"PROFILING OF THE PLATELET PROTEINS IN ATHEROSCLEROSIS RISK FACTOR GROUP: A COMPARATIVE ANALYSIS."

A DISSERTATION SUBMITTED

BY

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CERTIFICATE

This is to certify that the dissertation entitled **"Profiling Of The Platelet Proteins In Atherosclerosis Risk Factor Group: A Comparative Analysis"** submitted by **Binnu Gangadharan** in partial fulfillment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at **Thrombosis Research Unit**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695012.

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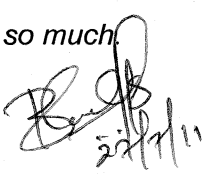

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ABBREVATIONS

%	Percent
[Ca]i	Calcium ion
ACD	Acid Citrate Dextrose
ADP	Adenosine diphosphate
CD	Cluster of Differentiation
cGMP	Cyclic Guanosine Monophosphate
CRP	C Reactive Protein
COX	Cyclooxygenase
DAG	Diacyl Glycerol
DTT	Dithiothreitol
GOD	Glucose Oxidase
GP	Glycoprotein
GPCR	G Protein Coupled Receptor
IL	Interleukin
IP3	Inositol Triphosphate
IU	International Unit
L	Ligand
µM	micromolar
ml	milliliter
mRNA	Messenger RNA
nM	nanomolar
NO	Nitric Oxide
oC	Degree Celsius

ORF	Open Reading Frame
P- Sel	P Selectin
PAF	Platelet Activating Factor
PAR	Protease Activated Receptor
PECAM	Platelet endothelial cell adhesion molecule
PDGF	Platelet-Derived Growth Factor
PF4	Platelet Factor 4
PIP2	Phosphatidyl Inositol 4, 5 bisphosphate
PMP	Platelet Microparticle
PMSF	Phenylmethylsulfonylflouride
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PVDF	Polyvinylidene Fluoride
SDS	Sodium Dodecyl Sulphate
SgIII	Secretogranin III
SSC	Side Scattering
TGF ß	Tumor Growth Factor-ß
TRAP	Thrombin Receptor Agonist Peptide
TXA2	Thromboxane A2
vSMC	Vascular Smooth Muscle Cells
vWF	von Willebrand Factor

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SYNOPSIS

Atherosclerosis is a chronic inflammatory disease influenced by circulating cells, including platelets. Platelet derived inflammatory mediators play an essential role in the pathogenesis of atherosclerosis, being involved from the primary phases of plaque development through leukocyte requirement to activate endothelium, to the eventual rupture of vulnerable atherosclerotic plaque with the formation of thrombus.

It is clear that platelets synthesize biologically relevant proteins in response to physiological stimuli that are regulated via gene expression programs at the translational level. Platelets activation leads to the release of many proteins, a large population of which are known inflammatory mediators (eg. CD40L, IL-1β, PF4 and RANTES) and studied extensively, whereas many of the other proteins released by activated platelets (eg Secretogranin III, Cyclophilin A, and Calumenin, have not been given much attention. The proteins get secreted by platelets can adhere to the vessel wall and promote atherosclerosis and -thrombosis.

Reports have established that diabetic patients are prone to atherosclerosis. It is also reported that hyperglycemia and high fibrinogen levels promotes atherosclerosis. Inflammation at the lesion site is the leading cause of cardiovascular death in diabetic subjects. Major percentage of releasate from activated platelets consists of inflammatory mediators and thus confirming their role in the progression of atherosclerosis.

Many proteins are known to be differently expressed in platelets when they are activated. It is not known how these factors could influence atherosclerosis progression. Studies have not been sufficient to identify their function to use one or more of these proteins as specific marker for platelet activation and a predictor of atherosclerosis. In order to identify the effects of such overexpressed proteins, difference between the proteins profiling of healthy and diabetic subjects may be a fascinating area of study. Proteins that

are not present in normal resting platelets but that exist in diabetic subjects need to be identified before they can be characterized.

Therefore this study was designed based on the hypothesis that activated platelets have a role in atherosclerosis and diabetic patients have an activated platelet proteome. In order to test this hypothesis, specific objectives were formed which includes:

- Analyze the platelet changes in each group, study their aggregation and adhesion variation.
- Study the specific markers for platelet activation (P Selectin, GpIIb).
- Check the presence of specific proteins (Secretogranin III and Cyclophilin A) in test subjects which are already been reported in activated samples.
- Study of platelet proteome of control, positive control and diabetic groups.

This dissertation is divided into 4 main chapters such as (i) Introduction; (ii) Materials and Methods (iii) Results and Discussion (iv) Summary & Conclusions.

Chapter I includes a brief introduction to the research topic and review of literature with citations. The reviewed literature proposes that a multitude of molecules released from activated platelets, membrane associated adhesive proteins and surface proteins present on the activated platelets etc are known to take part in the inflammatory response and/or vascular regeneration. Individuals having diabetics and hyper cholesterol are included in the risk factor groups of atherosclerosis. There are data which suggest platelets have a very important role in progression of atherosclerosis in diabetic subjects. However, this area is relatively less understood and needs further research to prove the involvement of specific platelets proteins in the onset of atherosclerosis in risk groups such as diabetics.

Chapter II gives the details of equipments and reagents used, along with sufficient descriptions of the procedures used in the study. Whole cell lysate was prepared by lysing cells in lysis buffer. Washed platelets were prepared by washing platelets 3-4 times in ACD-tyroids buffer. Platelets were activated with different concentration of thrombin and relesates were isolated from resting (control), thrombin activated and test subjects by high speed (50,000 g for 1h) ultracentrifugation. Platelets aggregation was studied using ADP and Collagen as a function of percentage transmittance. Adhesion was studied by SEM analysis. Platelets suspension was spread on fibrinogen and platelet adhesion and spreading was analyzed. Specific platelet activation marker CD62 and CD41 were analyzed by using flowcytometry. Platelet releasates were run on 12% acryamide gel for the separation of protein band. Western blot analysis was also done for two specific proteins. Two dimensional gel electrophoresis was done for the better comparison between control and test.

In Chapter III, results are represented with appropriate illustrations/ images and the findings are discussed in the light of current knowledge obtained from the literature. Results of present study clearly indicate that high glucose and fibrinogen level is an indication of hyperaggresive platelets. Response of platelets to agonist ADP and Collagen were different in study subjects, an indication that platelet functionality depends on pathological condition, as well as on medication, age and metabolism of individuals. Activation markers showed a concentration dependent increase in washed platelets. Thrombin activated platelets at two concentration (0.8IU and 1.0 IU/ml) showed almost same aggregation response and same percentage of activation marker. Thus 0.8IU/ml thrombin was selected as positive control for the further experiments. Whole cell lysate showed higher level of protein concentration when compare to releasates, gel analysis also showed clear difference between

the protein profile of lysate and releasate. SEM analysis also supports these results. Platelets from activated and test were spread with a sphere morphology and pseudopodia. In control adhesion and spreading was very less. SDS-PAGE from control, activated and test showed clear demarcation between protein profile. Western blot analysis confirms the presence of two critical protein in test. Two dimension gel also indicates the remarkable difference between control and test.

Results of the study suggest, that there are platelet proteins present in test, which are not previously reported in diabetic subjects. These and other proteins, which are demarcated in the study, may play a key role in the progression of atherosclerosis in diabetic subjects. This study is a preliminary study in this direction of the early search of suitable biomarker for the cardiovascular disease in the diabetic subjects.

CHAPTER 1

INTRODUCTION

I.1 BACKGROUND

Atherosclerosis is the underlying cause of several clinical manifestations, such as acute coronary syndromes, cerebrovascular disease and peripheral artery disease, which together are the leading cause of death worldwide. Circulating cells are main players in disease development including platelets which is a key regulator of the process. Inflammation in blood vessels is one of the main drivers of atherosclerosis. Development of atherosclerosis and thrombosis is promoted by the proteins which are responsible for the adhesion of activated platelets to the vessel walls. Platelets contain a number of preformed, morphologically distinguishable storage granules such as alpha-granules, dense granules and lysosomes, the contents of which are released upon platelet activation. The released molecules play an important role in hemostasis and thrombosis and have recently emerged as key regulators of inflammation. Platelets also synthesize biologically relevant proteins in response to physiological stimuli that are regulated via gene expression programs at the translational level. A small amount of constitutive protein synthesis (e.g., GPIIb/IIIa, vWF) occurs in non-stimulated platelets, synthesis of specific proteins is remarkably enhanced in response to activation. Many of these secreted and synthesized proteins are identified as the modulator of inflammation and present in the pathological conditions. In those who are diabetic, hypertensive and hyperlipidimic, circulating platelets are in activated state and are considered to be at the high risk for the development of atherosclerosis.

Diabetes is a well recognized risk factor for atherosclerotic cardiovascular disease and in fact most diabetic patients die from vascular complications. Studies have shown a consistent relationship between hyperglycemia and the incidence of chronic vascular complications in subjects with diabetes. Platelets are essential for haemostasis and abnormalities of platelet function may cause vascular disease in diabetes. Diabetic subjects have hyper

reactive platelets with exaggerated adhesion, aggregation and thrombin generation. In summary, the entire coagulation cascade is dysfunctional in diabetes. Hyperglycemia favors inflammation at the atherosclerotic sites and thus in the disease progression. Studies have revealed alteration in the platelet proteins in the diabetic subjects. Concentration of platelet proteins like PF4, β TG are also reported high in the diabetic subjects reflecting enhanced platelet activity.

Even though platelets lack nuclear DNA, they have a substantial and diverse transcriptome derived from the progenitor megakaryocytes and contain rough endoplasmic reticulum and polyribosomes, thereby retaining the capacity for protein biosynthesis from existing mRNA. While quiescent platelets exhibit only limited translational activity, platelet activation leads to signaling-dependent translation of existing mRNA. Studies have revealed platelets release numerous numbers of proteins which are usually not seen in normal circulation. Also during activation, they show an increase in amount of protein expression of normal circulating proteins. The presence of circulating activated platelets has been found in the circulating blood of patients with unstable atherosclerosis, stable coronary disease and hypercholesterolemia. Activated platelets in blood are prone to bind leukocytes, preferentially monocytes, to form platelet–leukocyte aggregates. Therefore, platelet activation is one of the major characteristics present throughout the atherosclerotic process. Platelets are now regarded as a primary source of inflammatory mediators involved in the original injury to the vascular endothelium that promotes plaque formation. At the site of vascular lesions, ECM proteins such as vWF and collagen are exposed to the blood. Platelet adhesion to the exposed matrix is considered to be the initial step in thrombus formation. Platelets adhere to vWF via the membrane adhesion receptor glycoprotein Ib/IX/V (GPIb/IX/V) and to collagen via GPVI. This results in platelet activation and transformation of the integrin receptors αIIbβ3 (GPIIb/IIIa, fibrinogen receptor) and α2β1 (collagen receptor), which firmly binds to the respective ECM components. Subsequently, platelets spread and

form a surface for the recruitment of additional platelets via fibrinogen bridges between two αIIbβ3 receptors. Secreted platelet proteins act in an autocrine or paracrine fashion to modulate cell signaling. Platelets also release several immune modulators such as platelet basic protein whose proteolytic product is neutrophil-activating peptide 2 (NAP-2), in addition to adhesion proteins such as platelet endothelial cell adhesion molecule (PECAM) that may support leukocyte migration. Thus, the platelet releasate contains factors of major significance in the development of atherothrombosis.

Platelet activation results in the local release of over 300 proteins, a large proportion of which are known inflammatory mediators. The central role of some—particularly CD40 ligand (CD40L), interleukin (IL)-1β, platelet factor 4, and RANTES—has been well established in the progression and stability of atherosclerotic disease, whereas number of novel proteins, which are secreted/synthesized by activated platelets have not been studied extensively. There may be platelet proteins which get expressed in diabetes subjects and contribute to disease progression over a period of time. Research in this area may lead to molecules that are not identified so far. Such proteins, if identified can be used as an early marker for the atherosclerois. To start with, it is important to analyze and detect difference in the protein profiling of the diabetic subjects vs healthy individuals. Subsequent molecular and functional characterization of differently expressed platelet proteins may throw some light for future research.

I.2 REVIEW OF LITERATURE:

I.2.1 Atherosclerosis and Platelets

Atherosclerosis is a complex process characterize basically by lipid retention, proteolytic injury and a chronic inflammatory response. The resulting pathological vascular remodeling involves endothelial cells, vascular smooth muscle cells, mononuclear cells, platelets, growth factors and cytokines [Blanco-Colio *et al*, 2006]. Vascular injury triggers a cascade of events that includes endothelial dysfunction, inflammation and vSMC activation proliferation and migration. This process leads the secretion of a number of proteins by different cells and the protein which is expressed by one type of cell may trigger a set of function of the other cell, e.g. PDGF released by platelets as well as by endothelial cells play a critical role in the migration and proliferation of SMCs [Betsholtz *et al,* 2001] . Myriad growth factors and cytokines can be detected in human vascular lesions. These mediators may be released by dysfunctional endothelial cells, inflammatory cells, platelets and vSMCs, mediating chemoattraction, cell migration, proliferation, apoptosis and matrix modulation [Victor *et al,* 2002]. The presence of circulating activated platelets was found in the circulating blood of patients with unstable atherosclerosis [Becker *et al,* 1994] stable coronary disease [Zanten *et al*, 1994] and hypercholesterolemia [Furman *et al*,1998]. Activated platelets in blood are prone to bind leukocytes, preferentially monocytes, to form platelet–leukocyte aggregates [Broijersen *et al*,1998]. Therefore, platelet activation is one of the major characteristics present throughout the atherosclerotic process.

Platelets, much more than a passive, circulating, anuclear cellular element, play a vital role in physiologic hemostasis by stemming blood loss and initiating tissue healing in response to vascular trauma. Platelets have storage granules, dense bodies, peroxisomes, and lysosomes dispersed throughout the cytoplasm and have various invaginations that acts as

transporters and channels during shape change with platelet activation, and adhesion.

Researches have shown the involvement of platelets in atherosclerosis and athero-progression in humans. The activation of circulating platelets is associated with enhanced wall thickening of the carotid artery in humans [Mohr & Grotta, 2004]

Atherosclerosis is a common disorder that specifically affects the medium and large arteries. It occurs when fat, cholesterol, and other substances build up in the walls of arteries and form hard structures called plaques. These plaques make artery narrower and less flexible, making it harder for blood to flow. If the coronary arteries become narrow, blood flow to the heart can slow down or stop. This can cause chest pain (stable angina), shortness of breath, heart attack, and other symptoms [Michael AC, 2010].

Pieces of plaque can break off and move through the affected artery to smaller blood vessels, blocking them and causing tissue damage or death (embolization). This is a common cause of heart attack and stroke. During these processes platelets are relevant factors acting with the endothelial and inflammatory cells. Interaction of platelets with endothelial cells is mediated by different platelet molecules such as P-sel, GPIIb/IIIa, CD40L, CD40 [Palomo *et al,* 2008].

Atherosclerosis without flow-limiting thrombosis is a slowly progressive disease. The usual mechanism responsible for the sudden transition from a stable, often clinically silent, disease to a symptomatic life-threatening condition is the denudation and erosion of the endothelial surface or plaque disruption followed by thrombosis. The majority of such acute vascular lesions resolve spontaneously through a repair phenomenon to hemostasis of coronary lesions [Zimarino & Caterina, 2008]. Platelet-derived chemokines like PF4 and other growth factors are found in human

atherosclerotic plaques. Current antiplatelet drugs (aspirin, clopidogrel) do not seem to have a major impact on athero-progression in humans. However, most antiplatelet strategies in high-risk patients have been applied for secondary prevention at a rather advanced atherosclerotic disease state. Clinical studies are required that evaluate the efficacy of a long-term antiplatelet strategy for primary prevention in high-risk patients at an early stage of atherosclerotic disease.

I.2.2 Platelet Activation In Atherosclerosis

Platelets major function is to arrest bleeding, but when blood vessels are injured, they adhere to the newly exposed subendothelial layer and to each other, forming a hemostatic plug. Platelets participate not only in primary but also in secondary haemostasis. The process involves many stages, such as platelet adhesion, aggregation and procoagulant activity [Andrew & Berndt 2004]. Both superficial and deep intimal injury disrupts the intact endothelium, which normally prevents the adherence of platelets by the production of the antiplatelet agents; nitric oxide and prostacyclin. When disruption of the endothelium exposes collagen, adherence of platelets to the subendothelium takes place both directly and via von Willebrand factor (vWF) and subsequently leads to platelet activation. Platelet adhesion is mediated by binding of platelet receptors to a number of arterial wall receptors, including subendothelial collagen (whose corresponding platelet receptor is Gp Ia/IIa), vWF (Gp Ib/IX and Gp IIb/III), and fibrinogen (Gp IIb/IIIa) and results in platelet activation.

However, the formation of hemostatic plug can be duplicated *in- vitro* in the presence of different agonists which includes ADP, Collagen, TRAP and thrombin. Most stimuli causes change in shape. This change involves first the formation of very fine pseudopodia (i.e, filopodia) from the rim of the disc, followed by a general "rounding up" of the platelet so that it becomes a spiny sphere, often with much broader pseudopodia. The accumulation of thrombin

at sites of vascular injury provides one of the major mechanisms of recruiting platelets into a hemostatic plug. Thrombin acts by activation of the G protein-coupled protease activated receptors on human platelets to initiate signaling cascades leading to increases in [Ca]i secretion of autocrine activators, trafficking of adhesion molecules to the plasma membrane and shape change, which all promote platelet aggregation [Brass, 2003].

At different phases of blood coagulation, thrombin is generated in an extremely wide range of concentrations, varying from picomolar and nanomolar amounts to the maximal level of 0·8–1·4 μmol/l activity of these thrombin concentrations correspond to 0·0001, 0·1 and 86–151 National Institutes of Health (NIH) units/ml, respectively, for thrombin with specific activity of 3000 NIH units/mg. Hence, during blood coagulation, platelets can be exposed to very low and very high thrombin concentrations, which may have different impact on platelet activation. Up-regulation of P-selectin (CD62P) is reported in human whole blood with 64 nmol/l thrombin [Leytin *et al,* 2007]. Expression of CD62, PMP and CD41 is also reported upon platelet activation.

Mild to moderate activation of platelets are known to be a threat for individuals with risk factors, because activated platelets express various membrane receptors which are instrumental for platelet-platelet, platelet-leukocyte and platelet –endothelial interactions. In addition, platelets release cytokines and growth factors which promote the process of inflammation and vascular changes. Even though platelets are anuclear they have the machinery to store mRNA and translate proteins [Spinelli *et al,* 2008]. Several membrane proteins are constitutively expressed in non-stimulated circulating platelets. However, synthesis of specific proteins is remarkably enhanced in response to activation leading to the release of more than 300 proteins and some of them are known to be inflammatory mediators. Out of these only very few proteins such as CD 40L and IL-1β, PF4, CRP and RANTES have been studied extensively to understand their role in various patho-physiological

conditions. These proteins may act in a concerted and finely regulated manner to influence wide range of biological functions such as cell adhesion, cell aggregation, chemotaxis, cell survival, proliferation, coagulation and proteolysis, all of which accelerates inflammatory processes and cardiovascular disease.

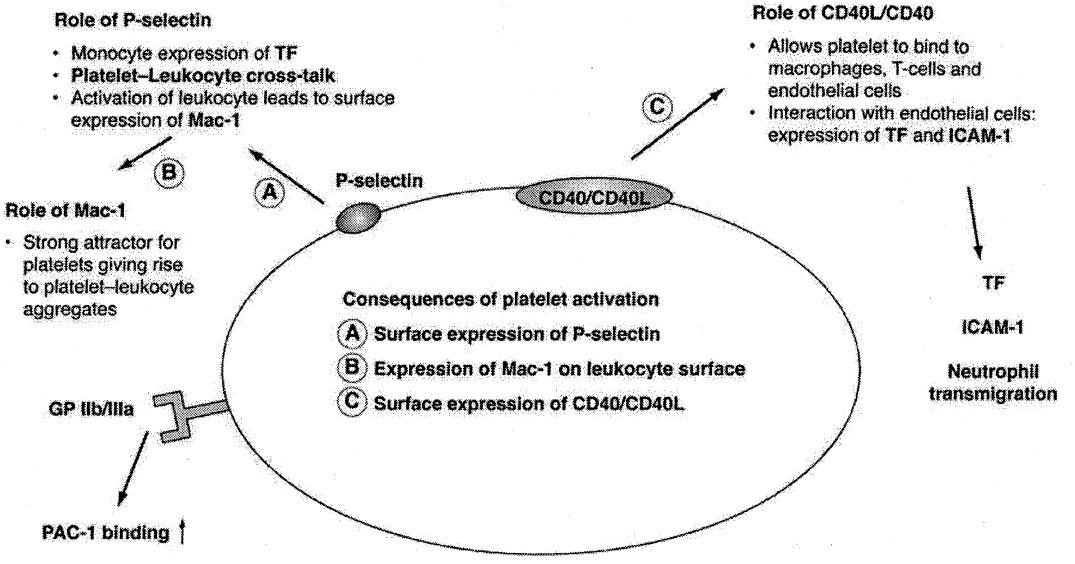

Fig.I.1 Cascade of events contributes to inflammation and thrombosis following platelet activation. (A) Following activation, degranulated platelets express P-selectin, enabling 'cross-talk' between platelets and leukocytes and resulting in leukocyte surface expression of integrins such as Mac-1. P-selectin expression on platelets also results in monocyte expression of tissue factor, and causes monocytes to release cytokines. (B) When Mac-1 is expressed on leukocytes, it is a strong attractor for platelets, giving rise to platelet–leukocyte aggregates. (C) Platelet activation also results in surface expression of CD40 and CD40 ligand (CD40L). CD40/CD40L interactions occur between platelets and the other main cell types involved in the pathophysiology of atherosclerosis (macrophages, T-cells and endothelial cells). TF, tissue factor; ICAM-1, intercellular adhesion molecule-1; Mac-1, leukocyte integrin (CD11b/CD18). PAC-1, antibody specific for activated form of the GP IIb/IIIa receptor.

I.2.3 Pathways of Platelet Activation

When the platelets adhere to the extracellular matrix, there is a rapid response to autocrine and paracrine signaling. The signaling molecules include adenosine diphosphate, (ADP), thrombin, epinephrine and thromboxane A2. These agonists are responsible for recruiting circulating platelets to form the hemostatic plug. Most of these agonists work via GPCR (G Protein Couples Receptor) [Brass, 2003]. Thrombin is the end product of the coagulation cascade, a series of steps in plasma that lead to the generation of thrombin which then causes the conversion of fibrinogen to fibrin which binds the clot together. Thrombin also activates platelets via two receptors on the platelet surface PAR-1 and PAR-4.

The final pathway for all agonists is the activation of the platelet integrin glycoprotein IIb/IIIa (αIIbβ3), the main receptor for adhesion and aggregation [Kulkarni *et al*, 2000]. Fibrinogen plays an important role in maintaining the stability of a thrombus, by bridging GPIIb/IIIa integrins between platelets; von Willebrand factor is necessary to facilitate inter-platelet bridges. There are mainly three pathways that control the reactivity of platelets and vascular endothelium. These are: the arachidonic acid–prostacyclin pathway, the l-arginine– nitric oxide pathway, and the endothelial ectoadenosine diphosphatase (ecto-ADPase) pathway [Wu & Thiagarajan, 1996]

The arachidoinic pathway involves the production of prostaglandin by COX 1 and 2 peroxidase which gets converted to prostacyclin and thromboxane A2 by prostacycline synthase and thromboxane synthase respectively. TXA2 is responsible for further platelet activation [Jin *et al*, 2005]. The vascular endothelium synthesizes Nitric oxide (NO) from the terminal guanidino nitrogen atoms of L-arginine [Palmer *et al*, 1988]. NO diffuses into platelets, stimulates the production of cyclic guanosine monophosphate (cGMP), and regulates cGMP–dependent protein kinases,

which causes a secondary decrease in intracellular [Ca]i flux. This reduction in intracellular [Ca]I levels suppresses the conformational changes in glycoprotein IIb/IIIa that is required for binding of the integrin to fibrinogen, thereby decreasing the number and affinity of fibrinogen binding sites on the platelet's surface [Moncada, 2006].

Once formed, TXA2 can diffuse across the membrane and activate other platelets. In platelets, there are two splice variants of the TXA2 receptor: TPα and TPβ, which differ in their cytoplasmic tail. TPα and TPβ couple to the proteins Gq and G12 or G13, all of which activate phospholipase C (PLC). This enzyme degrades the membrane phosphoinositides (such as phosphatidylinositol 4,5-bisphosphate [PIP2], releasing the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG). DAG activates intracellular protein kinase C (PKC), which causes protein phosphorylation. The release of IP3 increases cytosolic levels of [Ca]i, which is released from the endoplasmic reticulum. ADP is released from platelets and red cells. Platelets express at least two ADP receptors, P2Y1 and P2Y12, which couples to Gq and Gi, respectively. The activation of P2Y12 inhibits adenylate cyclase, causing a decrease in the cyclic AMP (cAMP) level, and the activation of P2Y1 causes an increase in the intracellular [Ca]i level. The effects of agonists mediated by the decrease in cAMP levels and increase in intracellular [Ca]i levels lead to platelet aggregation through the change in the ligand-binding properties of the glycoprotein IIb/IIIa (αIIbβ3), which acquires the ability to bind soluble adhesive proteins such as fibrinogen and von Willebrand factor [Giovanni, 2007]. The release of ADP and TXA2 induces further platelet activation and aggregation.

The small-peptide sequence arginine– glycine–aspartic acid (RGD) of the adhesive proteins binds to the glycoprotein IIb/IIIa receptor. Fibrinogen contains two RGD sequences on its α-chain, one in the N-terminal region and the other in the C-terminal region. The study of fibrinogen −/− mice has shown that von Willebrand factor alone is not sufficient to achieve stable platelet

aggregation [Ni *et al*, 2000] and supports the hypothesis that the concurrent binding of von Willebrand factor to glycoprotein IIb/IIIa and glycoprotein Ibα allows for initial contact between platelets, whereas fibrinogen is necessary for a permanent linkage between activated glycoprotein IIb/IIIa on adjacent platelets to ensure stable aggregate formation. Platelet activation and plaque progression is also enhanced by releasing adhesive ligands, mainly P-Selectin. This protein is usually seen on platelet membranes and is potent in mediating platelet endothelium interaction [Ruggeri, 2002]. Signaling by P-selectin stimulates monocytes and macrophages to produce chemoattractants and growth factors. P-selectin glycoprotein ligand 1(PCGL) on the monocyte surface initiates the formation of platelet–monocyte aggregates and outside-in signaling that induces the transcription of COX-2 [Dixon *et al*, 2006]. Prolonged adhesion dependent signaling promotes the expression of interleukin-1β. This cytokine enhances the stability of COX-2 mRNA, thereby promoting synthesis of the enzyme which results in further activation of platelets. Agonists can cause the shedding of small micro particles. Its been reported that at the site of vascular injury, P Selectin which is expressed during platelet activation triggers the recruitment of microparticles bearing PSGL1[Morel *et al*, 2006]. Microparticles have also been implicated in the upregulation of COX-2–dependent prostanoid formation in monocytes and endothelial cells. Activated platelets also release chemokines that can trigger the recruitment of monocytes [Hundelshausen, 2001] or promote their differentiation into macrophages. PF4, a platelet-specific chemokine released upon platelet activation, induces the expression of E-selectin by endothelial cells[Yu *et al.,*. 2005]. Platelets are a rich source of stimulators and inhibitors of angiogenesis process.

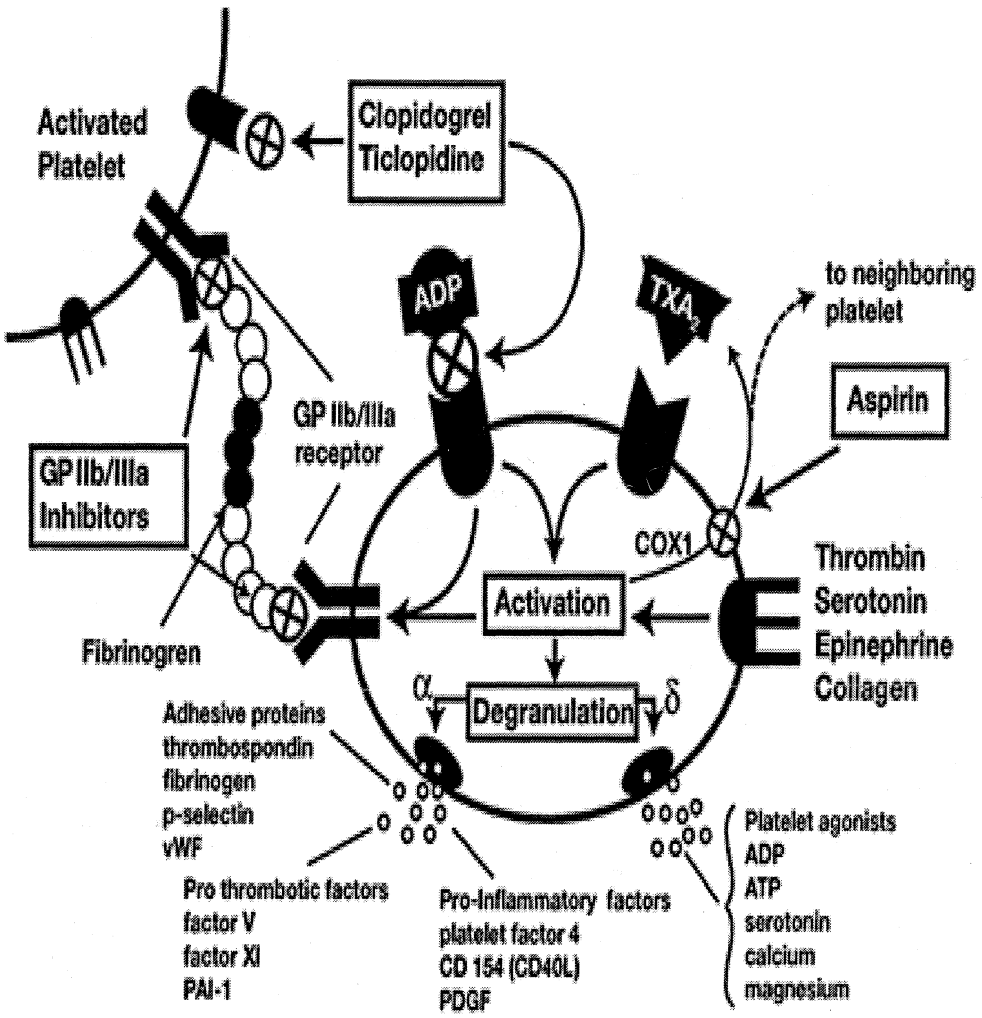

Fig 2 : Mechanism of platelet activation
[interventions.onlinejacc.org/cgi/content-nw/full/1/2/111/FIG1]

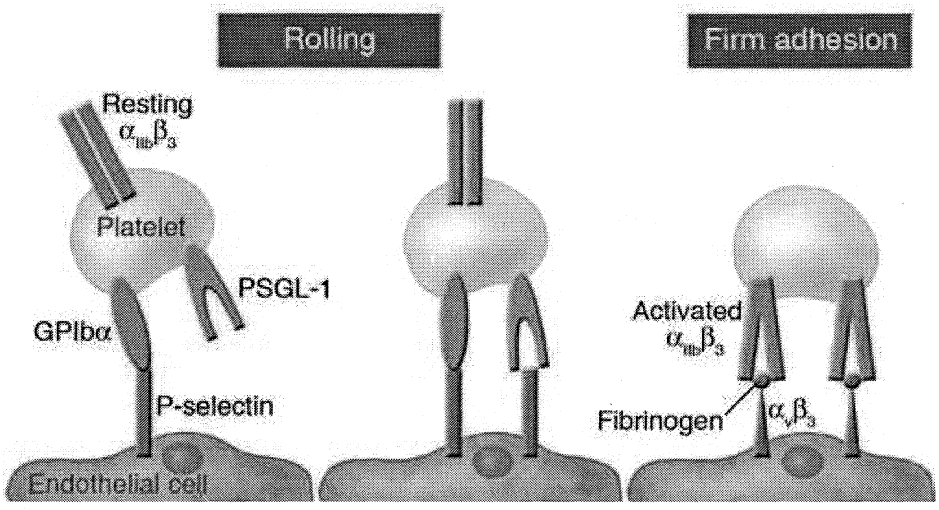

Fig3 Platelet-endothelium adhesion. Activated endothelium surface expresses P-selectin. Platelet surface receptors GPIbα and PSGL-1 interact with endothelial P-selectin and mediate platelet rolling. Subsequent firm adhesion is mediated through β3 integrins.

Activated endothelium surface expresses P-selectin. Platelet surface receptors GPIbα and PSGL-1 interact with endothelial P-selectin and mediate platelet rolling. Subsequent firm adhesion is mediated through β3 integrins. IL-1β is a major mediator of activated endothelium by platelets. IL-1β modifies the release of chemotactic proteins and also increases endothelial expression of adhesion molecules such as ICAM. IL-1β also promotes neutrophil and monocyte adhesion to endothelium [Gawaz *et.al*, 2000].

I.2.4 The Platelet Secretome

Platelets contain alpha, dense and lambda granules. Once the platelets get activated, they secrete the inner contents of these granules into circulating system which further triggers the platelet activation. The dense granules contain ADP, ATP, Calcium while the alpha granules contain platelet factor 4 (PF4), Transforming Growth Factor β1 (TGF β1), Platelet Derived Growth Factor (PGDF), fibronectin, Von Willibrand Factor (vWF), and coagulation factors. PDGF is the major growth factor that is involved in

stimulating vascular smooth muscle cell migration and proliferation. PDGF is chemotactic and activates monocytes. Therefore, PDGF has long been speculated to be an important participant in the development of atherosclerosis. Endothelial cells lack PDGF receptors and are unresponsive to PDGF. Therefore, PDGF may mainly target other vascular cells in the vessel wall. Macrophages and foam cells also produce PDGF throughout the progression of atherosclerosis [Huo, 1989]. Lambda granules show similarity to lysosomes and contain hydrolytic enzymes. Platelets lack nucleus but they have mRNA within them. Once they get triggered, the mRNA which were resting during normal conditions, start translation and hence results in novel proteins or over or down regulation of already existing proteins.

Studies have shown the existence of more than 300 proteins secreted during activation alone. The research group observed that 63% of the proteins were not known to be released from platelets, they may have got contaminated with intracellular platelet lysis. Few other proteins were released by secretory cells alone such as tubulin CAP1[Garin *et al.*, 2001].They found about 81 proteins seen consistensly in platelet releasate, out of which 46 were detected at mRNA level. The platelet transcriptome may largely reflect that of the parent megakaryocyte [Shaw *et al*, 1984]. The messages for many secreted proteins may, therefore, be transcribed in the megakaryocyte and passed to the daughter platelet cells. Other proteins released that do not have a corresponding mRNA may be endocytosed by platelets, for instance, fibrinogen and albumin [Coppinger *et al.*, 2004]. While absent in normal vasculature, a variety of newly described platelet derived proteins that may promote atherogenesis (e.g., secretoganin III, calumenin, cyclophilin A) are present in human atherosclerotic tissue. Cyclophilin A has been studied as a multifunctional protein that is upregulated in a variety of inflammatory conditions. It has been classified as a immunophilin and has a variety of intracellular functions including intracellular signaling, protein trafficking and regulation of other protein activities [Satoh *et al*, 2010]. Secretogranin III stimulates monocyte adhesion to the vessel wall and their transendothelial

migration. Cyclophilin A acts as an autocrine stimulator for extracellular signal-reduced kinase (ERK1/2) and vascular smooth muscle proliferation, events regulating the progression of atherosclerosis [Jin, 2000]. Therefore presence of these proteins in circulation may be considered an early indication for atherosclerosis.

The recent integration of proteomics into biochemical and biological platelet research has proved to be a powerful tool in understanding platelet function. Although platelet proteomics is a young field, remarkable advances have already been accomplished. Thus, proteomics and analysis of activation-dependent protein synthesis open a new and promising direction of platelet research and may disclose novel molecular mechanisms of platelet mediated inflammation and atherothrombosis.

I.2.5 Diabetes and Atherosclerosis

Atherosclerosis is the most common complication of diabetes. Epidemiologic and pathophysiologic evidence suggest a number of possible reasons for this. They include alterations in lipoproteins, platelets, soluble clotting factors, the balance of prostacyclin-thromboxane, blood pressure regulation, and arterial smooth muscle cell metabolism and proliferation. Many of these alterations may accompany hyperinsulinemia and may account for the recent evidence that hyperinsulinemia is a risk factor for atherosclerosis [Steiner, 1981]. It has been reported that macrophages that were differentiated from human peripheral blood monocytes in the presence of high glucose concentrations showed increased expression of cell-surface CD36 secondary to an increase in translational efficiency of CD36 mRNA. Similar data was obtained from primary cells isolated from human vascular lesions, and it was found that glucose sensitivity is a function of ribosomal re-initiation following translation of an upstream open reading frame (uORF). Increased translation of macrophage CD36 transcript under high glucose conditions

provides a mechanism for accelerated atherosclerosis in diabetics [Griffin, 2001].

I.2.6 Platelet Behavior in Diabetes

Tests of platelet behavior *in vitro*, particularly aggregation and retention and *in-vivo* tests such as measurement of platelet survival and plasma levels of beta-thromboglobulin were reported to be frequently abnormal in diabetic patients, particularly in those with vascular disease. The concept has therefore arisen that platelet hyper-reactivity is one factor responsible for diabetic microangiopathy. Whereas there is experimental and histological evidence for the mediation of platelets in the pathogenesis of atherosclerosis, direct evidence of platelet involvement in microangiopathy is scanty. In diabetic subjects, some changes in platelet behavior are reversed by improved glycaemic control. Evidence that platelets are involved in the pathogenesis of diabetic microangiopathy therefore remains circumstantial [Patton & Passa, 1983]. Apart from this, alterations in the platelet functions and proteins synthesized/secreted by platelets are also reported.

Platelets in diabetic individuals adhere to vascular endothelium and aggregate more readily than those in healthy people. Loss of sensitivity to the normal restraints exercised by prostacyclin (PGI2)) and nitric oxide (NO) generated by the vascular endothelium presents as the major defect in platelet function. Insulin is a natural antagonist of platelet hyperactivity. It sensitizes the platelet to prostaglandin I2 (PGI2) and enhances endothelial generation of PGI2 and nitric oxide (NO). Thus, the defects in insulin action in diabetes create a milieu of disordered platelet activity conducive to macrovascular and microvascular events [Vinik *et al.*, 2001].

It has now been demonstrated in animal models that platelet microthrombi are found in small retinal vessels after months of experimental diabetes. Collectively, these findings demonstrate that alterations in platelet

and endothelial function that favor thrombosis occur early in the diabetic state and may contribute to microvascular disease [Colwell *et al*, 1983]. The development of endothelial cell dysfunction with suppression of nitric oxide and prostacyclin synthesis combined with platelet resistance to the anti-aggregatory effects of these hormones leads to loss of control over platelet activation. In addition, hyperglycaemia and glycation have marked effects on fibrin structure function, generating a clot which has a denser structure, resistant to fibrinolysis. The combination of increased circulating coagulation zymogens, inhibition of fibrinolysis, changes in fibrin structure/function and alterations in platelet reactivity creates a thrombotic risk clustering which underpins the development of cardiovascular disease [Grant, 2007].

In addition to enhancing the initiation of atherogenesis, diabetes promotes plaque instability. Diabetic endothelial cells elaborate cytokines that decrease the de novo synthesis of collagen by vascular smooth muscle cells [Hussain, 1996]. Diabetes also enhances the production of matrix metalloproteinases that lead to breakdown of collagen. Hyperglycemia is known to enhance inflammation at the atherosclerotic site. Inflammation may be caused by inflammatory mediator secreted by platelets in response to high level of glucose, which in-turn results in the progression of atherosclerosis.

There are reports stating the modification in the platelet proteins in diabetic subjects as well as in thrombin activated platelets. Platelet proteins get glycated and phosphorylated in the diabetic subjects [Springer, 2008]. Carbonylation of platelet proteins occurs as consequence of oxidative stress and thrombin activation, and is stimulated by ageing and type 2 diabetes [Alexandru, 2005]. Protein glycation in diabetes leads to the platelet resistance to aspirin, thus altering the functional behavior of platelets [Watala , 2005]. The alteration in the platelet proteins and the proteins which synthesize or get secreted in response to high glucose level may play an important role in the progression of atherosclerosis.

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I.2.7 Summary

The reviewed literature shows that in the case of vascular injury or in other pathological conditions platelets get activated and play a key role in the progression of disease. The local *in-vivo* concentration of agonists especially thrombin increases as a response to the physiological stimuli and causes platelet activation. A multitude of molecules released from activated platelets, membrane associated adhesive proteins and surface proteins on the debris of activated platelets etc. are known to take part in the inflammatory responses and/or vascular regeneration. Studies have shown modification/alteration in the platelet proteins up on activation. The fact that diabetic subjects are more prone to atherosclerosis may be because of the inflammatory mediators released by the platelets in those individuals.

It is been estimated that diabetic or other groups with risk factor have their platelet in circulation in an activated state. Activated Platelets synthesize a large number of proteins which are otherwise absent in resting platelets. Apart from this the expression level of few proteins increases upon activation. Many of these proteins are shown to be present at the atherosclerotic site and contribute to the progress of disease

I.2.8 Gap Area

Many proteins are known to be differently expressed in platelets when they are activated. It is not known how these factors could influence atherosclerosis progression. Studies have not been sufficient to identify their function to use one or more of these proteins as specific marker for platelet activation and a predictor of atherosclerosis. In order to identify the effects of such over expressed proteins, difference between the proteins profiling of healthy and diabetic subjects may be a fascinating area of study. Proteins that are not present in normal resting platelets but that exist in diabetic subjects need to be identified before they can be characterized. If there is major

difference in protein content such proteins need to be identified and characterized for their functional role and understand how it may act on endothelial cell or vascular wall.

1.2.9 Hypothesis

Hypothesis of the present study is:

i) Activation status of platelets in healthy and diabetes subjects may be distinguished by analysis of platelet adhesion to fibrinogen, estimating response to agonists, by quantitative analysis of secretion and by estimation of activation marker expression on cell surface.

ii) Further, differences in secreted protein between healthy and diabetic subjects may exist and simple separation and analysis techniques may be applied to pick up major proteome variants.

I.2.10 Objectives

- ✓ Study platelet aggregation in healthy volunteers (control) and diabetic subjects (test) using aggregometry
- ✓ To detect expression of activation marker, P-selectin in control and test using flow cytometry.
- ✓ Analyze the adhesion of platelets (control and test) to fibrinogen by scanning electron microscopy.
- ✓ Wash platelets free from plasma proteins, activate and isolate secreted proteins by ultracentrifugation of platelet suspension.
- ✓ Analyze quantitative difference in secreted protein using Lowry's protein assay.
- ✓ To separate the released fraction and analyze the protein separated on SDS-PAGE

- ✓ To analyze Western blot of two known proteins secretogarnin and cyclophilin A (inflammatory proteins) test and control to establish the difference in activation status.
- ✓ Two- Dimensional electrophoresis and comparative analysis of platelet proteome in control, and test samples.

CHAPTER II

MATERIALS AND METHODS

II.1 Materials

Thrombin, ADP, Collagen, Low Molecular Weight Marker (Sigma), Glutaraldehye (Nice Chemicals), Acetic Acid (SDFC), Citric Acid(BDH), TEMED(Sigma), Triton X 100 (Sigma), Glycerol (Nice Chemicals), Glycine(Sigma) , Lauryl Sulfate (Sigma), Acrylamide (Sigma), Tris base (Sigma), PMSF(Sigma) , APS(Sigma), Idoacetamide(Sigma), CHAPS(Sigma), Bromo Phenol Blue(Sigma), Urea(Sigma), DTT(Sigma), Silver Nitrate (Chem Labs), Phenol Reagent (Nice Chemicals), Antibodies to Secretogranin and Cyclophilin A (Abcam), 4CN (Sigma), Sodium Carbonate (Merck), Sodium Chloride (Sigma), Dextrose(Sigma), Hydrogen Peroxide (Sigma), Flurosphere (Beckman Coulter).

II. 2 Isolation and Preparation of Washed Platelets

Blood was collected from human volunteers after informed consent and mixed with the anticoagulant, ACD at a ratio of 8.5:1.5. The blood sample was centrifuged at 750g for 20 minutes using a table-top centrifuge (*Heraeus Labofuge 300, UK)*. Platelet rich plasma (PRP) was aspirated along with the white blood cells using a Pasteur pipette into a fresh tube. One part ACD was added to nine parts PRP (by volume) and mixed gently. The cells were pelleted by spinning at 750*g* for 10 minutes and the supernatant was discarded. The pellet was re suspended in one part ACD and nine parts Tyrode's buffer. The platelets were washed 3-4 times and last wash supernatant was confirmed the absence of contaminating plasma proteins in the platelet releasates.

II.3 Isolation and Preparation of Whole Cell Platelet Lysate

Washed platelets of the control and activated groups were prepared as mentioned in the section II.2. Equal volume of lysis buffer was added to the activated samples and kept at thirty minutes in ice for incubation. The

samples were centrifuged at 16000g for five minutes, supernatant was obtained and resuspended in 200µl of PBS and was stored at 4°C.

I.4Thrombin Induced Activation of Platelets

Washed platelets were incubated in Ca-Tyrode's buffer for 30 min before they were activated using agonists. Different concentrations of thrombin (agonist) were added to make a final concentration of 0.1 IU, 0.4 IU, 0.8 IU and 1 IU. 100µl platelet suspensions were used for each assay and sample was labeled immediately with fluorochrome-conjugated antibodies and analyzed using flow cytometry.

Released platelet proteins were isolated by centrifugation of activated or resting platelets at 50,000*g* at 4° C for 1h in ultracentrifuge (Beckman Coulter). The supernatant thus obtained was carefully aspirated out; protein was estimated and stored as aliquots at -80° C for further analysis.

II.5 Estimation of Released Platelet Protein

The concentrations of protein present in the samples prepared as per the procedures described in *II.2* and *II.3,* were determined using Lowry's method [Lowry *et al*, 1951]. Reagent A was prepared by dissolving 1% sodium carbonate in 0.1 N NaOH. Reagent B was prepared by dissolving 0.5 % $CuSO_4$ in 1% Sodium Potassium Tartarate. Reagent C was prepared from Reagent A and Reagent B by mixing it in a ratio of 50:1 and was incubated with samples for ten minutes. Folins Reagent was added and incubated in dark for half an hour. The samples were diluted using 1X PBS. Absorbance at 600 nm was taken using UV-Visible Spectrophotometer (*Hewlett Packard Diode array 8453, Germany*). (The recipe for reagent preparation is given in the APPENDIX).

II.6 Glucose Estimation

Two ml of blood was collected from test subjects in Fluoride- Oxalate tubes. Blood was centrifuged at 750 *g* for 10 minutes and glucose was estimated in plasma by glucose oxidase (GOD) enzymatic method. One ml of glucose oxidase reagent was mixed with 10 µl of plasma and incubated at room temperature for 20 minutes. The color obtained was measured using a green filter (505nm) in (ERBA-Chem 7 Biochemistry analyzer. Germany).

II.7 Platelet Adhesion to Fibrinogen by SEM Analysis

Platelet adhesion (from healthy, activated and test) to the fibrinogen was estimated by SEM (Scanning Electron Microscopy) analysis. Slides were coated with a fibrinogen concentration of 60µg. Once dried, platelets were smeared in such a way so as to form a uniform layer. This was followed by successive dehydration steps involving washing with different concentrations of alcohol (30%, 50%, 70%, 90% and 100%) for 15 minutes each. Samples were lyophilized and platinum coating was done. The morphological changes were observed through SEM with a magnification of 10000X.

II.8 Aggregation Studies

Response of platelets (from healthy and diabetic subjects) to the agonist ADP (10µM) and collagen (1µg) was estimated by optical method using whole blood aggregometer (Chronolog-700 aggregometer, USA). Test was performed as per the standard protocol. In brief, 450 µl of count adjusted (2.5×10^8 /ml) platelet rich plasma (PRP) from each samples were collected and agonist was added after adjusting the baseline with platelet poor plasma (PPP). Test was allowed to run for 5 minutes and then stopped, as per the established standard procedure. Slope and amplitude were determined using the calculate option in AGGRO/LINK8.

II.9 Estimation of Fibrinogen

Clottable fibrinogen in PPP of test and control was estimated by clotting assay using commercially (Diagnostica Stago, France) available reagent Fibriprest on Start 4 semi- automated coagulation analyzer (Diagnostica Stago) as per the manufacturer's instruction. Standard assayed reference plasma (SARP) was used for calibration and the curve generated using diluted plasma was used for automated calculation of fibrinogen by the equipment software.

II.10 Estimation of Activation Markers

Unlabelled resting platelets were used for adjusting the gate position based on FSC and SSC. Staining was done for CD62 platelets and CD41 using fluorochrome-labeled antibodies obtained from Beckman Coulter (USA). Protocol was developed for analysis by FSC and SSC analysis of unstained platelets and by selecting the negative gate for fluorescence to detect positive cells. After incubation of samples with antibodies as per manufacturers instruction samples were with 3.7% formaldehyde and made up to 1ml with PBS. The data was then analyzed using a single laser (488 nm) 3-color flow cytometer (Epics XL Beckman coulter, USA).

II.11 Analysis of Proteins

Protein profile from each group was analyzed using SDS PAGE. 20 µg of each secreted protein sample was loaded onto 12 % Acrylamide gel and was run at a constant voltage 100.The gel was developed with the silver staining protocol. Gels were fixed in formaldehyde for overnight followed by thorough washing in distilled water. After fixation gel was incubated with glutaraldehyde for 5min and washed 3-4 times with distilled water. Gels were then incubated with ice cold DTT (0.1%) for 30 min and rinsed thoroughly followed by the addition of 0.1% $AgNO_3$ for 30min. Gels were developed by

adding (3%) Na_2CO_3 and formaldehyde. Reaction was stopped by the addition of 6% acetic acid. Gels were documented with gel doc system.

II.12 Western Blot Analysis of Specific Proteins

The blots were developed using primary monoclonal antibodies against GPIIb, secretograin and Cyclophilin A (Abcam). The secondary antibody used was Anti mouse IgG. In Brief, 200µg protein sample was loaded for SDS-PAGE. After the run, gel was wet in transfer buffer for 5 minutes. Gel was transferred to PVDF membrane by using (Pharmacia MultiphorII) for 50 min at a constant voltage ($0.8/cm^2$). After the run, membrane was washed thrice with PBS with an interval of 15 min each. A strip was used for amido black staining to ensure efficiency of transfer. Strips from the blots were then blocked in BSA, washed in PBS and incubated with primary Ab (1:1000) for overnight. Blot was incubated with HRP conjugated secondary Ab for 1 h and was then developed by using 4-Chloronaphthol.

II.13 Two Dimensional Electrophoresis Analysis for Spotting the Proteins

Proteins were first separated on the basis of their isoelectric point. 200µg of each sample was loaded on to the immobilized (IPG) strips with 3-10 pH gradient. Two ml mineral oil was pored on to the strip and strips were then allowed to rehydrate at room temperature for overnight. After rehydration, the following voltages were applied to the IPG strips by using Protean IEF cell (Biorad, Hercules, CA, USA): 2500 volt for 4hrs, 4000 volt for 5hrs, 5000 volt for 6hrs and 8000 volt for 5hrs and finally 500 volt for 12 hrs. Strips were stored at -80°C until second dimension separation. After first dimension each strip was equilibrated in a buffer containing 6M Urea, 50mm Tris pH 8.8, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 1% DTT for 15min at room temperature. This is followed by a second equilibration for 15min using same buffer, except that DTT was replaced with 2.5% iodoacetamide to prevent

thiol reoxidation. Second dimension electrophoresis was run on a 13 X 13 cm 12% SDS-PAGE by using protean II System (Biorad Hercules, CA, USA), at 25mA/gel. The gels were developed using silver staining protocol as described in II.11, and documented.

CHAPTER 3
RESULTS AND DISCUSSIONS

RESULTS AND DISCUSSION

The preliminary experiments in this study were carried out to establish that the test group (diabetic) volunteers were diabetic at the time of blood collection. Diabetes in the blood samples of the volunteers was confirmed by estimation of glucose. The results of the random blood glucose estimation are given in table 1. All the volunteers were under medication due to which blood glucose was under control. However, the average showed values higher than the normal. To establish if the test group is under risk for cardiovascular disease development, one known risk factor- plasma fibrinogen assay was also included in the study. The values for the test (diabetic) and control (healthy) groups are given in table 1.

Table 1 Levels of glucose and fibrinogen in study subjects

	Control	**Test 1**
Glucose (mg/dl)	120 ± 3.8	247.66±89.30
Fibrinogen (g/l)	2.0± 0.1	2.70±0.36

Results are presented as Mean ± SD (n=6).

Fibrinogen level in test blood plasma was higher as compared to control. The fibrinogen level is an essential component of inflammation, coagulation and fibrinolysis and the entire coagulation gets disturbed in diabetes. High Fibrinogen concentration might be a risk marker for cardiovascular disease in diabetes because it is an expression of an existing thrombophilia [Barazzoni *et al*, 2003]. Fibrinogen is a known risk factor for the cardiovascular diseases and elevated plasma fibrinogen is considered as a marker for cardiac disorders. Studies have shown correlation between the elevated level of fibrinogen and thrombin generation [Huseyin *et al*, 2011]. Thus, increase fibrinogen concentration in the test samples may be directly related to the local release of thrombin in the diabetic subjects, which

activates the platelets and promote the synthesis or secretion of proteins, which are otherwise absent in the normal circulation.

Once the diabetes and risk of atherosclerosis development in the test group was established, the first objective of the study was to evaluate test and control samples for the activation status of the platelets in circulation. This objective was achieved by comparing platelet adhesion, aggregation, and secretion which are the three major functions of platelets. In addition, activation status of circulating platelets was also monitored by measuring the expression level of two known markers; CD62 and CD41 using flow cytometric analysis.

III.1 Aggregation Response of platelets to the agonist ADP and Collagen in control and test:

Since platelets play a major role in atherosclerosis, the increased risk of cardiovascular events among diabetic patients may be, in part, due to altered platelet function. To study this, platelet response to agonists ADP and collagen were tested by using optical method from control and test.

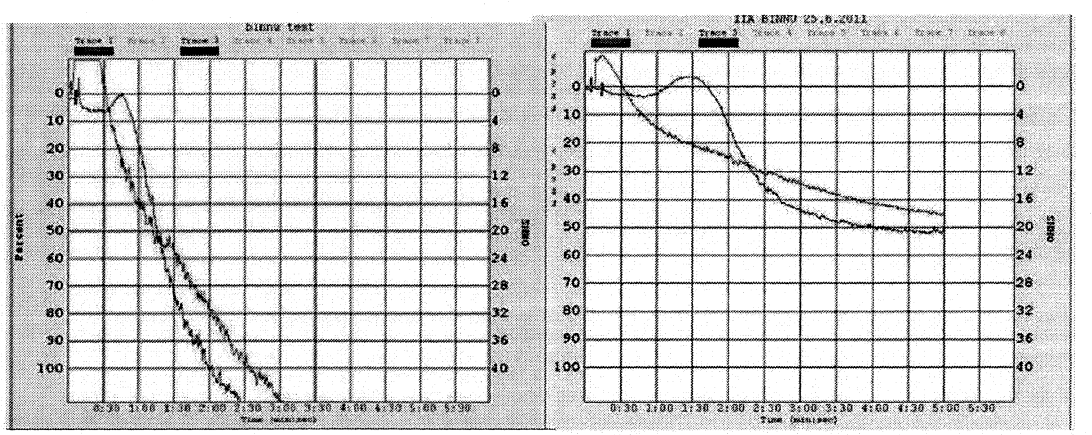

Fig.III.1 Representative figures for the platelet aggregation from the test and control samples

The left panel shows the tracing of one of the test sample. It may be appreciated that platelets responded and showed hyper reactivity to both ADP and collagen and as a result, the percentage transmission in test cuvette reached more than 100% in 3 min after adding the agonist. It means that all the platelets in test has got recruited into aggregate and resulted in light transmission more than the reference cuvette which contained PPP. In the control sample in 5min after agonist stimulation the percentage transmission was below 50% in both cases. Therefore, all the platelets in control did not get activated and recruited into the aggregates. Therefore, PRP remained turbid even after 5 min. The average and standard deviation of all subjects in the study are shown in Table 2.

Table 2. Platelet response to the agonist ADP and Collagen

SAMPLE	ADP (%)	Collagen (%)
Control	45± 4.25	49±4.3
Test	73±6.7	65± 5.2

Results are presented as Mean ± SD (n=6).

Table 3. Platelet response to the agonist thrombin

0.8IU/ml	1.0IU/ml
56.7± 6.5	59 ±7.2

Results are presented as Mean ± SD (n=6).

This data clearly establishes that the test group platelets are hyper-responsive, probably due to a pre-activation status in circulation. When an agonist is added then the response became very fast. This can be explained as synergistic action in platelets, when platelets are activated first with a very mild dose of an agonist, and further exposed to a sub threshold concentration

of another agonist, they become hyper responsive. In table 3, the data on the response of washed platelets to thrombin is also presented. These tests were done because thrombin activated platelet releasate is planned for use as positive control for proteomic analysis. With 0.8 IU thrombin, there was good aggregation. Therefore, complete secretion of platelet proteins may be expected at this thrombin dose.

There is an ample evidence that platelets from diabetic subjects are larger and hyper reactive and cause increased aggregation, and increased platelet-dependent thrombin generation. In this study, we found that platelet response to the agonists were not similar in test samples. Few test samples showed hyper-reactivity whereas the others showed aggregation compared to the control. This may be related to the progression of diabetes, age of the subjects and medication. Recent study on platelet function from diabetic subjects with and without vascular complication also showed comparable aggregometry response to control (healthy) [Peterson & Gormsen, 2009]. Platelet aggregation in diabetic patients and non-diabetic patients appears to be related directly to blood glucose levels [Johns BD *et.al*,1986] as well as to the fibrinogen level. It is suggested that elevated fibrinogen level may be closely related to the abnormality of platelet functions in diabetic subjects, which may lead to the vascular lesions. Our findings also support that levels of glucose and fibrinogen were high in test samples which showed hyper-aggregation.

Aggregation response of the platelets to the agonist from the diabetic and non-diabetic subjects on medication (Aspirin and Clopidogrel) was in a dose dependent manner [Cornelissen *et al*, 2006]. Only after high dose of clopidogrel, platelets from the diabetic group showed resistance or low response, which suggest that platelet response to agonist is also depend on the medication and physical state of the subjects. The test samples selected in this study were also on medication to control blood glucose level. The different response of platelets to different agonist may be due to the

medication. A direct anti-platelet effect of insulin has been demonstrated by many groups; studies have shown that insulin-induces attenuation of the thrombin-induced Ca^{2+} response and platelet aggregation as well as the release of ADP [Randriamboavonjy, 2004], reports from groups assessing the same responses are inconsistent. Part of the controversy may be attributed to the fact that responses to insulin are highly heterogeneous; indeed, clear populations of "responders" and "nonresponders" have been identified in several studies and can be related to numerous factors including physical condition.

III.2 Detection of activation marker CD62 and CD41 in control and test samples using flowcytometry:

To demonstrate the effect of agonist concentration on activation marker, P-sel (CD62) and CD41 was analyzed by flowcytometry. It was observed that expression level of P-sel increases as the concentration of agonist (Table 4) whereas CD41 expression decreases with the increasing concentration of agonist. The decrease in CD41 antibody binding is found to be consistent (unpublished data). This finding is considered to be due to microparticle shedding on activation of platelets. Microparticles may carry part of the membrane with glycoprotein receptors and probably removes the receptors such as CD41 and CD62. Therefore, a reduction in CD41 binding may be caused as the platelets get activated above a certain level. Decrease in the CD41 levels with the higher agonist concentration may be due the membrane shedding or conformational change in the platelets, which results in the unavailability of binding site for the antibody [Gawaz *et al*, 1999]. Another reason may be the binding of GPIIb to available fibrinogen and thus patients with diabetes mellitus have increased platelet-surface expression of glycoprotein Ib (GP Ib), which mediates binding to von Willebrand factor, and GP IIb/IIIa, which mediates platelet-fibrin interaction and represents the final common pathway of platelet activation, leading to platelet aggregation

decrease in the expression on activation. This was further confirmed by using a control (non-activated) sample with CD41 antibody and result showed the high level of expression compared to that of activated samples.

Table 4: Flowcytometry Analysis in PRP

SAMPLE	CD62	CD41
Control	0.23± 0.13	93.2±2.1
Test	0.83±0.64	74.12± 5.8
Thrombin activated (0.8IU/ml)	2.86± 1.16	63.69± 6.64

The increase in platelet activation marker CD62 was concentration dependent till 0.8IU/ml. There was no significant difference between the 0.8IU and 1.0IU/ml thrombin activated platelets. Hence 0.8IU was selected for further study and taken as a positive control for proteomic analysis.

Flowcytometry analysis for washed platelets showed increase in activation, which was dependent on thrombin concentration. High percentage of CD62 and CD41 was found in thrombin activated samples in a concentration dependent manner (Fig.III.3). Fig III.2 and III.4 are the representative figure for the flowcytometry analysis in washed platelets from resting, 0.8 IU thrombin activated and test samples. Here the individual graph represents fluorochrome on X-axis and side scatter on Y-axis. Side scatter represents the granularity whereas X-axis represents the % of fluorochrome positive cells. These figures clearly indicate that CD62 and CD41 positive cells are much higher in test when compared to control. In washed platelets CD41 expression increases. This may be due to the fact that fibrinogen is not present and antibody might have bound to its antigen.

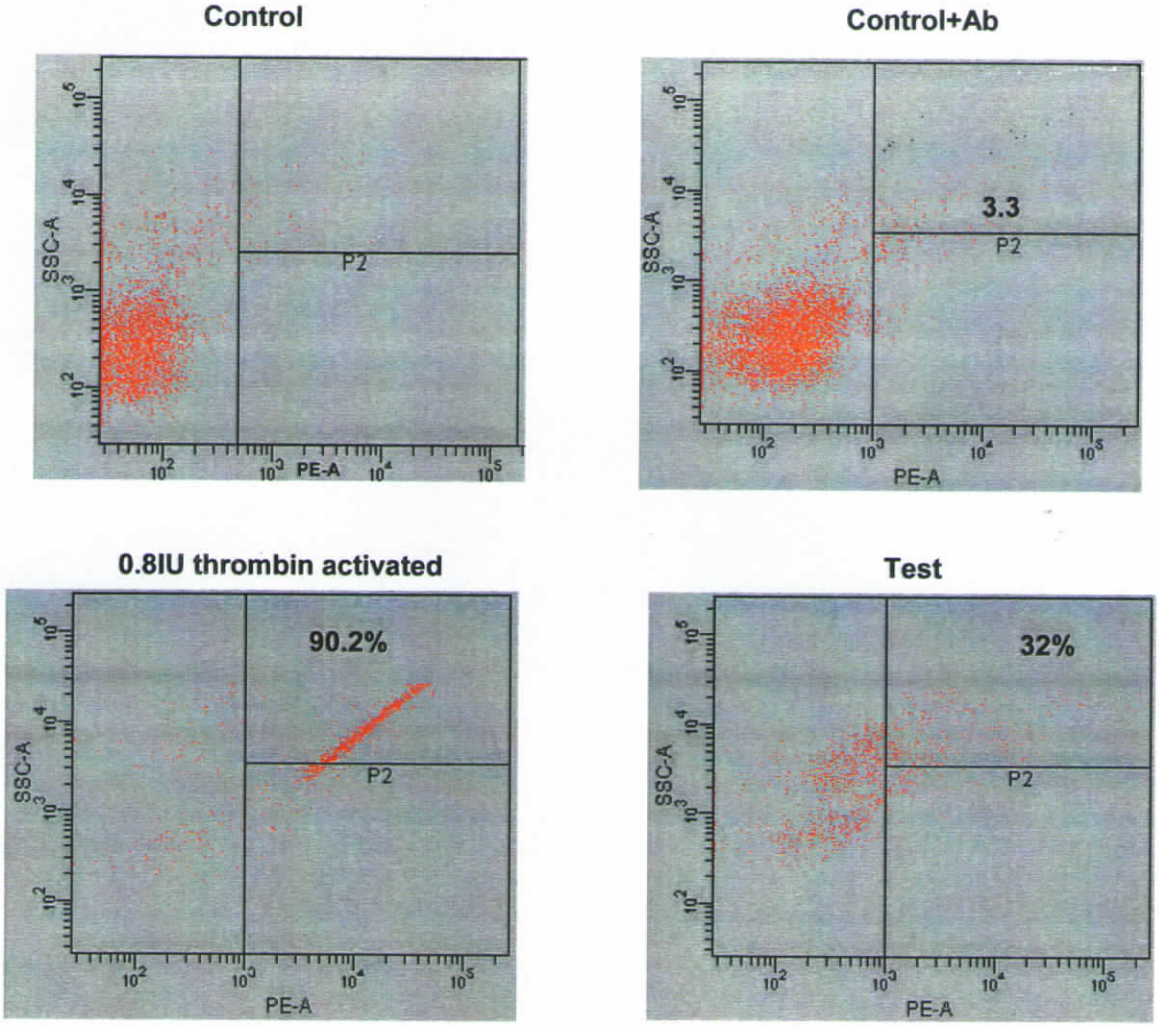

Fig III. 2. CD62 expression in washed platelets

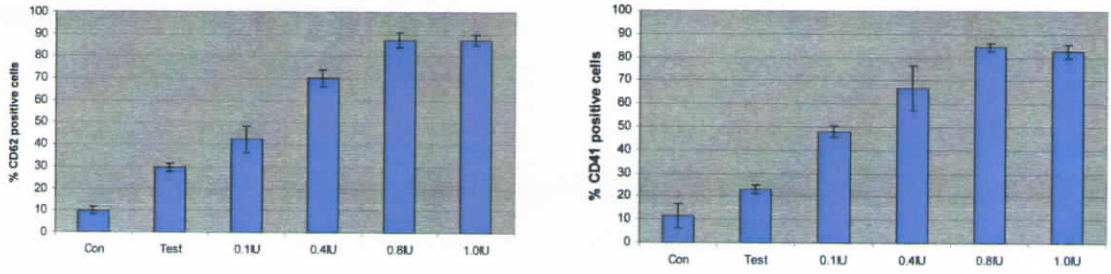

Fig III. 3. % Expression of CD62 and CD41 positive cells in Washed resting, activated and test platelets

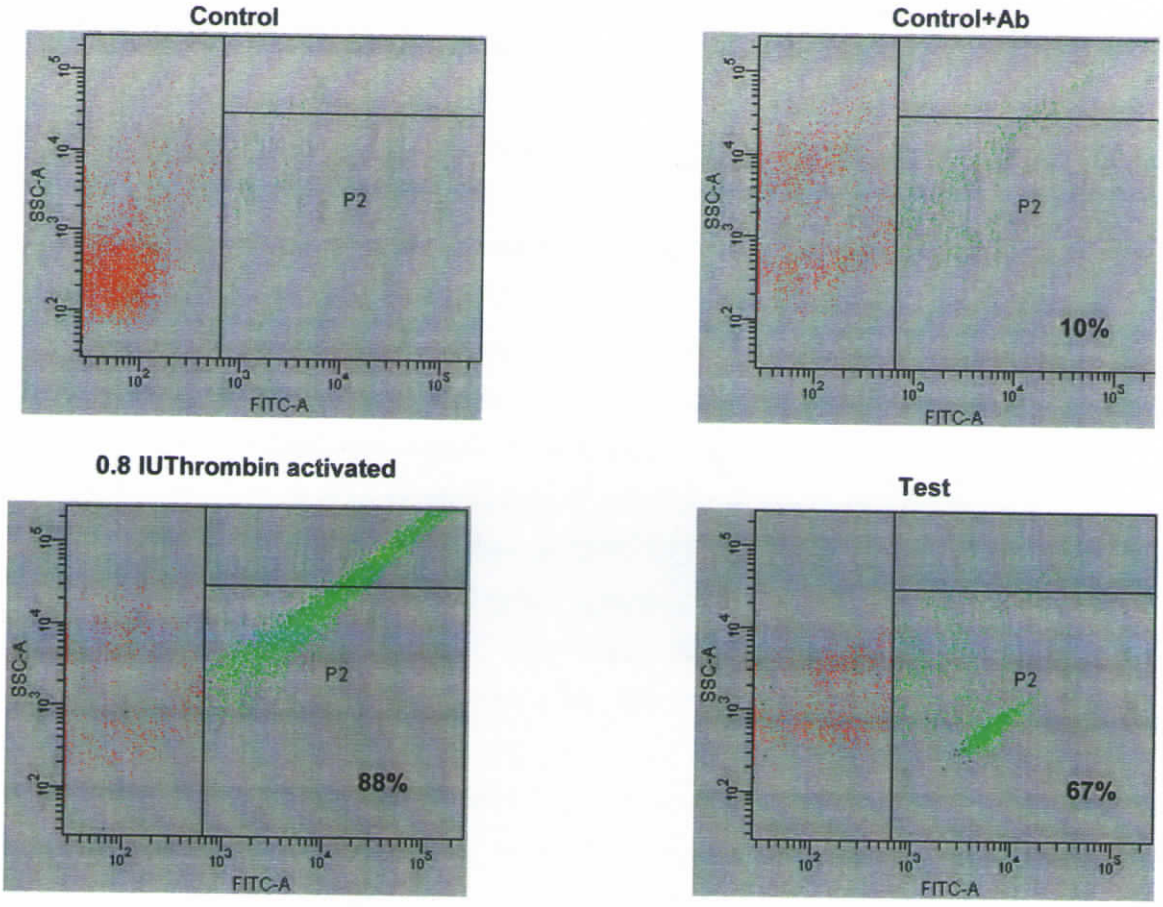

Fig III.4. CD41 expression in washed platelets

Subjects with diabetes mellitus have increased platelet-surface expression of glycoprotein Ib (GP Ib), which mediates binding to von Willebrand factor, and GP IIb/IIIa, which mediates platelet-fibrin interaction and represents the final common pathway of platelet activation, leading to platelet aggregation. P-sel is a membrane marker which gets released even on mild activation of platelets. In the diabetic subjects as well as in the high risk groups like hypertension, hypercholesterolimia, platelets are reported in their activated stage. To confirm this, expression level of P-sel (CD62) and GPIIb (CD41) were tested using fluorochrome tagged antibody (CD62 PE conjugated and CD41 FITC conjugated) against P-sel and GPIIb. Results

indicate activation of platelets in test samples compared to the control but the expression level was less when compare to positive control (i.e 0.8IU/ml). This suggests that circulating platelets in test are in activated stage

III.3 Adhesion of platelets by SEM analysis:

The objective of this experiment was to see the adhesion and spreading of platelets from control, positive control and test samples on fibrinogen matrix. Platelet activation is marked by platelet shape change (discoid shape to round with extended pseudopods and aggregates) and spreading on adhesive substrate. Thus morphological change associated with the activation is studied by spreading of platelets on the fibrinogen matrix. In the present study we found that platelet adhesion and aggregation was maximum in thrombin activated group (positive control) followed by test. In control samples no aggregates were found. Platelets were spread on the fibrinogen matrix.

Fibrinogen and thrombin bind together and form fibrin, which acts as a mesh to hold platelets (Pretorius *et al, 2011)*. In normal individuals, thrombin is present in very minute amount which doesn't have a fate of binding with fibrinogen to make fibrin. Lack of fibrin network doesn't allow platelets to adhere which in turn results in absence of clot and very less risk of atherosclerosis. While in the case of activated platelets, they adhere easily to the fibrinogen leading clot formation. In the present study we found platelets aggregates on positive control and test samples (Fig III. 5). Previous studies have shown that the ultra-structural changes in clot formation occur in patients in whom there are changes in the coagulation system due to, for example, an inflammatory condition. It is also reported that fibrin network in all diabetic patients had thickened masses of thin, minor fibres over the major fibres, a profile typical of an inflammatory condition [Pretorious *et al*, 2011] and now it is known that inflammation and inflammatory proteins are the major culprit for the atherosclerosis.

Proteins secreted by activated platelets may have a major contribution in the disease progression. Platelet spreading with pseudopodia and aggregation are comparable in test and positive control (thrombin activated) which is evident from fig III 5 a-c. In both cases platelets were plenty with clump formation on fibrinogen coated surface. In the case of the control (healthy subjects) platelets were very rarely found on the surface. The adhered cell was found to be single with no significant pseudopod formation or spreading (Fig III 5b). These observations indicated that platelets from diabetic subjects behaved similar to the thrombin activated positive control platelets. These observations are correlating well with the aggregation results and indicate that platelets of diabetic subjects are in activated state.

Activation status is sufficient for adhesion and spreading to fibrinogen coated surface. All these data suggest that any further exposure to agonist is likely to induce the platelet to secrete more of the stored proteins. However, it is not clear if these activated platelets have more variants of protein or quantity of protein in their storage granule.

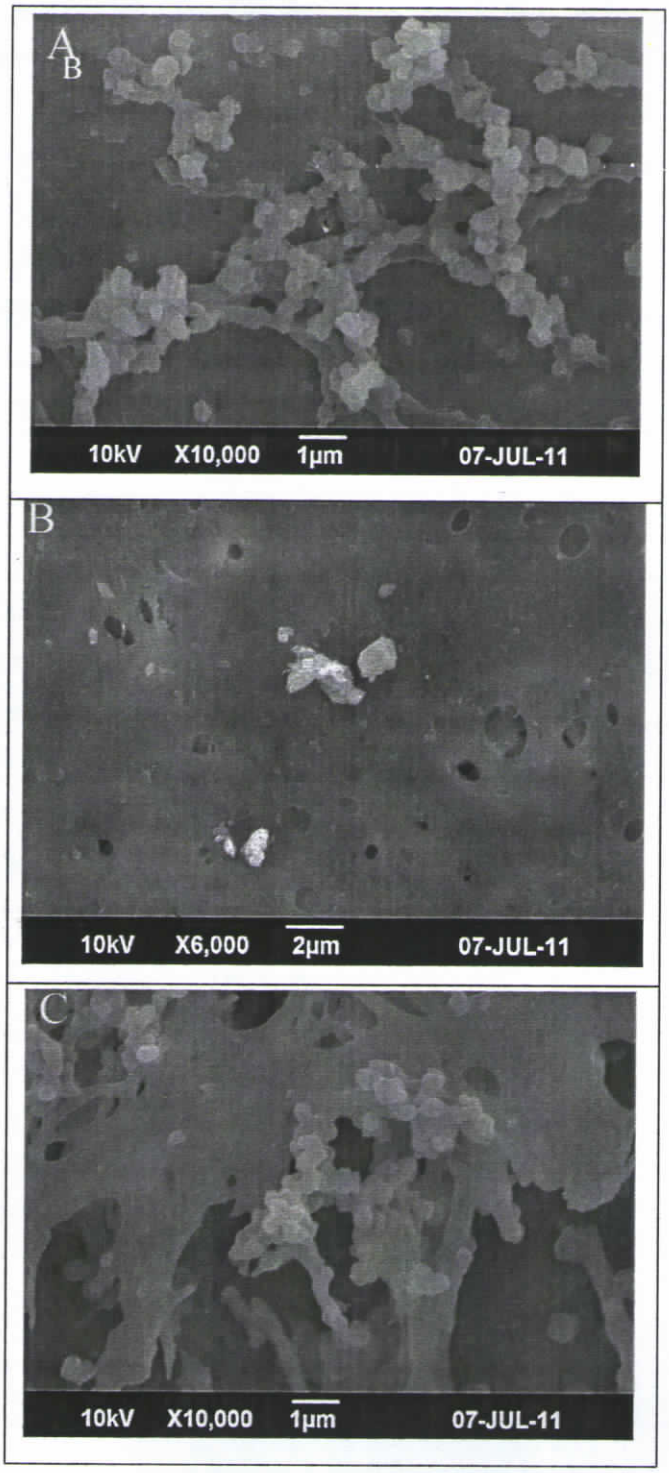

Fig III.5. Representative SEM pictures of platelets spreading on fibrinogen matrix. A, thrombin activated platelets; B, control; C, test

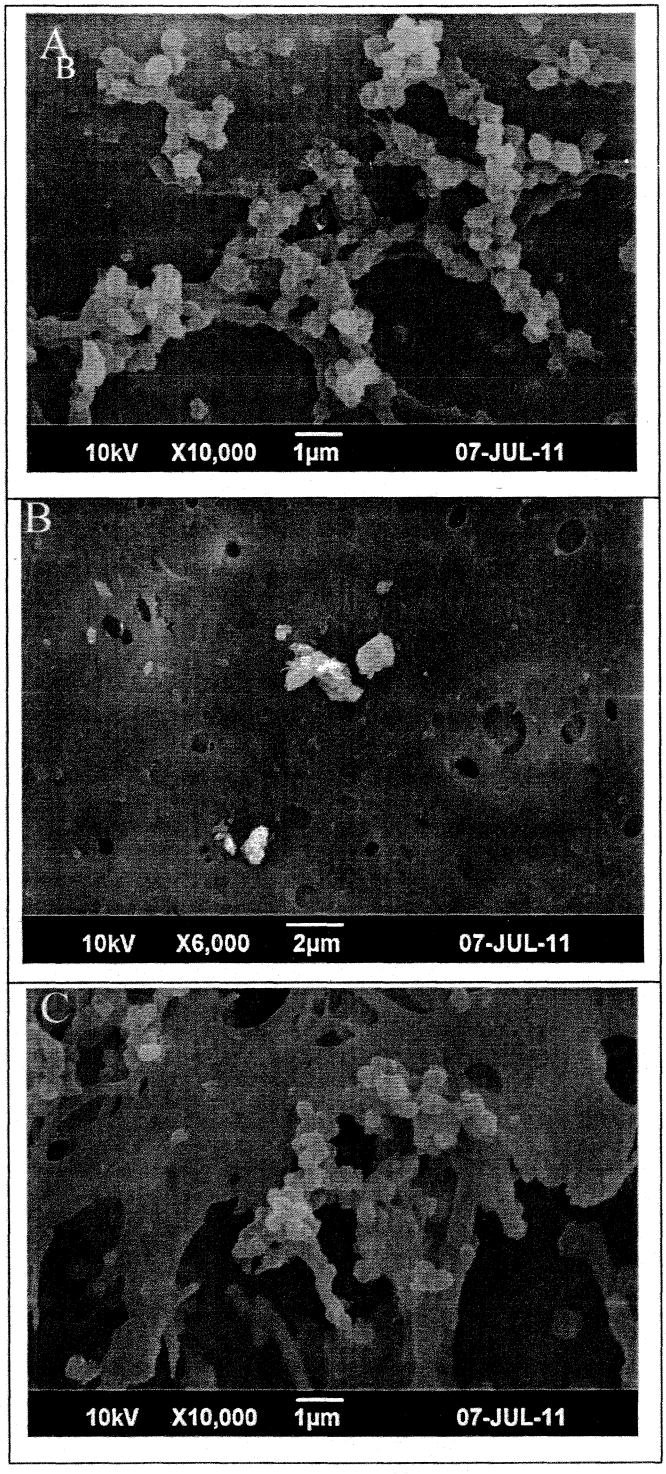

Fig III.5. Representative SEM pictures of platelets spreading on fibrinogen matrix. A, thrombin activated platelets; B, control; C, test

III. 4(a). Whole cell lysate for analyzing the difference between secretome and total protein present in the platelets:

Platelets were lysed in cell lysis buffer, protein content was determined by lowry method. It was found that whole protein content in platelets were much higher (table 5) than what is secreted by platelets. Though this study is basically focused on the proteins which get secreted and circulate in the blood stream, so that can be identified in the early stage of the progression of disease, but the presence of protein at the site of lesions cannot be neglected. Hence, whole proteome of resting and activated (with different concentration of thrombin) platelets was analyzed and it was found that Platelets in resting stage also content almost same no of proteins as in activated stage (Fig III 6), but most of the proteins gets more expressed upon activation. The interesting finding is that, though there is not much difference in the whole cell lysate proteome, the difference in the secretome from resting and activated platelets was remarkable This indicates that most of the proteins are already present in the platelets, but get secreted only upon activation. Presence of few bands only in the activated relasates showed that synthesis of proteins in response to stimuli also take place in the platelets.

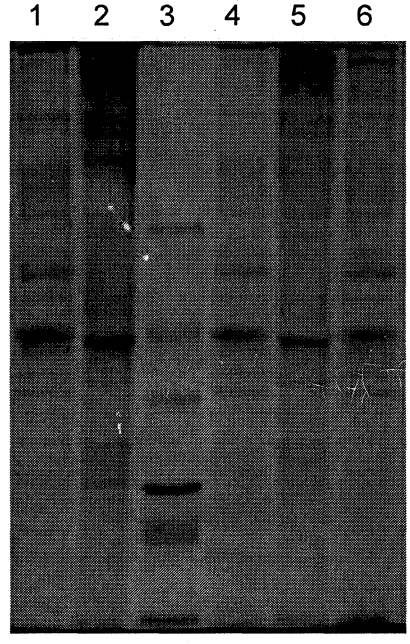

Fig III 6 : SDS PAGE for whole proteome of resting and activated platelets Lane1:0.8IU, Lane2:1IU, Lane3 LMW,. Lane 4: resting platelets, Lane5: 0.1IU and Lane 6: 0.4IU

III.4(b). Wash platelets free from plasma proteins, to activate and isolate secreted proteins by ultracentrifugation of platelet suspension

To isolate the platelet relasates platelets were washed in ACD tyroid buffer 3-4 times. Last wash supernatant was analyzed to eliminate the possibility of contaminating plasma proteins in the platelet relasates. This fraction was loaded to the 10% gel along with the isolated platelet fraction and it was found that no contaminating plasma proteins are present in the isolated platelet releasates fractions (Fig III 7a). Samples were activated with different concentration of thrombin (0.1, 0.4, 0.8 and 1IU/ml). Platelet releasates were separated from control, activated as well as from test samples by doing high speed ultracentrifugation at 50,000g for 1h at 4° C .After centrifugation cleared supernatant (which contents released proteins from platelets) was aspirated carefully. Protein was estimated in each sample immediately by Lowry's protocol. After protein estimation samples were aliquot as (20 µg and 200µg) and stored at -80 ° C for the further experiments

III.4 (c) Quantitative estimation of protein secreted by platelets:

Releasates obtained after III 4 (a), were then analyzed for their protein content. Amount of secreted proteins were significantly high in the activated and test samples when compare to control. This indicates that upon activation platelet synthesize or secretes its granule contents in the circulation. Test also showed marginally high amount of released protein which supports the hypothesis that in diabetic subjects platelets circulates in its active stage, which results in the synthesis or secretion of high amount of protein (Table 4).

Table 5. Protein Estimation in control, activated and test

Sample	Protein concentration(µg/10µl)
Control	5.2± 0.70
Thrombin Activated (0.1 IU/ml)	8.6±3.3
Thrombin Activated (0.4 IU/ml)	10.2±2.9
Thrombin activated (0.8IU/ml)	14.9±4.0
Thrombin activated (1.0IU/ml)	15.3±5.5
Test	10.3±1.13
Whole Cell Lyste	19.3±3.6

Results are expressed as Mean ± SD (n=6)

III.5 Separation of platelet proteins by SDS-:

To optimize the thrombin concentration as well as to see the effect of mild to moderate activation on platelet relasate profile, platelets were activated with different thrombin concentration. Protein were separated and quantified as described in III 4a and III 4b.

Twenty µg of protein from each samples (control and activated) was loaded on 12% gel. When different concentrations of thrombin were used, intensity of protein bands were proportional to the thrombin concentration without any notable change in the protein profiling. There was no significant change observed in band intensity of 0.8 and 1.0IU/ml thrombin activated samples Our result indicates that for almost all dominating bands band intensity increases as the concentration of agonist increases (Fig III 7 b). Thus some of the proteins get released more and more in response to activation.

The first gel represents the protein profile of activated, control and washed platelets and the second gel is the representation of the protein profiling of test samples. Protein gets separated on the basis of their

molecular weight. It was observed that more number of proteins gets secreted between the molecular wt range of 10-30 KDa and 50-70 KDa. We also found the presence of two bands in both activated as well as in the test samples, between 20-24 KDa, which is corresponded to the sectrtogranin and cyclophilin A. To confirm this western blot was developed for these two proteins.

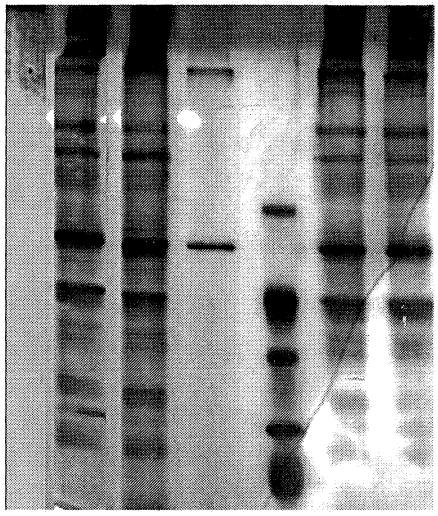

Fig III 7a. SDS PAGE showing platelets secretome from thrombin activated platelets. Lane 1: washed platelets, Lane2: 0.8IU, Lane3: 1IU, Lane4: Control, Lane 5: LMW, Lane6: 0.4IU and Lane7: 0.1IU

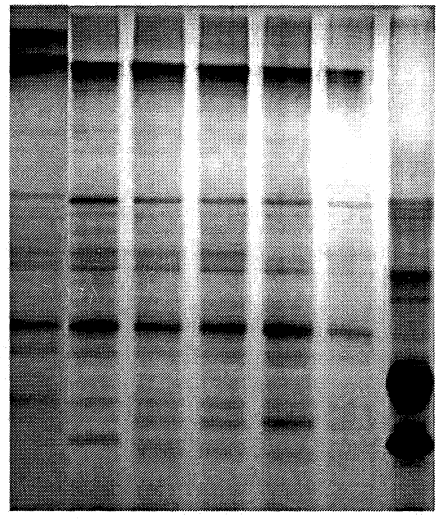

Fig III 7b. SDS PAGE showing platelets secretome from activated and test samples. Lane 1: 0.8 thrombin activated, Lane2-6: Test, Lane7: LMW

Detailed molecular, cellular, animal and human studies have provided incredible insights into the structure and function of platelets, both under normal physiologic conditions as well as in a variety of disease states. However, molecular changes underlying for the change in platelets morphology and function have not been studied much. Identification of proteins involved in the signaling pathways leading to a variety of platelet responses *in vivo* are yet to be fully characterized. Since platelets are readily available, are easily isolated in relatively large numbers, lack nuclei and genomic DNA, and have a limited RNA pool, proteomic techniques are ideally suited for the analysis of platelets. Indeed, several proteomic analysis

techniques have taken a lead in identifying the proteomic content of platelets, as reported in several exciting publications [Moebious et al, 2005]. Despite these efforts, a number of proteomic components of the human platelets remain to be identified, especially notable when considering that the human platelet proteome has been predicted to contain approx. 2000–3000 unique proteins. Due to the limitations of available techniques and continuous altered behavior of platelets in response to different patho-physiological conditions, identification of all proteins is not possible in a single stretch. Modification, alteration, secretion and synthesis of platelet proteins are in response to one pathological condition is different from the other but may be correlated pathological stage. All these proteins may act in synergistic way or may promote the synthesis, alteration, modification and secretion of other proteins. Thus the present study is the preliminary approach to see the difference in the protein profiling of diabetic subjects from control and it can be further extended for the identification of several proteins which gets expressed in diabetic subjects by using more advanced LC-MS or MALDI-Tof.

Platelet proteome gets modified along with the progression of number of diseases and these proteins are found to be play a critical role in the progression of diseases, hence gives an opportunity to researchers to consider them as an early marker for the disease. For eg PF4 increases in a variety of tumors and considered as a biomarker for tumor [Cervi *et al.*,2008].

III.6 Western Blot analysis for two inflammatory proteins:

In the previous experiments (SDS-PAGE) bands were observed at the position between 20-24KDa corresponding to the Cyclophilin (21KDa) and Secrtogranin III (24KDa). These two proteins play important role in the progression of cardiovascular diseases. Due to the closeness in the molecular weight, it was difficult to distinguish these two proteins, and thus the presence of these proteins cannot be assured by SDS-PAGE alone. To confirm whether these two proteins are secreted on activation, western blot analysis was done.

Results showed presence of secretogranin as well as cyclophilin A in activated as well as in test samples (Fig III 8), an indication that platelets are critical in inflammation.

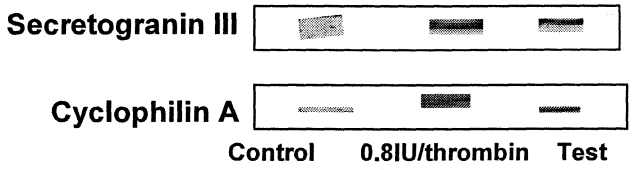

Fig III 8 Representative blot for SgIII and Cyclo A

Sg III and cyclophilin A not previously recognized to be present in or released by platelets, were examined further because they are of potential interest in the pathogenesis of atherosclerosis. These two proteins were localized to platelets by western blotting.

Sg III is a member of the chromogranin family of acidic secretory proteins, previously shown only to be localized to storage vesicles of neuronal and endocrine cells [Rong *et.al*, 2002, Taupenot *et.al*, 2003]. Sg II, a close homolog also found in platelet releasate [Coppinger *et.al*, 2004] is present in neuroendocrine storage vesicles and is the precursor of the neuropeptide secretoneurin, which has a tissue distribution and function similar to the proinflammatory neuropeptides, substance P and neuropeptide Y. Secretoneurin stimulates monocyte adhesion to the vessel wall followed by their transendothelial migration [Kehler *et.al*, 2002].

. Cyclophilins are peptidyl-propyl *cis-trans*-isomerases that act intracellularly both as catalysts and chaperones in protein folding [Gothel *et al,*1999] and have extracellular signaling functions such as the induction of chemotaxis and adhesion of memory CD4 cells [Sherry *et al*, 1998]. Recently, cyclophilin A was found to be secreted by vascular smooth muscle cells in response to oxidative stress, where it acted in an autocrine manner to stimulate extracellular signal-regulated kinase (ERK1/2) activation and vascular smooth muscle proliferation [Jin *et al*, 2000]. Therefore, cyclophilin A

released from activated platelets may stimulate the migration and proliferation of smooth muscle cells, a process implicated in the development of atherosclerosis.

Thus the finding of this study showed the presence of Sg III and Cyclophilin A in the test (diabetic subjects) and demonstrated the risk of atherosclerosis in them. These proteins were not present in all test samples, indicates that these are regulated by the level of activation. Whether Sg III and Cyclophilin A secreted by platelets play same function or different is not known and whether it is synthesized by platelets or exocytose like other proteins (albumin) is also not clear.

III.7: 2D gel electrophoresis for platelet proteins:

Two dimensional gel electrophoresis is a promising technique for the identification of proteins with same molecular weight. This is being used for the analysis of platelet protein since 1986 [Hanesh *et al*, 1986] and since then numbers of novel protein has been added to the platelet proteome. Platelet proteome is vast and it is expected to be made up of 2000-3000 proteins. Till now not even half of this is being identified.

The basic difficulty in identification is alteration in the platelet proteins in different physiological/patho-physiological conditions. It is not only the synthesis of protein that varies on stimulation, but includes absorption as well as secretion of the proteins. This study aimed to identify the difference between the 2D profiles of platelets protein from control, activated and test secretome.

The figure III.9 represents the 2D gel from control and test. X-.axis shows the pI of the Immobilized pH gradient strips (IPG) and Y-axis represents the molecular weight marker. Rehydration buffer was modified to deplete the more abundant proteins. Twenty two protein spots were marked and 2 D gel showed a remarkable difference in control and test. Though the

gels are not so clear, difference between control and test can be identified easily. NO of spots are also more in test, but due to horizontal striking identification is difficult. Striking may be due to the gap between the gel and casting plates or may be due to high salt concentration. Optimization is needed for better quality of gel.

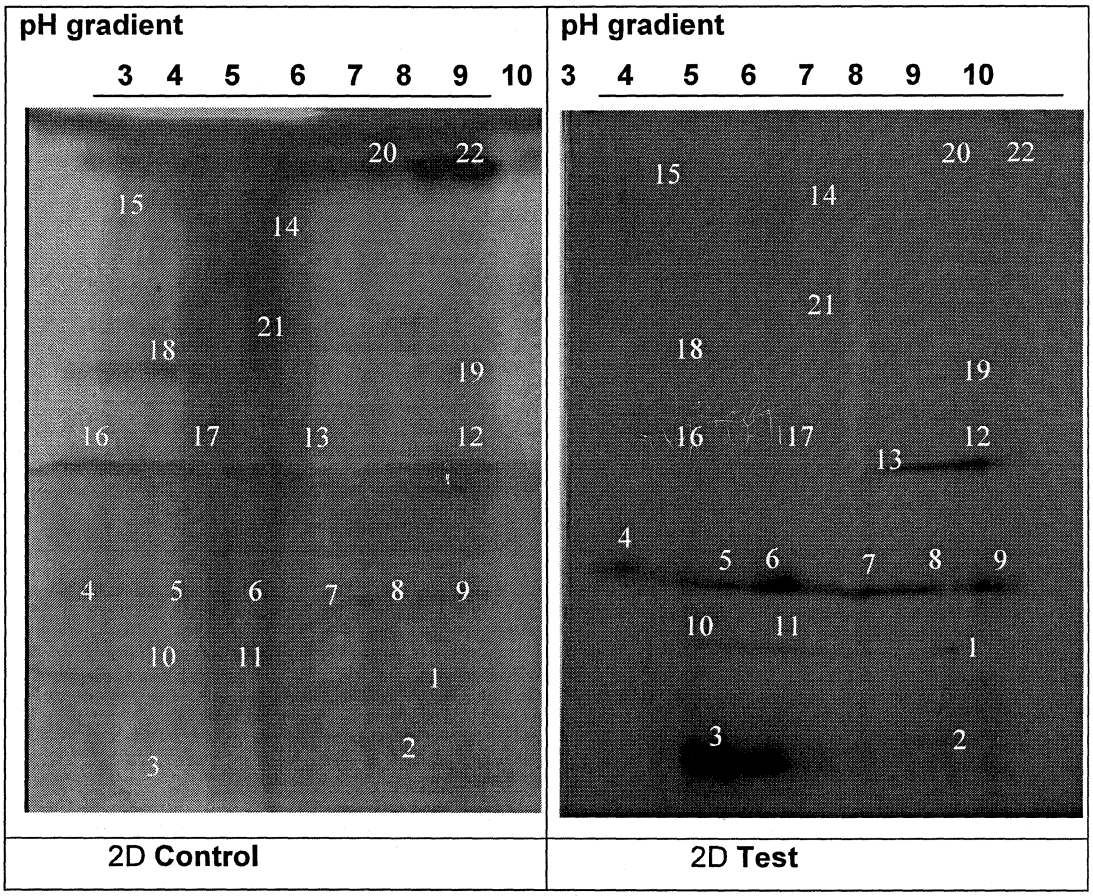

Fig III 9. Representative 2D gels of control and test

Figure III 9 show the presence of protein at between 20-24KDa at position 1,10 and 11, corresponding to the pI 9 and 5.2. Cyclophilin A also has a pI value of 9.5 and secretoganin with its two isoforms has a pI valve of 5.2. So the presence of spots at these positions may be corresponding to the Secretogranin III, II and cyclophilin A. These bands were found to be present

only in test, while absent in control, confirming the finding of SDS- PAGE and in confirmation with western blot results..

Proteins at position 4-9 are present in both the gel, but with a very high level expression in test. These proteins may be correspondence to the protein with almost same molecular weight but different pI. These may be Apo A1, its isoforms, 14.3.3protein, TMP-4-ALK protein corresponding to the MW 26, 28 and 27. These proteins were highly expressed in test, explaining their role in the disease.

Hepatoglobin, Osteonectin, antitrypsin and actin are also almost same molecular wt proteins, but differs in pI. Actin and haepatoglobin are having basic pI values. Thus bands observed at position 12,13 may be corresponding to this two proteins. The protein present at position 12 in control has two spots nearby, which is absent in test. This may be due to the post-translational modification of protein. Intensity of these bands are very high in test, an indication of the upregulation of these protein in activated platelets.

There are few proteins at position 14,18,20,22 and 16 which are present in control, but not in test may be an indication of down regulation of these proteins in activated stage, These proteins may be a group of anti-inflammatory protein, which is inhibited in diabetic subjects. Thrombospondin and albumin (position 18, 20) are present in both control as well as in test, but expression is higher in control.

The explanation is based on the assumption of pI and molecular weight of proteins. Proteins identification can be done only by using MALDI-Tof or GC-MS. In this study, we aimed to see the difference in protein profile between the two groups (healthy and diabetic). Further studies can target identification of these new as well as differentially expressed proteins. Once identified, their role in atherosclerosis progression can be studied by seeing

effect of these proteins in cell-cell interaction, cell proliferation and in inflammation.

Hence the present study demonstrated the higher level of protein expression in the test group as well as activated sample when compared to that in the control. Alteration or post translational modifications were also observed for few proteins. Thus the subjects with high glucose and fibrinogen level are more prone to atherosclerosis. The major limitation of 2D is reproducibility, thus more number of samples needs to be done for better understanding. This is a preliminary study to show that the main culprit in test (diabetic subjects), which make them more prone to atherosclerosis, may be the platelet proteins. The role of different identified proteins in inflammation needs to be addressed.

CHAPTER 4

SUMMARYAND CONCLUSION

SUMMARY

Platelets are the first circulating blood cells that interact and adhere to vascular lesions. They are one of the prime factors for the development of atherosclerosis. Despite their biological significance, platelet proteome is revealed less, specifically in pathophysiological conditions. Alteration/ post translational modification in protein is well known for proteins. Proteins which are in circulation may have more impact than the localized proteins. Protein which synthesize/secretes in response to stimulus, may stimulates number of other proteins and signaling pathways. Thus change of one pathological condition to other may be marginally dependent on the proteins which get secreted or synthesized. The platelet releasate are thought to be major culprit for the cardiovascular disease in diabetic subjects, as the presence of inflammatory proteins secreted in response to high glucose level are found to be present in atherosclerotic lesions in diabetic subjects.

Present study was aimed to see the difference between the resting (control) and diabetic (test) platelet releasates. In the first set of experiment screening was done to confirm that all subjects considered as test were diabetic. Fibrinogen level in test blood plasma was higher as compared to control. The fibrinogen level is an essential component of inflammation, coagulation and fibrinolysis and the entire coagulation gets disturbed in diabetes. Results showed high level of fibrinogen in test, an indication of the risk factor for the atherosclerosis in diabetes.

Since platelets play a major role in atherosclerosis, the increased risk of cardiovascular events among diabetic patients may be, in part, due to altered platelet function. Platelet function studies were done and results showed hyperaggregation from the subjects having high level of glucose and fibrinogen.

Concentration of thrombin a strong agonist, varies from a very small concentration to very high concentration in different patho-physiological conditions. Platelets were activated with different concentration of thrombin to find out the optimum concentration and 0.8IU/ml activated platelets were selected as the positive control for the study. At this concentration platelets showed good aggregation also, that indicated release of maximum proteins from the platelets.

Subjects with diabetes mellitus have increased platelet-surface expression of glycoprotein Ib (GP Ib), which mediates binding to von Willebrand factor, and GP IIb/IIIa, which mediates platelet-fibrin interaction and represents the final common pathway of platelet activation, leading to platelet aggregation. P-sel is a membrane marker which gets releases even on mild activation of platelets. In the diabetic subjects as well as in the high risk groups like hypertension, hypercholesteriomia, platelets are reported to be in their activated stage. Flow cytometry analysis of tests in our study also showed high % of activation marker confirming the presence of activated platelets in the circulation. To demonstrate the effect of agonist concentration on activation marker, P-sel (CD62) and CD41 was analyzed by flow cytometry. It was observed that expression level of P-Sel and CD41l increases as the concentration of agonist increases in washed platelets.

Fibrinogen and thrombin bind together and forms fibrin, which acts as a mesh to hold platelets in normal individuals; thrombin is present in very minute amount which doesn't have a fate of binding with fibrinogen to make fibrin. Lack of fibrin network doesn't allow platelets to adhere which in turn results in absence of clot and very less risk of atherosclerosis. While in the case of activated platelets, they adhere easily to the fibrinogen leading clot formation. In the present study we found platelets aggregates on positive control and test samples SEM analysis also showed morphological difference in the test and control samples. The control platelets didn't adhere to the fibrinogen coating indicating they are not activated, while in case of activated

samples and test samples, platelets were adhesion to the fibrinogen coating observed with a marked morphological changes. This confirmed that the platelets are producing membrane proteins which bind to the fibrinogen, making it as a mesh/network for the platelets to hold on. Morphologically, they developed extensions, shape got ruptured and they formed aggregates indicating their activation.

Protein content was analyzed from whole cell lysate, washed resting, activated and test platelets and it was found that released protein concentration increases as the concentration of agonist increases. Test releasates also showed marginally high protein content when compared to control.

Detailed molecular, cellular, animal and human studies have provided incredible insights into the structure and function of platelets, both under normal physiologic conditions as well as in a variety of disease states. However, molecular changes underlying for the change in platelets morphology and function have not been studied much. Identification of proteins involved in the signaling pathways leading to a variety of platelet responses *in vivo* are yet to be fully characterized. In this study we found remarkable changes in the protein pattern of control and test and a similarity in activated and test. This was further confirmed by the presence of two inflammatory proteins Secretogranin III and Cyclophilin A by western blot analysis. Such extracellular platelet proteins may prove suitable as therapeutic targets. 2D analysis of control, tests and activated subjects revealed that number of proteins in test gets altered during the progression of disease. Proteins are up-regulated and down-regulated depending up on the physiological condition.

CONCLUSION

The present study can be concluded by stating that platelets in diabetic subject's circulates in its active state and secretes/synthesized number of proteins.

- Proteome of test is comparable with that of thrombin activated group, an indication that physiological concentration of agonist increases in diseased condition.
- These activated platelets showed high efficiency of adhesion and aggregation which is the prime cause of atherosclerosis. The proteome analysis also revealed similarity in activated and test samples.
- This study may be considered as a preliminary research for the identification of the difference between control and diabetic subject. Thus, the targeting of one or more proteins which gets expressed as an early inflammation marker in circulation may provide a novel means of diagnose atherosclerosis in risk factor group at a early stage.

FUTURE PROSPECTS

- ✓ To identify the proteins secreted in the early stages of diabetes and characterize them using MALDI TOF/ LC-MS.
- ✓ To study the expression profile of specific proteins, namely, Secretogranin III and Cyclophilin A when platelets get activated.
- ✓ To study the role of these proteins in proliferation and inflammation of endothelial and smooth muscle cells.

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APPENDIX

1. Acid Citrate Dextrose

For 100mL

Trisodium citrate	-	2.20g
Citric acid	-	0.80g
Dextrose	-	2.50g

Made upto 1L using deionized water and filter

Stored at 4-8°C

2. Tyrode's Buffer

For 1L

Dextrose	-	1g
MgCl2	-	0.199g
KCl	-	0.402g
NaCl	-	8.12g
Tris base	-	1.756g

Made upto 1L using deionized water

pH was adjusted to 7.4

Filtered and stored at 4-8°C

3. Phosphate Buffered Saline (1X PBS)

For 1L

NaCl	-	8.166g
Na2HPO4	-	1.419g
KH2PO4	-	0.204g

Made upto 1L using deionized water

Filtered and stored at room temperature

4. Cell Lysis Buffer

Triton X Buffer	-	10µl
PMSF	-	0.3484mg
Leupeptin	-	100µl
Trypsin Inhibitor	-	0.5mg

5. Reagents for the Estimation of Protein

Reagent A

2% Na2CO3 in 0.1N NaOH

Reagent B

0.5% CuSO4.5H2O in 1% potassium tartarate

Reagent C

10mL Reagent A + 0.2mL Reagent B

Reagent D

1mL Folin-Ciocalteau Reagent + 2mL deionized water

6. Reagents for SDS PAGE

Preparation of Resolving gel for 15mL:

Deionized Water	-	5mL
30% Acrylamide mix	-	6mL
1.5M Tris HCl (pH 8.8)	-	3.8mL
10% SDS	-	0.15mL
10% APS	-	0.15mL
TEMED	-	0.004mL

Preparation of Stacking gel for 10mL:

Deionized Water	-	6.8mL
30% Acrylamide mix	-	1.7mL
Tris HCl (pH 6.8)	-	1.25mL

10% SDS	-	0.1mL
10% APS	-	0.1mL
TEMED	-	0.004mL

Gel Loading Buffer (1X) – For 10mL:

Deionized Water	-	3.55mL
0.5M Tris HCl (pH 6.8)	-	1.25mL
Glycerol	-	2.5mL
10% SDS	-	2mL
0.5% Bromophenol blue	-	0.2mL

Coomassie Brilliant Blue Solution - for 100mL:

Brilliant Blue R-250	-	0.1g (0.1%)
Acetic acid	-	10mL (10%)
Methanol	-	40mL (40%)
Distilled Water	-	50mL

Destaining Solution for 1L:

Methanol	-	250mL (25%)
Acetic acid	-	70mL (7%)
Distilled Water	-	680mL

7. Reagents for Silver Staining:

Silver Staining fixative for *100ml:*

Methanol	-	50mL
Acetic acid	-	10mL
Glycerol	-	250µL
Deionized Water	-	30mL

Glutaraldehyde (10%) 40mL, 25% glutaraldehyde was made upto 100mL using deionized water.

Developer:

3% Na_2CO_3

0.02% Formaldehyde

1M Citric acid (2.1014g citric acid was made upto 10mL using deionized water.)

8. Reagents for Western Blotting:

Transfer buffer for 100ml:

Tris	-	0.58gm
Glycine	-	0.29gm
SDS	-	0.1gm
Methanol	-	20ml
Distilled Water	-	80ml

Amido Black for 15ml:

0.1 % Amido Black	-	0.015gm
45% Methanol	-	6.75ml
10% Acetic Acid	-	1.5ml
Distilled Water	-	6.75ml

Distaining Solution for 15ml:

90% Methanol	-	13.5ml
2% Acetic Acid	-	0.3ml
Distilled Water	-	1.5ml

Chloronaphthol :

Dissolve 0.3 gm Chloronaphthol in 10 ml absolute alcohol and store at -20° C.

9. Reagents for 2D Electrophoresis:

1st Dimension rehydration buffer for 10 ml:

8M Urea	-	4.8gm
2% CHAPS	-	200mg
Ampholytes	-	20µl
DTT	-	77mg
BPB	-	0.1mg

2nd Dimesion equilibration buffer I for 50 ml:

DTT	-	1gm
SDS	-	1gm
Urea	-	18gm
1.5 M Tris(pH 8.8)	-	12.5ml
Glycerol	-	10ml
Distilled Water-	-	27.5ml

2nd Dimesion equilibration buffer II for 50 ml:

Iodoacetamide	-	1.25ml
SDS	-	1gm
Urea	-	18gm
1.5 M Tris(pH 8.8)	-	12.5ml
Glycerol	-	10ml
Distilled Water	-	27.5ml