flow cytometry are majorly used to measure surface protein expression.

2.2.1 P-selectin expression

P-selectin is a 140 kDa glycoprotein evenly distributed in the platelet membrane[25]. The platelet α-granules contain P-selectin on their membrane[26]. Upon activation, platelets express these P-selectin on their membrane due to the fusion of α-granules[27]. Therefore, P-selectin is usually considered as a major activation marker[28].

The major role of P-selectin is to mediate platelet rolling on the endothelium[29;30]. Endothelial cells express P-selectin glycoprotein ligand 1 (PSGL-1) on their surface, a receptor for P-selectin that assists in the rolling process[31]. Other clinical investigations have shown that, P-selectin also plays important roles in thrombogenesis by assisting in the formation of large stable aggregates[32;33]. P-selectin also mediates inflammatory responses during cardiovascular diseases.

Due to its significance in propagation of cardiovascular diseases, a number of studies have been conducted to quantify the amount of P-selectin expression on platelets under different shear stress conditions. Significant P-selectin expression was detected by Goto *et al*. after exposing platelets to the shear rate of 10,800 s^{-1} [34]. To study the effect of different shear stress level on P-selectin expression, Lu *et al*. included four different shear conditions of 0, 100, 150, 200 dyne/cm^2 for an exposure time of 120 s. Their study had shown an increased P-selectin expression with increase of shear stress level[35]. Zhang *et al.,* after exposing platelets at 100 dyne/cm^2 for less than 10 - 20 s, concluded that high shear stress for shorter duration was not sufficient to cause significant P-selectin expression[36]. Results from their study established that platelet activation not only depends on the magnitude of shear stress but also on shear exposure duration. Yin *et al*. used 2.4 and 9 dyne/cm^2 constant shear stress to stimulate platelets for 30 min, and found that both shear stress conditions were able to induce changes in platelet surface P-selectin expression[37]. However, little work has been done to examine platelet activation and associated P-selectin expression under physiologically relevant dynamic shear conditions.

2.2.2 GPIbα expression

GPIbα is a platelet surface glycoprotein with a molecular weight of approximately 170 kDa[38]. It is a key functional component in the GPIb-IX-V complex, which is involved in platelet aggregate formation under high shear stress. The other components in GPIb-IX-V complex (Figure 2.4) include GPIbβ, GPIX and GPV, which facilitate in firm adhesion of platelets.

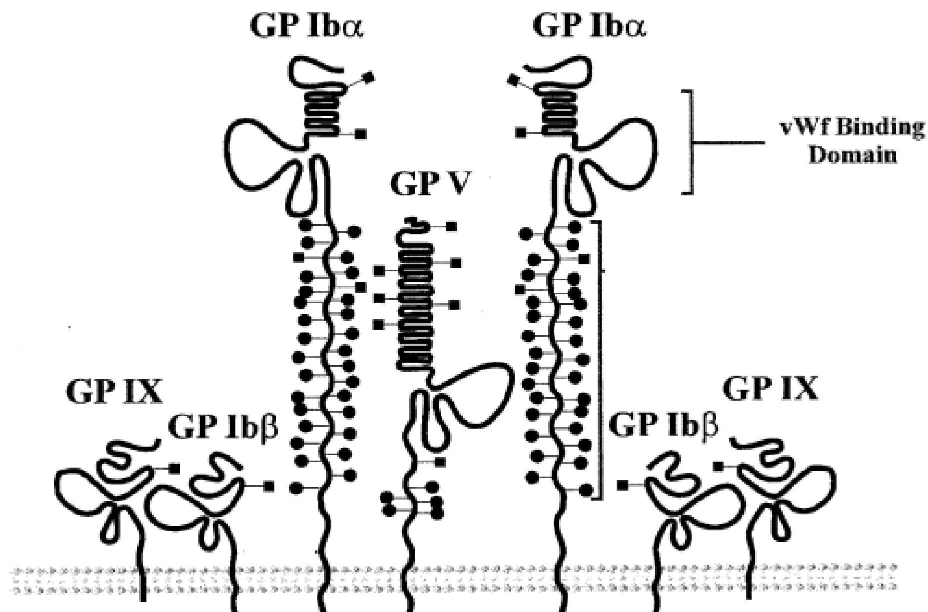

Fig 2.4: Structure of GPIb-IX-V complex[39].

GPIbα is the main binding domain of the GPIb-IX-V complex. There are about 25,000 GPIbα molecules expressed on resting platelet surface[40]. The major function of GPIbα is to interact with the immobilized vWF of the plasma or sub-endothelium matrix under high shear conditions. This interaction enables platelet to roll on the sites of vascular injury, reduces their velocity, and increases the time of interaction, thus facilitating in a firm adhesion[41]. In addition, GPIbα binds to its counter-receptor P-selectin, expressed on activated platelets and endothelial cells[42]. Also, this glycoprotein acts as a high affinity binding site for thrombin[43]. Upon binding, GPIbα undergoes conformational structural changes and promote platelet activation[44]. The other major response to this signal is to activate GPIIb/IIIa, which can bind to fibrinogen or vWF and promote platelet aggregation under pathological conditions[45;46].

Shear stress acts as a potent agonist on GPIbα activation. Therefore, a lot of research has been carried out so far to study the effect of shear stress on the expression of GPIbα on platelet surface. Leytin *et al*. ,using different shear stress of 0, 10, 44, 117 dyne/cm^2 for 90 s, showed that GPIbα expression remained unchanged in response to those shear stresses[47]. However, high shear stresses of 204, 388 dyne/cm^2 for the same duration (90 s) caused a significant down regulation of GPIbα. Another study by Li *et al*. also found no significant change in the expression of GPIbα at relatively low shear rates (100, 150, 1000 s^{-1}). But significant changes were observed at high shear rate (3000 s^{-1} for 7 min exposure)[48]. At this high shear rate, they observed an increased level of GPIbα for the first 1 min, which gradually decreased with time. To relate the modulation of GPIbα expression to platelet response, Chow *et al*. added vWF exogenously to platelets and applied shear stress at 30, 60, 90, and 120 dyne/cm^2 for 2 min. This addition significantly increased the binding of vWF to GPIbα as shear stress increased[49]. Results were further verified by using aurin tricarboxylic acid (ATA) to inhibit the binding of vWF to GPIbα, which resulted in a complete inhibition of platelet aggregation. This indicates that GPIbα is absolutely required for initial adhesion and subsequent aggregation[50]. Though GPIbα has crucial contribution in platelet responses, not many studies have been conducted to study the effect of physiologically relevant dynamic shear stresses on modulation of platelet GPIbα expression.

2.3 Platelet Microparticles (PMP)

Platelet microparticles (PMP) are the most abundant microparticles found in blood circulation[51]. They were first discovered by Wolf *et al*. who described them as "platelet dust" due to their size[52]. Compared to platelets, they are very small and their size ranges between 0.1-1μm. PMP are mainly produced by activated platelets through membrane shedding. However, apoptosis can also generate PMP[53;54].

2.3.1 PMP physiology

PMP have negatively charged phospholipids on their outer membrane and receptors for factor Va and Xa (coagulation factors)[55;56]. In Addition, they also express glycoproteins like GPIbα, GPIIb/IIIa, and P-selectin on their surface, similar to that of platelets[57;58]. PMP membrane possesses almost all the properties of platelet membrane.

2.3.2 Effect of shear stresses on PMP generation

A lot of research has been conducted to study effects of different physiological and pathological agonists on PMP generation. Sims *et al*., investigated PMP generation from different agonists including adenosine diphosphate (ADP), thrombin, collagen, and calcium ionophore (A23187)[59]. Their study revealed that all of these agonists enhanced PMP production; however, their potency level varied. Using some of these agonists and high shear stress (108 dyne/cm^2 for 6 min) on platelets, Nakamura *et al*. found an elevated PMP generation[60].

Occurrence of high shear stress is most common in diseased blood vessels (near the vessel narrowing), which may increase the probability of PMP generation. Tangential

shearing due to fluid forces on platelets can induce activation, leading to pseudopod formation from their surface membrane. Fracture of these pseudopods under elevated hydrodynamic drag can cause PMP generation[61]. Further, under high shear stresses, binding of GPIbα receptors on platelet surface to immobilized vWF can also induce PMP generation. The GPIbα binding triggers the opening of calcium channels, leading to increased calcium influx that activates calpain. Calpain activation then leads to PMP generation. The role of GPIbα binding in PMP generation was established by Pontiggia *et al*. in their recently published work where they blocked GPIbα with an inhibitor (monoclonal antibody, Mab Ib-23) and found 50% decrease in PMP formation[62].

Miyazaki *et al*. studied the effect of low and high shear stresses on PMP generation[63]. Initially, they exposed platelet rich plasma (PRP) to shear stress at 6 dyne/cm^2 for 15 s. This was followed by either 12 dyne/cm^2 (normal) or 108 dyne/cm^2 (high) exposure for 5 min. Within 30 s of exposure to normal or high shear, a significant increase in PMP generation was observed. Further, under high shear condition, the PMP generation continuously increased with time. To study the effect of high arterial shear stress of severely stenotic regions, Holme *et al*. exposed whole blood to a range of shear stress of 13, 80, 315 dyne/cm^2 for varying durations between 0.075 and 3.045 s. A significant increase in PMP release (5 to 15 fold increase compared to resting platelets) was observed from platelets that were exposed to 315 dyne/cm^2 (after 0.075 s of exposure)[64]. Nomura *et al*., investigated the combined effect of high shear stress (108 dyne/cm^2 for 5 min) and cytokines on PMP generation. They found that in the presence of high shear stress, cytokines (Interleukin 6, thrombopoietin) enhanced PMP generation[65]. Another study conducted by Nomura *et al.* indicated that high shear (108

dyne/cm^2 for 6 min) induced PMP have a positive feedback mechanism which can further mediate the release of inflammatory cytokines that contribute to the thrombus development[13]. However, most of these studies investigated only the effect of constant high shear stress on PMP generation, failing to quantify the effect of physiologically relevant shear stress.

2.3.3 Role of PMP in disease progression

PMP plays important roles in hemostasis and thrombus formation due to their procoagulant activity and ability to activate platelets and EC[66;67]. Compared to activated platelets, PMP exhibits 50-100 times higher procoagulation activities[57;68]. This is due to the presence of phosphatidylserine and concentrated coagulation proteins in PMP[69]. Sheriff *et al.*, generated PMP by exposing platelets initially to the shear stress of 70 dyne/cm^2 (high) for 40 s followed by 1 dyne/cm^2 (low) for 15 min. They measured the procoagulant activity, at the end of both high shear exposure and low shear, as a function of thrombin generation using PAS assay. Their results revealed that 50% of the total procoagulant activity was due to the generated PMP during shear and the rest was from the platelets[16]. Generated PMP can adhere to subendothelial matrix and promote further platelet adhesion in the presence of shear stress[70]. Using a rabbit model, Merten *et al.* found 3 to 5 fold increase in PMP adhesion in sites of vessel injury[71]. Activated platelets binding to these PMP also increased 4 fold in a dose-dependent manner.

Increased amount of PMP in blood circulation can perpetuate many diseases like transient ischemic attack, hypertension, aortic valve stenosis, and myocardial infarction[72-74]. On the other hand, significantly reduced levels of PMP can also be harmful as it can

lead to mild bleeding disorder[59]. That is why, it is highly essential to investigate the effect of physiologically relevant shear stress on PMP generation.

2.4 Endothelial cell (EC)

Endothelial cells are the cells that comprise the innermost lining of the blood vessels that play a major role in maintaining hemostasis. Under physiological conditions, EC are involved in maintaining vascular permeability, regulating transport of different substances across the vessel wall (such as nutrients), providing an atheroprotective surface, and forming new blood vessels (during wound healing or angiogenesis). However, EC activation or damage induced by vascular injury, presence of biochemical (such as cytokines) and biomechanical (such as shear stress) agonists, as well as interaction between EC and platelets can affect these normal activities of EC. Activated EC participate in thrombosis, atherosclerosis, and inflammation by secreting mediator proteins and adhesion molecules.

EC are directly exposed to blood flow and experience the tangential shear force induced by the flow on the vessel wall, which can greatly modulate their function. Arterial shear stress ($>15 dyne/cm^2$) promotes atheroprotective gene expression, whereas altered low shear stress ($<4 dyne/cm^2$) can promote athereogenic surface[12]. Shear stress also regulates the secretion of endothelium derived vasodilation factors like nitric oxide (NO) and prostacyclin (PGI_2) (inhibit platelet adhesion, activation, and aggregation)[75;76]. In addition, EC express tissue factor (TF), an inflammatory marker, which can trigger the coagulation cascade. The expression of TF is greatly altered by shear stress[77]. In a recent study by Yin *et al*., EC were exposed to three different pulsatile shear stress conditions -

Normal (varies between 0.05 and 1Pa), stenosis (varies between 0.1 and 6 Pa), and recirculation (between 0 and 0.4 Pa) shear for 30 min and TF expression was measured. Their results revealed that low shear induced a significant increase in TF, while high shear resulted in a moderate decrease in TF expression[78].

In addition to shear stress, a number of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interferon-γ (IFN-γ) can induce EC activation. Interestingly, these cytokines also act as a positive feedback mechanism to further enhance cytokine production by EC[79]. Nawroth *et al*. found incubating bovine aortic EC with TNF-α (synthesized by macrophages) enhanced procoagulant activity of EC, increased TF expression, and changed the atheroprotective EC layer to an atherogenic surface[80]. TNF-α is also associated with several cardiovascular diseases that can increase EC permeability and induce the secretion of platelet activating factor (PAF)[81-84].

2.5 Platelet and Endothelial cell (EC) interaction under shear stress

EC and platelets coexist in the blood circulation system and their interaction plays important roles in pathogenesis of thrombosis and atherosclerosis[85;86]. During a vascular injury, EC - platelet interaction triggers the activation and propagation of coagulation cascade, resulting in decreased blood loss and thereby maintaining hemostasis. However, under pathological conditions, the altered shear stress can induce both EC and platelet activation, which can significantly alter their interactions. Frenette *et al.* reported that activated platelets directly tether and roll on EC through P-selectin - PSGL-1 binding[29]. Massberg *et al*., during postischemic reperfusion, observed that platelets roll and adhere

firmly to EC surface of microvessels[87]. Roling of platelet P-selectin in promoting atherosclerosis was further established by Burger *et al.*[88]. A number of previous studies have established the relation of shear stress with platelet P-selectin and EC PSGL-1 expression, thereby relating the role of altered shear stress in EC - platelet interaction[31;89].

Furthermore, platelet adhesion to vessel wall is enhanced when EC become damaged and extracellular matrix proteins (like vWF, collagen) are exposed. Platelets can also adhere to EC through GPIbα-vWF (under high shear stress condition) and GPVI-collagen binding, which on the other hand can initiate platelet activation[90;91]. Exposing a monolayer of HUVEC to 2, 8, and 12 dynes/cm^2 for 6 and 15 hr, Galbusera *et al*. found significant increase in vWF release that can result in an increased GPIbα-vWF binding[92]. Secretion of PAF by activated EC can further accelerate platelet adhesion and activation. Activated platelet can generate PMP, which can also interact with EC. Macey *et al*. has shown that the interaction between flowing whole blood with TNF-α treated inflamed EC enhanced PMP generation[93]. Nomura *et al*. in their study showed that high shear stress (108 dyne/cm^2 for 5 min) activated platelets and generated PMP enhanced cytokines production and inflammatory gene expression in EC[13]. Factor Xa, which is present in activated platelets and PMP, was reported to induce cytokine production and adhesion molecule expression on EC[94]. Activated platelets can also induce inflammatory responses and procoagulant phenotype on EC[95;96]. Alternatively, they can contribute to wound healing and angiogenesis by releasing growth factors such as platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF)[97].

Yin *et al*. reported a significant increase in EC intercellular adhesion molecule-1 (ICAM-1) expression, a marker of EC activation and mediator in inflammation, in both

high (varies between 0.1 and 6 Pa) and low (between 0 and 0.4 Pa) shear stress[78]. In another study, Bombeli *et al*. revealed that the blocking of EC ICAM-1 resulted in significantly reduced platelet adhesion to EC[98]. Both studies suggested that platelet adhesion to EC can increase under both high and low shear stress.

2.6 Coronary circulation

Coronary arteries supply oxygen and nutrients to the cardiac muscles. Two main branches of the coronary artery (left and right) originate from the base of ascending aorta (shown in Fig 2.5) and branches out downward[99]. Coronary arteries are tethered to the surface of the heart and their complex vasculature induces highly disturbed blood flow conditions. They are highly susceptible to atherosclerotic lesions which can lead to coronary heart diseases (CHD). Some of the geometrical complexities like bifurcation, taper, twist, and the myocardial curvature induce complex flow patterns like skewness, separation and recirculation etc.

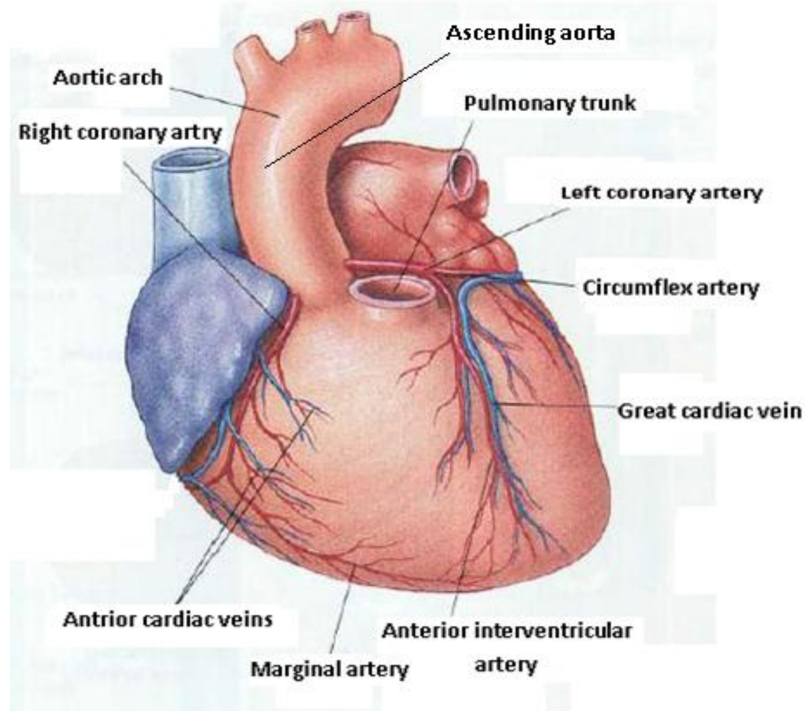

Fig 2.5: Human heart with coronary arteries[99].

Under physiological conditions, the geometry of vasculature along with the pulsatile nature of blood flow governs the variation in shear stress distribution. However, under diseased conditions, especially CHD, blood flow pattern may get further disturbed due to the presence of vascular occlusion. Platelets, traveling through the stenotic regions, may experience huge shear stress gradients that can activate them instantaneously. Also, after passing through a stenotic region, platelets may get trapped inside the recirculation zone where they are exposed to very low shear stress for a longer duration. This environment can also lead to platelet activation. Furthermore, inside the recirculation zone, platelets may have an elongated duration to interact with vascular EC promoting EC activation. As described before, the activation of both platelets and EC depends on both shear stress magnitude and time of exposure[100]

CHAPTER III

MATERIALS AND METHODS

3.1 Shear stress

In order to investigate the effect of shear stress on cell response *in vitro*, some of these hemodynamic shear conditions could be simulated using a cone-plate shearing device.

3.1.1 Cone-Plate shearing device

The hemodynamic cone-plate shearing device used in this study, was a modified design of the original cell shearing device designed by Blackman *et al.*[101]. Cone-plate device is capable of generating a uniform shear stress environment. The magnitude of shear stress generated is directly proportional to the rotational speed of the cone, which is controlled by a microstepper motor connected to a computer. The required shear stress profiles, corresponding to certain angular velocities, were programed through a BASIC program. Figure 3.1 represents a general schematic diagram of a cone-plate shearing device and Figure 3.2 depicts the cone and plate shearing device used for the current study.

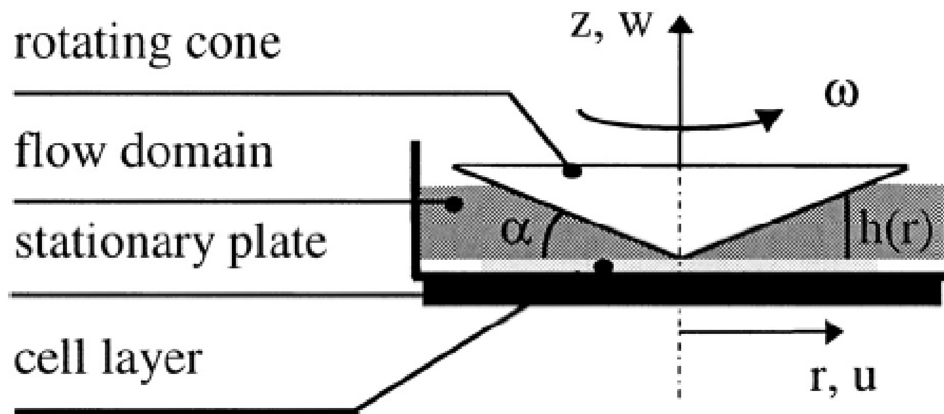

Fig 3.1: Schematic diagram of the cone-plate shearing device generating uniform shear stress on the flow domain and on the endothelial cell layer (figure obtained from Buschmann *et al.*[102]).

The cone was made of ultra-high molecular weight polyethylene (UHMWPE) and was directly connected to the motor. The angle of the cone was 2˚ and the diameter was 3.466 cm. All experiments were conducted in 60 mm (diameter) polypropylene petri dishes. Without the presence of endothelial cells, platelets were sheared at room temperature; with the presence of endothelial cells, platelets were sheared at 37°C.

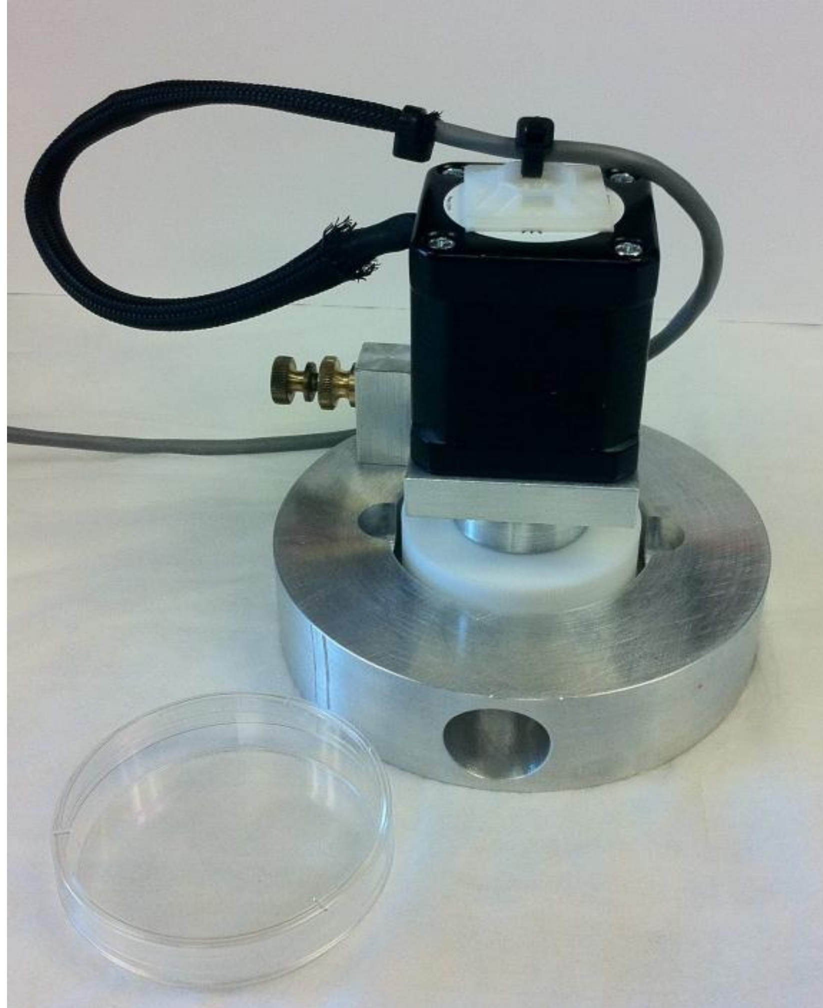

Fig 3.2: Hemodynamic single cone-plate shearing device used to shear platelets in the presence/absence of endothelial cells.

3.1.2 Platelet Shear stress

Platelets were exposed to two different types of shear stresses (constant and dynamic). After applying shear stress, both shear induced platelet activation and platelet microparticle (PMP) generation was measured.

3.1.2.1 Constant shear stress

Platelets were stimulated at four different constant shear stress (0.1, 0.3, 1, and 3 Pa) conditions. Shear stress levels were chosen so as to correlate the obtained results with the results from previous studies.

3.1.2.2 Dynamic shear stress

To investigate the effect of dynamic shear stresses on platelet activation, three different physiologically relevant shear stresses (normal, recirculation, and stenosis) were used. As shear stress could not be measured directly *in vivo*, numerical simulations were employed to compute shear stress distribution in a human left coronary artery. In one of the previous research work conducted in our lab, a 3D computational fluid dynamics model of a human left coronary artery was developed and the shear distribution inside the vessel was estimated under both physiological and pathological conditions. Normal shear, which varies between 0.1 and 1 Pa, mimics the physiological shear stress condition in a healthy artery. Recirculation shear stress (0.06 to 0.4 Pa) mimics the low unidirectional pathological shear stress that platelets are exposed to while they are trapped in a recirculation zone past a stenosis. Stenosis shear represents the shear stress applied to platelets while they pass through a 60% stenosis: platelets were exposed to elevated shear stress at 6.5 Pa for a duration of 0.1 sec, at a frequency of once every 90 sec; for the rest

of the time, platelets were exposed to normal pulsatile shear stress (0.1 to 1 Pa). Fig 3.3 depicts these three dynamic shear stress conditions.

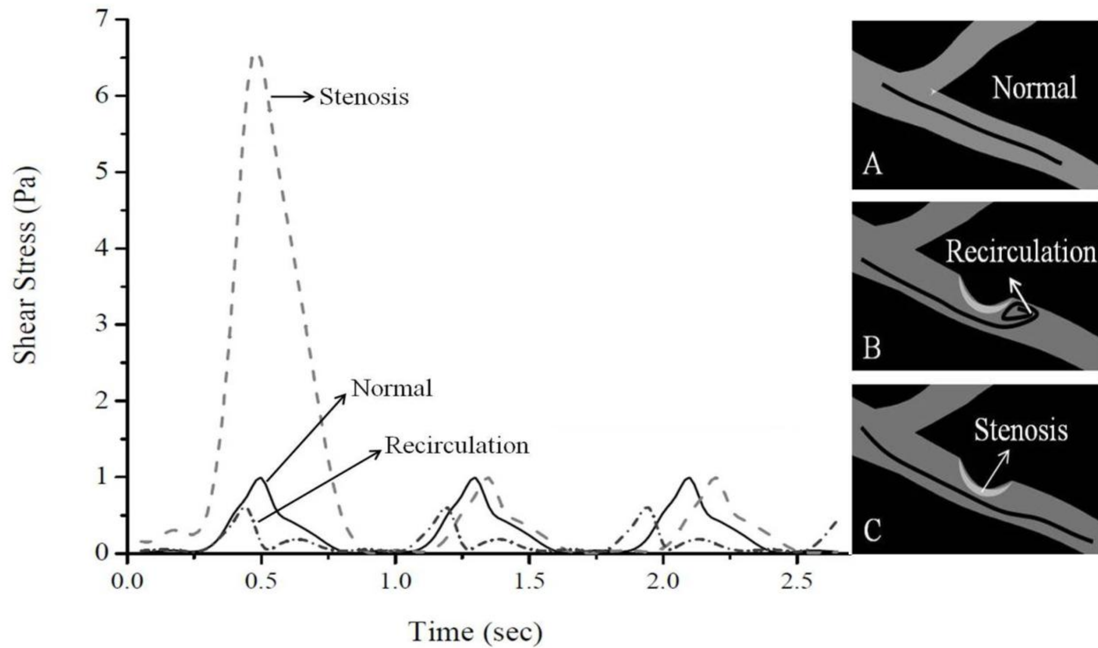

Fig 3.3: Shear stress history of platelets passing through the left coronary artery under physiological and pathological conditions. Insert A, B, C at the right side shows the corresponding trajectory of the passing platelet[78].

3.2 Materials

3.2.1 Buffers used

The different buffers used in this study are listed below:

1) HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) buffered modified Tyrode's solution (HBMT)

HBMT (final pH 7.4) contains 137 mM sodium chloride, 2.7 mM potassium chloride, 0.36 mM monobasic sodium phosphate, 12 mM sodium bicarbonate and 2 mM magnesium chloride, 0.2% BSA, 5.5 mM of 2% dextrose and 0.01mL of HEPES Buffer (0.01 M – pH 7). In some experiments, HBMT was used to make washed platelets.

2) Platelet buffer

Platelet buffer (pH 7.4) is made with 135 mM sodium chloride, 5 mM D-(+)-Glucose, 2.7 mM potassium chloride, 0.5 mM sodium phosphate dibasic, 1 mM magnesium chloride, 1 mM sodium citrate, 0.1% bovine serum albumin, and 10 mM HEPES. In other experiments, washed platelets were prepared using platelet buffer (especially for the constant shear experiments for platelet activation measurement).

3) Annexin V binding buffer

This buffer contained 0.01 M HEPES, 0.14 M sodium chloride, and 2.5 mM calcium chloride. The final buffer solution was maintained at a pH of 7.4. Annexin V binding buffer was used to dilute the samples to read in flow cytometry for measuring phosphatidylserine (PS) expression.

4) P-selectin binding buffer

It contained 50 mM sodium phosphate, 100 mM potassium chloride, 150 mM sodium chloride, 5% glycerol, and 0.2% BSA. The buffer pH was maintained at 7.4. Platelet samples in suspension, stained for CD62P expression, were diluted in the P-selectin binding buffer before analyzing in the flow cytometery.

5) Phosphate buffer saline (PBS)

PBS, pH level of 7.4, contained 9% sodium chloride, 1 M sodium dibasic phosphate, 0.5 M sodium monobasic phosphate. Warm PBS (37°C) was used in most experiments to wash the cells.

3.2.2 Washed Platelet

Platelet rich plasma (PRP), anticoagulated with 0.32% sodium citrate, was purchased from Oklahoma Blood Institute (Oklahoma City, OK). PRP was centrifuged at 3,000 rpm (1,100x*g*) for 9 min in order to isolate platelets. After aspirating the supernatant, platelet pellets were resuspended in either platelet buffer or HBMT. Platelet buffer was used to prepare washed platelets in constant shear experiments to quantify platelet activation (P-selectin and GPIbα expression). Whereas, in all other experiments HBMT was used. The final concentration of the washed platelet was adjusted to 250,000/µl and samples were used within 6 hr after preparation.

3.2.3 Endothelial Cells (EC)

Human coronary artery endothelial cells (HCAEC) were purchased from ScienceCell Research Laboratories (Carlsbad, CA) and were grown to confluence on

fibronectin (2 μg/cm^2) (Sigma-Aldrich) coated 60 mm culture petri dishes. The cells were maintained in endothelial cell medium (ECM) (ScienceCell), supplemented with 5% FBS, 1:100 penicillin/streptomycin (Invitrogen Corporation, CA) and 1:100 endothelial cell growth supplement (ECGS) (ScienceCell). HCAEC were used between passages 1 to 7. To study the effect of activated EC (by cytokine) on platelet activation and microparticle generation, confluent HCAEC were further treated over night with 1 ng/mL of human recombinant tumor necrosis factor TNF-α (Sigma-Aldrich) before use.

3.3 Platelet activation

Shear stress induced platelet activation was quantified using P-selectin and GPIbα expression. The quantification of platelet activation was conducted by flow cytometry (Accuri Cytometers Inc., Ann Arbor, MI).

3.3.1 P-selectin expression

Washed platelets were exposed to various shear stresses (both constant and dynamic shear) for 1 hr and platelet samples were removed from the cone and plate shearing device every 15 min for CD62P expression measurement. Samples were incubated with FITC conjugated monoclonal mouse anti-human P-selectin anti-body (CD62P, 1:50 dilution, Ancell Corporation, Bayport, MN) for 30 min at room temperature. Platelet samples were then diluted in CD62P buffer solution (1:10) and read immediately using a flow cytometer (Accuri C6). FITC conjugated MOPC antibody (1:100 dilution, Ancell Corporation) was used to detect non-specific binding in all experiments. Untreated platelets were used as negative control. 20 μM TRAP (Thrombin

Receptor Activator Peptide, Sigma-Aldrich) activated platelets were used as positive control to represent platelets that were fully activated (5 min, RT).

3.3.2 GPIbα expression

To measure surface GPIbα expression, timed platelet samples were collected once every 15 min (3 hr for constant shear stress and 1 hour for dynamic shear stress). Samples were incubated with FITC conjugated mouse anti human monoclonal GPIbα (CD42b, 1:10 dilution, Abcam, Cambridge, MA) antibody for 30 min on ice. Samples were then diluted in platelet buffer (in constant shear experiments) or in HBMT (in dynamic shear experiments) and read using flow cytometry. FITC conjugated MOPC antibody (1:100 dilution, Ancell Corporation) was used to detect non-specific binding in all experiments. 20 μM TRAP (5min, RT) activated platelets were used as the positive control to represent fully activated platelets.

3.3.3 Mathematical modeling

To establish a mathematical relationship between platelet activation (surface CD62P expression and GPIbα expression) and shear stress exposure, CD62P and GPIbα data from different constant shear experiments were imported to MATLAB R2009a (version 7.8.0). The *in vitro* CD62P data (obtained at every 15 min interval for 1 hr) were used to compute the variation of CD62P expression over a period of 1 hr. Initially, CD62P expression at different time points for a particular shear stress was generated using MATLAB. Four separate graphs (indicating four different shear stress conditions of 0.1, 0.3, 1, and 3 Pa) were plotted to obtain the interpolated values of CD62P expression at every 5 min interval. These interpolated data were imported to the surface plot toolbox

in MATLAB, and a 3D surface plot was obtained combining all four shear conditions over a time of 1 hr. As different shear stress magnitudes and time intervals were both interpolated, the surface plot provides a visualization of variation in CD62P expression with both amount of shear stress and time.

In a similar procedure (as explained above), the results from *in vitro* experiments that investigated the variation in GPIbα expression (at every 15 min interval for 3 hr) were used to generate another 3D surface plot to establish a mathematical relationship between time, shear stress and GPIbα expression.

3.4 Platelet microparticle (PMP) generation

Washed platelets were exposed to different shear waveforms (constant and dynamic) for a duration of 30 min. Post shearing, 1.5 mL of sheared platelets sample was collected. Platelet samples were then centrifuged at 4,000 rpm (1,500x*g*) for 15 min at room temperature. Discarding the platelet pellets, 1.2 mL of supernatant was collected carefully and was subjected to a subsequent centrifugation at 13,500 rpm (17,000x*g*) for 2 min at 4ºC. After removing the supernatant, PMP pellets were resuspended in 100 µl of cold Annexin V binding buffer. Samples were then incubated with FITC conjugated Annexin V antibody (Invitrogen Corporation, Carlsbad, CA) at 1:20 dilution. Annexin V was used to measure the PS expression on the PMP surface. After 15 min of incubation at room temperature, samples were then diluted by adding 400 µl Annexin V binding buffer. Samples were then immediately analyzed by flow cytometry for PMP production measurement. Based on the size from forward and side scatter plot, suitable gates were established separately for platelets (using resting platelets) and PMP (shown in Fig 3.4).

A minimum of 30,000 events were counted within the PMP gate. PMP were distinguished from platelets based on their size (smaller than single platelet) and positive Annexin V binding (PS expression). Untreated washed platelets were used as the negative control. Platelets treated with TRAP (20 µM, 5 min, RT) or a homogenizer (35,000 rpm, 30 sec, RT) were used as the positive control.

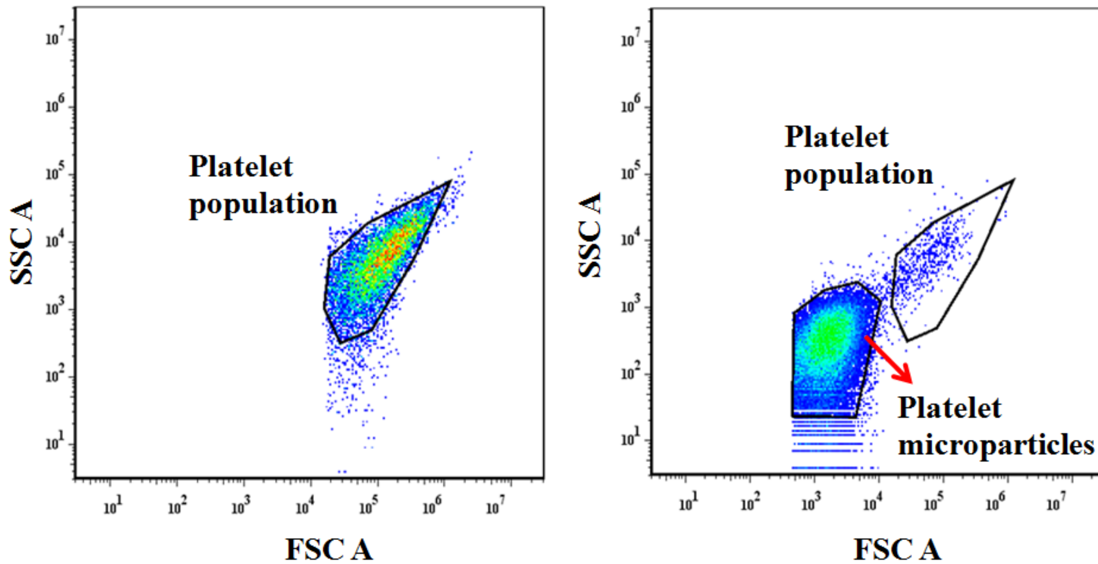

Fig 3.4: Pseudocolor scatter plots (side scattered area vs forward scattered area) from flow cytometry, showing different gates used to detect platelet and PMP population.

3.5 Endothelial cell (EC) and platelet interaction

Using the cone-plate shearing device, three dynamic shear stresses (normal, recirculation and stenosis) were applied on washed platelets (for 60 min in platelet activation experiments and for 30 min in PMP generation experiments) in the presence of confluent HCAEC. EC were washed twice with warm PBS. Washed platelets (37°C), at 250,000 /µL, were then placed on top of EC and sheared at 37°C. The study was planned to investigate the interaction between platelets and EC under various shear conditions. To

investigate the role of damaged (activated) EC on platelet behavior under flow conditions, EC were treated with TNF-α overnight before experiments.

3.5.1 Platelet activation

Post shearing, platelet activation was quantified by measuring their surface P-selectin and GPIbα expression (as described earlier) in the presence of EC. Every 15 min, platelet samples were collected and incubated with FITC conjugated anti-CD62P or anti-CD42b antibody to quantify the P-selectin and GPIbα expressions respectively. Resting platelets without contact with EC were used as control and TRAP treated platelets were used as positive controls. MOPC was used to detect background binding of mouse antibodies.

3.5.2 PMP generation

Post shearing, platelets were isolated. The amount of PMP generated with the presence of shear stress and EC was quantified following the same procedure as described before.

3.6 Statistical Analysis

From flow cytometry, the all mean fluorescence values and percentage shift in population within a particular gate under constant and dynamic shear stress conditions were obtained. All these values were normalized with respect to that of resting platelets (control), in order to account for the variation in platelet donors and experimental randomization. The data was analyzed using single factor ANOVA and if any significant difference was detected, Student-Newman-Keuls *post hoc* method was implemented for

further multiple comparisons. To examine the combined effect from shear stress, shear exposure time, and their interaction on P-selectin and GPIbα expression, data was analyzed using two-way ANOVA. Three-way ANOVA was used to examine the effect from shear stress, shear exposure time, and platelet-EC interaction on surface P-selectin and GPIbα expression. The data were considered as significant if $P<0.05$. Statistical software "Primer of Biostatistics" (version 4.02) was used for single factor ANOVA tests and Statistical Analysis System software (SAS 9.2) was used for two-way and three-way ANOVA.

CHAPTER IV

RESULTS

4.1 Platelet activation in absence of EC

4.1.1 P-selectin expression

Platelets were exposed to constant and dynamic shear stresses. Platelet activation was quantified by cell surface P-selectin (CD62P) expression.

4.1.1.1 Constant shear stress

Platelets (250,000/µl) were exposed to constant shear stress at 0.1, 0.3, 1, and 3 Pa for a duration of 60 min in a hemodynamic cone and plate shearing device. Sheared platelets were sampled every 15 min and incubated with FITC conjugated mouse anti-human CD62P antibody for 30 min and analyzed using flow cytometry. The all mean fluorescence values obtained were normalized to that of resting platelets (not exposed to shear stress) due to the variation in platelet activation level among donors. Figure 4.1 shows normalized all mean fluorescence intensities of platelet P-selectin expression at different shear stress levels as function of shear exposure time. Two-way ANOVA

indicated that shear stress amplitudes have significant effect ($P<0.05$) on platelet CD62P expression. Table 4.1 summarizes the P values of this analysis and Table 4.2 summarizes the statistically significant P values of the combined effect of shear stress and shear exposure time on P-selectin expression. These results demonstrated that higher shear stress (1 and 3 Pa) and longer exposure time (60 min) tend to enhance platelet activation.

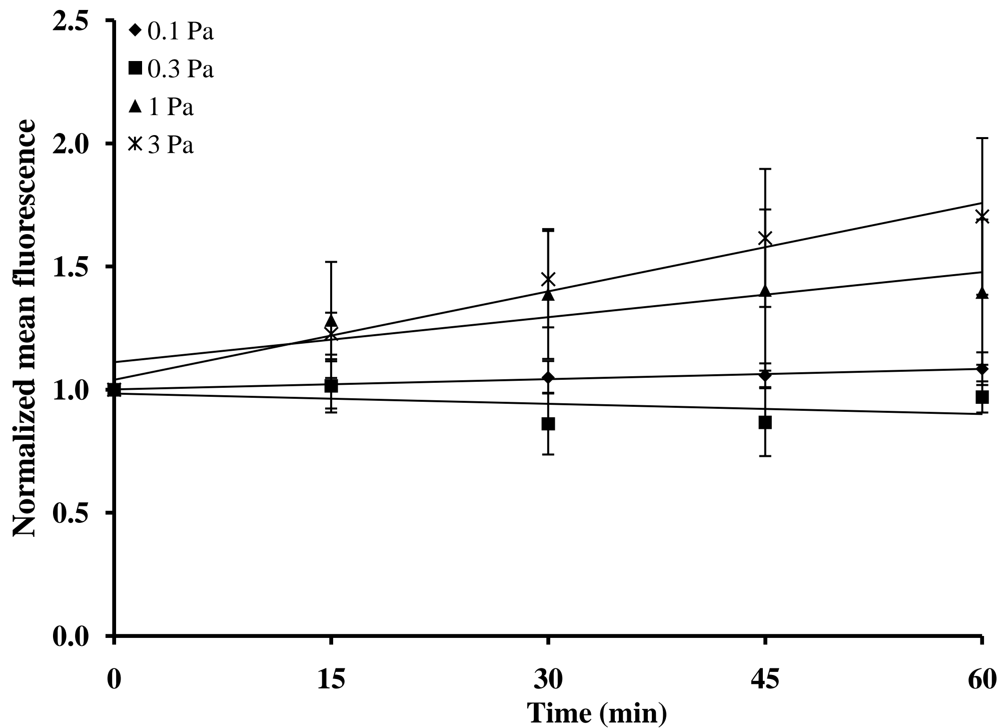

Fig 4.1: P-selectin expression by activated platelets were measured at shear stress magnitude of 0.1, 0.3, 1, and 3 Pa at every 15 min interval for 60 min exposure time. All platelet activation data were normalized to that of resting platelets and presented as Mean ± SD. 1 and 3 Pa induced significantly ($P<0.05$) higher platelet activation at longer shear exposure time (60 min).

Table 4.1: Summary of the P values obtained from two-way ANOVA for P-selectin expression under constant shear stress. N.S. represents not significant.

	P value
Shear Stress (Pa)	<0.0001
Time (min)	N.S.
Shear Stress*Time	N.S.

Table 4.2: Combined effect of shear stress magnitude and shear exposure time on platelet activation (P-selectin expression). N.S. indicates "not significant".

	Time (15 min)		
Shear stress (Pa)	0.3	1	3
0.1	N.S.	N.S.	N.S.
0.3		N.S.	N.S.
1			N.S.

	Time (30 min)		
Shear stress (Pa)	0.3	1	3
0.1	N.S.	N.S.	0.0063
0.3		0.0072	0.0005
1			N.S.

	Time (45 min)		
Shear stress (Pa)	0.3	1	3
0.1	N.S.	N.S.	0.0002
0.3		0.0069	<0.0001
1			0.0171

	Time (60 min)		
Shear stress (Pa)	0.3	1	3
0.1	N.S.	N.S.	<0.0001
0.3		0.0351	<0.0001
1			0.0034

In addition, the rate of change of P-selectin expression over 1 hr of shearing was calculated as the slope (rate of CD62P expression) of the best fit regression line of normalized mean fluorescence values (Table 4.3). Statistical analysis of these slopes using single factor ANOVA indicated that shear stress amplitude had a significant effect on the rate of CD62P expression ($P<0.05$). Student-Newman-Keuls *post hoc* method was used to compare between shear stresses. The results demonstrated that 3 Pa (slope 0.0120 min^{-1}, n=4) induced significantly higher ($P=0.001$) platelet activation compared to 0.1 Pa (0.0014 min^{-1}, n=5), 0.3 Pa (-0.0014 min^{-1}, n=3), and 1 Pa (0.0044 min^{-1}, n=6).

Figure 4.2 depicts the representative histograms of platelet population positive for CD62P expression. MOPC was used to detect non specific binding. Shear stress at 3 Pa induced (at the end of 60 min shear exposure duration) a higher shift in platelet population (7.71%) compared to 0.1 Pa (5.21%), 0.3 Pa (4.02%), and 1 Pa (4.31%). The results indicate that the platelets that are exposed to high shear stress for longer duration show a significant increase in platelet activation, matching the all mean fluorescence observations.

Table 4.3: Rate of change in P-selectin expression. Slope of all mean fluorescence values of platelet activation (P-selectin expression) over a time interval of 60 min at various shear stress magnitudes. Slope of 3 Pa induced significantly (*P*=0.016) higher platelet activation than 0.1, 0.3, and 1 Pa.

Shear Stress	0.1 Pa (n=5)	0.3 Pa (n=3)	1 Pa (n=6)	3 Pa (n=4)
CD62P expression rate (Mean ± SD)	0.0014 ± 0.0007	-0.0014 ± 0.0017	0.0044 ± 0.0045	0.0120 ± 0.0053

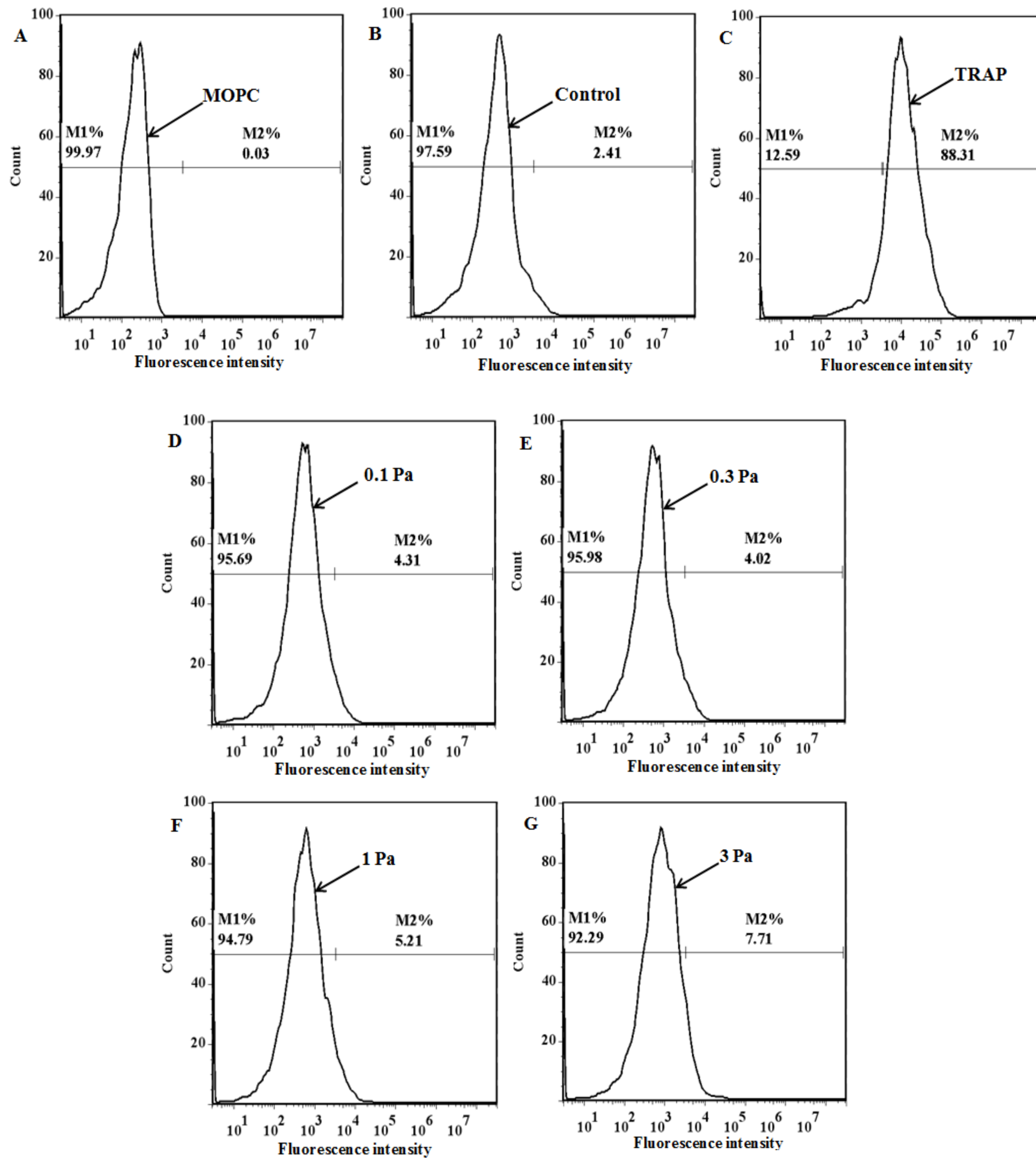

Fig 4.2: Flow cytometry plots of activated platelet population. A, B, C – MOPC, control and TRAP samples (incubated with CD62P antibody) respectively. D, E, F, G – sheared platelet samples incubated with CD62P antibody at shear stress magnitude of 0.1, 0.3, 1, and 3 Pa respectively (represents shift at 60 min).

4.1.1.2 Dynamic shear stress

Washed platelets (250,000/μl) were exposed to three different dynamic shear stresses: normal, recirculation (low pulsatile shear), and stenosis shear (high pulsatile shear) for 60 min in the cone plate shearing device. Platelet samples were collected every 15 min and incubated with P-selectin (CD62P) antibody as described before. Figure 4.3 represents normalized all mean fluorescence values of P-selectin expression under dynamic shear stresses for 60 min. Two-way ANOVA revealed that neither shear stress magnitude nor shear exposure time had an effect on platelet activation ($P>0.05$, n=5). Figure 4.4 shows typical histograms of platelet population positive for CD62P staining.

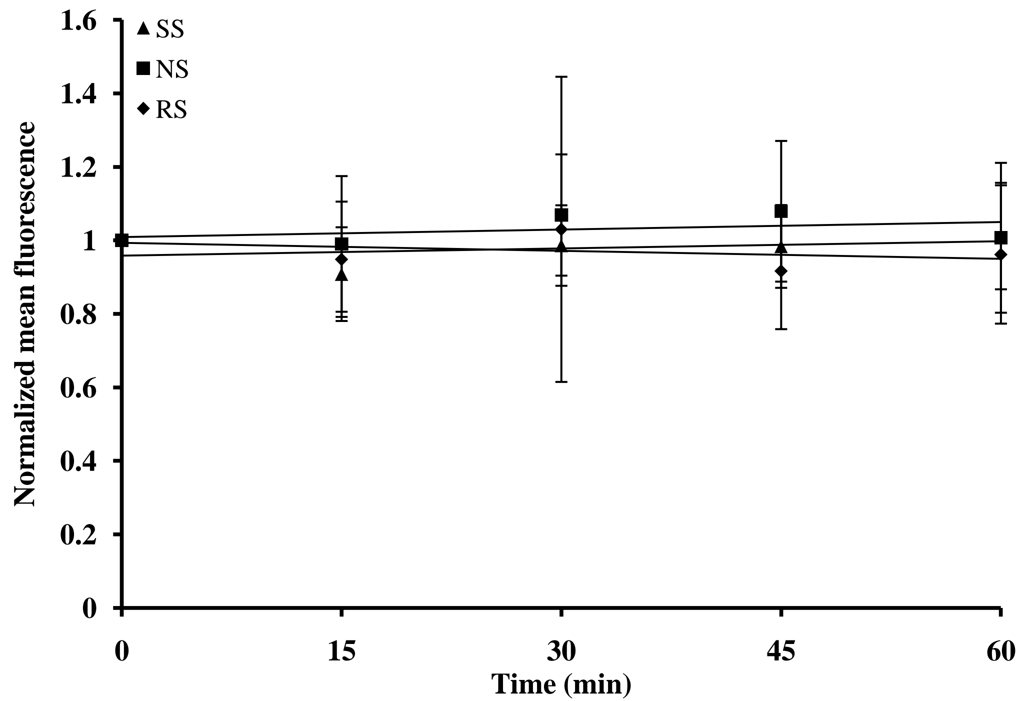

Fig 4.3: Platelets were exposed to normal, recirculation, and stenosis shear stresses and P-selectin expression by activated platelets was quantified every 15 min for 1 hr. All the mean fluorescence values were normalized to that of resting platelet. Data are presented as Mean ± SD. Statistical analysis represented that there were no significant effect of dynamic shear stress magnitude and exposure time on platelet surface P-selectin expression. (NS – Normal Shear; RS – Recirculation Shear; SS – Stenosis Shear).

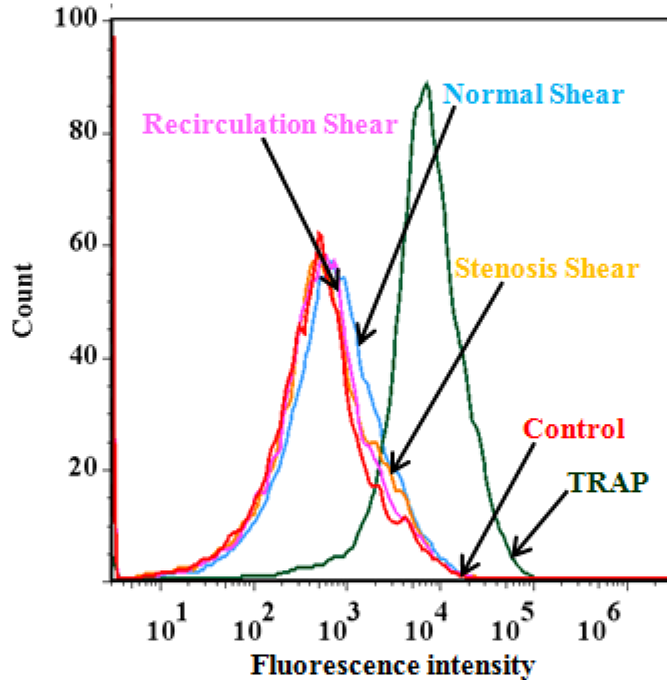

Fig 4.4: Representative histograms of activated platelet population shift under different treatment conditions. Resting platelets were used as negative experimental control and TRAP treated platelets were used as positive control.

4.1.1.3 Mathematical model of P-selectin expression

A 3D surface plot representing platelet surface P-selectin (CD62P) expression as a function of shear stress magnitude and shear exposure duration was developed using MATLAB. Figure 4.5 depicts the 3D surface plot of CD62P expression. The mathematical model can be used to predict the relative amount of P-selectin expression under different shear conditions (amplitude and exposure time). The model demonstrated that platelets exposed to low shear stress for longer duration (60 min) or high shear stress for shorter duration (15 min) did not induce enough platelet activation (P-selectin expression). However, a significant increase in P-selectin expression (platelet activation)

was observed with a combined increase in shear stress magnitude and shear exposure time.

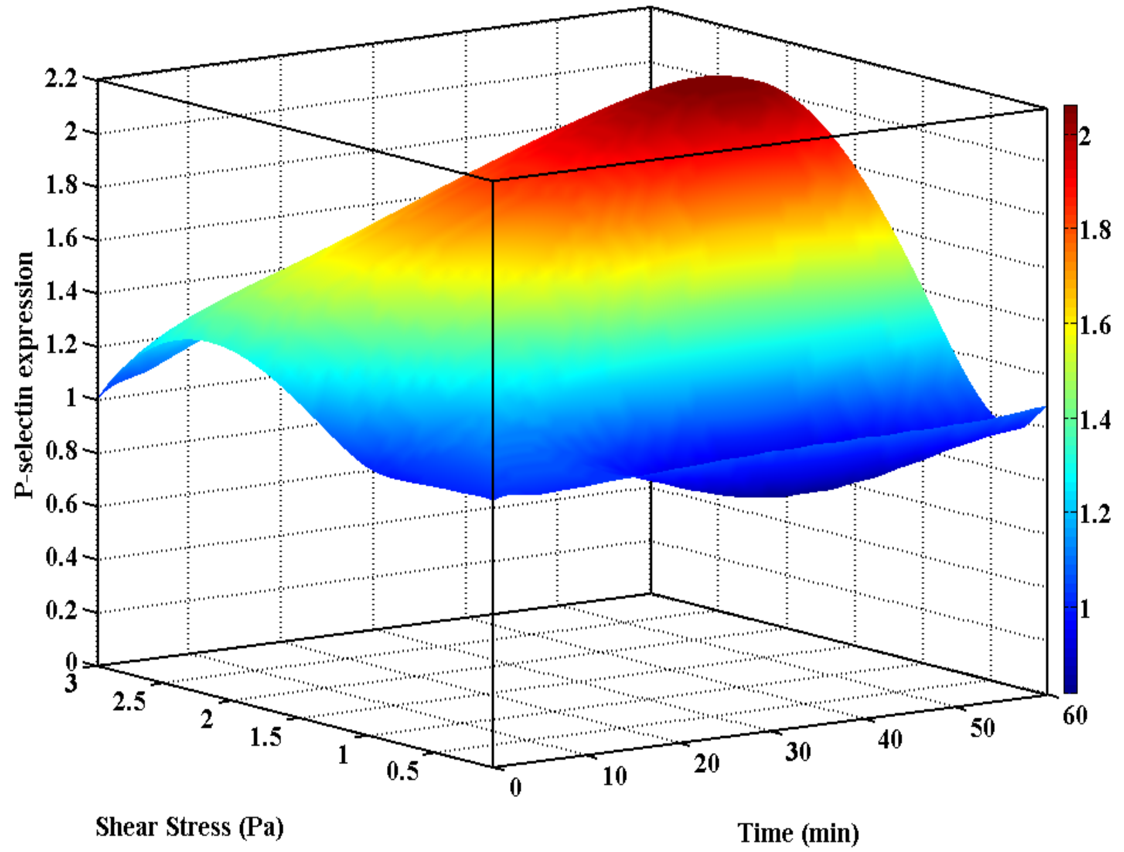

Fig 4.5: Mathematical model of surface P-selectin expression as a function of shear stress magnitude and shear exposure duration. The model was constructed based on platelet surface P-selectin expression under varying constant shear stress magnitude (0.1, 0.3, 1, and 3 Pa) and their exposure time (varying between 0 to 60 min).

4.1.1.4 Mathematical model validation

The 3D mathematical representation of surface P-selectin expression (Fig 4.5) was generated (using MATLAB) based on the experimental results under constant shear stress conditions. In order to validate this model, the P-selectin expression from dynamic shear experiments were compared to the estimated value from the mathematical model. P-selectin expression values was plotted as a function of shear stress-exposure time integral (Fig 4.6) for each shear condition and the mathematical expression for the values was obtained using the curve fit tool in Microsoft Excel. The shear stress-exposure time integral values of all three dynamic shear conditions was calculated (values presented in table 4.4) and was used as an input to the steady shear mathematical expression and corresponding P-selectin expression (at the end of 60 min) was calculated.

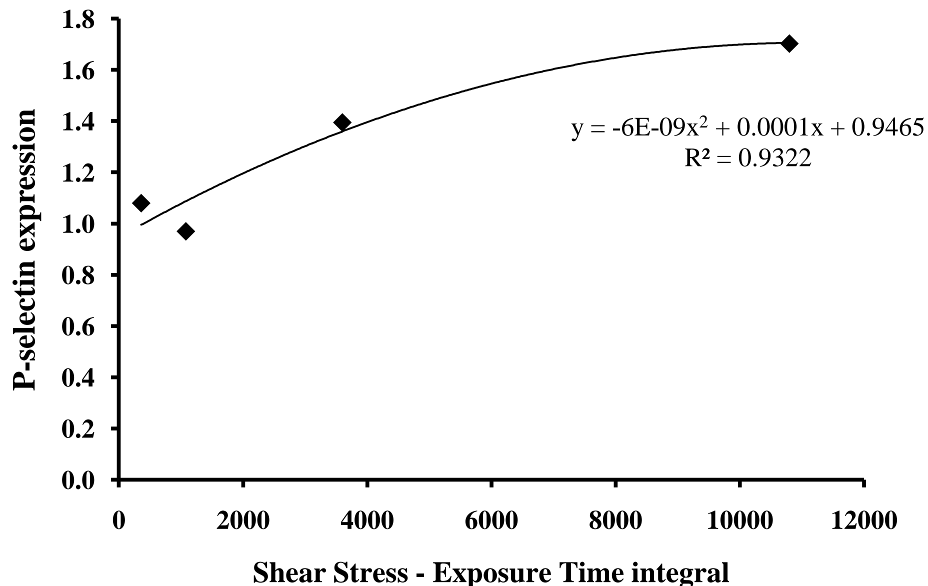

Fig 4.6: Representation of P-selectin expression under different shear stress-exposure time.

Table 4.4: Mathematical model validation through comparison between experimental and predicted values

Shear Stress	Shear Stress-Time integral (dyne s/cm^2)	Experimental value	Predicted value	% error
NS	1980	1.007	1.121	11.32
RS	828	0.962	1.025	6.5
SS	2088	1.012	1.129	11.56

From the table, it can be seen that the results from dynamic shear experiments are similar to that of the predicted values from the mathematical model. The results are within an acceptance error range considering the pulsatility of the shear waveform and the physiological significance of the shear exposure duration (60 min).

4.1.2 GPIbα expression

Platelet surface GPIbα expression was measured after exposing platelets to constant and dynamic shear stresses.

4.1.2.1 Constant shear stress

Platelets were exposed to constant shear stress with magnitudes of 0.1, 0.3, 1, and 3 Pa and their surface GPIbα expression was measured. Timed platelet samples were collected every 15 min and incubated with FITC conjugated anti-human GPIbα antibody. The all mean fluorescence intensity values were normalized to that of resting platelets. Figure 4.7 represents normalized all mean fluorescence values of GPIbα expression as a function of shear stress (0.1, 0.3, 1, and 3 Pa) and shear exposure time (at every 15 min for 3 hr). The results demonstrated that, at the end of 30 min, shear stress at 3 Pa (1.56 ± 1.03, n=4) led to a higher GPIbα expression than shear stresses at 1 Pa (1.34 ± 0.48, n=6), 0.3 Pa (1.10 ± 0.36, n=6), and 0.1 Pa (1.08 ± 0.14, n=5). However, after 30 min of shear exposure, GPIbα expression started to drop significantly. At the end of 60 min, platelets exposed to shear stress at 3 Pa had a significant reduction in GPIbα expression (0.31 ± 0.05, n=4) compared to other shear conditions (1.07 ± 0.46, n=6 for 1 Pa; 1.23 ± 0.63, n=6 for 0.3 Pa and 1.09 ± 0.65, n=4 for 0.1 Pa). As the shear exposure time increased, platelet GPIbα expression continuously decreased. Two-way ANOVA was conducted to investigate the role of exposure time and shear stress magnitude on GPIbα expression. The results indicated that both shear stress and shear exposure time, as well as the interaction between the two had significant effect on GPIbα expression. Table 4.5 summarizes the statistically significant P values of these results.

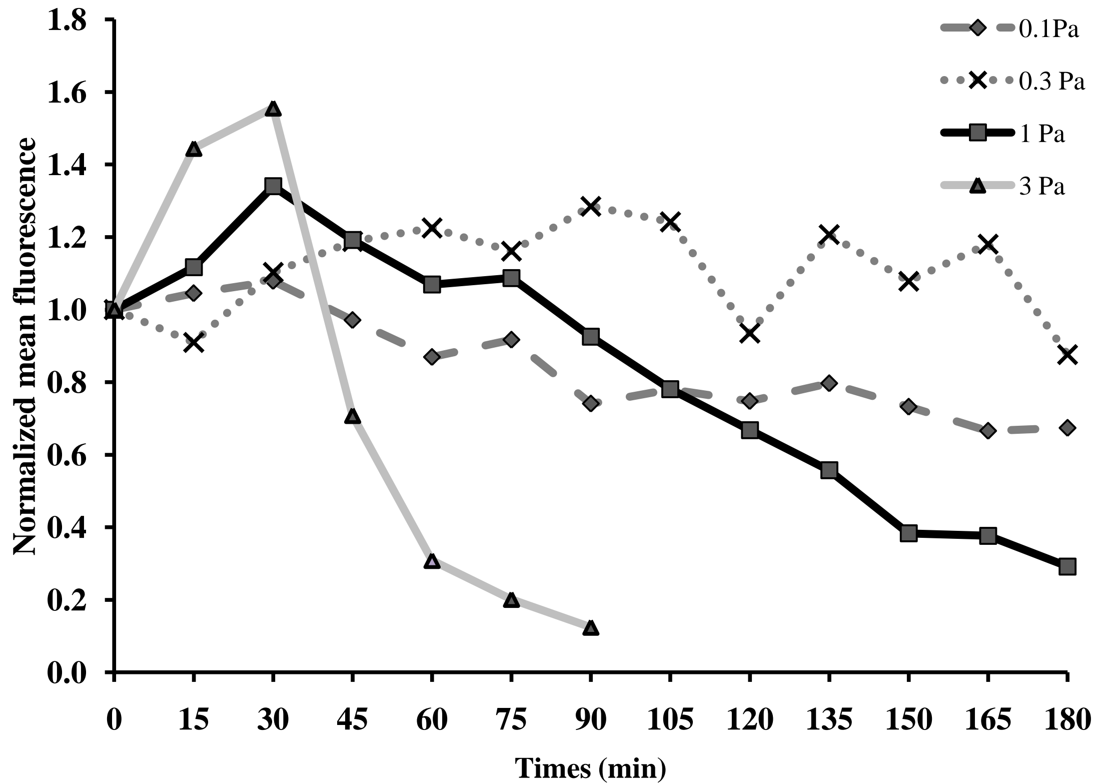

Fig 4.7: Platelets were activated under 0.1, 0.3, 1, and 3 Pa shear stress conditions for 3 hr and the activation was measured by surface GPIbα expression. All data were normalized to that of control platelet and mean values were presented. At 30 min, there was increase in GPIbα expression with increase in shear stress magnitude. After this point of time, there was significant downfall of GPIbα expression with increase in shear stress magnitude (for 1 and 3 Pa) and shear exposure time.

Table 4.5: Summary of the statistically significant P values obtained from two-way ANOVA for GPIbα expression under constant shear stress.

	P value
Shear Stress (Pa)	<0.0001
Time (min)	<0.0001
Shear Stress*Time	0.0002

Table 4.6 represents the interaction effect of shear stress and shear exposure time on GPIbα expression. Figure 4.8 are the histogram plots of GPIbα positive platelet populations under various conditions. Percentage shift of platelet population under different shear stress conditions were represented only at 30 min and 1 hr. MOPC treated platelet sample (for non-specific binding) showed a population shift of 0.05%, whereas, control platelet and TRAP treated samples indicated a shift of 12.23% and 37.41% respectively. At the end of 30 min shearing, percentage shift of GPIbα positive platelet population was 12.89% (for 0.1 Pa), 14.63% (for 0.3 Pa), 33.40% (for 1 Pa), and 28.84% (for 3 Pa), which became 35.44% (for 0.1 Pa), 36.04% (for 0.3 Pa), 21.09% (for 1 Pa), and 6.46% (for 3 Pa) respectively after 1 hr shearing. These results demonstrated that mild shear stress stimulation (0.1 and 0.3 Pa) did not affect platelet surface GPIbα expression, while elevated shear stress (especially 3 Pa) impaired GPIbα expression.

Table 4.6: Combined effect of shear stress magnitude and shear exposure time on GPIbα expression. N.S. indicates “not significant”.

	Time (60 min)		
Shear stress (Pa)	0.3	1	3
0.1	N.S.	N.S.	0.038
0.3		N.S.	0.0005
1			0.0036

	Time (90 min)		
Shear stress (Pa)	0.3	1	3
0.1	N.S.	N.S.	0.0222
0.3		N.S.	<0.0001
1			0.0022

	Time (180 min)		
Shear stress (Pa)	0.3	1	3
0.1	N.S.	N.S.	-
0.3		0.0165	-
1			-

Fig 4.8: Fluorescence histogram of percentage shift in platelet population (flow cytometry plot). A, B, C - MOPC, control, and TRAP samples respectively. D, E – Sheared sample at 0.1 Pa at 30 min and 60 min respectively. F, G - Sheared sample at 0.3 Pa at 30 min and 60 min respectively. H, I - Sheared sample at 1 Pa at 30 min and 60 min respectively. J, K - Sheared sample at 3 Pa at 30 min and 60 min respectively.

4.1.2.2 Dynamic shear stress

Platelets (250,000/µl) were exposed to normal, recirculation (low pulsatile shear), and stenosis shear (high pulsatile shear) stress for 60 min in the cone plate shearing device. GPIbα expression was measured every 15 min by incubating sheared platelet samples with FITC conjugated GPIbα (CD42b) antibody. Normalized values of GPIbα expression (the all mean fluorescence values were normalized to that of resting platelets) are depicted in Figure 4.9. Two-way ANOVA indicated that the different shear stress patterns did not have any significant effect on GPIbα expression. Figure 4.10 demonstrates plots of histograms of CD42b positive platelet population for resting and sheared platelets. MOPC was used to detect non-specific binding. TRAP treated platelets were used as positive control.

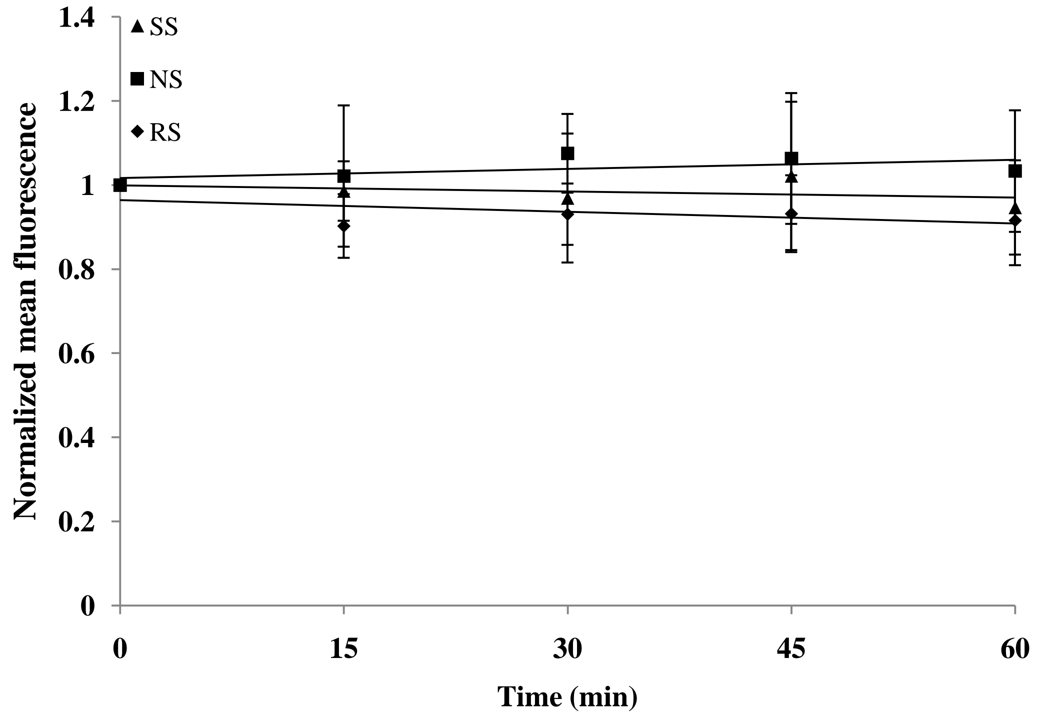

Fig 4.9: GPIbα expression by activated platelets, every 15 min for 1 hr, was measured under normal, recirculation, and stenosis shear stress conditions. All values were normalized to that of control platelets (platelets of 0 min time point). Data are presented as Mean ± SD. No significant differences were observed when platelets were exposed to dynamic shear stress conditions for 1 hr. (NS – Normal Shear; RS – Recirculation Shear; SS – Stenosis Shear).

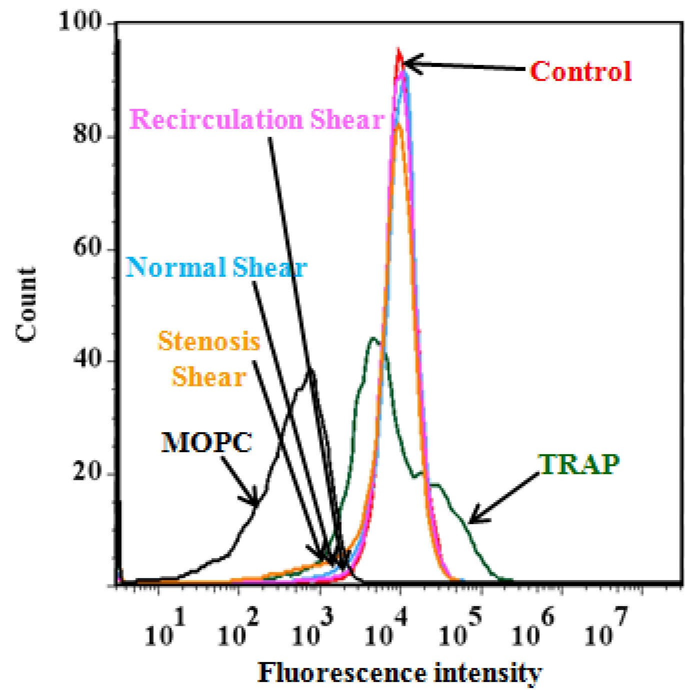

Fig 4.10: Activated platelet population of MOPC and TRAP treated samples, control, and platelet samples exposed to different shear stress (normal, recirculation, and stenosis) conditions.

4.1.2.3 Mathematical model of GPIbα expression

A 3D surface plot was developed (using MATLAB) for GPIbα expression. This model was developed to present normalized GPIbα expression as a function of constant shear stress conditions (0.1, 0.3, 1, and 3 Pa) over a period of 3 hrs which could be used to estimate platelet surface GPIbα expression with various shear stress amplitudes and shear exposure time.

The model demonstrated that exposure to low shear stress for longer duration did not induce significant change in GPIbα expression. However, exposure to high shear stress for longer duration resulted in an initial increase and then a sharp decrease of GPIbα expression (Figure 4.11).

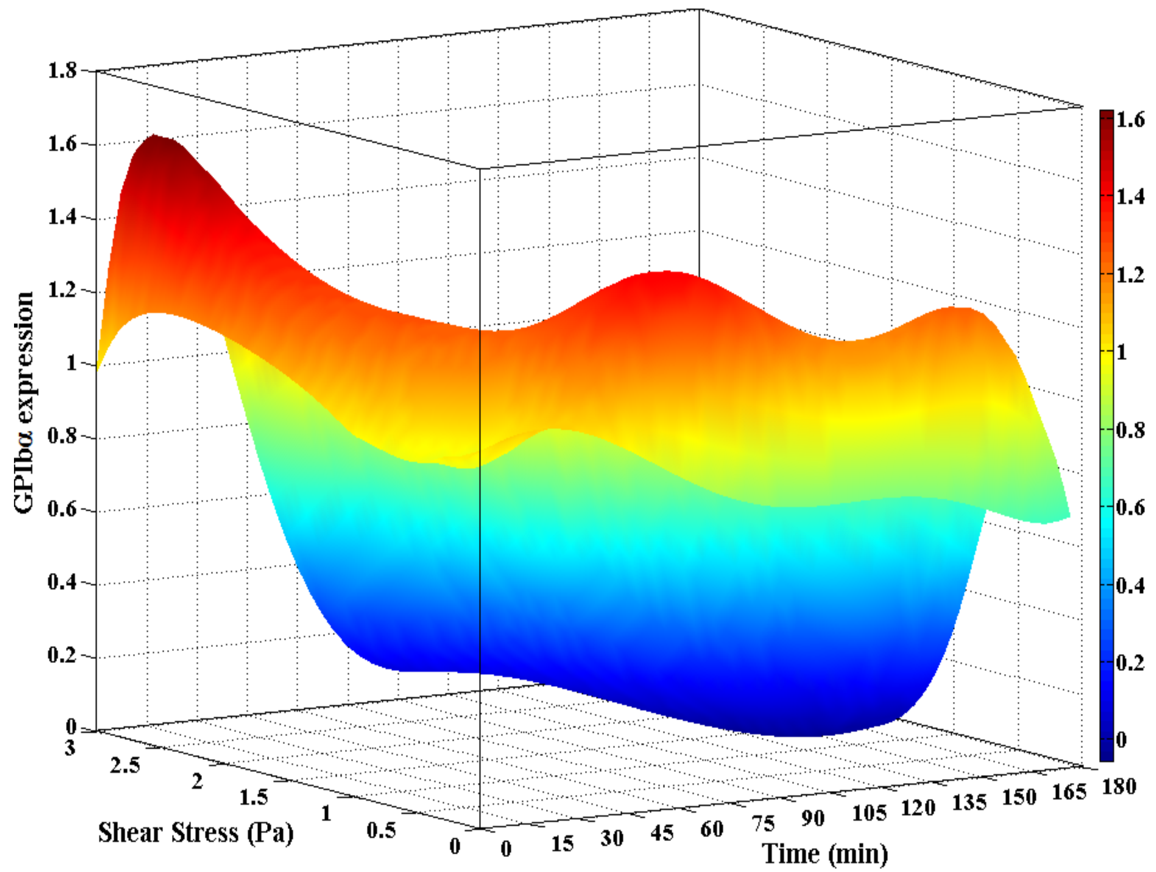

Fig 4.11: Mathematical model of GPIbα expression as a function of shear stress magnitude and shear exposure time. The model was constructed using the constant shear stress magnitude (0.1, 0.3, 1, and 3 Pa), shear exposure time (varying over a range of 3 hr) and corresponding GPIbα expression.

4.1.2.4 Mathematical model validation

The 3D model for evaluating GPIbα expression for constant shear stresses was generated using MATLAB (Fig 4.11). This mathematical model was validated using the *in vitro* experimental values of GPIbα expression from dynamic shear experiments. A mathematical expression for constant shear stress values was obtained using Microsoft Excel after plotting the GPIbα expression as a function of shear stress-exposure time integral (Fig 4.12). The calculated values (table 4.7) of shear stress-exposure time integral of the dynamic shear conditions were fit into the constant shear mathematical expression and the corresponding GPIbα values were calculated.

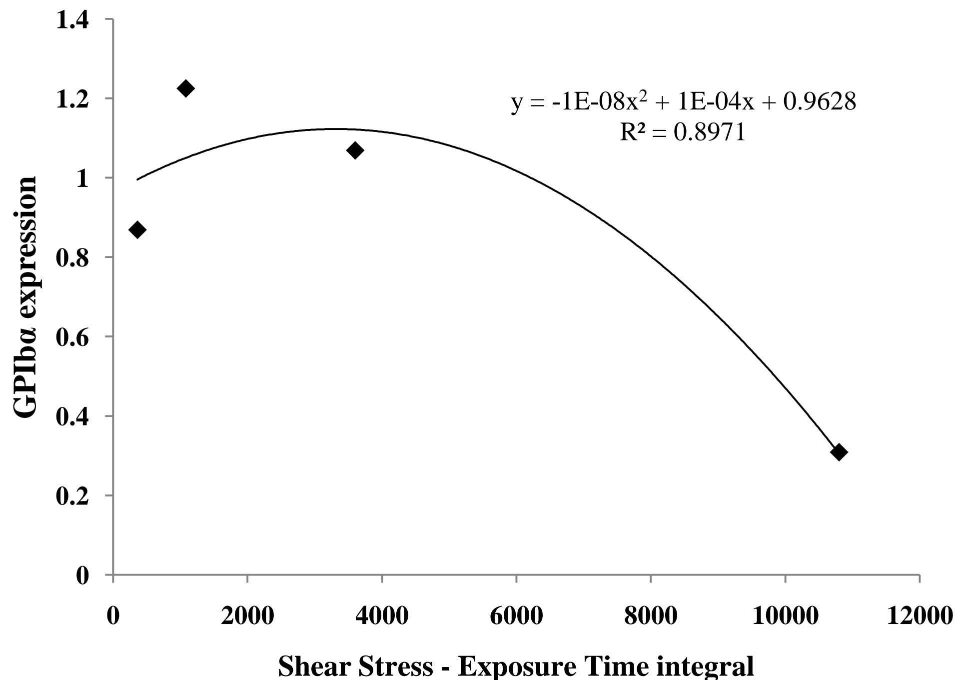

Fig 4.12: Representation of GPIbα expression under different shear stress-exposure time.

Table 4.7: Mathematical model validation through comparison between experimental and predicted values

Shear Stress	Shear Stress-Time integral	Experimental value	Predicted value	% error
NS	1980	1.033	1.122	8.6
RS	828	0.916	1.039	13.42
SS	2088	0.947	1.128	19.11

The comparison table shows that, results from dynamic shear experiments and the predicted values from the mathematical expression are similar and the results are within an acceptable error range. The reasons for the error could be the pulsatile nature of the shear waveforms and the 60 min shear exposure duration.

4.2 Platelet microparticle (PMP) generation in absence of EC

PMP generation was quantified after exposing platelets to both constant and dynamic shear stress conditions for duration of 30 min.

4.2.1 Constant shear stress

Washed platelets (250,000/μl) were exposed to constant shear stress at 0.1, 0.3, 1, and 3 Pa for 30 min. Post shearing, platelet samples were analyzed using flow cytometry for PMP generation. Gates were established (based on size) for platelet (using resting platelets) and PMP population. In the PMP gate, 30,000 total events were counted. PMP were identified as positive for Annexin V binding (Figure 4.13). The results were analyzed using one-way ANOVA and Student-Newman-Keuls *post hoc* method. Increased PMP numbers were observed under all shear stress conditions compared to control ($P<0.05$, Figure 4.14). Platelets, homogenized (positive control) at 35,000 rpm, induced significant increase in PMP generation (even higher PMP generation compared to the shear stresses). However, TRAP was shown as the positive control in all the PMP generation bar plots, as it was used as positive control in all other experiments. Shear stress at 3 Pa (3439±964, n=6) induced a significant ($P=0.016$) increase in PMP generation compared to 0.1 Pa (2300±427, n=8), 0.3 Pa (2401±525, n=6), and 1 Pa (2281±491, n=6).

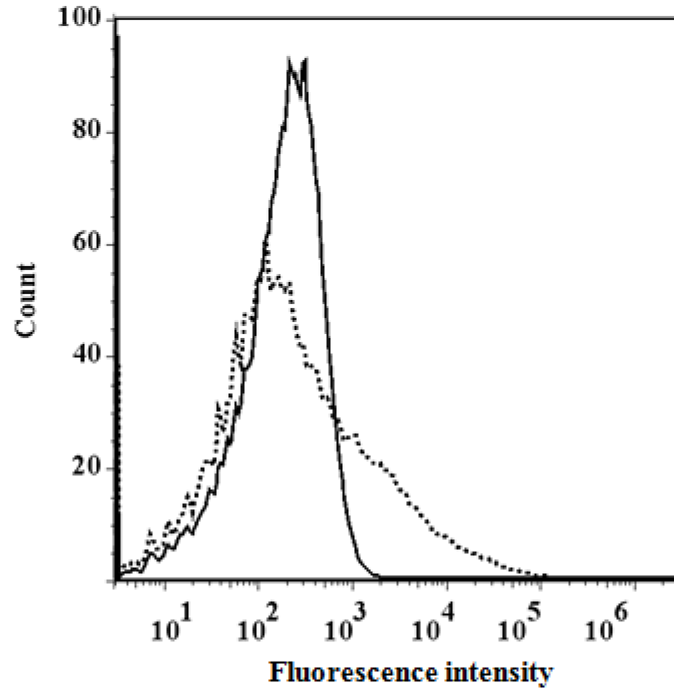

Fig 4.13: Fluorescence histogram of PMP population (Annexin V positive) generated due to shear stress exposure compared to MOPC treated (negative control) PMP population.

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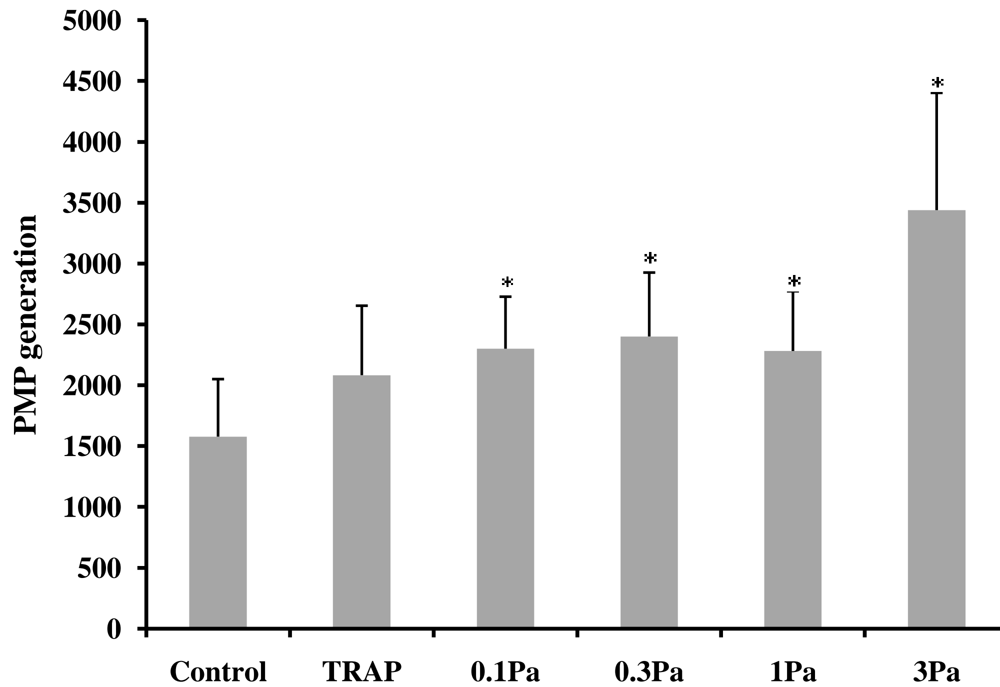

Fig 4.14: Platelets were exposed to different constant shear stress (0.1, 0.3, 1, and 3 Pa) for 30 min and PMP generation (due to platelet activation) was quantified based on Annexin V binding and their size. PMP generation increased, compared to the control, at all shear stress magnitude and due to TRAP treatment.

4.2.2 Dynamic shear stress

PMP generation was quantified following the same procedures as mentioned earlier after exposing platelets to dynamic shear stress conditions. The results (Figure 4.15) demonstrated that all three dynamic shear stresses induced a significant increase in PMP generation ($P<0.05$) compared to resting platelets.

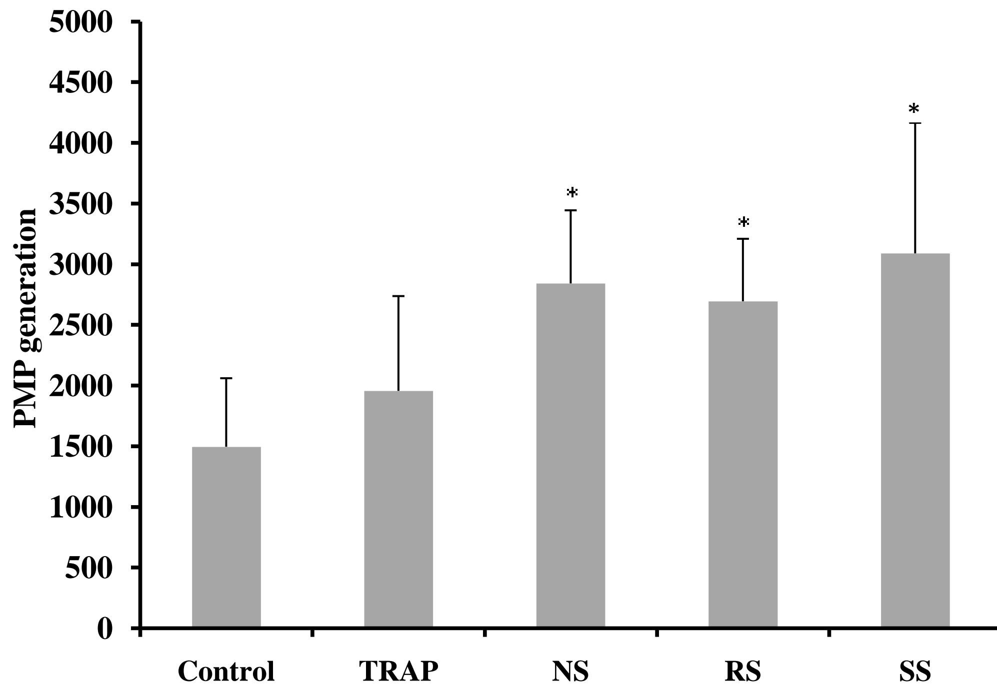

Fig 4.15: PMP generation due to washed platelet exposure to dynamic shear stresses (normal, recirculation, and stenosis) for 30 min. PMP generation increased, compared to the control, at all shear stress magnitude and for TRAP treatment. (NS – Normal Shear; RS – Recirculation Shear; SS – Stenosis Shear; * – significant difference).

4.3 Platelet activation in presence of EC

4.3.1 P-selectin expression under dynamic shear stress

To understand the effect of platelet-EC interaction on platelet activation (surface P-selectin expression) under dynamic shear stress conditions, washed platelets (250,000/μl) were stimulated to three different dynamic shear stresses in the presence of confluent untreated and treated (TNF-α treated overnight) HCAEC for 60 min. Timed platelet samples were collected from the platelet-EC co-shearing system every 15 min and platelet activation was measured by platelet surface P-selectin expression. All mean fluorescence values were normalized to that of resting platelets. Normalized P-selectin expression of platelets, platelets with untreated EC, and platelets with treated EC under different dynamic shear stress conditions (normal, recirculation, and stenosis respectively) are represented by Figure 4.16, 4.17, 4.18. Statistical analysis (represented by table 4.8, 4.9, 4.10) was conducted using three-way ANOVA to examine the effects from shear stress pattern, shear exposure time, platelet-EC interaction on platelet surface CD62P expression. The results demonstrated that stenosis shear stress has a significant effect (P=0.0235) on P-selectin expression at any time point compared to the recirculation shear. However, cell interaction, shear exposure time or the combinations of the three factors did not have any significant effect on P-selectin expression.

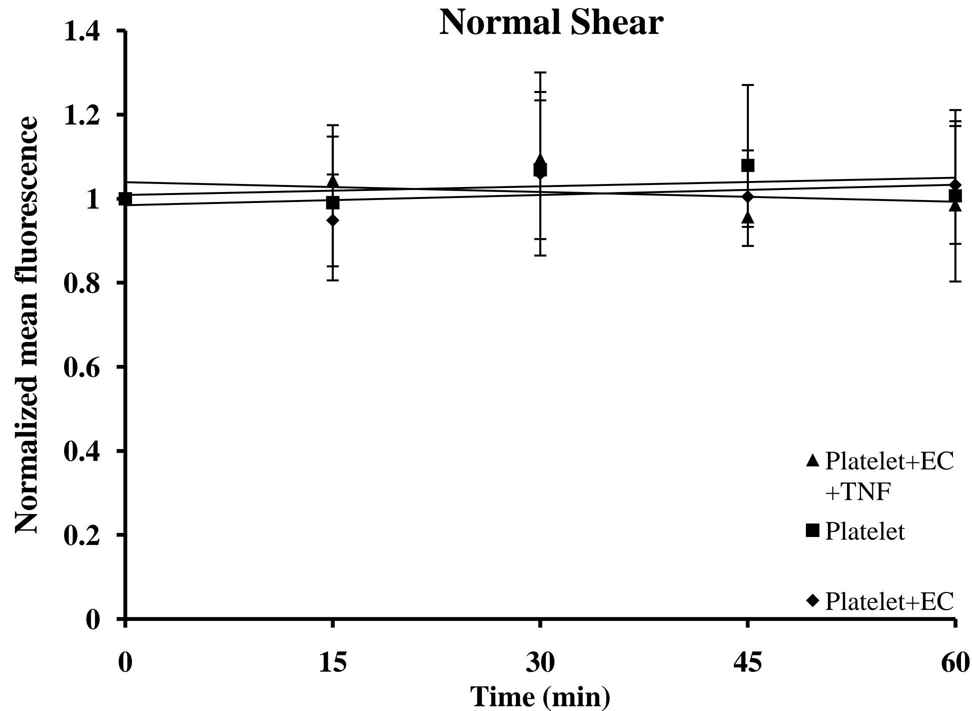

Fig 4.16: Washed platelets alone, in presence of untreated EC, and treated EC (TNF-α treated) were exposed to normal shear stress condition separately and P-selectin expression were measured every 15 min for 1 hr for platelet activation. Data are presented as Mean ± SD after normalizing all mean fluorescence values with that of platelet (before shear exposure). Statistical data analysis by three-way ANOVA indicated significant (P=0.0235) increase in P-selectin expression only under different shear stress conditions. No significant effect of exposure time or cell interaction was observed on P-selectin expression.

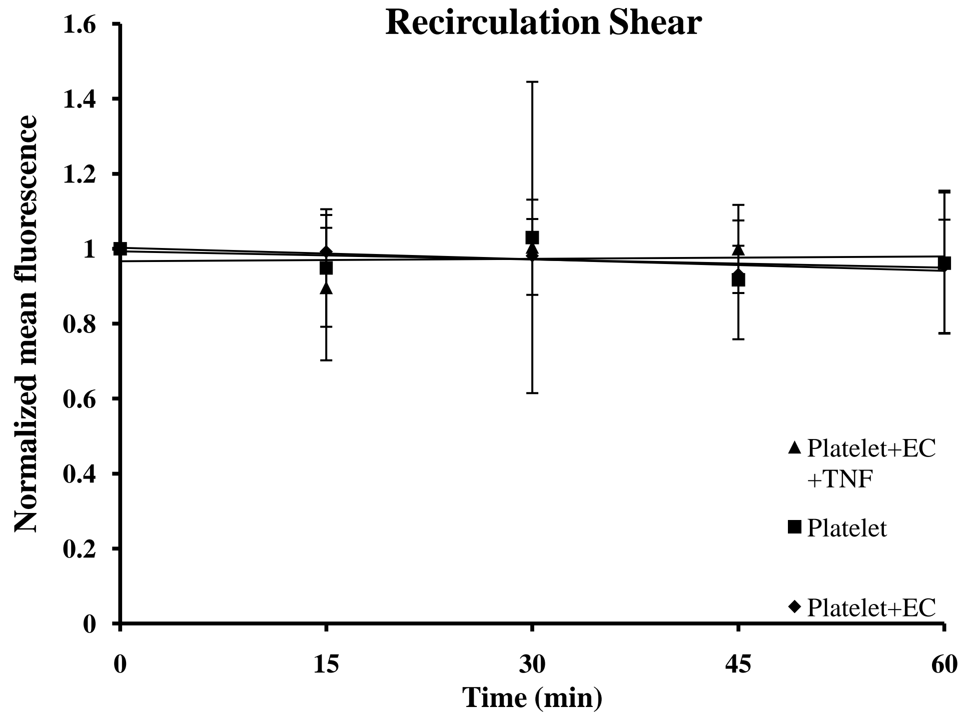

Fig 4.17: Comparison of platelet activation (P-selectin expression) under recirculation shear stress exposure of platelet, platelet with untreated EC, and platelet with TNF-α treated EC. Normalized mean fluorescence data are presented as Mean ± SD.

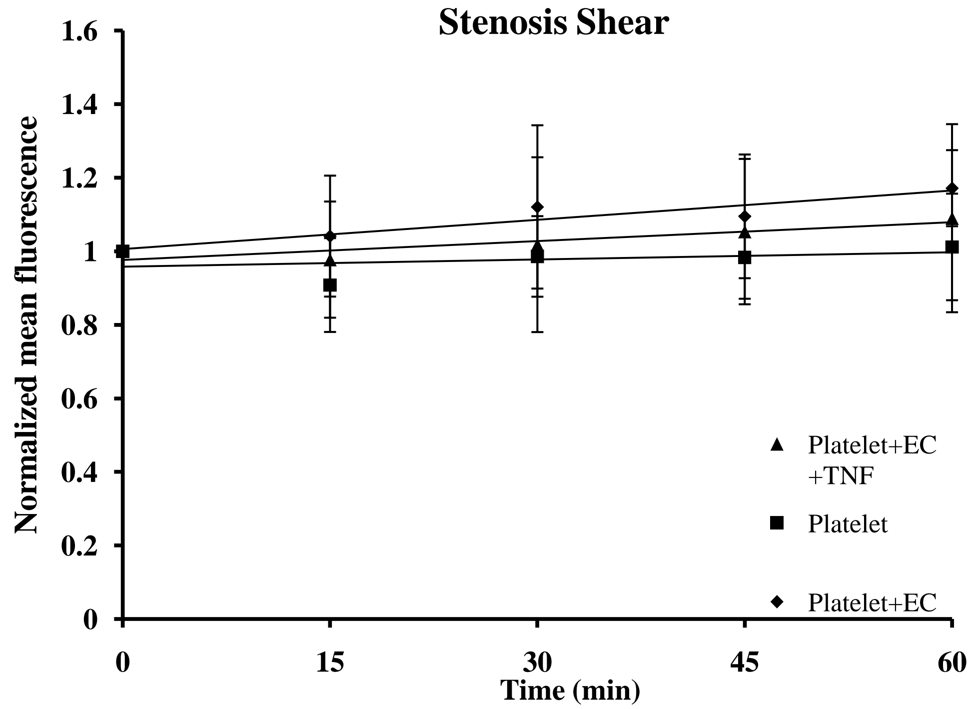

Fig 4.18: P-selectin expression of platelets when they were exposed to stenosis shear stress in absence of EC, in presence of untreated EC, and in presence of TNF-α treated EC. All mean fluorescence values were normalized to that of resting platelet and are presented as Mean ± SD.

Table 4.8: Effect of cell interaction on platelet activation (CD62P expression):

Cell Interaction	Platelet+untreated EC	Platelet + TNF-α treated EC
Platelet	0.2159	0.4388
Platelet + untreated EC		0.6258

Table 4.9: Effect of shear stress on P-selectin expression:

Shear Stress (Pa)	Recirculation Shear (RS)	Stenosis Shear (SS)
NS	0.0789	0.5993
RS		0.0235

Table 4.10: Effect of time of shear exposure on surface P-selectin expression:

Time (min)	30	45	60
15	0.0891	0.4808	0.1930
30		0.3173	0.6876
45			0.5492

4.3.2 GPIbα expression under dynamic shear stress

Platelet activation was measured as a function of GPIbα expression over a period of 60 min under different dynamic shear stress conditions. Platelets were also exposed to these shear conditions in presence of both untreated and TNF-α treated EC. Platelet samples were collected every 15 min and platelet surface GPIbα expression was quantified using anti-CD42b antibody. All mean fluorescence values were normalized with respect to that of resting platelets. Figure 4.19, 4.20, and 4.21 represent the normalized GPIbα expressions under normal shear, recirculation shear, and stenosis shear stress conditions respectively at all three conditions (resting platelets, platelet with untreated EC, and platelet with TNF-α treated EC). To analyze the effects of platelet-EC interaction, shear stress, and shear exposure time on GPIbα expression, three-way ANOVA was conducted. Table 4.11, 4.12, and 4.13 represent the statistical analysis. Results from the analysis show that cell interaction (Table 4.9) and shear exposure time (Table 4.11) both had significant effects on GPIbα expression.

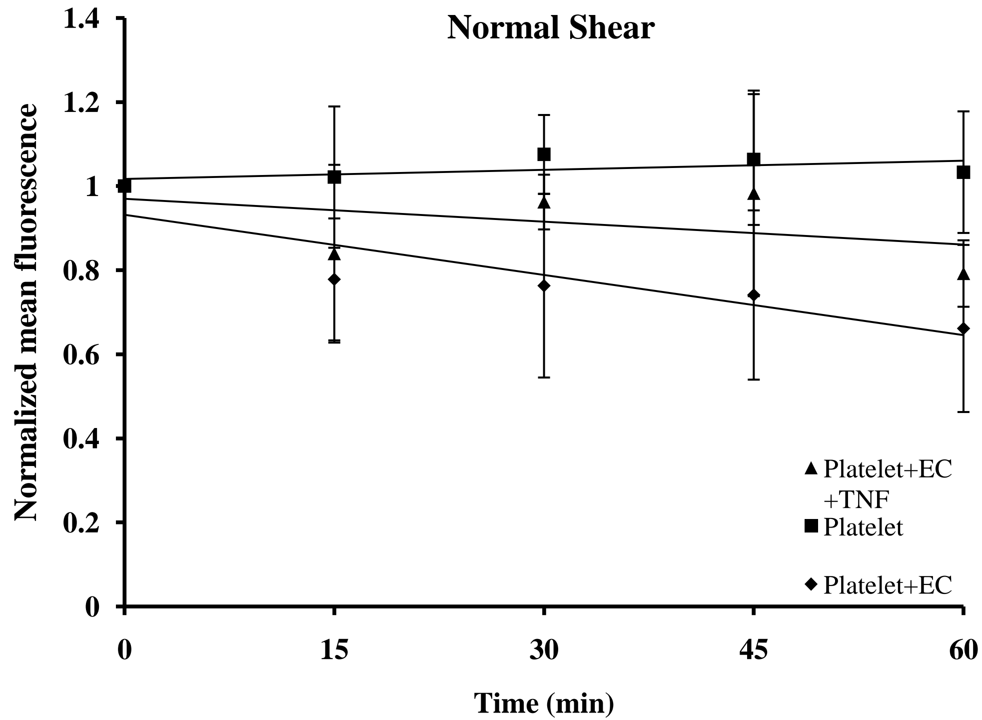

Fig 4.19: Comparison of surface GPIbα expression (every 15 min for 1 hr) of platelets when they were exposed (alone, in presence of untreated and treated EC respectively) to normal shear stress. All mean fluorescence values were normalized to that of corresponding resting platelets (not exposed to shear stress) and are represented as Mean ± SD. Statistical analysis by three-way ANOVA demonstrated significant effect of time and cell interaction on GPIbα expression, but no significant effect of shear stress.

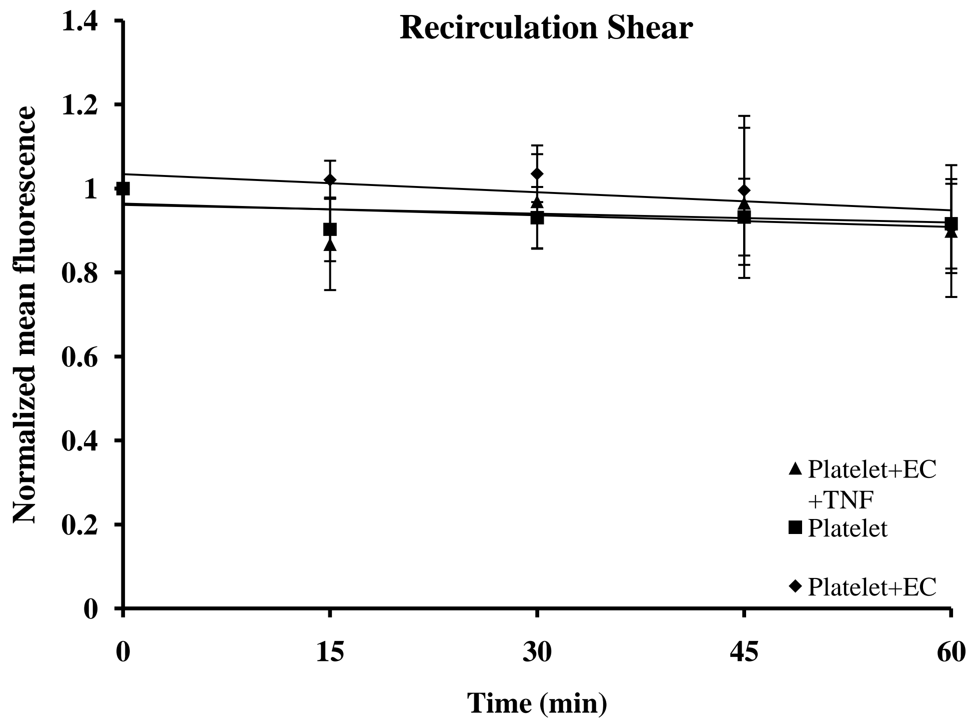

Fig 4.20: GPIbα expression of platelets under recirculation shear stress when platelets were exposed to that low shear in absence of EC, in presence of untreated and treated EC. Normalized values of all mean fluorescence values were used (Mean ± SD).

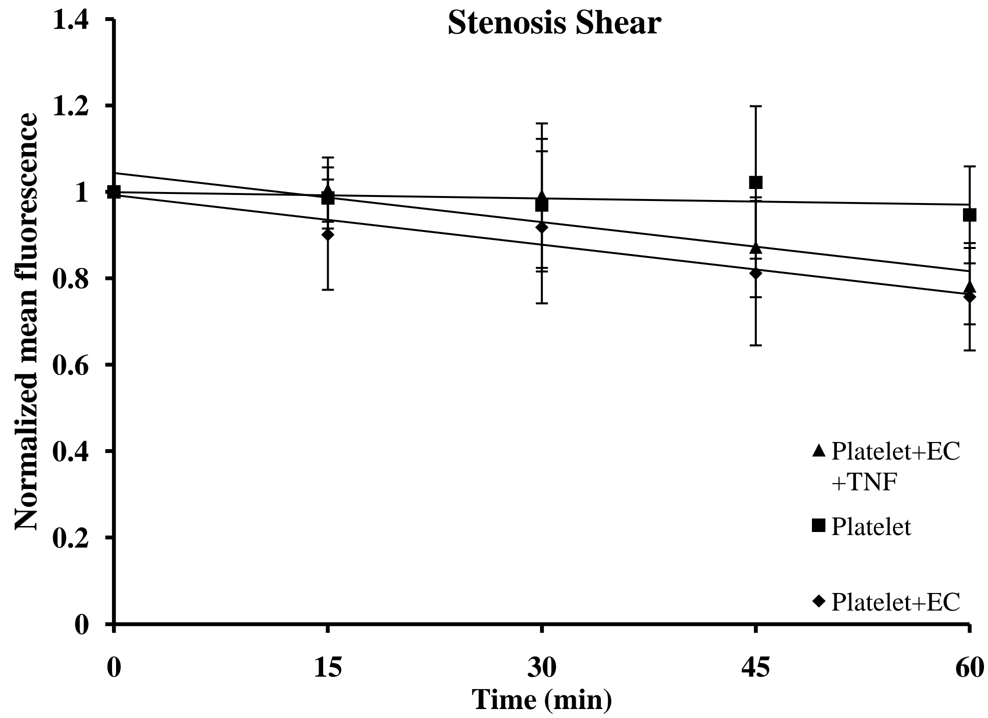

Fig 4.21: Washed platelets, platelets with untreated and treated EC were sheared under stenosis shear stress and surface GPIbα expression was measured. Data are presented as normalized mean fluorescence (with that of control platelet at 0 min) ± SD.

Table 4.11: Effect of cell interaction on GPIbα expression:

Cell Interaction	Platelet+untreated EC	Platelet + TNF-α treated EC
Platelet	<0.0001	0.054
Platelet + untreated EC		0.026

Table 4.12: Effect of shear stress on surface GPIbα expression:

Shear Stress (Pa)	Recirculation Shear (RS)	Stenosis Shear (SS)
Normal Shear (NS)	0.061	0.945
Recirculation Shear (RS)		0.071

Table 4.13: Effect of shear exposure time on GPIbα expression:

Time (min)	30	45	60
15	0.491	0.863	0.009
30		0.387	0.001
45			0.014

4.4 PMP generation in the presence of untreated EC

Washed platelets were exposed to dynamic shear stresses in presence of untreated EC. After 30 min of shear exposure, PMP generation was measured as described earlier. PMP generation plot (Figure 4.22) demonstrates increased number of PMP under all treatment conditions compared to the control (platelets at 0 min). Analysis of normalized values (with respect to control platelets) at different conditions revealed statistically significant ($P<0.05$) increase in PMP generation compared to the control.

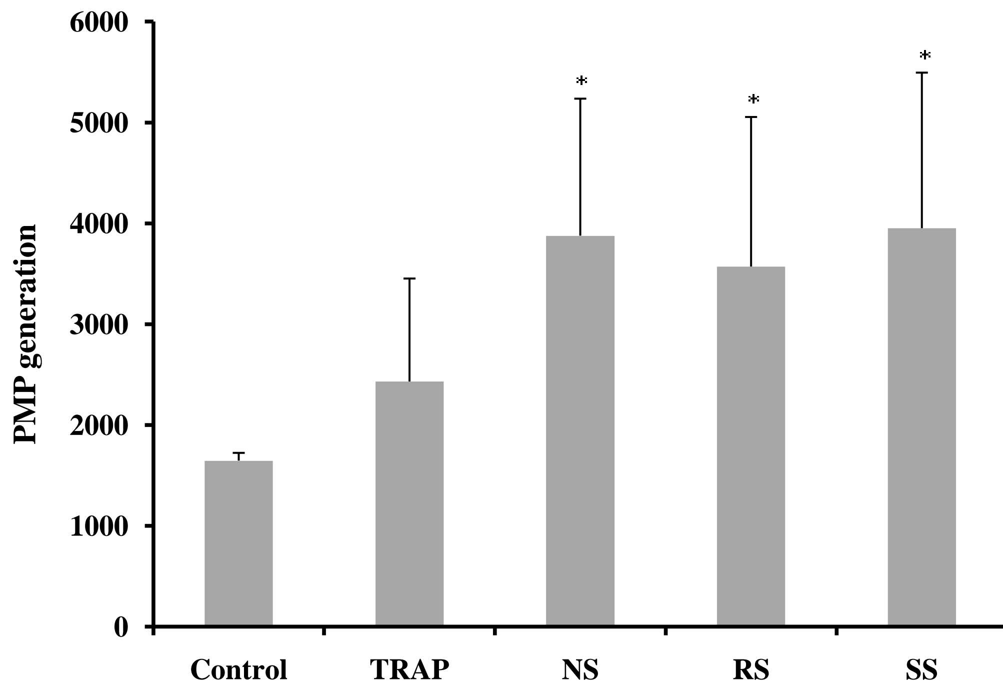

Fig 4.22: PMP generation after 30 min exposure of platelets to dynamic shear stresses (normal, recirculation, and stenosis) in presence of untreated EC. PMP generation increased at all shear stress magnitude and for TRAP treatment, compared to the control. (NS – Normal Shear; RS – Recirculation Shear; SS – Stenosis Shear; * – significant difference).

4.5 PMP generation in the presence of TNF-α treated EC

Dynamic shear stresses were applied on washed platelets, placed on TNF-α treated EC (activated), for 30 min duration. Post shearing, PMP generation was measured as described above. Significantly increased number of PMP were observed with sheared platelets compared to resting platelets (Figure 4.23). ANOVA analysis showed that under stenosis shear stress condition, PMP generation was significantly higher ($P<0.05$) than the other two shear conditions.

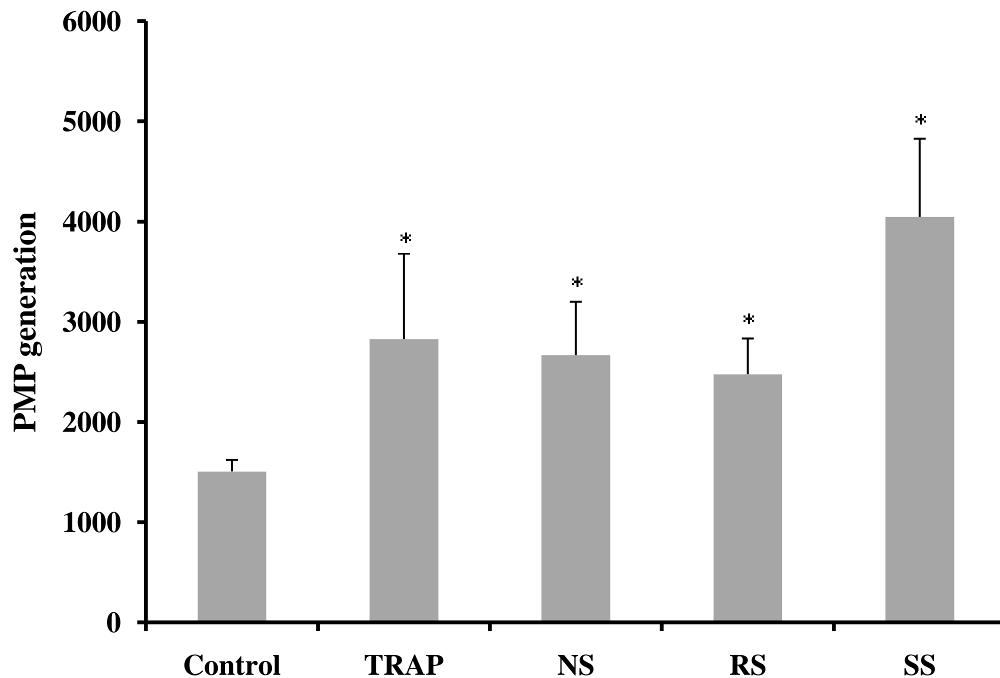

Fig 4.23: PMP generation due to platelet exposure (in presence of activated EC by TNF-α treatment) to dynamic shear stresses (normal, recirculation, and stenosis) for 30 min. PMP generation increased, compared to the control, at all shear stress magnitudes. TRAP treated washed platelet also showed enhanced PMP generation. (NS – Normal Shear; RS – Recirculation Shear; SS – Stenosis Shear; * – significant difference).

CHAPTER V

DISCUSSION

Altered blood shear stress plays an important role in platelet activation and can lead to thrombosis and pathogenesis of many cardiovascular diseases. Platelets are constantly exposed to dynamic shear stress induced by blood flow, which modulates their functions and behaviors. Communication between vascular endothelial cells (EC) and platelets also affects platelet functions and activities. Many studies have been conducted to study platelet responses to altered shear stresses. However, very few of these studies investigated the effects of physiologically relevant dynamic shear stress on platelet behavior, especially when EC were included in the shearing system. The major goal of this study was to subject platelets to physiologically relevant shear stress patterns in the absence/presence of normal or cytokine (TNF-α) stimulated EC and to investigate platelet activation and PMP generation. Placing both platelets and EC in a physiologically relevant dynamic shear stress environment would bring more insight into the role of shear stress and platelet-EC communication on platelet activities *in vivo*.

A hemodynamic cone and plate shearing device was used in this study to

effectively replicate the necessary shear stress conditions. Constant shear stress of 0.1, 0.3, 1, and 3 Pa for different durations (60-180 min) were applied to platelets in constant shear stress experiments. In dynamic shear stress experiments, pulsatile shear stresses (estimated by numerical simulation[103]) in a normal left coronary artery, around a 60% stenosis, and in the recirculation zone past the stenosis were used to stimulate platelets for a duration of 60 min. After different shear exposure, platelet responses were measured by platelet surface P-selectin and GPIbα expression using flow cytometry. In a similar manner, platelet microparticle generation was also quantified. Surface glycoproteins (P-selectin and GPIbα) and PMP generation were further measured after shearing platelets in presence of confluent HCAEC (untreated or treated with TNF-α) under dynamic shear stress. During these experiments, the setup temperature was maintained at 37°C (as body temperature is approximately 37°C and is required to maintain a healthy EC) while the platelet experiments (platelets sheared in absence of EC) were conducted at room temperature. However, results from some preliminary experiments, in our lab, indicated that there was no variation in platelet responses due to temperature change.

EC were grown on fibronectin coated plates, to 1) partially mimic the ECM and 2) allow EC's to adhere to the plates rapidly. This was used during the entire experiment paradigm (as platelet and EC were sheared for 1 hr duration). A literature survey shows that, the presence of fibronectin does not affect EC responses as a study by Qi *et al*. reported that EC cultured on both uncoated and fibronectin-coated plates induced similar EC responses[104]. However, other studies also reported that EC response varies when they are cultured on different substrates including collagen, gelatin[105]. Therefore, this study is

limited by the substrate that cells were grown on and we cannot extend our results to other substrates, such as collagen, or the native extracellular matrix.

P-selectin are stored on the α-granule membrane of resting platelets which becomes expressed on the platelet membrane upon activation. They can mediate thrombus formation by assisting in rolling of platelets on EC. In this study, it was observed that platelet surface P-selectin expression responded differently to different shear stress patterns. Platelets exposed to constant shear stress with elevated amplitude (1 Pa and 3 Pa) had a significant increase in P-selectin expression as shear exposure time elongated. Platelets exposed to low amplitude constant shear stress (0.1 and 0.3 Pa) did not have any changes in P-selectin expression even after 60 min of shear exposure. Thus, it is evident that platelet surface P-selectin expression is more sensitive to elevated shear stress under constant shear conditions. A similar result was observed in a study conducted by Rubenstein *et al*. which reported significant platelet activation due to higher shear stress exposure for a duration of 40 min[19]. Results from the dynamic shear experiments revealed that none of the shear stress patterns (normal, recirculation & stenosis shear) had any significant effect on P-selectin expression. Under normal and recirculation shear conditions, shear stress varies between 0.1 to 1 Pa and 0.06 to 0.4 Pa respectively; whereas, for stenosis shear stress, the shear magnitude reaches as high as 6.5 Pa for a short duration of 0.1 sec (once in every 90 sec) and then goes back to normal shear magnitude for the rest of the time. The reason for no significant change in P-selectin expression, under dynamic shear stress, could be attributed to their relatively low shear magnitude and variation in shear stress over time. Moreover, the study by Rubenstein *et al.* proved that platelets exposed to same shear stress-exposure time get activated to the

same level[19]. This could be another reason for no significant change in P-selectin expression under normal and stenosis shear stress, as the shear stress-exposure time value for normal and stenosis shear stress are 1980 and 2088 dyne s/cm^2 respectively. For stenosis shear, no significant change in P-selectin expression could also be due to exposure of high shear stress for a very short duration, which is not probably sufficient to activate platelets. These results suggest that, even though the presence of a stenosis can greatly affect local flow and shear conditions, it may not be enough to induce any significant platelet activation.

Apart from P-selectin expression, platelet surface GPIbα expression was also measured under both constant and dynamic shear stress conditions. GPIbα mediates in firm adhesion of activated platelets to EC (which release vWF upon activation) via vWF-GPIbα interaction. From constant shear experiments, it was observed that expression of GPIbα decreased significantly (after an initial increase for 30 min) under elevated shear stress (3 Pa). However, low shear stress (0.1 and 0.3 Pa) did not induce any significant changes. For constant shear stress at 1 Pa, longer exposure duration induced a significant reduction in GPIbα expression. One of the reasons for this impairment could be attributed to thrombin generation. A study by Yin *et al*. reported that thrombin generation increases significantly at higher shear stress levels with longer shear exposure duration (30 min)[37]. Reported by another study by Michelson *et al.*, thrombin was shown to down regulate GPIbα expression[106]. Therefore, the reduction in GPIbα could possibly be due to the increase in thrombin generation (with increasing shear exposure duration). Significant decrease in GPIbα was also observed under constant shear stress conditions by Leytin *et al*.s' group who attributed clustering of GPIbα on the platelet membrane as one of the

reason for decrease of the glycoprotein's expression[47]. However, dynamic shear stress did not have any significant effect on platelet GPIbα expression. This is probably due to the same reason of low shear magnitude and variation in shear stress over time for different shear conditions, similar shear stress-exposure time value for normal and stenosis shear stress, and very short exposure of high shear stress for stenosis shear.

To investigate the effect of platelet-EC interaction on platelet activation, platelets were exposed to dynamic shear stresses in the presence of untreated EC. Interaction of platelets with untreated EC led to significant increase in P-selectin expression under elevated (stenosis) shear stress condition. However, platelets sheared in the presence of TNF-α activated EC, under stenosis shear, expressed less amount of P-selectin compared to platelets interacting with untreated EC and no significant increase was observed. Pathologically, these results would signify that, the presence of activated/damaged EC (induced by TNF-α) is probably inducing a protective mechanism that inhibits platelet activation, suggesting that the presence of a stenosis is not the only factor that will affect platelet activation; the communication between platelets and endothelial cells is also very important. To understand the combined effect of shear stress pattern, shear exposure time, and platelet-EC interaction on P-selectin expression, a three-way ANOVA analysis was conducted. The results from the analysis showed that P-selectin expression increased significantly when platelets were exposed to stenosis shear stress even at 15 min and remained high up to 60 min of shear exposure. The significant increase, under stenosis shear stress, was observed both in the presence or absence of EC (treated or untreated). However, platelets in the presence or absence of treated or untreated EC could not generate significant amount of P-selectin under normal and recirculation shear stress

conditions. This implies that P-selectin expression may be more sensitive to shear stress magnitude.

For GPIbα expression, it had been observed that exposure of platelets to different dynamic shear stress conditions, in the presence of untreated EC, led to a significant decrease in GPIbα expression. However, the decrease was not so significant for platelets interacting with TNF-α activated EC. To study the effect of shear stress, shear exposure time, and cell interaction on GPIbα expression, three-way ANOVA analysis was implemented. The results revealed that decrease in GPIbα expression was affected by shear exposure duration and cell interaction, but not by shear stress amplitude. Significant decrease in GPIbα expression was observed at longer shear exposure duration (at 60 min) under all shear stress magnitudes when platelets were sheared in the absence or presence of untreated or treated EC. These results indicated that the mechanism of GPIbα expression may be different from that of P-selectin expression (platelet activation). While P-selection expression seems to be more sensitive to shear stress amplitudes, GPIbα expression may be more sensitive to shear exposure time and the involvement of EC.

In addition, PMP generation was also measured after exposing platelets to constant and physiologically relevant dynamic shear stress conditions. PMP were identified based on their size (smaller than platelets) and positive Annexin V binding. Under both constant and dynamic shear stress conditions, PMP generation increased significantly. Elevated shear stress of 3 Pa induced significantly more PMP generation when compared to low amplitude constant shear stress (0.1, 0.3, and 1 Pa). From dynamic shear stress *in vitro* experiments, similar results were obtained for stenosis shear stress (high shear): enhanced PMP generation was observed compared to the other two

shear conditions. This could be attributed to higher platelet activation under elevated shear conditions. As shear stress can fracture the pseudopods of activated platelets, it can lead to PMP formation. Therefore, PMP generation is sensitive to shear stress amplitude; the presence of elevated shear stress can enhance PMP generation.

The presence of vascular EC further contributed to PMP generation. Some of the previous studies claimed that vWF-GPIbα interaction, influx of extracellular calcium could be the reasons for PMP generation under high shear stress[63;90]. Platelets interacting with untreated EC generated more PMP than that generated when platelets were exposed to shear stress alone. This may indicate that, in addition to shear stress, the communication between platelets and EC also contributes to PMP generation. Interestingly, PMP generation decreased when platelets were sheared under normal and recirculation shear stresses in the presence of TNF-α treated EC. One possible reason for this behavior could be the role of some kind of protective mechanism/cascade that was triggered by the presence of TNF-α which resulted in the inhibition of PMP generation. However, this mechanism also depends on the shear stress magnitude, as it was effective only under normal and low shear stress conditions, while high (stenosis) shear stress still induced an elevated PMP generation. In a similar study by Macey *et al.*, increased PMP formation was observed, under flow conditions, after exposing blood to activated EC[93]. All these results indicate that PMP generation is sensitive to shear stress, especially to elevated shear stress.

Overall, platelet activation (P-selectin expression), GPIbα expression, and PMP generation can all be affected by blood flow induced shear stress. Constant shear stress and dynamic (pulsatile) shear stress have different effects on these reactions. Therefore,

merely using high or low constant shear stress to study platelet functions does not seem to be feasible, since the shear conditions *in vivo* are generally dynamic and more complex. Including EC into the flow environment makes our study more physiologically relevant. Our results indicated that the communication between platelets and EC indeed contributed significantly to platelet activities. Surprisingly, when EC were activated or damaged by TNF-α, platelets did not get further activated but somewhat had a decreased P-selectin expression and increased GPIbα expression, indicating certain protective mechanism was triggered.

CHAPTER VI

CONCLUSION

In vitro experiments were conducted to investigate the effects of constant (0.1, 0.3, 1, and 3 Pa) and dynamic shear stress (normal, recirculation, and stenosis shear stress) on platelet activities and PMP generation by exposing platelets to these stresses, in a hemodynamic cone and plate shearing device, in the presence or absence of confluent human coronary artery EC (treated with TNF-α or untreated). Our results demonstrated that elevated constant shear stress significantly increased in P-selectin expression (platelet activation) and impaired platelet GPIbα expression (important glycoprotein in adhesion and aggregation). PMP generation also increased as shear stress increased. However, dynamic shear stresses could not induce any significant changes in P-selectin or GPIbα expression, though generated significant amount of PMP. Communication between EC and platelets, under elevated pulsatile shear stress, affected platelet activation further as platelets sheared in the presence of untreated EC expressed significantly enhanced amount of P-selectin and decreased amount of GPIbα under stenosis shear condition. No significant changes were observed in either of the expressions under recirculation or normal shear condition. Platelets sheared with TNF-α

treated EC, under dynamic shear stresses, also did not induce any significant changes; though P-selectin expression increased and GPIbα expression was impaired. Platelets interacting with both treated and untreated EC induced significant increase in PMP generation. However, platelets interacting with TNF-α treated EC produced less amount of PMP than platelets sheared with untreated EC under normal and recirculation shear stress.

All these results indicated that altered shear stress can enhance platelet activation, impair GPIbα expression, and cause PMP generation. Furthermore, the presence of untreated EC augmented these platelet responses, which indicated that platelet-EC interaction could modulate platelet function and activity. Activated platelets and PMP (generated due to platelet activation) may interact with EC and mediate the progression of cardiovascular diseases. Thus, the results support our hypothesis that altered shear stress induced by disturbed blood flow conditions can activate platelets, which can be enhanced due to platelet-EC interactions, leading to the pathogenesis of various cardiovascular diseases.

REFERENCES

1. Adams R, rown T, ai S. Heart Disease and Stroke Statistics—2010 Update: A Report From the American Heart Association. Circulation 2010;121:e1-e170.

2. White JG. Anatomy and structural organization of platelets. In: RW Colman JHVMES, ed. Hemostasis and Thrombosis: Basic principles and chemical practice. 1994:397-413.

3. Harrison P, Cramer EM. Platelet alpha-granules. Blood Rev. 1993;7:52-62.

4. Hellums JD. 1993 Whitaker Lecture: biorheology in thrombosis research. Ann.Biomed.Eng 1994;22:445-455.

5. Gawaz M. Role of platelets in coronary thrombosis and reperfusion of ischemic myocardium. Elsevier 2004;61:498-511.

6. Suhasini Kulkarni SMDCLYCRMFPMKAHASISHFLaSPJ. A revised model of platelet aggregation. The journal of Clinical Investigation 2011;105:783-791.

7. Paul S.Frenette CVDLWKJSSBKJHHDVaDDW. P-selectin Glycoprotein Ligand 1 (PSGL-1) Is Expressed on Platelets and Can Mediate Platelet-Endothelial Interactions In Vivo. Journal of Experimental Medicine 2000;191:1413-1422.

8. Kerstin Jurk and Beate E.Kehrel. Platelets: Physiology and Biochemistry. Seminars in Thrombosis and Hemostasis 2005;31:

9. Phillips DR, Charo IF, Parise LV, Fitzgerald LA. The platelet membrane glycoprotein IIb-IIIa complex. Blood 1988;71:831-843.

10. Chatzizisis YS, Giannoglou GD. Coronary hemodynamics and atherosclerotic wall stiffness: a vicious cycle. Med.Hypotheses 2007;69:349-355.

11. Strony J, Phillips M, Brands D, Moake J, Adelman B. Aurintricarboxylic acid in a canine model of coronary artery thrombosis. Circulation 1990;81:1106-1114.

12. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. JAMA 1999;282:2035-2042.

13. Nomura S, Tandon NN, Nakamura T et al. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. Atherosclerosis 2001;158:277-287.

14. Brown CH, III, Leverett LB, Lewis CW, Alfrey CP, Jr., Hellums JD. Morphological, biochemical, and functional changes in human platelets subjected to shear stress. J.Lab Clin.Med. 1975;86:462-471.

15. Sakariassen KS, Holme PA, Orvim U et al. Shear-induced platelet activation and platelet microparticle formation in native human blood. Thromb.Res. 1998;92:S33-S41.

16. Sheriff J, Bluestein D, Girdhar G, Jesty J. High-shear stress sensitizes platelets to subsequent low-shear conditions. Ann.Biomed.Eng 2010;38:1442-1450.

17. Ramstack JM, Zuckerman L, Mockros LF. Shear-induced activation of platelets. J.Biomech. 1979;12:113-125.

18. Wurzinger LJ, Opitz R, Blasberg P, Schmid-Schonbein H. Platelet and coagulation parameters following millisecond exposure to laminar shear stress. Thromb.Haemost. 1985;54:381-386.

19. Rubenstein DA, Yin W. Quantifying the effects of shear stress and shear exposure duration regulation on flow induced platelet activation and aggregation. J.Thromb.Thrombolysis. 2010;30:36-45.

20. O'Connor CM, Gurbel PA, Serebruany VL. Usefulness of soluble and surface-bound P-selectin in detecting heightened platelet activity in patients with congestive heart failure. Am.J.Cardiol. 1999;83:1345-1349.

21. Minamino T, Kitakaze M, Sanada S et al. Increased expression of P-selectin on platelets is a risk factor for silent cerebral infarction in patients with atrial fibrillation: role of nitric oxide. Circulation 1998;98:1721-1727.

22. Einav S, Bluestein D. Dynamics of blood flow and platelet transport in pathological vessels. Ann.N.Y.Acad.Sci. 2004;1015:351-366.

23. Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. J.Biol.Chem. 1985;260:11107-11114.

24. Leytin V, Mody M, Semple JW, Garvey B, Freedman J. Flow cytometric parameters for characterizing platelet activation by measuring P-selectin (CD62) expression: theoretical consideration and evaluation in thrombin-treated platelet populations. Biochem.Biophys.Res.Commun. 2000;269:85-90.

25. Dunlop LC, Skinner MP, Bendall LJ et al. Characterization of GMP-140 (P-selectin) as a circulating plasma protein. J.Exp.Med. 1992;175:1147-1150.

26. Stone JP, Wagner DD. P-selectin mediates adhesion of platelets to neuroblastoma and small cell lung cancer. J.Clin.Invest 1993;92:804-813.

27. Merten M, Thiagarajan P. P-selectin expression on platelets determines size and stability of platelet aggregates. Circulation 2000;102:1931-1936.

28. Hsu-Lin S, Berman CL, Furie BC, August D, Furie B. A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. J.Biol.Chem. 1984;259:9121-9126.

29. Frenette PS, Johnson RC, Hynes RO, Wagner DD. Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. Proc.Natl.Acad.Sci.U.S.A 1995;92:7450-7454.

30. Larsen E, Celi A, Gilbert GE et al. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. Cell 1989;59:305-312.

31. da Costa MP, Garcia-Vallejo JJ, van Thienen JV et al. P-selectin glycoprotein ligand-1 is expressed on endothelial cells and mediates monocyte adhesion to activated endothelium. Arterioscler.Thromb.Vasc.Biol. 2007;27:1023-1029.

32. Yokoyama S, Ikeda H, Haramaki N et al. Platelet P-selectin plays an important role in arterial thrombogenesis by forming large stable platelet-leukocyte aggregates. J.Am.Coll.Cardiol. 2005;45:1280-1286.

33. Merten M, Chow T, Hellums JD, Thiagarajan P. A new role for P-selectin in shear-induced platelet aggregation. Circulation 2000;102:2045-2050.

34. Goto S, Ichikawa N, Lee M et al. Platelet surface P-selectin molecules increased after exposing platelet to a high shear flow. Int.Angiol. 2000;19:147-151.

35. Lu Q, Malinauskas RA. Comparison of Two Platelet Activation Markers Using Flow Cytometry After In Vitro Shear Stress Exposure of Whole Human Blood. Artif.Organs 2010

36. Zhang JN, Bergeron AL, Yu Q et al. Duration of exposure to high fluid shear stress is critical in shear-induced platelet activation-aggregation. Thromb.Haemost. 2003;90:672-678.

37. Yin W, Rubenstein DA. Dose Effect of Shear Stress on Platelet Complement Activation in a Cone and Plate Shearing Device. Cellular and Molecular Bioengineering 2009;2:274-280.

38. Ruggeri ZM. The platelet glycoprotein Ib-IX complex. Prog.Hemost.Thromb. 1991;10:35-68.

39. Lopez JA, Berndt MC. The GPIb-IX-V complex. Platelets. 2002:85-104.

40. Bergmeier W, Piffath CL, Goerge T et al. The role of platelet adhesion receptor GPIbalpha far exceeds that of its main ligand, von Willebrand factor, in arterial thrombosis. Proc.Natl.Acad.Sci.U.S.A 2006;103:16900-16905.

41. Berndt MC, Shen Y, Dopheide SM, Gardiner EE, Andrews RK. The vascular biology of the glycoprotein Ib-IX-V complex. Thromb.Haemost. 2001;86:178-188.

42. Romo GM, Dong JF, Schade AJ et al. The glycoprotein Ib-IX-V complex is a platelet counterreceptor for P-selectin. J.Exp.Med. 1999;190:803-814.

43. Dong JF, Sae-Tung G, Lopez JA. Role of glycoprotein V in the formation of the platelet high-affinity thrombin-binding site. Blood 1997;89:4355-4363.

44. Nesbitt WS, Kulkarni S, Giuliano S et al. Distinct glycoprotein Ib/V/IX and integrin alpha IIbbeta 3-dependent calcium signals cooperatively regulate platelet adhesion under flow. J.Biol.Chem. 2002;277:2965-2972.

45. Andrews RK, Gardiner EE, Shen Y, Whisstock JC, Berndt MC. Glycoprotein Ib-IX-V. Int.J.Biochem.Cell Biol. 2003;35:1170-1174.

46. Jurk K, Kehrel BE. Platelets: physiology and biochemistry. Semin.Thromb.Hemost. 2005;31:381-392.

47. Leytin V, Allen DJ, Mykhaylov S et al. Pathologic high shear stress induces apoptosis events in human platelets. Biochem.Biophys.Res.Commun. 2004;320:303-310.

48. Li M, Cong Y, Deng X, Hu J, Qin X. [Impact of shear stress on expression of platelet membrane glycoproteins]. Zhonghua Yi.Xue.Za Zhi. 2002;82:267-270.

49. Chow TW, Hellums JD, Moake JL, Kroll MH. Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. Blood 1992;80:113-120.

50. Konstantopoulos K, Chow TW, Turner NA, Hellums JD, Moake JL. Shear stress-induced binding of von Willebrand factor to platelets. Biorheology 1997;34:57-71.

51. Horstman LL, Ahn YS. Platelet microparticles: a wide-angle perspective. Crit Rev.Oncol.Hematol. 1999;30:111-142.

52. Wolf P. The nature and significance of platelet products in human plasma. Br.J.Haematol. 1967;13:269-288.

53. Holme PA, Brosstad F, Solum NO. The difference between platelet and plasma FXIII used to study the mechanism of platelet microvesicle formation. Thromb.Haemost. 1993;70:681-686.

54. Bulboaca, Adriana and Bulboaca, A. Platelet microparticles from their features to stroke implication. ROMANIAN JOURNAL OF NEUROLOGY 9(1), 13-17. 2010.
Ref Type: Journal (Full)

55. Holme PA, Brosstad F, Solum NO. Platelet-derived microvesicles and activated platelets express factor Xa activity. Blood Coagul.Fibrinolysis 1995;6:302-310.

56. Monkovic DD, Tracy PB. Functional characterization of human platelet-released factor V and its activation by factor Xa and thrombin. J.Biol.Chem. 1990;265:17132-17140.

57. Sims PJ, Faioni EM, Wiedmer T, Shattil SJ. Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. J.Biol.Chem. 1988;263:18205-18212.

58. George JN, Pickett EB, Saucerman S et al. Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. J.Clin.Invest 1986;78:340-348.

59. Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. J.Biol.Chem. 1989;264:17049-17057.

60. Nomura S, Nakamura T, Cone J, Tandon NN, Kambayashi J. Cytometric analysis of high shear-induced platelet microparticles and effect of cytokines on microparticle generation. Cytometry 2000;40:173-181.

61. Yano Y, Kambayashi J, Shiba E et al. The role of protein phosphorylation and cytoskeletal reorganization in microparticle formation from the platelet plasma membrane. Biochem.J. 1994;299 (Pt 1):303-308.

62. Pontiggia L, Steiner B, Ulrichts H et al. Platelet microparticle formation and thrombin generation under high shear are effectively suppressed by a monoclonal antibody against GPIba. Thromb.Haemost. 2006;96:774-780.

63. Miyazaki Y, Nomura S, Miyake T et al. High shear stress can initiate both platelet aggregation and shedding of procoagulant containing microparticles. Blood 1996;88:3456-3464.

64. Holme PA, Orvim U, Hamers MJ et al. Shear-induced platelet activation and platelet microparticle formation at blood flow conditions as in arteries with a severe stenosis. Arterioscler.Thromb.Vasc.Biol. 1997;17:646-653.

65. Nomura S, Imamura A, Okuno M et al. Platelet-derived microparticles in patients with arteriosclerosis obliterans: enhancement of high shear-induced microparticle generation by cytokines. Thromb.Res. 2000;98:257-268.

66. Mallat Z, Hugel B, Ohan J et al. Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. Circulation 1999;99:348-353.

67. Barry OP, Pratico D, Lawson JA, FitzGerald GA. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. J.Clin.Invest 1997;99:2118-2127.

68. Sinauridze EI, Kireev DA, Popenko NY et al. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. Thromb.Haemost. 2007;97:425-434.

69. Gilbert GE, Sims PJ, Wiedmer T et al. Platelet-derived microparticles express high affinity receptors for factor VIII. J.Biol.Chem. 1991;266:17261-17268.

70. Owens MR, Holme S, Cardinali S. Platelet microvesicles adhere to subendothelium and promote adhesion of platelets. Thromb.Res. 1992;66:247-258.

71. Merten M, Pakala R, Thiagarajan P, Benedict CR. Platelet microparticles promote platelet interaction with subendothelial matrix in a glycoprotein IIb/IIIa-dependent mechanism. Circulation 1999;99:2577-2582.

72. Diehl P, Nagy F, Sossong V et al. Increased levels of circulating microparticles in patients with severe aortic valve stenosis. Thromb.Haemost. 2008;99:711-719.

73. Lee YJ, Jy W, Horstman LL et al. Elevated platelet microparticles in transient ischemic attacks, lacunar infarcts, and multiinfarct dementias. Thromb.Res. 1993;72:295-304.

74. Gawaz M, Neumann FJ, Ott I, Schiessler A, Schomig A. Platelet function in acute myocardial infarction treated with direct angioplasty. Circulation 1996;93:229-237.

75. Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. Am.J.Physiol 1986;250:H1145-H1149.

76. Frangos JA, McIntire LV, Eskin SG. Shear stress induced stimulation of mammalian cell metabolism. Biotechnol.Bioeng. 1988;32:1053-1060.

77. Lin MC, Almus-Jacobs F, Chen HH et al. Shear stress induction of the tissue factor gene. J.Clin.Invest 1997;99:737-744.

78. Yin W, Shanmugavelayudam SK, Rubenstein DA. The effect of physiologically relevant dynamic shear stress on platelet and endothelial cell activation. Thromb.Res. 2010

79. Cotran RS, Pober JS. Cytokine-endothelial interactions in inflammation, immunity, and vascular injury. J.Am.Soc.Nephrol. 1990;1:225-235.

80. Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. J.Exp.Med. 1986;163:740-745.

81. Levine B, Kalman J, Mayer L, Fillit HM, Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. N.Engl.J.Med. 1990;323:236-241.

82. Yokoyama T, Vaca L, Rossen RD et al. Cellular basis for the negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian heart. J.Clin.Invest 1993;92:2303-2312.

83. Brett J, Gerlach H, Nawroth P et al. Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. J.Exp.Med. 1989;169:1977-1991.

84. Mantovani A, Bussolino F, Dejana E. Cytokine regulation of endothelial cell function. FASEB J. 1992;6:2591-2599.

85. Prentice CR. Pathogenesis of thrombosis. Haemostasis 1990;20 Suppl 1:50-59.

86. Libby P. The interface of atherosclerosis and thrombosis: basic mechanisms. Vasc.Med. 1998;3:225-229.

87. Massberg S, Enders G, Leiderer R et al. Platelet-endothelial cell interactions during ischemia/reperfusion: the role of P-selectin. Blood 1998;92:507-515.

88. Burger PC, Wagner DD. Platelet P-selectin facilitates atherosclerotic lesion development. Blood 2003;101:2661-2666.

89. Yong AS, Pennings GJ, Chang M et al. Intracoronary shear-related up-regulation of platelet P-selectin and platelet-monocyte aggregation despite the use of aspirin and clopidogrel. Blood 2011;117:11-20.

90. Reininger AJ, Heijnen HF, Schumann H et al. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. Blood 2006;107:3537-3545.

91. Kulkarni S, Dopheide SM, Yap CL et al. A revised model of platelet aggregation. J.Clin.Invest 2000;105:783-791.

92. Galbusera M, Zoja C, Donadelli R et al. Fluid shear stress modulates von Willebrand factor release from human vascular endothelium. Blood 1997;90:1558-1564.

93. Macey MG, Wolf SI, Lawson C. Microparticle formation after exposure of blood to activated endothelium under flow. Cytometry A 2010;77:761-768.

94. Senden NH, Jeunhomme TM, Heemskerk JW et al. Factor Xa induces cytokine production and expression of adhesion molecules by human umbilical vein endothelial cells. J.Immunol. 1998;161:4318-4324.

95. Henn V, Slupsky JR, Grafe M et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 1998;391:591-594.

96. Slupsky JR, Kalbas M, Willuweit A et al. Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. Thromb.Haemost. 1998;80:1008-1014.

97. Mohle R, Green D, Moore MA, Nachman RL, Rafii S. Constitutive production and thrombin-induced release of vascular endothelial growth factor by human megakaryocytes and platelets. Proc.Natl.Acad.Sci.U.S.A 1997;94:663-668.

98. Bombeli T, Schwartz BR, Harlan JM. Adhesion of activated platelets to endothelial cells: evidence for a GPIIbIIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), alphavbeta3 integrin, and GPIbalpha. J.Exp.Med. 1998;187:329-339.

99. Nath J, Martini F. The Blood. In: Pearson International, ed. Fundamentals of Anatomy and Physiology. 2009:681-718.

100. Kroll MH, Hellums JD, McIntire LV, Schafer AI, Moake JL. Pletelets and Shear Stress. The journal of the American Society of Hematology 1996;88:1525-1541.

101. Blackman BR, Barbee KA, Thibault LE. In vitro cell shearing device to investigate the dynamic response of cells in a controlled hydrodynamic environment. Ann.Biomed.Eng 2000;28:363-372.

102. Buschmann MH, Dieterich P, Adams NA, Schnittler HJ. Analysis of flow in a cone-and-plate apparatus with respect to spatial and temporal effects on endothelial cells. Biotechnol.Bioeng. 2005;89:493-502.

103. Shanmugavelayudam SK, Rubenstein DA, Yin W. Effect of geometrical assumptions on numerical modeling of coronary blood flow under normal and disease conditions. J.Biomech.Eng 2010;132:061004.

104. Qi J, Kreutzer DL, Piela-Smith TH. Fibrin induction of ICAM-1 expression in human vascular endothelial cells. J.Immunol. 1997;158:1880-1886.

105. Pratt BM, Form D, Madri JA. Endothelial cell-extracellular matrix interactions. Ann.N.Y.Acad.Sci. 1985;460:274-288.

106. Michelson AD. Thrombin-induced down-regulation of the platelet membrane glycoprotein Ib-IX complex. Semin.Thromb.Hemost. 1992;18:18-27.

APPPENDIX

BASIC PROGRAM USED IN CONE

AND PLATE SHEARING DEVICE

Constant shear stress experiment

rem 2667 is 2.4 dynes/cm2 and 10004 is for 9 dynes/cm2

```
joff
sposx 0
accx 0
B = 1111
velx B
jogx
0
end
$
```

Dynamic shear stress experiment

Normal Shear

```
joff
300
sposx  0
accx 0

A = 332
B = 308
C = 360
velx A
jogx
wait 250

100
A = A + B
velx A
```

```
wait 10
if A > 11108 then goto 200
goto 100

200
A = A - C
velx A
wait 10
if A > 340 then goto 200

goto 300

end
$
```

Recirculation Shear

```
joff
300
sposx  0
accx 0

A = 83
B = 77
C = 90
velx A
jogx
wait 250

100
A = A + B
velx A
wait 10
if A > 2777 then goto 200
goto 100

200
A = A - C
velx A
wait 10
if A > 85 then goto 200

goto 300

end
$
```

Stenosis Shear

```
joff

N = 0

300

sposx  0
accx 0

N = N+1

A = 332
B = 308
C = 360
velx A
jogx
wait 250

100
A = A + B
velx A
wait 10

if A > 11108 then goto 200
goto 100

200
A = A - C
velx A
wait 10

if A > 340 then goto 200

if N < 70 then goto 300

velx 72000
jogx
wait 1000
N = 0

goto 300

end
$
```

CONFERENCE ABSTRACTS

Discrete phase modeling of platelet aggregation and adhesion in the left coronary artery (Published at 2010 BMES Conference)

Wei Yin, Rajesh Hariharan, Farzana Rouf, Saravan Shanmugavelayudam, David A. Rubenstein

Platelet activation and aggregation/adhesion can be affected by local blood flow. The goal of this study was to combine a CFD model and in vitro studies to estimate platelet shear stress and their aggregation/adhesion potential. A 3D left coronary artery model with a realistic geometry was built, with blood as a non-Newtonian fluid, and platelets as a discrete phase. Transient flow was solved using a k-ω turbulence model, under normal and stenosis conditions. Platelets were tracked using Lagrangian formulation and their shear stress history was calculated. The results demonstrated that with normal flow, platelets were exposed to pulsatile shear stress varying between 0 and 13 dynes/cm^2, while with a stenosis, platelet shear stress could reach 120 dynes/cm^2. In parallel, various shear stress was applied to platelets in a cone and plate shearing device and platelet surface glycoprotein Ibα (GPIbα) was measured by flow cytometry. The results indicated that exposure to low shear stress did not affect platelet GPIbα expression, while exposure to elevated shear stress led to a fast increase in GPIbα expression, followed by a gradual decay. A mathematical model was built based on this relationship, which was then fed back into the CFD model. Platelet aggregation/adhesion potential was estimated by the amount of GPIbα, the distance between two activated platelets and the distance between activated platelets and damaged blood vessel wall. This model significantly advanced the role of numerical simulation in understanding cellular responses to blood flow induced shear stress.

Effect of dynamic shear stress on platelet microparticle generation (Published at 2011 BMES Conference)

Wei Yin, Farzana Rouf and David A. Rubenstein

Introduction: Platelet microparticles (PMP) are small vesicular particles that are released from activated platelets. They retain certain platelet membrane glycoproteins such as GPIb, GPIIb/IIa and P-selectin. They are more pro-coagulant than activated platelets and play important roles thrombosis and atherosclerosis. Altered shear stress induced by disturbed blood flow is one of the risk factors that can cause platelet activation and PMP release. A lot of studies have been focused on the effect of elevated shear stress on PMP generation and activities. However, shear stress conditions *in vivo* are more complicated than just high or low shear stress. Due to the pulsatile nature of blood flow, platelets are continuously exposed to dynamic shear stress. It is not clear how PMP production is affected by dynamic shear conditions. Furthermore, vascular endothelial cells (EC) contribute significantly to the behavior of platelets under flow conditions. Therefore, investigating PMP generation in a physiologically relevant and dynamic shear environment with the presence of EC can help us to better understand how altered shear stress and the interaction between platelets and vascular EC contribute to platelet activation, PMP generation and cardiovascular disease development.

Materials and Methods: Washed platelets were prepared from fresh platelet rich plasma by centrifugation and exposed to various shear stress waveforms. Through a computer controlled dynamic cone and plate shearing device, washed platelets were stimulated by four constant stresses (0.1, 0.3, 1 and 3 Pa) and three pulsatile shear stresses (normal, low and high) for 60 minutes. Normal pulsatile shear stress varied between 0.1 and 1 Pa, mimicking shear stress in a normal left coronary artery; low pulsatile shear stress varied between 0.06 to 0.4 Pa, mimicking that in a recirculation zone. For high pulsatile shear stress, platelets were exposed to elevated shear stress at 6.5 Pa for 0.1 sec at a frequency of once every 90 seconds, mimicking the situation when platelets pass a 65% stenosis in the left coronary artery every 90 seconds. Confluent human coronary artery EC (untreated or treated with TNF-α overnight) were added to some of the dynamic shear experiments to investigate the role of EC on PMP generation. Post shearing, platelets were centrifuged at 1500xg for 15 min at room temperature. Platelet pellets were discarded and the supernatant containing PMP was collected and further centrifuged at 17,000xg for 2 min at 4°C. PMP pellets were then resuspended and particle surface Annexin V binding was measured using flow cytometry. 30,000 total events were counted. PMP were identified by their size in the forward and side scatter plot, and positive staining for Annexin V.

Results and Discussion: Our results are summarized in Table 1, which demonstrate that shear

Table 1. Number of PMP generated among 30,000 events using flow cytometry (n=6).

	Constant - 0.1 Pa	Constant – 0.3 Pa	Constant – 1 Pa	Constant – 3 Pa
Platelets	2300±427	2400±525	1996±877	3439±964
	Normal Pulsatile	Low Pulsatile	Elevated Pulsatile	
Platelets	2841±602	2693±515	3170±1135	
Platelets +EC	4320±1071	4598±1389	3950±1542	
Platelets +EC+TNF-α	2665±533	2475±356	4045±780	

stress at any level was able to induce PMP generation. Elevated shear stress (3 Pa) led to a significant increase in PMP generation compared to low amplitude constant shear stresses Normal and low amplitude pulsatile shear stress had similar effects on PMP generation as the low constant shear, while high pulsatile shear stress enhanced PMP generation significantly, even though the exposure to the elevated shear stress was very short (<0.1 sec). Furthermore, the

communication between platelets and EC affected PMP generation. When platelets were exposed to dynamic shear stresses with the presence of untreated EC, the number of PMP generated increased significantly under all shear conditions, indicting the interaction between platelets and EC regulated platelet activities. When platelets were sheared in the presence of EC pre-treated with TNF- α, only high pulsatile shear stress induced a further increase in PMP generation, but not the normal or low amplitude pulsatile shear stress.
Conclusions: Our results indicated that PMP generation was sensitive to shear stress amplitude. Even short exposure to elevated shear stress could lead to significant amount of PMP release. Also, the interaction between platelets and EC plays a role in PMP generation. However, when EC are activated or damaged, certain protective mechanism might be triggered to prevent PMP generation, but only under mild shear loading conditions.
Acknowledgements: We would like to thank American Heart Association for supporting this project.

Activated endothelial cells enhance platelet responses to dynamic shear stress conditions (Pulished at 2011 EB conference)

Wei Yin, Farzana Rouf and David A. Rubenstein

The goal of this study was to investigate the effects of various dynamic shear stress on platelet activation, with or without the presence of endothelial cells (EC). Washed platelets were exposed to constant (0.1, .03, 1 and 3 Pa) or physiologically relevant pulsatile shear stress (normal - 0.1~1 Pa, low- 0.05~0.5 Pa, high-0.1~6 Pa) for 60 min in a cone and plate shearing device. Platelet surface CD62P and CD42b expression was measured using flow cytometry. Elevated constant shear stress (1 and 3 Pa) can significantly enhance platelet surface CD62P expression, and impair platelet CD42b expression. While pathological pulsatile shear stress, even at an elevated level (6 Pa), was not able to achieve the same effect. However, the presence of coronary artery EC induced a pronounced change – elevated pulsatile shear stress significantly increased platelet CD62P expression and impaired CD42b production. Interestingly, this change was significantly enlarged when EC were pre-treated with TNF-α. These results suggested that pathological shear stress was only able to activate platelets through the help of endothelial activation, induced by either pathological shear condition itself, or chemical agonists.

VITA

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Candidate for the Degree of

Master of Science

Thesis: THE EFFECTS OF PHYSIOLOGICALLY RELEVANT SHEAR STRESS AND PLATELET-ENDOTHELIAL CELL INTERACTION ON PLATELET ACTIVATION AND PLATELET MICROPARTICLE GENERATION

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Scope and Method of Study: The major scope of this study was to investigate the effects of physiologically relevant shear stresses and platelet-endothelial cell (EC) interactions on platelet activation, and platelet microparticle (PMP) generation. *In vitro* experiments were conducted to investigate the effects of constant (0.1, 0.3, 1, and 3 Pa) and dynamic shear stress (normal, recirculation, and stenosis shear stress) on platelet activities and PMP generation. Platelets were exposed to these stresses, in a hemodynamic cone and plate shearing device, in the presence or absence of confluent human coronary artery EC (untreated or treated with TNF-α). Platelet activation was measured based on platelet surface P-selectin expression. Platelet surface glycoprotein GPIbα expression and platelet microparticle generation were also measured simultaneously using flow cytometry.

Findings and Conclusions: Experimental results indicated that altered shear stress can enhance platelet activation, impair GPIbα expression, and cause PMP generation. Furthermore, the presence of untreated EC augmented these platelet responses, which indicated that platelet-EC interaction could modulate platelet function and activity. These results support the hypothesis that altered shear stress induced by disturbed blood flow conditions can activate platelets, which can be enhanced due to platelet-EC interactions, leading to the pathogenesis of various cardiovascular diseases.

ADVISER'S APPROVAL: Dr. Wei Yin