Comparative Analysis of Platelet Proteome and Role of Circulating Platelet Proteins in Endothelial Dysfunction Among Diabetic and Healthy Individuals

A THESIS SUBMITTED

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

TRIVANDRUM – 695 011

DECLARATION

I, **Serene Hilary**, hereby declare that I personally carried out the work depicted in the thesis entitled "**Comparative analysis of platelet proteome and role of circulating platelet proteins in endothelial dysfunction among diabetic and healthy individuals**" under the direct supervision of **Dr. Anugya Bhatt, Scientist C, Thrombosis Research Unit**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala, India. External help sought are acknowledged.

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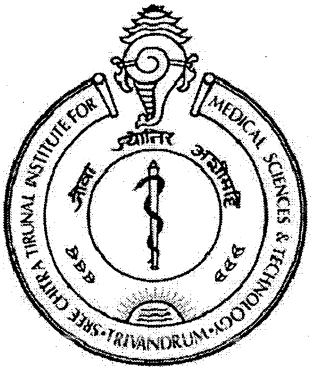

CERTIFICATE

This is to certify that the dissertation entitled "**Comparative analysis of platelet proteome and role of circulating platelet proteins in endothelial dysfunction among healthy and diabetic individuals**" submitted by **Serene Hilary** in partial fulfilment 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 695 012.

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"COMPARATIVE ANALYSIS OF PLATELET PROTEOME AND ROLE OF CIRCULATING PLATELET PROTEINS IN ENDOTHELIAL DYSFUNCTION AMONG HEALTHY AND DIABETIC INDIVIDUALS"

Submitted by

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For

Master of Philosophy

Of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM- 695 011

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Acknowledgements

Foremost, I thank Almighty for being the pillar of my strength during the demanding phase of my work.

I feel extremely honoured to have worked under the supervision of Dr. Anugya Bhatt, Scientist C, Thrombosis Research Unit. I take this opportunity to express my profound sense of gratitude for her guidance, monitoring and constant encouragement throughout the course of my project.

My heartfelt gratitude goes out to Dr. Lissy K. Krishnan whose contributions, valuable suggestion and encouragement helped me coordinate my work from time to time.

My sincere thanks to The Director (SCTIMST), Head (BMT Wing) and Deputy Registrar (SCTIMST) for providing all the facilities to carry out my project in this reputed institution.

I thank Dr. Jyothidev Kesavdev, Dr. Arun, Ms. Gopika, Ms. Binushma and all other staffs members of the Research Unit at Jyothidev Diabetes and Research Centre, Trivandrum for providing me the blood samples for my study.

I thank all study subjects who willingly donated their blood and supported my work.

I express my sincere gratitude to Dr. Abdul Jaleel K. A. (Senior Consultant, Proteomic Facility) and Mr. Arun Surendran in Rajiv Gandhi Centre for Biotechnology, Trivandrum for carrying out the MALDI-Tof analysis.

I am indebted to the members of Thrombosis Research Unit, Ms. Priyanka, Mr. Renjith S, Ms. Mary Vasantha, Mr. Renjith P. Nair, Ms Tara, Mr. Unnikrishnan, Ms. Subha, Ms Soumya, Ms. Renu Ramesh and Ms. Indu for their support and co-operation during my project period.

Few words will not suffice if I am to acknowledge the extent of care and support given to me by my husband, parents and my dearest friends, without which this project would not have materialised.

Thank you all for being there for me!

Serene Hilary

Abbreviations

%	Percentage
µg	Micro gram
µl	Micro litres
ACD	Acid citrate dextrose
AcLDL	Acetylated low density lipoprotein
ADP	Adenosinediphosphate
AGE	Advanced glycation end products
bFGF	basic-Fibroblast Growth Factor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBB- R250	Coomassie brilliant blue-R250
CD	Cluster of Differentiation
CVD	Cardiovascular disease
DEPC	Diethylpyrocarbonate
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothretol
ECM	Extra-cellular matrix
ECs	Endothelial cells
EDRF	Endothelium derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
ENA-78	Epithelial Neutrophil Activating Protein- 78
eNOS	Endothelial nitric oxide synthase
FACS	Flourescence activated cell sorting
FBS	Foetal bovine serum
FP	Forward primer
FSC	Forward Scatter

g	Gravity
GAPDH	Glutaraldehyde 3-phosphate dehydrogenase
GP	Glycoproteins
HBSS	Hank's balanced salt solution
HUVEC	Human umbilical vein endothelial cells
IDA	International Diabetic Association
IGF-1	Insulin like Growth Factor-1
IMDM	Iscove's modified Dulbecco's medium
IPG	Immobilised pH gradient
kDa	Kilo Dalton
MALDI-Tof MS	Matrix assisted laser desorption/ionisation- time of flight mass spectroscopy
MIP-1α	Macropahge Inflammatory Protein-1α
mRNA	Messenger RNA
NO	Nitric oxide
ºC	Degree Celsius
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen Activator Inhibitor- 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDEGF	Platelet Derived Epithelial Growth Factor
PDGF	Platelet Derived Growth Factor
PI	Propidium iodide
PMSF	Phenylmethylsulphonylflouride
PPP	Platelet poor plasma
PRP	Platelet rich plasma
RANTES	Regulated upon activation, healthy T cell expressed and secreted

ROS	Reactive oxygen species
RP	Reverse primer
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SFM	Serum free media
SSC	Side Scatter
TEMED	Tetramethylethylenediamine
TF	Tissue Factor
TGF-β	Tumor Growth Factor-β
TGS	Tris-glycine-SDS
tPA	Tissue plasminogen activator
VEGF	Vascular Endothelial Growth Factor
vWF	von Willibrand Factor
β-TG	β – Thromboglobulin

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Synopsis

Cardio-vascular disease is one of the major causes of death world-wide. Diabetes increases atherothrombotic risk due to many factors. It is reported that platelets in diabetic subjects are activated under physiological conditions. The hyperactivity of platelets in diabetic subjects could be attributed to different factors like increased fibrinogen content, altered membrane fluidity, accumulation of advanced glycation end products, over expression of adhesion molecules etc. Platelets contain several morphologically distinguishable storage granules which stores cytokines and other mediators of cellular response. These contents are released into circulation upon activation of platelets. Hence in diabetic individuals several of such proteins are increased in circulation which may have an inflammatory response on the endothelial cells lining the vessel walls.

The important feature in the pathogenesis of atherosclerosis is endothelial dysfunction. This feature is common to both diabetes and atherosclerosis and is observed in the initial stages of both diseases. Alterations in the normal function of endothelium have been proven to initiate the plaque development. The presence of differently expressed platelet proteins in circulation of diabetic individuals may be responsible in causing endothelial dysfunction. Differential expression of proteins of platelets in diabetic individuals and its role in causing endothelial dysfunction has not been studied extensively. Hence the goal of this project was to isolate and characterise platelet proteome and circulating platelet proteins from diabetic and healthy groups and study the role of these proteins in causing endothelial dysfunction.

This dissertation is divided into 4 chapters. Chapter I gives a brief outline of the study, review of literature, brief description of the gap area, hypothesis and objectives. Chapter II gives the details of all equipments and reagents used along with sufficient description about the procedures used for the study. Results of the study are divided into two chapters (III and IV) and

are represented with appropriate images/graphs/tables. The observations are discussed in the light of current knowledge in literature. Chapter V summarises the results of the study and conclusion are highlighted.

The reviewed literature proposes that platelet secretion play an important role in the progression of atherosclerosis. Hyperactivity of platelets in diabetes is also reported in literature. But studies into how these proteins initiate atherothrombotic risk is highly lacking as the effect of these platelet proteins in plasma in causing endothelial dysfunction has not been probed so far. Blood is the most popular source for biomarkers. Once the culprit proteins secreted by platelets are identified and characterised, it may pave way for use as a biomarker for cardiovascular disease in diabetic individuals.

Therefore this study was designed based on the hypothesis that the platelet proteome of diabetic and healthy individuals vary due to differently expressed proteins and these active platelets secrete their granule contents into the circulation. These proteins present in circulation may interact directly with the endothelial cells and can cause EC dysfunction. In order to test the hypothesis objectives set were:

- To isolate secreted platelet proteins from plasma of diabetic and healthy control.
- To isolate whole platelet proteome from diabetic and healthy control.
- To compare the difference in plasma proteins and whole platelet proteome in diabetic and control subjects by SDS-PAGE and 2D gel electrophoresis.
- To analyse the expression of platelet proteins in diabetic subjects by MALDI-Tof.
- To study the effect of these proteins in causing endothelial dysfunction by PCR analysis of different markers:
    - Antithrombotic marker- tPA, eNOS
    - Prothrombotic marker- vWF

The study subjects were screened by the blood glucose level and HbA1 c percentage. Ethanol precipitation was used to deplete the high abundant plasma proteins. Comparison of plasma proteins from both groups was carried out in SDS-PAGE and 2-D gel electrophoresis. Whole platelet proteome difference from both groups was studied using 2-D gel electrophoresis. Finally the effect of circulating platelet proteins in endothelial dysfunction was analysed by apoptosis assay using flow cytometry and gene expression studies using PCR.

Results of the present study clearly showed 30% ethanol was the optimum concentration to deplete the plasma of abundant proteins. On comparison of plasma proteome from both groups it was noted there was significant difference between the two groups. Diabetic plasma contained more number of proteins as compared to healthy controls. Similar differences were observed in platelet proteome from both the groups implying that differently expressed proteins in plasma are from platelet source. When plasma from diabetic subjects were immobilised on cell culture matrix, apoptosis of EC was evident as compared to control culture conditions. In gene expression studies it was observed that ECs turned prothrombotic on long term exposure to diabetic circulating platelet proteins as evidenced from the upregulation of vWF gene with the down-regulation of both eNOS and tPA

Based on the study conducted it can be concluded that the protein profile of platelets from control and diabetic are highly variant. These differently expressed proteins in diabetic platelets are secreted into the circulation. Such proteins can interact with ECs and cause vascular damage and shift the gene expression pattern of these cells to favour prothrombotic phenotype. Thereby, the proteins demarcated in the study may play a key role in the progression of atherosclerosis in diabetic subjects. Further studies are required to pinpoint to the specific platelet proteins and how it initiates the changes in ECs.

Chapter I

Introduction

1.1 Background

The World Health Organisation estimates that over 347 million people in the world are diabetic and in India the number is even more alarming. According to the Diabetes Atlas 2006 published by the International Diabetes Federation, the number of people with diabetes in India currently around 40.9 million is expected to rise to 69.9 million by 2025 unless urgent preventive steps are taken [Sicree *et al.*, 2006]. In another study on mortality rates in India carried out among the urban population it was seen that mortality rate was about 18.9% in diabetic individuals as compared to 5.4% in non-diabetic individuals. Of this 52.9% of death in diabetic group was because of cardiovascular diseases [Mohan *et al.*, 2006]. Cardiovascular disease is already recognised as the single leading cause of death world-wide and is projected to remain the same. Most cardiovascular complications can be prevented by addressing the contributory factor like diabetes, high blood pressure, tobacco use, unhealthy diet, physical inactivity, and raised lipids. Among all the other risk factors diabetes stands out as the major contributor to the development of this disease.

Diabetes, which is characterised by the presence of elevated fasting glucose concentration in blood, increases atherothrombotic risk due to many factors. It is seen that platelets in diabetic subjects are activated under normal physiology of the individual. The hyperactivity of platelets in diabetic subjects could be attributed to different factors like increased fibrinogen content, altered membrane fluidity, accumulation of advanced glyation end products, over expression of adhesion molecules etc, all of which are metabolic changes associated with diabetes. All these factors work hand in hand to accentuate the risk of atherosclerosis in diabetic individuals.

The most important feature in the pathogenesis of atherosclerosis is endothelial dysfunction. Similar change in the functions of endothelium is also seen in diabetic conditions. Alterations in the normal endothelial function can initiate plaque development which later leads to constriction of the vein and rupture of the plaque thereby recruiting the already activated platelets in circulation to form abnormal thrombus thereby resulting in myocardial infarction. Activated platelets release a number of proteins into circulation which comes in direct contact with endothelial cells. Though platelets proteins play a very crucial role in the progression of diabetes and associated cardiac diseases, platelet proteome is not being explored much to see the effect of these proteins on endothelium.

Platelet proteome is vast and vary with respect to changes in the metabolism caused by any disease. The role of platelets in abnormal thrombus formation in cardio vascular disease (CVD) has already been noted by many researchers proving that platelet plays a significant role in the disease. The difference in the secretory function of platelets with respect to diabetes can be used for the development of biomarkers of CVD in these individuals. It is suspected that the low abundant proteins secreted by platelets in plasma of diabetic individuals plays significant role in causing endothelial dysfunction. The study on such low abundant proteins may help to identify biomarkers for CVD in diabetic individuals. Based on this hypothesis, this study is aimed to analyse differences in platelet protein profile between diabetic and healthy individuals and to study effect of these proteins on endothelial cells.

1.2 Review of literature

Platelets are blood cells that facilitate thrombosis by adhering to the endothelium. Under normal physiology of an individual platelets are inactive but when activated, these cells promote thrombosis. Activated platelets release a wide variety of proteins into the plasma. Secreted platelet proteins in plasma can mediate signalling and initiate several different functions in

autocrine or paracrine mode. Some of these proteins are prothrombotic while others regulate cell proliferation through cell signalling. The platelet proteome is extensive and the entire protein content has not been characterised and their roles have not yet been elucidated. Future studies are needed to throw light upon the circulating platelet proteins in plasma and its functions.

Individuals with diabetes, have increased platelet reactivity. Diabetes promotes oxidative stress and inflammation which results in endothelial dysfunction. This can promote activation of platelets by decreasing the production of nitric oxide (NO). Since platelets are active in diabetic subjects there is a risk for accelerated atherosclerosis. Activated platelets in diabetic subjects play a pivotal role in the initiation, development and progression of atherosclerotic plaque. Independent studies have pointed out the presence of platelet proteins at the plaque site in atherosclerosis. The roles of these proteins have not been studied in detail. Cardiovascular disease is the leading cause of death in patients with diabetes. Since the risk factor of atherosclerosis in diabetic subjects is more, early detection is the key to avoid complication that can result later. A better understanding of the platelet proteome involved in diabetic atherosclerosis can help to characterise them for use as biomarkers to identify high risk factor group.

1.2.1 Platelet structure and function

Platelets are small anucleated cells that are derived from fragmentation of precursor megakaryocytes. Although platelets lack genomic DNA they contain megakaryocyte derived mRNA and transitional machinery that is required for protein synthesis. The lifespan of platelets in circulation is about 10 days. These cells are discoid in shape and they are the second most abundant corpuscle in blood with a cell number ranging anywhere between $150\text{-}450\times10^{9}$/L under normal physiological conditions [Harrison, 2005]. The main function of platelets is in clot formation although they are involved in many other pathophysiological processes. The shape and size of platelets is such that they are capable of reaching the edge of blood vessels and this

helps in their functions as the surveyor for vascular damage. On exposure to vascular injury platelets undergo a highly regulated set of responses like adhesion, spreading, protein release function, aggregation, exposure of a procoagulant surface, microparticle formation and clot retraction [George, 2000]. All these functions work hand in hand for thrombosis at the site of damage to prevent blood loss. Any defect in these functions can result in increased risk of bleeding or increased platelet reactivity that can result in inappropriate thrombus formation. Such impaired thrombus can develop within atherosclerotic lesions which can lead to stroke and myocardial infarction.

1.2.2 Platelet activation

Normal endothelium prevents the adherence of platelets by production of anti-platelet agents. But in case of vascular injury, which is manifested by the exposure of collagen due to disruption of the endothelium, platelet adherence occurs at the site which in turn activates them. Platelet adhesion to the extracellular matrix is the first step in thrombus formation. Platelets roll, adhere and spread on collagen matrix to form an activated platelet layer. The adhesion of platelets to injury site is mediated by glycoprotein (GP) Ib/V/IX receptor complex on the platelet surface to von Willebrand factor (vWF) and GPVI and GPIa to collagen at sites of injury. Under normal physiological conditions, soluble vWF does not interact with GPIb/V/IX [Soslau *et al.*, 2001]. However, when immobilised on exposed collagen at sites of injury, it becomes a strong adhesive substrate. Following the adhesive response other mediators of clot formation like ADP, thrombin serotonin, epinephrine and thromboxane A2 amplify and sustain the initial platelet response. Most of these agonists operate through G-protein coupled receptors [Offermanns, 2006]. The binding of these agonists initiates platelet activation which is resulted by a series of events such as hydrolysis of membrane phospholipids, mobilization of intracellular calcium and phosphorylation of important intracellular proteins. These mediators accentuate the repair process by recruiting circulating platelets from the flowing blood to form the thrombus.

There are several different manifestations to platelet activation. These include platelet shape change, expression of pro-inflammatory molecules such as P-selectin and soluble CD40 ligand (sCD40L), expression of platelet procoagulant activity, and conversion of GPIIb/IIIa (αIIβ3-integrin) into an active form, which allows platelet aggregation and the potential for pathologic thrombosis. Local accumulation of such agents results in formation of a haemostatic plug [Hodivala *et al.*, 1999]. GPIIb/IIIa is the main receptor for adhesion and aggregation. Several adhesive substrates bind to GPIIb/IIIa. Fibrinogen bridges GPIIb/IIIa between activated platelets and contributes to thrombus stabilisation. vWF is necessary to facilitate inter-platelet bridges [Ni *et al.*, 2000]. Under normal physiological conditions the platelets are kept inactive by the vascular endothelium. Endothelial cells control the reactivity of platelets by three different pathways; the arachidonic acid-prostacyclin pathway, the L-arginine-nitric oxide pathway and the endothelial ecto-adenosine diphosphatase pathway [Jin *et al.*, 2005]. Quiescent platelets contain the pre-mRNA for Tissue-Factor (TF), which is the primary initiator molecule of the coagulation cascade. On activation of platelets signal dependant splicing of TF pre-mRNA takes place. This consequently leads to the synthesis of TF protein which converts prothrombin to thrombin and fibrinogen to fibrin [Schertz *et al.*, 2006].

Thrombin is rapidly generated at the site of vascular injury and in addition to cleaving of fibrinogen it is a very potent activator of platelets. Thrombin can activate platelets even at extremely lower concentrations [Offermanns, 2006] even lower than those required for the activation of the coagulation cascade. ADP is another important platelet activating agent. ADP is stored in and secreted from dense granules in platelets and interacts with its platelet receptors $P2Y_{12}$ on quiescent platelets thereby amplifying the thrombus formation [Dorsan *et al.*, 2004]. Release of thromboxane A2 from adherent platelets enhances recruitment and aggregation to the primary plug and activates platelets during both protective haemostasis and pathologic thrombus formation [Cheng *et al.*, 2002].

1.2.3 Platelet proteins in plasma

Platelets are anucleated and in spite of the absence of nucleus in these cells the biosynthesis of proteins by platelets has been documented [Schwertz *et al.*, 2010]. The protein synthesising capacity of platelets has been attributed to the inheritance of stable mRNA from megakaryocytes [Kieffer *et al.*, 1987]. Proteins synthesis occurs in circulating platelets and its capacity is 10 times stronger than newly formed platelets. Upon activation of platelets, they release their granule contents as well as newly synthesized proteins into the plasma which can further enhance the response to the injury.

Platelets contain a large number of preformed storage granules which are distinguished as α-granules, dense granules and lysosomes. Dense granules contain high levels of adenine and guanine nucleotides, divalent cations and serotonins. Other molecules present in dense granules are histamine and ATP which are involved in prothrombotic response and inflammation [Momi and Gresele, 2002]. The α-granules are the major granules present in the platelets. They contain different proteins like platelet factor-4, plasminogen activator inhibitor-1, β thromboglobulin, fibrinogen, fibronectin, thrombospondin, vWF and several growth factors like platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived epidermal growth factor (PDEGF) and insulin-like growth factor-1 (IGF-1) [Reed, 2002]. Apart from these molecules α-granules also contain chemokines RANTES, macrophage inflammatory protein-1α (MIP-1α), β-thromboglobulin (β-TG) and epithelial neutrophil activating protein-78 (ENA-78) [Power *et al.*, 1995]. Lysosomes mainly consist of proteases and glycohydrolases that facilitate extravasation of leucocytes at the site of platelet accumulation [Rendu and Brohard-Bohn, 2001]. During activation two different membrane vesicles are given off by platelets; cell surface derived microvesicle and exosomes of endosomal origin [Heijnen, 1999]. The protein content of microvesicle is similar to that of activated plasma membrane and these are involved in procoagulant and inflammatory response. The functions

of exosomes released by platelets are still to be elucidated. Exosomes are released by other cells of the immune system and they are found to have immunomodulatory functions.

In a recent study it is reported that up on thrombin stimulation platelets releases more than 300 proteins. It was found in this study that many of these proteins were not previously associated with platelets [Coppinger *et al.*, 2004]. Platelet proteome is wide and it is consist of more than 2000 proteins. But synthesis of new proteins changes with the pathological conditions. These changes can be identified easily in circulating plasma. Plasma is a remarkable rich and diverse medium, containing millions of antibodies and thousands of other proteins with a multitude of biological activities ranging from coagulation/ complement activation and modulation of receptor mediated signal transduction to modulation of different pathological conditions. For decades, plasma has been used as a diagnostic medium and proteins from plasma have been identified as biological marker for different diseases. Blood plasma is an exceptional proteome in many respects. Studies have been done to identify the plasma proteins which can be used as a biomarker for coronary diseases. It is reported that platelet proteome changes when they are activated with different agonists like arachidonic acid, collagen and thrombin [Majek *et al.,* 2010]. Platelets up-on activation releases its granule contents into plasma, these platelet proteins are low abundant in plasma and can be identified only when high abundant plasma proteins are depleted.

The recent integration of proteomics into biochemical and biological platelet research has proved to be a powerful tool in understanding platelet proteome. 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.

1.2.4 Platelets in diabetes and atherosclerosis

Diabetes is a disease characterised by the increase in fasting blood glucose concentration. It can be caused by the decrease in insulin production or resistance to insulin or both. Atherosclerosis is the development of artheromatous plaques in the inner lining of the arteries and is characterised by chronic inflammation and occlusion of the lumen of the blood vessels. This condition can be caused by several different factors like elevated cholesterol or blood pressure, smoking, diabetes or mutations in genes related to the depositions in the vessel wall [Humphries and Morgan, 2004]. The site of atherosclerosis is the arterial wall where thickening takes place. This site is characterised by the presence of fat, cholesterol, calcium, lipoproteins and cells such as macrophages (foam cells) derived from the blood [Herczenik and Gebbink, 2008]. Diabetes and atherosclerosis are convergent due to the role played by platelets in both conditions. A community based prospective study showed that compared with non-diabetic individuals, most people with diabetes have elevated risk of coronary heart disease [Folsom *et al.*, 2003]. It is seen that platelet reactivity is high in diabetic subjects and this increases the risk of atherosclerosis.

The high platelet reactivity in diabetic subjects is manifested by increased platelet aggregation and adhesiveness. There are various factors that are attributed to this difference. It has been reported that platelets in diabetic subjects had reduction in membrane fluidity which can be result of glycation of membrane proteins and increased cytosolic calcium levels. There is significant alteration of calcium – magnesium homeostasis in platelets. An increase in arachidonic acid metabolism is present which leads to increased thromboxane A2 synthesis. This directly results in increased platelet sensitivity [Natarajan *et al.*, 2008]. Proteomic analysis of human platelets showed very high levels of acetyl CoA in the platelet of diabetic subjects. This correlates the increase in synthesis of thromboxane A2 and malonyl dialdehyde and lipid peroxidation as well as the spontaneous, thrombin or collagen induced aggregation of platelets [Banfi *et al.*, 2010]. The platelets in

diabetic subjects also have decreased prostacyclin and NO production. NO and prostacyclin normally inhibit platelet endothelial interaction and mediates vasodilatation. This mechanism is impaired in diabetic subjects [Xiang *et al.*, 2006]. Over expression of activation dependant adhesion molecules like P-selectin or GPIIb/IIa is yet another manifestation of platelets in diabetic subjects that directly lead to increased aggregation of platelets in diabetic condition [van Gils *et al.*, 2009].

Other contributory factors include the increased level of fibrinogen content in plasma. When the fibrinogen level is increased in diabetic subjects and due to the pro-coagulatory effect of other factors abnormal clots are easily formed. There is also increased level of plasminogen activator inhibitor-1 (PAI-1) in diabetic subjects. The increased concentration of PAI-1 leads to decreased fibrinolysis of the abnormal clots that are formed thus accentuating the risk of atherosclerosis [Festa *et al.*, 2006]. Another factor responsible for platelet hyperactivity in diabetic subjects is the accumulation of advanced glycation end products (AGE). A recent study has demonstrated that AGE generated under hyperglycaemic conditions can interact with CD36 on platelets. This interaction can trigger CD36 dependant JNK2 activation and thereby enhance platelet aggregation and thrombus formation [Zhu *et al.*, 2012]. A similar study has reported that misfolded proteins can activate platelets by a process mediated by scavenger receptor CD36 and agglutination receptor GPIbα [Herczenik *et al.*, 2007]. In diabetes subjects, high blood glucose level might contribute to amyloid properties in albumin that can be attributed to why the platelets in diabetes patients show high reactivity [Korporaal *et al.*, 2005]. It has also been reported that after activation with various stimuli platelets express amyloid-β proteins and these were detected in platelet-derived microparticle from healthy individuals. The level of these amyloid proteins in atherosclerotic patients was found to be high. These particles are sources of tissue factor which is an important initiator of thrombosis [Korga *et al.*, 2006]. Activated platelets release number of mitogenic factors which affects growth, proliferation and migration of

monocytes to the subendothelial layer of the artery wall [Langer & Gawaz 2008]. There are upcoming evidences that proteome of platelets differ from healthy and diabetic individuals. And this difference in platelet proteome and its secretory functions may also be different in these two groups. Such events may be responsible for triggering the formation of atherosclerotic plaques.

1.2.5 Endothelial cell functions

The endothelial cells (ECs) are the chief regulators of vascular homeostasis, interacting with both circulating cells as well as cell present in the vessel wall. ECs form the interface between blood and tissue and are susceptible to changes in blood composition and in blood flow. ECs respond to all these changes and play a central role in the mechanisms underlying the development of vascular disorders. One of the principle functions of EC is that it forms a selective barrier between the blood and tissue and this endothelial lining presents a large surface area for the exchange of materials [Baldwin and Thurston, 2001]. The inner surface of the endothelium in blood vessels is both anti-coagulant and anti-thrombotic under normal conditions. This state of the blood vessel is maintained by the variety of molecules secreted by the ECs which regulates both blood coagulation and platelet functions. Damaged vessel or exposure of ECs to certain cytokines or inflammatory stimuli makes them attain procoagulant or prothrombotic phenotype. A variety of anti-platelet molecules are secreted by the ECs of which the major ones are prostacyclin and nitric oxide (NO). Both these molecules prevent the aggregation of platelets by increasing the cAMP content in these cells. Prostacyclin and NO are continually released by ECs but their synthesis can be increased by stimulation of ECs by other molecules that are involved in the coagulation process (bradykinine, thrombin etc.) [Michiels, 2003].

Another important function of endothelium is fibrinolysis. ECs participate in fibrinolysis by releasing tissue-Plasminogen Activator (tPA) and urokinase. tPA converts plasminogen into plasmin which degrades the thrombi by digesting the fibin mesh. tPA is continuously secreted by ECs

under normal conditions but they release urokinase only when activated. It is also interesting to note that the natural inhibitor of tPA, Plasminogen Activator Inhibitor type-1 (PAI-1) is also secreted by ECs. The ratio of tPA and PAI-1 is always in favour of PAI-1 which is altered by certain cytokines again shifting the role of ECs to procoagulant type [Sidelmann *et al.*, 2000]. The ECs once activated can shift the balance of endothelial properties to favour platelet aggregation and clot formation. This is achieved by the induction of procoagulant/prothrombotic factors following the suppression of anti-coagulant mechanisms. Once activated by cytokines the ECs can activate platelets by releasing two mediators, Platelet-Activating Factor (PAF) and vWF. PAF is a strong platelet activator and promotes platelet adhesion to endothelial cells. ECs are the major source of vWF. ECs secrete vWF continuously into the plasma and the subendothelial matrix. This protein is also stored in high amounts in the Weibel-Palade bodies in the cytoplasm and are mobilised rapidly in response to agonists like thrombin [van Mourik *et al.*, 2002].

1.2.6 Nitric Oxide (NO) and Endothelial nitric oxide synthase (eNOs)

The principle function of ECs is to regulate the vasculature tone. The vascular tone is maintained due to the release of various molecules by ECs. One of the most important vasodilatory agent released by ECs is NO, the other being prostacyclin. This not only acts as a vasodialatory agent but inhibits inflammation and has anti-aggregation effect on platelets. Nitric oxide was originally identified as endothelium-derived relaxing factor (EDRF). Nitric oxide is constitutively secreted by ECs, but its production is modulated by a number of exogenous chemical and physical stimuli [Duran *et al.,* 2010]. ECs generate NO through the oxidation of L-arginine to L-citrulline by NO synthases and the NO thus generated has very short half life. Three different isoforms of NO synthase has been identified of which one isoform, endothelial nitric oxide synthase (eNOS) or the Nos3 gene product, the predominant one in the vessel wall, is constitutively active but it stimulated by receptor-dependant agonists [Stamler *et al.*, 1992]. Under normal conditions eNOS is

inactive and remains membrane bound by virtue of inhibitory interaction with caveolin, the principle structural protein in caveolae. Receptor mediated agonist stimulation by molecules like bradykinin, acetylcholine, thrombin, histamine etc., leads to rapid enzyme activation and release from plasma membrane [Marletta, 2001]. Shear stress is also another important modulator of eNOS activity. Shear stress causes rapid increase in the release of NO which is attained by rapid activation eNOS and upregulation of eNOS gene expression [Xiao *et al.*, 1997].

1.2.7 Endothelial dysfunction in diabetes and atherosclerosis

Physiological impairment in diabetes like platelet hyperactivity, impaired fibrinolysis, tendency for thrombosis and coagulation, increased inflammation and endothelial dysfunction maybe linked to the increase in atherosclerotic vascular disease in these individuals. But unlike other contributory factors on the list, endothelial dysfunction is an important factor linking diabetes and atherosclerosis. The first evidence in human of impaired endothelium dependant vasodilation in the presence of atherosclerosis was shown by Ludmer *et al.*, in 1986. Their study also demonstrated that endothelial dysfunction is present in the early stages of atherosclerosis. So it can be inferred that endothelial dysfunction may be initiating factor for the development of CVD in diabetic subjects, in whom the impairment of the endothelium is present throughout the course of the disease.

The mechanism underlying endothelial dysfunction in diabetic subjects varies. The elevation of blood glucose was the first recognised abnormality in people with diabetes. The ECs which are sensitive to changes in blood parameters effectively sense the increased glucose level and rapidly become dysfunctional. Dysfunction in response to increased glucose in blood is seen as early as 6 hours [Beckman *et al.*, 2001]. Another effect of hyperglycemia is the increased production of reactive oxygen species (ROS) by endothelium and activation of protein kinase C. Both these molecules have been reported to play a vital role in causing endothelial dysfunction in hyperglycemic

conditions [Hink *et al.*, 2001]. Increased production of ROS decreases the availability of NO as these superoxides scavenge NO to form peroxynitrite. The decrease in the natural vasodilatory substance produced by the endothelium combined with the increased production of vasoconstrictors by endothelial cells is all hallmark of endothelial dysfunction which finally ends in development of atherosclerosis [Zou *et al.*, 1999].

Insulin resistance is the first event that precedes diabetes and it occurs in the endothelium. It is already proves that insulin resistance promotes atherosclerotic risk [Reaven *et al.*, 1988]. Inflammation is another manifestation of atherogenic process. It is strongly linked to both insulin resistance and diabetes. The first step in atherosclerosis is the migration of T-lymphocytes into the vascular intima. These cells then produce cytokines and chemokines and recruit monocytes and vascular smooth muscle cells into the plaque site. These cells then scavenge LDL and become foam cells and form a mass in the region which later forms the atherosclerotic plaque. Hence it is understood that endothelial dysfunction enhances each of these early processes through the activation of inflammatory transcription factors [Nomura *et al.*, 2000].

All of the metabolic disturbances described above impair the ECs activity and make the vascular environment favourable for the development of atherosclerosis. Diabetic condition in humans compromises most of the anti-atherosclerotic processes and thereby increasing the risk of cardio vascular disease in these individuals.

1.2.8 Endothelial dysfunction and platelet proteins

Endothelial dysfunction is an early event in CVD as well as in associated diseases like diabetes and hypertension. Mechanisms lying behind are well studied and are still an area of interest for the scientific community working in the respective field. However, a very important factor i.e platelets protein and their role in the progression of diseases were left behind and gaining attention recently. Platelets are able to modulate the function of

endothelial and vascular smooth muscle cells via the direct release of growth factors and pro-inflammatory chemokines. The microparticles produced by the platelets function as transcellular delivery system for micro RNAs.

Functionality of ECs depends on microenvironment, which includes matrix and growth factors which is crucial for EC survival. In pathological conditions microenvironment gets altered which causes EC dysfunction. In *in-vitro* studies it is important to maintain the physiological matrix for EC growth. To control adhesion, growth, viability, differentiation, and function of the cells for a longer period it's necessary to provide natural ECM. It is reported that matrix composition affects the EC differentiation. EC remains functional for a longer time if cultured on fibrin growth factor composite coated dishes when compared with gelatin coated dishes [Prasad KC and Krishnan LK, 2005].

Diabetes causes the dysfunction of several vascular physiology components which includes endothelium, vascular smooth muscle cells and platelets. This type of dysfunction is due to the development of oxidative stress in hyperglycemia. The endothelium plays a key role in the regulation of arterial tone and blood flow.

Up on vessel wall injury platelets rapidly adhere to the exposed subendothelial matrix. This is mediated by cellular receptors present on platelets or endothelial cells and various adhesive proteins such vWF, collagen and fibrinogen. The activated platelet recruits additional platelets to the injured site and resulting in the generation of a platelet aggregate which forms a stable platelet plug. The interaction of platelet with endothelial cells induces the release of P-selectin from the platelet α granules and the Weibel Palade bodies form the endothelial cells.

1.2.9 Summary

Platelets and endothelial cells are both important in maintaining the primary haemostasis. Both these cells have secretory function and the cross talk between these cells maintains vascular homeostasis. In diabetic

individuals platelets show hyperactivity due to changes in the blood parameters like hyperglycaemia, increased production of superoxides and cytokines etc and secrete number of proteins in circulation which can interact directly on ECs. These proteins may shift the normal balance of ECs and may convert them to prothrombotic/ procoagulant state. This elevates the risk of atherosclerotic plaque development in diabetic individuals. Surveys have already shown that there is increased mortality rate from CVD in diabetic individuals. The most effective method to alleviate the problem of increased mortality in diabetic subjects due to CVD is to identify the early marker for CVD in diabetic individuals. Platelet proteins can be identified in circulation and differently expressed platelet proteins found in plasma of diabetes subjects may therefore serve as a biomarker.

1.3 Gap Area

It is reported that proteome of platelets varies in different pathological conditions. The synthesis and secretory functions of platelet may vary in diabetic conditions as platelets in diabetes are reported to be active in circulation. Due to this platelets may release several proteins into the circulation of diabetic individuals which maybe absent in the normal vasculature. Though role of these proteins are not studied much it is possible that they may be the key regulators for cardio-vascular disease progression. It is also reported that endothelial dysfunction occurs in the initial stages of diabetes and later progresses to the development of atherosclerotic plaques. But not many studies have been initiated in this aspect to investigate whether circulating platelet proteins in diabetes can cause endothelial dysfunction. A proteomic approach to analyse these proteins found in blood of diabetic subjects in necessary to understand cardio-vascular disease progression in diabetes.

1.4 Hypothesis

The activated platelet proteins in diabetic conditions secrete their proteins into circulation. These differently expressed proteins in plasma of diabetic individuals may interact with the endothelial cells and can cause EC dysfunction. Analysis of plasma proteome of diabetic subjects and comparison to normal healthy subjects is the first step for identification of the differently expressed proteins. Effect of these proteins could be studied by tracking the shift in expression levels of endothelial cell in the presence of these proteins immobilized on the culture matrix.

In order to test the hypothesis the specific objectives were defined as shown below:

1.5 Objectives

- To isolate secreted platelet proteins from plasma diabetic and healthy control.
- To isolate whole platelet proteome from diabetic and healthy control.
- To compare the difference in plasma proteins and whole platelet proteome in diabetic and control subjects by SDS-PAGE and 2D gel electrophoresis.
- To analyse the differently expressed of platelet proteins in diabetic subjects by MALDI-Tof
- To study the effect of these proteins in causing endothelial dysfunction by PCR analysis of different markers:
    - Antithrombotic marker- tPA, eNOS
    - Prothrombotic marker- vWF

Chapter II

Materials and Methods

2.1 Materials

Thrombin, Fibrinogen, Gelatin, VEGF, IMDM (Gibco), FBS (Gibco), Sodium bicarbonate (Sigma), Antibiotics (Gibco), Glucose (Sigma), Trypsin EDTA (Gibco), Collagenase (Gibco), Potassium chloride (Merck), Potassium dihydrogen phosphate (Merck), Sodium chloride (Sigma), Disodium hydrogen phosphate (Merck), Acetylated DiI complex low density lipoprotein from human plasma (Molecular Probes), Ammonium chloride (Sigma), Triton X-100 (Sigma), Anti-vWF antibody- goat polyclonal IgG (Santacruz Biotechnology), Mouse anti-goat polyclonal IgG conjugated with Texas Red (Santacruz Biotechnology), Sodium hydroxide (Merck), Potassium sodium tartarate (Sigma), Copper sulphate (Merck), Folin's Phenol (SDFCL), Acrylamide (Sigma), Bisacrylamide (Sigma), Trizma base (Sigma), Hydrochloric acid (Merck), SDS (Sigma), Ammonium persulphate (Sigma), TEMED (Sigma), Glycine (Sigma), Bromophenol Blue (Sigma), Glycerol (Sigma), Beta-mercaptoethanol (Sigma), Coomassie Brilliant Blue- R250 (Sigma), Glacial Acetic Acid (SDFCL), Methanol (Merck), Sodium thiosulphate (Sigma), Silver Nitrate (CHEM LABS), Sodium carbonate (Merck), Formaldehyde (SDFCL), Citric acid (Merck), Sodium citrate (Merck), Dextrose (Sigma), Calcium chloride (Merck), Magnesium chloride (Merck), Sodium dihydrogen phosphate (Merck), Absolute ethanol (Merck), EZBlock Protease Inhibitor Cocktail, (BioVision), PMSF (Sigma), EDTA (Merck), 2-D Clean-up kit (Bio-Rad), 2-D Quantification kit (GE Healthcare), 2-D Rehydration Buffer (Bio-Rad), Equilibration Buffer I and Equilibration Buffer II (Bio-Rad), Urea (Sigma), DTT (Sigma), 3-10 pI 7cm IPG strips (GE Healthcare), 4-7 pI 7 cm IPG strips (Bio-Rad), Mineral oil (Bio-Rad), Overlay Agarose (Bio-Rad), Diethypyrocarbonate (Sigma), Trizol reagent (Invitrogen), Chloroform (SDFCL), Isopropanol

(Merck), Qubit RNA Assay Kit (Molecular Probes), Oligo $(dT)_{20}$ (Invitrogen), dNTPs (Invitrogen), 5X first strand buffer (Invitrogen), DTT 0.1M (Invitrogen), RNase OUT (Invitrogen), Superscript III RT (Invitrogen), 10X PCR Buffer (Invitrogen), 50mM $MgCl_2$ (Invitrogen), Taq polymerase Enzyme (Invitrogen), Primers for human eNOS gene (IDT), Primers for human tPA gene (IDT), Primers for human vWF gene (IDT), Primers for human GAPDH gene (IDT). qPCR Master Mix for SYBR assay (Eurogentec).

2.2 Study Subjects

Blood samples for the study were collected from Jyothidev Diabetes and Research Centre, Trivandrum after obtaining the Ethical Committee approval. 20ml blood was collected from each group in tubes with anticoagulant ACD.

Inclusion criteria for the study: Diabetic individuals of the age group 45-65 with fasting blood glucose level more than 120mg/dl and HbA1 c level more than 6%.

Exclusion criteria for the study: Individuals below the age of 45 and patients pertaining to the age group of the study if suffering from cardiovascular complication or on anti-platelet drugs were excluded.

2.3 Preparation of whole platelet proteome and platelet proteins in plasma

Protein preparations of whole platelets, platelet proteins in plasma were made in order to confirm the presence of released platelet proteins in plasma.

2.3.1 Ethanol precipitation of plasma

To isolate the platelet protein from circulating plasma, plasma was collected by centrifuging blood at 1000×g for 15 min following which 1mM PMSF and 1mM EDTA were added to the plasma. To these mixture different

concentrations of ethanol ranging from 10-50% was used to precipitate out the high molecular weight proteins. The plasma was allowed to precipitate in ice for 3 hours following which it was transferred to -20°C for overnight incubation. The suspension was then centrifuged at 12,000×g for 20 minutes at 4°C. Supernatant was stored and used for SDS PAGE and 2D gel electrophoresis. Ethanol concentration was optimized on the basis of presence of low molecular weight protein in supernatant. The supernatant was collected and run on 10% polyacrylamide gel and bands were observed by staining gel the with coomassie stain/silver stain.

2.3.2 Ethanol precipitation of activated control, control and diabetic plasma

Once optimized, 30% ethanol was used for the further studies. Platelets proteins were isolated from the plasma of control, diabetic, and thrombin activated platelets (positive control). 0.8 IU thrombin was used to activate the platelets in PRP. In brief, PRP was collected and activated with a final concentration of 0.8IU thrombin, incubated at 37°C for 30 minutes. Plasma was separated and precipitated with 30% ethanol. Supernatant was stored for SDS PAGE.

2.3.3 Preparation of washed platelets

Blood was collected from control and diabetic group was centrifuged at 750×g for 5 minutes using a table-top centrifuge (Heraeus Labofuge 300, UK). Platelet rich plasma (PRP) was aspirated using a Pasteur pipette into a fresh tube and 1% ACD was added to it. This was then centrifuged at 200×g for 10 minutes in order to pellet down any remaining RBCs and WBCs. The supernatant was then transferred to another fresh tube and was centrifuged at 1200×g for 10 minutes. The supernatant was discarded and the platelets which forms the pellet was washed thrice with ACD: Tyrode's Buffer in the ratio of 1:9, regaining the pellet after wash by centrifuging at 1200×g for 10 minutes.

2.3.4 Whole platelet proteome preparation

Washed platelets were resuspended in 200µL of lysis buffer (20mM Tris pH 7.5, 300mM NaCl, 4mM EDTA, 2% Triton X-100 containing EZBlock Protease Inhibitor Cocktail). This was vortexed briefly and incubated in ice for 45 minutes with intermittent vortexing every 10 minutes. Following incubation in ice the mixture was centrifuged at 10,000×g for 10 minutes. The supernatant was collected into a fresh eppendorf tube for SDS-PAGE. Alternatively the platelets after ACD-Tyrode's wash was resuspended in 400 µL 2D rehydration buffer and sonicated under cold conditions for isolating the whole cell proteome for 2D gel electrophoresis.

2.4 Protein Quantification

2.4.1 Lowry's method of protein quantification

The concentrations of protein present in the samples prepared as per the procedures described in section 2.3, 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 1mL of this solution was added to 100 µL of the sample and was incubated for ten minutes.100 µL of Folin's Reagent was added and incubated in dark for half an hour. The samples were diluted using 1X PBS prior to the assay. Absorbance at 600 nm was taken using UV-Visible Spectrophotometer (Hewlett Packard Diode array 8453, Germany).

2.4.2 Protein quantification using 2-D Quant Kit

The concentration of protein for 2-D gel electrophoresis was determined using 2D quant kit, (GE Health care), according to the manufactures protocol. In brief, proteins were precipitated and the samples were kept for centrifugation at 10,000×g for 5 minute which sediments the

protein. Hundred µl copper solution and 400µl deionised water were added to the protein and vortexed. The samples were incubated for 15 minutes in room temperature after adding the working colour reagent for colour development. The samples were read at 480nm in a spectrophotometer and concentration was calculated from the standard plot generated.

2.5 SDS-PAGE

For this study, 10% SDS poly acrylamide gels were used to separate the proteins. The stacking gel and resolving gel were prepared using the standard protocol and the samples with equal protein concentrations (20 - 40µg) were loaded on the gel. The proteins samples were run on 100V until it crossed the stacking and the voltage was increased to 120V upon reaching the resolving gel. Once the die front has run out the electrophoresis was stopped and the gel was carefully removed from the glass plates and the stacking gel was cut out. The gel was washed with distilled water and incubated in coomassie stain (10% acetic acid, 40% methanol, 50% distilled water and 0.1g CBB-R250) overnight with shaking. The gels were destained in destaining solution (10% acetic acid, 40% methanol and 50% distilled water). Alternatively, the gels were silver stained as described in section 2.7. Gels were documented using Alpha Imager Gel Documentation System 2000 and analyzed with Alpha Imager Analysis System.

2.6 2-Dimensional Gel Electrophoresis

2.6.1 Platelet proteome samples

To optimise the conditions for 2-D gel electrophoresis 3-10pl 7cm IPG strips were immobilised with platelet proteins (150µg) by passive rehydration. Different focusing conditions were tried in order to optimize the proper focusing of protein samples on strips. After completion of iso-electric focussing the second dimension was carried out in a 10% polyacrylamide gel. The IPG strips were equilibrated using equilibration buffer 1 and 2 for 10 minutes each. The strips were immersed in tris-glycine-SDS buffer for a few

seconds before loading on the polyacrylamide gel. Strips were overlaid with 0.5% agarose in TGS buffer and allowed to solidify. Electrophoresis of the samples was carried out at 100V until the die front had run out. The gels were silver stained and documented using Alpha Imager Gel Documentation System 2000 and analyzed with Image Master 2D Platinum image analysis soft ware version 7.0.

2.6.2 30% ethanol precipitated plasma samples

To optimise the focusing condition and sample concentration for 2-D gel electrophoresis of plasma 4-7 pI 7cm IPG strips were immobilised with varying plasma protein concentration ranging from (200-400µg) by passive rehydration and focused at different conditions. After completion of iso-electric focussing the second dimension was carried out in a 10% polyacrylamide gel. The IPG strips were equilibrated using equilibration buffer 1 and 2 for 10 minutes each. The strips were immersed in tris-glycine-SDS buffer for a few seconds before loading on the polyacrylamide gel. Strips were overlaid with 0.5% agarose in TGS buffer and allowed to solidify. Electrophoresis of the samples was carried out at 100V until the die front had run out. The gels were silver stained and documented using Alpha Imager Gel Documentation System 2000 and analyzed with Image Master 2D Platinum image analysis soft ware version 7.0.

2.7 Silver staining of polyacrylamide gels

2.7.1 Protocol using glutaraldehyde fixation

The polyacrylamide gel was incubated in a solution of distilled water: methanol: acetic acid at ratio of 4:5:1 containing 0.25% glycerol for 1 hour. After incubation, the gel was washed three times for 5 minutes with distilled water. Next the gel was exposed to 10% glutaraldehyde for 10 minutes and washed thoroughly with the distilled water several times until the odour of glutaraldehyde has gone. Gel was then sensitised using 0.1% ice cold DTT for 20 minutes and rinsed thoroughly with distilled water. The gel was then

incubated in 0.1% silver solution for 30 minutes. The remnant silver solution in the gel is washed off with distilled water after incubation. The gel is then developed using 3% sodium carbonate solution containing formaldehyde and once the optimum band intensity has been attained the reaction was stopped using 6% citric acid solution.

2.7.2 Protocol compatible with mass spectroscopy

The polyacrylamide gel was washed with distilled water following electrophoresis. The gel was incubated in the first fixing solution (50%methanol, 10% acetic acid) for 30 minutes with shaking. The gel was transferred to the second fixing solution (5% methanol, 1% acetic acid) immediately after the first one and incubated for 15 minutes with shaking. Following the second fixation step the gel was rinsed with the third fixing solution (50% methanol) for 1 minute. After all three fixation steps the gel was washed with distilled water 3 times for 10 minutes. The gel was left in distilled water overnight with shaking after 3 washes. After overnight washing the gel was sensitized using the sensitizing solution (0.02% sodium thiosulphate) for 90 seconds with shaking. The gel was then rinsed with distilled water 3 times for 30 seconds. Next the gel was incubated in chilled 0.2% silver nitrate solution overnight. After overnight incubation the silver solution was removed and the gel was washed with distilled water 3 times for 1 minute. The gel was developed using the developer solution (6% sodium carbonate containing sodium thiosulphate and formaldehyde) until bands had developed to the required intensity. The reaction was stopped using the stopper (6% acetic acid) and the gel washed with distilled water and analysed.

2.8 Sample preparation for MALDI-Tof analysis

Two proteins spots which were differently expressed (one control, one diabetic) in whole platelet proteome was picked at random and processed for MALDI-Tof analysis. In brief, gel pieces were excised, minced and destained. It was washed thrice with wash solution (50mM ammonium bicarbonate and

50% acetonitrile) until the gel has turned transparent. The gel pieces were dehydrated in 100% acetonitrile and rehydrated using protease solution (20µg/mL trypsin in 50mM ammonium bicarbonate) and digested overnight at 37°C. The sample was subjected to centrifugation at 12,000×g for 30 seconds and the supernatant was collected. The pellet was resuspended with extraction solution (60% acetonitrile and 0.1% TFA) remanant peptides were extracted from the pellet by centrifuging at 12,000×g for 30 seconds. The supernatants were pooled together and lyophilised. The lyophilised samples were submitted to RGCB for MALDI-Tof analysis.

2.9 HUVEC Isolation and culture

2.9.1 Coating of cell culture dishes with fibrin matrix

The growth, development and signalling of cultured cells strongly depends on the surface upon which the cells are seeded. Extra cellular matrix is essential for the survival of ECs in culture. So in order to expand ECs in culture it is essential to provide these cell an appropriate matrix which supports its growth. For this purpose the cell culture dishes were coated with a fibrin composite. In brief, the dishes were incubated with 5 IU/mL thrombin for 30 minutes at 37°C in CO_2 incubator. The solution was then discarded and the dishes were coated with a fibrin composite of 10mg/mL fibrinogen, 0.2% gelatin and 50µg/mL VEGF. The clot was allowed to form for 30 minutes by incubating the dishes at 37°C in CO_2 incubator. Following this the dishes were lyophilised and used for cell culture.

2.9.2 Isolation of HUVEC from umbilical cord

Umbilical cord was collected from local maternity hospital in Trivandrum (after informed consent), in HBSS substituted with 10mg/ml glucose and antibiotics (1000U/mL benzyl penicillin and 1000µg/ml streptomycin). Human umbilical vein endothelial cells (HUVEC) were isolated and grown as per the method of Prasad et al., 2005. In brief, the cord was thoroughly clean and fresh cuts were made on both ends of the cord. The

lumen of the vein was washed with HBSS and was then filled with 0.5% collagenase and IMDM (serum free) in the ratio of 1:1. The cord was placed in a beaker filled with HBSS soaked cotton and incubated at 37°C in 5% CO_2 incubator for 12 minutes. After incubation the cells were flushed out of the vein by passing serum free IMDM simultaneously dislodging the cells by gentle massaging on the surface of the lumen. The cell suspension was collected and centrifuged at 400×g for 5 minutes. The pellet was resuspended in IMDM medium containing 20% FBS and growth factors and seeded on to fibrin growth factor composite coated tissue culture dish (as described in section 2.8.1) and was incubated at 37°C in 5% CO_2 incubator. Medium change was given after 24 hour. Cells were sub-cultured and cells from 2-4 passages were used for the study.

2.9.3 Trypsinization

Trypsinization was done once the cells had attained 80% confluency. For trypsinization the medium was removed and cells were washed with serum free medium. 0.25% Trypsin EDTA was added to the culture flask and the cell were incubated for 2 minutes at 37°C in 5% CO_2 incubator. The trypsin activity was arrested after incubation by adding serum containing media and the cells were collected in to a sterile centrifuge tube. After centrifugation at 400×g for 5 minute, the cells were resuspended in complete medium for HUVEC and seeded onto fibrin growth factor composite coated tissue culture dish.

2.10 Characterisation of HUVEC

2.10.1 Acetylated LDL uptake assay

Ac-LDL is taken up by macrophages and endothelial cells via the scavenger cell pathway of LDL metabolism and this feature can be used to identify endothelial cells based on their increased metabolism of AcLDL. DiI-Ac-LDL, Acetylated Low Density Lipoprotein (labelled with 1,1'-dioctadecyl – 3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) labels both vascular

endothelial cells and macrophages When cells are labelled with DiI-Ac-LDL, the lipoprotein is degraded by lysosomal enzymes and the DiI (fluorescent probe) accumulates in the intracellular membranes. Cells from 2-5th passage were used for the study. In brief cells at 80% confluency were incubated with 10µg/ml of DiI conjugated acetylated low density lipoprotein from human plasma (DiIAcLDL, Molecular Probes) for 4 hours in 5% CO2 incubator. After incubation, the medium was removed and cells were washed with serum free IMDM medium. Images were taken using N2 filter in fluorescence microscope (Lieca DM IRB).

2.10.2 Immunostaining for vWF

vWF is a multimeric plasma glycoprotein produced uniquely by endothelial cells and megakaryocytes and this is constitutively secreted by endothelial cells and stored in the Weibel-Palade bodies of endothelial cells. vWF is widely used as specific endothelial cell marker. The cellular expression of vWF was analysed by seeding 10,000 cells/ well onto a four-well plate. Upon reaching 80% confluency the cells were fixed using 3.7% formaldehyde in PBS for 30 minutes. The cells were rinsed three times with PBS after 30 minutes incubation. Next the cells were quenched by incubating in 0.27% ammonium chloride /0.38% glycine in PBS for 20 minutes. The cells were washed with PBS after incubation. The cells were then permeabilised with 0.5% Triton X-100 for 5 minutes after which it is washed with PBS. The cells were incubated with primary antibody (vWF goat polyclonal IgG, Santacruz Biotechnology) in the dilution of 1:100 in 0.5%BSA in PBS for 1 hour. Following incubation the cells were washed to remove any unbound antibody. The cells were then incubated with secondary antibody (Mouse anti goat IgG polyclonal conjugated with Texas Red, Santacruz Biotechnology) in the dilution of 1:200 in 0.5%BSA in PBS for 1 hour. After incubation the cells were again washed with PBS and images were taken using green filter in florescent microscope (Lieca DM IRB).

2.11 Effect of circulating platelet proteins in HUVEC

2.11.1 Coating of platelet proteins in tissue culture dishes

In order to study the effect of circulating platelet proteins in plasma on endothelial cells, plasma precipitated with 30% ethanol was used. As previous studies have indicated that 100µg is the effective concentration, this concentration was fixed for the experiment. To analyse the effect of slow release of this protein, the cell culture dishes were coated with fibrin composite as described in section 2.8.1 with 100µg 30% ethanol precipitated control and diabetic plasma per dish. The dishes were lyophilised for 3 hours and stored at 4°C.

2.11.2 Apoptosis Assay

To study the effect of circulating platelet proteins in ECs viability HUVEC was grown on 100µg control plasma and diabetic plasma coated dishes (section 2.10.1). Fibrin growth factor composite dish served as the control for the experiment. Apoptosis assay was carried out using Vybrant Apoptosis Assay Kit from Molecular Probes. In brief, 20,000cells/cm^2 was seeded on control plasma and diabetic plasma coated dishes and after 2 hours of seeding the unattached cell were removed. Cell were allowed to grow for 48 hours in the dishes after which it was trypsinised and processed for apoptosis assay as per manufacturer's protocol. Briefly, cells were washed with ice cold PBS twice and incubated with annexin and PI for 15 minutes.

After the incubation flow cytometry analysis of the samples was carried out using BD FACS ARIA and BD FACS DIVA software. For flow cytometry studies 20,000 event were acquired. The compensation was done using single stained annexin and PI cells. Unstained cells were used to gate the population. Gating of the population was done based on the mean fluorescence intensity and the apoptotic cells were gated to quadrant 4. Experiment was done in triplicate and the percentage of dead cells was

calculated in control plasma group and diabetic plasma group as compared with the reference.

2.11.3 RNA Isolation and cDNA synthesis

HUVEC was seeded onto control and diabetic plasma coated and normal fibrin composite coated cell culture dishes. RNA was isolated from the three groups using TRIzol reagent (Invitrogen, USA) as per manufacturers protocol. In brief, Trizol reagent was added to the cells and incubated for 5 minutes in room temperature. The solution was retrieved and chloroform was added to this mixture and was mixed by inversion. This was then vigorously mixed for 15 seconds. The sample was incubated at room temperature for 2-3 minutes and centrifuged at 12,000×g for 15 minutes at 4°C. 100% isopropanol was added to the aqueous phase and incubated at room temperature for 10 minutes. This was then centrifuged at 12,000×g for 10 minutes at 4°C and the RNA pellet was washed with 75% ethanol and centrifuged at 7500×g for 5 minutes at 4°C. RNA was air dried for 5 -7 minutes and resuspended in DEPC treated water. The isolated RNA was quantified using Qubit Flurometer-2. 1µg of RNA was taken for cDNA synthesis using Thermal Cycler (Master Cycler, Eppendorf, USA). The reaction volume of 20µL contained Oligo $(dT)_{20}$, 1µg of RNA, dNTPs 0.1 M DTT, RNase OUT and Superscript III RT. The synthesised cDNA was used for gene expression studies.

2.11.4 Semi-quantitative PCR

The cDNA synthesised were taken for expression studies for the genes vWF, eNOS and tPA. GAPDH was selected as the house-keeping gene for the study. The primer sequences were taken from literature. The sequence of the primers used, its amplicon size and annealing temperatures are given in table 1.The PCR mixture for each assay contained 10X PCR buffer- 2.5µl, 50mM $MgCl_2$- 0.75 µl, 10mM dNTP- 0.5 µl, forward primer (FP)- 0.5 µl, reverse primer (RP)- 0.5 µl, Taq polymerase- 0.2 µl and cDNA- 2 µl in a final volume of 25 µl. Duplicates of PCR reaction from each group (reference HUVEC, control

plasma group and diabetic plasma group) was carried out. Electrophoresis of the PCR products were carried out in 1% agarose and the gels were documented using Alpha Imager Gel Documentation System 2000. The relative band intensity difference between the samples were analysed for the genes using Alpha Imager Analysis System.

2.11.5 Real- Time PCR

Real-time qPCR analysis was carried out to compare the mRNA expression difference of eNOS, tPA and vWF in ECs after exposure to control and diabetic plasma for 72 hours in culture. The experiment was carried out using a Chromo4 system (MJ Research, USA). All reactions were carried out in a total volume of 25μL containing 12.5μL qPCR master mix (Eurogentec), 200nM forward primer, 200nM reverse primer and 2μL template cDNA. For each gene, quality and specificity was assessed by examining PCR-melt curves following the reactions. The cDNA copy numbers of the target gene were analysed after normalising to the copy numbers of GAPDH. The experiment was carried out in duplicates. Difference in mRNA expression of diabetic plasma coated dishes was compared to that of control plasma coated dishes.

2.12 Statistical analysis

Statistical analysis was done by Student's t- test using Graph Pad online software for all the quantitative parameters to identify significance of difference between the control and test groups. All data are presented as Mean. ±SD. P value of <0.05 was considered as statistically significant.

GAPDH - 210bp Amplicon size, Annealing temperature- 58ºC
FP- 5'GCTTGTCATCAATGGAAATCCC3' RP- 5'TCCACACCCATGACGAACATG3'
eNOS- 486bp Amplicon size, Annealing temperature- 58ºC
FP- 5'AGCTGTGCTGGCATACAGGA3' RP- 5'ATGGTAACATCGCCGCAGAC3'
tPA- 319bp Amplicon size, Annealing temperature- 53ºC
FP- 5'ATGGGAAGACATGAATGCAC3' RP- 5'GAAAGGGGAAGGAGACTTGA3'
vWF- 310bp Amplicon size, Annealing temperature- 55ºC
FP- 5'CACCATTCAGCTAAGAGGAGG3' RP- 5'GCCCTGGCAGTAGTGGATA3'

Table 1: Details of primers used for PCR analysis

Chapter III

Results and Discussion:

Isolation and Characterization of Circulating Platelet Proteins

3.1 Screening of subjects

The study was aimed to see the difference between the platelet proteome of diabetic individuals from the healthy controls and then to see the effect of these proteins on endothelial cells. Samples were collected from Jyothidev Diabetes and Research Centre after ethics committee approval and informed consent. Blood was collected and brought to the lab within 30min of collection to prevent platelet activation.

Samples were screened according to the recommendations of International Diabetic Association (IDA). According to IDA, an individual is diabetic if fasting glucose level is higher than 120mg/dl and glycosylated haemoglobin (HbA1 c) level is greater than 6%. HbA1 c gives the average level of glucose over a period of 3 months. If an individual was having persistently high levels of glucose in the blood it will be reflected in the HbA1 c concentrations [Kim *et al.,* 2011].

Six samples from each group (diabetic and healthy control group) were collected. Inclusion criteria for the study were HbA1c level and fasting glucose level as per IDA (table 2). The two groups for the study showed significant difference with respect both aspects (figure 1). Subjects were only on diet control. Patients on anti-platelet drugs were excluded from the study, as these drugs may alter the platelet activity and thus the protein expression. Age matched, healthy subjects were taken as control.

	Control	Diabetic
HbA1 c Percentage	5.36 ± 0.17	8.5 ± 2.67*
Fasting Blood Glucose	88 ± 3.39	186.6 ± 59.18*

Table 2: HbA1 c percentage and fasting blood glucose in diabetic and control group. Values are given as Mean ± SD (n=5). P<0.05

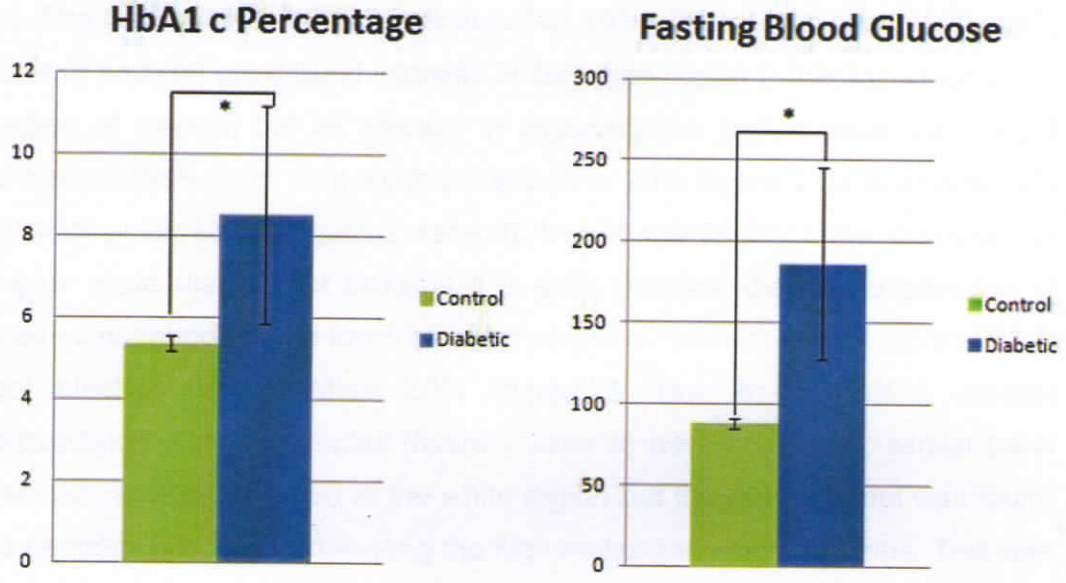

Figure 1: Comparison of HbA1 c percentage and FBS between diabetic and control group. Values are given as Mean ± SD (n=5). P<0.05

3.2 Standardisation of ethanol precipitation of plasma

To optimize the isolation of the circulating platelet proteins from plasma and to deplete the high abundant plasma protein, plasma samples from both the groups were precipitated with different concentration of ethanol ranging from 10-30% for different time intervals 1-3 hours. Supernatant was run on 10% SDS-PAGE. Platelets proteins are low molecular weight proteins and present in plasma in low abundance.

It was observed that in all the three concentrations of ethanol (10-30%) incubation period had little or no effect on depletion of plasma. Band

intensities did not change significantly on analysing the three time intervals of the same concentration of ethanol (Figure 2: Lane 1, Lane 2 and Lane 3). Specific regions in the protein lane were selected to compare the intensity difference between 10%, 20% and 30% ethanol precipitation. Region marked red indicates proteins between 250-100kDa, yellow region indicated proteins 100-48kDa, blue region has proteins between 48-25kDa, and region marked white represents proteins with molecular weight below 25kDa (Figure 2: lane 1). The data given in table 3 indicates that 10% ethanol precipitation (figure 2: lane 3) showed good band intensity in the white region (<25kDa) which is the region of interest but its efficacy in depleting the higher molecular weight proteins where much less when compared to 20% (figure 2: lane 6) and 30% ethanol precipitation (figure 2: lane 9). Due to the thicker band intensities at higher molecular weight proteins it is quite possible that the expression of these low abundant and low molecular weight proteins can get masked in 2-D gel electrophoresis. When 20% (figure 2: lane 6) and 30% ethanol precipitated plasma samples (figure 2: lane 9) were compared, similar band intensities were observed in the white region but the 30% ethanol was found to be more effective in depleting the high molecular weight proteins. This was evident from the difference in the band intensity in the yellow region which showed considerable decrease for 30% ethanol precipitation.

Although 30% ethanol precipitation of plasma yielded better depletion than 10% and 20% ethanol the proteins in the higher molecular weight range were not effectively depleted. In order to improve the depletion efficiency ethanol precipitation of plasma was carried out with 40% and 50% ethanol. From table 4, it can be noted that bands in the higher molecular weight range between 250-75kDa were effectively removed by 40% (figure 3: lane 3) and 50% ethanol (lane 4) as compared to crude plasma (lane1).

The depletion of higher abundant proteins was found to be increasing with the increase in ethanol concentration ranging from 30-50%. But higher concentration of ethanol also resulted in the precipitation of low molecular

weight proteins (<25kDa). Thus 30% ethanol was found to be optimum concentration for isolating low molecular weight platelet proteins from plasma.

To confirm that the loss of lower abundant proteins were minimal with 30% ethanol precipitation, SDS-PAGE analysis was carried out to compare the proteins between the supernatant and pellet of ethanol precipitation (figure 4). From table 5 it can be noted that the band intensity of pellet proteins below 25kDa is increasing as the concentration of ethanol increases from 30% to 50% (Figure 4: lane 1, lane 2 and lane 3). This results correlate with the proteins in supernatant. It can be observed that band intensity in the same region is decreasing with increasing concentrations of ethanol (Figure 4: lane 4, lane 5 and lane 6). Although the higher molecular weight protein depletion in 30% is not as effective as 40% or 50% but there is minimal loss of proteins from the area of interest. Hence 30% ethanol precipitated samples were used for further studies.

The proteome of plasma is wide and contains proteins from different cellular origin. These proteins are rich source for the biomarker research, as they are in circulation and easy to isolate. Platelets proteins play a critical role in diabetes and associated diseases. Platelet proteome changes in pathological conditions and may get reflected in circulating plasma. In this study we attempted to isolate circulating platelet proteins from the plasma by ethanol precipitation method and confirmed that these proteins are from platelet origin by comparing precipitated samples with whole platelet proteome from the same donor. It was clear from the gel that proteins isolated after 30% ethanol precipitation is from platelet origin (figure 5).

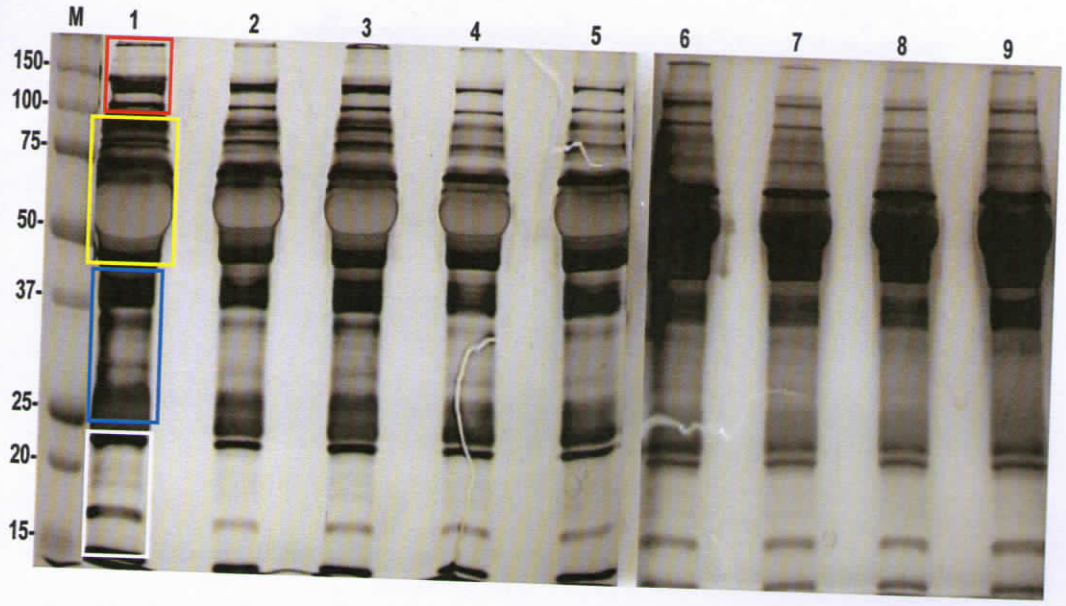

Figure 2: Ethanol precipitation of plasma with 10%, 20% and 30% ethanol. Loading order from LHS: Marker, 1: 10% ethanol-1 hour incubation, 2: 10% ethanol-2 hour incubation, 3: 10% ethanol-3 hour incubation, 4: 20% ethanol-1 hour incubation, 5: 20% ethanol-2 hour incubation, 6: 20% ethanol-3 hour incubation, 7: 30% ethanol-1 hour incubation, 8: 30% ethanol-2 hour incubation and 9: 30% ethanol-3 hour incubation (Molecular weights expressed in kDa)

Region	Percentage intensity of bands		
	10%- 3 hours	20%- 3 hour	30%- 3 hour
250-100 kDa	38%	36.2%	25.8%
100-48 kDa	38.7%	33.5%	27.8%
48-25 kDa	35.4%	32.9%	31.7%
<25 kDa	37.5%	34.9%	27.6%

Table 3: Band intensity difference between 10%, 20% and 30% ethanol precipitated plasma samples.

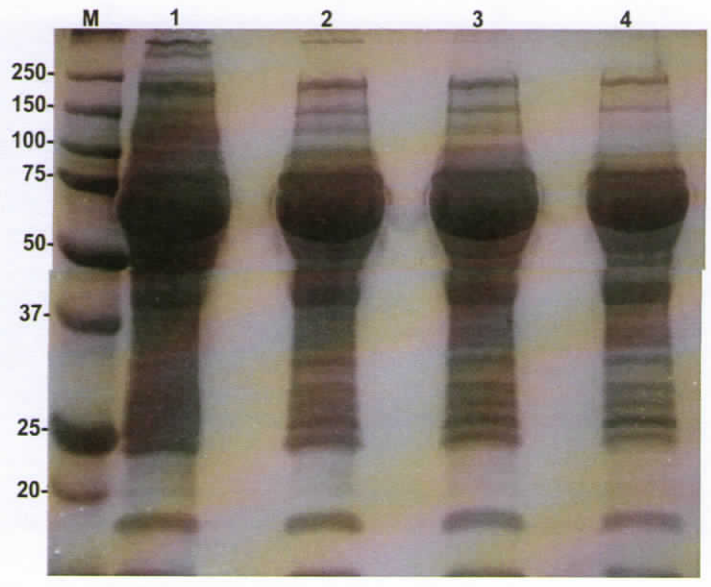

Figure 3: Ethanol precipitation of plasma with 30%, 40% and 50% ethanol concentrations. Loading order from RHS: Marker, 1: crude plasma, 2: 30% ethanol precipitated plasma, 3: 40% ethanol precipitated plasma, 4: 50% ethanol precipitated plasma. (Molecular weights expressed in kDa)

Region	Percentage intensity of bands			
	Crude	30%	40%	50%
250-100 kDa	33.8%	25.7%	24.2%	16.3%
100-48 kDa	36.3%	26.9%	21.7%	21.7%
48-25 kDa	35.7%	23.4%	21.0%	19.9%
<25 kDa	38.2%	25.8%	19.2%	16.8%

Table 4: Band intensity difference between 30%, 40% and 50% ethanol precipitated plasma

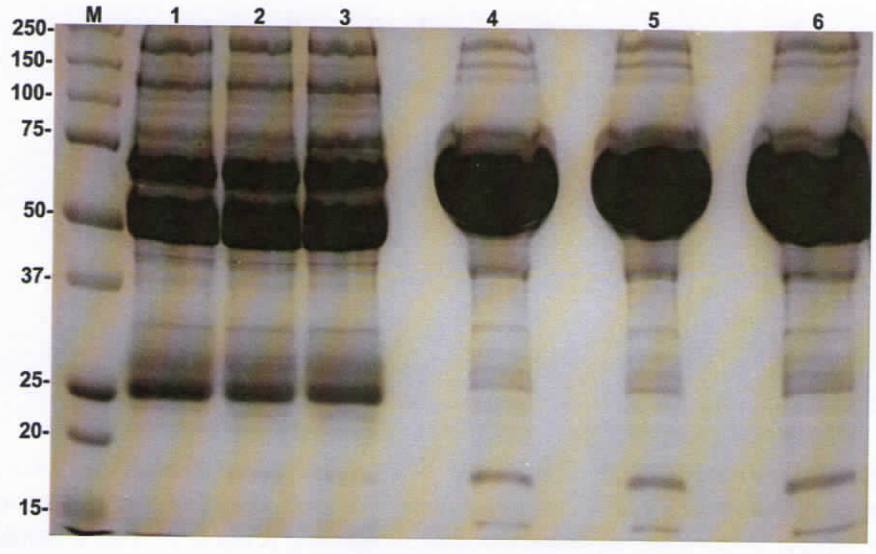

Figure 4: Comparison of ethanol precipitated plasma supernatant and pellet. Loading order: Marker, 1: 30% ethanol precipitated plasma pellet, 2: 40% ethanol precipitated plasma pellet, 3: 50% ethanol precipitated plasma pellet, 4: 30% ethanol precipitated plasma supernatant, 5: 40% ethanol precipitated plasma supernatant, 6: 50% ethanol precipitated plasma supernatant (Molecular weight expressed in kDa)

Region	Percentage intensity of bands					
	Pellet			Supernatant		
	30%	40%	50%	30%	40%	50%
250-100 kDa	31.7%	33.8%	34.5%	35.4%	33.7%	30.9%
100-48 kDa	31.2%	34.2%	34.6%	39.6%	36.7%	23.7%
48-25 kDa	29.1%	34.7%	36.2%	36.4%	33.4%	30.2%
<25 kDa	32.4%	34.2%	33.4%	34.5%	33.4%	32.1%

Table 5: Band intensity difference between 30%, 40% and 50% ethanol precipitated plasma supernatant and pellet

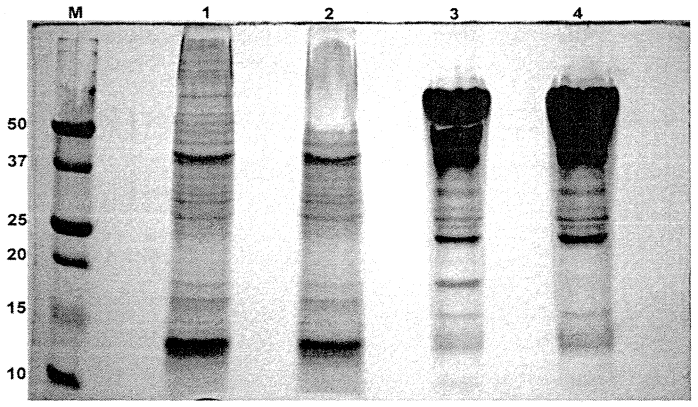

Figure 5: Whole platelet lysate and 30% ethanol precipitated samples from the same donor. Loading order: Marker, 1: Control platelet lysate, 2: diabetic platelet lysate, 3: control plasma, 4: diabetic plasma (Molecular weight expressed in kDa)

3.3 Ethanol precipitation of activated control plasma, control plasma and diabetic plasma

To study the difference in plasma proteome of control and diabetic subjects SDS-PAGE of 30% ethanol precipitated plasma from both the groups was carried out. 0.8IU thrombin activated platelets were used as a positive control. Platelets were activated in PRP and plasma was collected and precipitated with 30% ethanol to see the protein profile of activated platelets. This was compared with diabetic samples to ensure the activation of platelets in the circulation of diabetic subjects. For analysis of the gel (figure 6) eight distinctive bands (shown in lane 1) below 37kDa were picked to compare the intensity difference between control and diabetic groups. Molecular weights and percentage intensity difference between the four samples are provided in table 6. Upon analysing each bands selected it can be observed that there is increase in the intensity of the bands in diabetic when compared to control plasma. It is also interesting to note that there is difference in the protein content between activated control plasma and control plasma (figure 6: lane 3 and lane 4). This difference can be attributed to the activation of platelets with thrombin resulting in release of granule proteins by platelets into plasma

thereby increasing the protein content in activated control plasma sample when compared to control plasma. These results indicate that in diabetic subjects, platelets remains activated in circulation and release its protein content in circulating plasma.

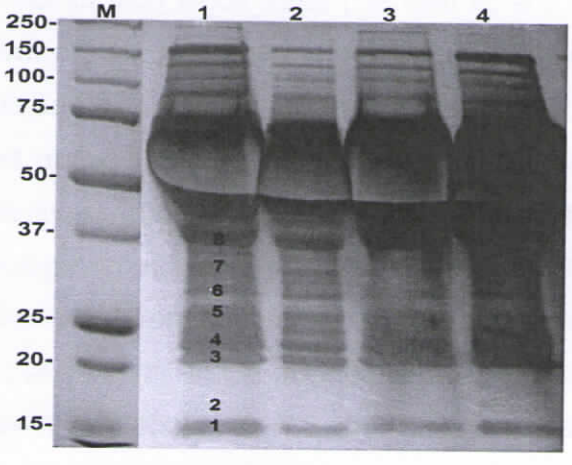

Figure 6: Comparison of 30% ethanol precipitated plasma samples of control, activated control and diabetic samples. Loading order: Marker, 1: control plasma, 2: activated control plasma, 3: diabetic plasma 1, 4: diabetic plasma 2 (Molecular weights expressed in kDa).

Band No.	Molecular weight	Percentage intensity of bands			
		Control plasma	Activated control plasma	Diabetic plasma 1	Diabetic plasma 2
1	13.07 kDa	17.6	26.3	29.8	26.3
2	14.74 kDa	19.38	23.1	28.02	29.5
3	20.15 kDa	15.0	21.5	31.2	32.3
4	21.83 kDa	14.37	22.9	32.03	30.7
5	29.60 kDa	15.64	23.52	29.52	31.32
6	34.47 kDa	15.54	24.12	29.92	30.42
7	36.35 kDa	15.41	23.33	31.03	30.23
8	37.68 kDa	12.21	19.33	33.93	34.53

Table 6: Band intensity difference of plasma proteins between control plasma, diabetic plasma and activated control plasma.

3.4 2-Dimensional gel electrophoresis

Two Dimension gel electrophoresis is a powerful and widely used method for the analysis of complex mixtures of proteins extracted from cells, tissues and other biological samples such as blood. This is being used for the analysis of platelet protein since 1986 [Hanash *et al.*, 1989] 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.In a study conducted about 300 proteins were identified from platelet releasates. 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. In the present study whole platelet proteome and circulating platelet proteome of the control and diabetic subjects was compared to identify the differently expressed proteins.

3.4.1 Whole platelet proteome from control and diabetic subjects

Platelet proteome has been investigated using 2-D gel electrophoresis for more than two decades [Frobel *et al.*, 2013; Tucker *et al.*, 2009; Senis and Garcia 2012]. Analysis of platelet biochemistry is largely dependent on protein analysis as platelets are anucleated cells providing no scope for PCR based studies. To analyze the whole platelet proteome different focusing conditions were tried and optimized (Step 1- 250V- Rapid- 15 minutes, Step 2- 4000V- Gradual- 1 hour, Step 3- 6000V- Rapid- 20000 VHr^{-1}, Step 4- 500V- Hold) for a 7 cm (3-10 pI IPG strip). Silver staining protocol was also modified in order to get a better resolved gel.

Washed platelets from both study groups were sonicated and 150 µg proteins in 2-D sample buffer was immobilised on IPG strips by passive rehydration. After focusing the IPG strip for 20000VHr^{-1} second dimension was carried out in 10% polyacrylamide gel (figure 7). Samples were run in triplicates. Representative gels are given in figure 8. Gels were analyzed by using Image Master 2D Platinum gel analysis software version 7.0. Images

were analyzed for spot intensity, overlapping and difference in spot match, total number of proteins spot in each gel. Results are shown in figure 9. It is clearly demonstrated by gel analysis that there are significant differences between the two groups.

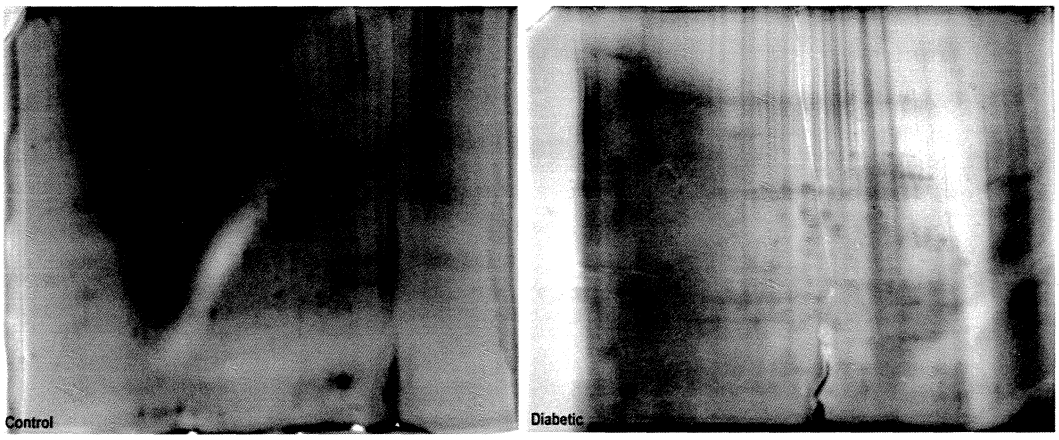

Figure 7: 2-D gel electrophoresis of platelet proteome (1). Focussing conditions: Step 1- 250V- Rapid- 15 minutes, Step 2- 4000V- Gradual- 1 hour, Step 3- 4000V- Rapid- 15,000 VHr^{-1}, Step 4- 500V- Hold. Right panel shows diabetic platelet proteome and left panel shows control platelet proteome.

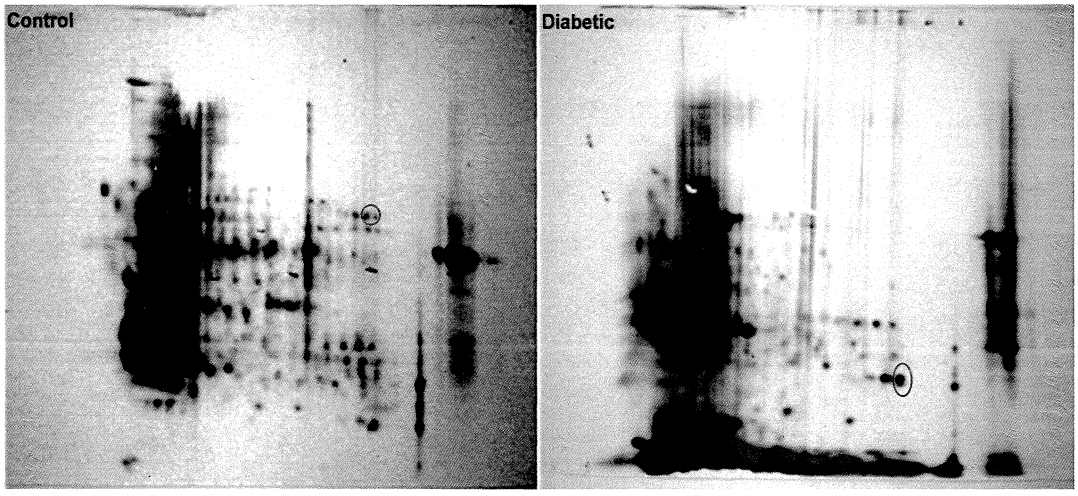

Figure 8: 2-D gel electrophoresis of platelet proteome (2). Focussing conditions: Step 1- 250V- Rapid- 15 minutes, Step 2- 4000V- Gradual- 1 hour, Step 3- 6000V- Rapid- 20,000 VHr^{-1}, Step 4- 500V- Hold. The right panel shows diabetic platelet proteome and left panel shows control platelet proteome. Red circles in the images indicate the gel spots picked for MALDI-Tof analysis

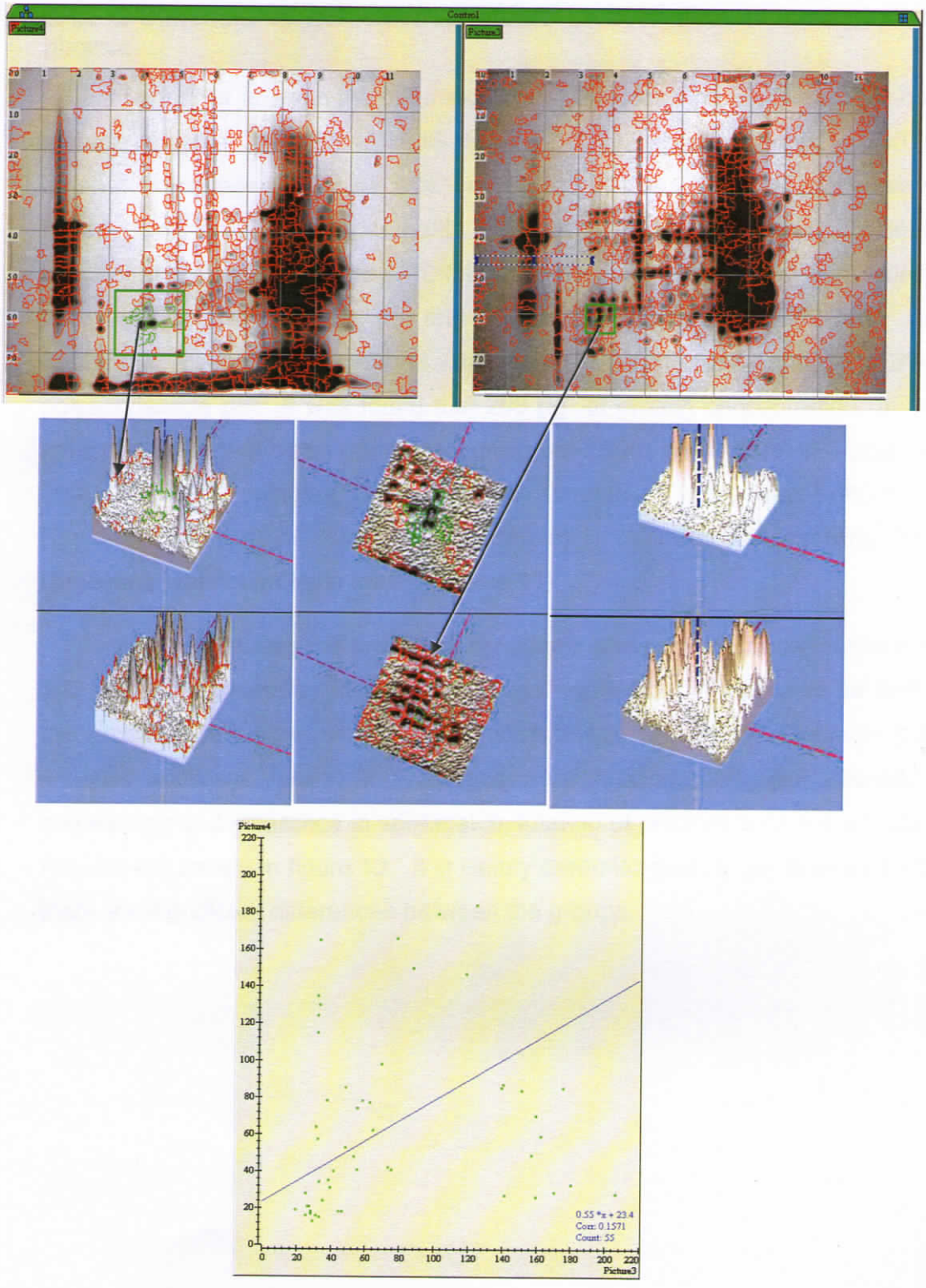

Figure 9: Comparative analysis of 2-D gels of platelet proteome. 3-D analysis of the gels shows difference in protein profile. 1206 spots were identified in both gels out of which 55 spots were matched and analysed. Intensity based analysis showed 22% matches. Other spots were expressed but at different intensity.

3.4.2 2-Dimensional gel electrophoresis of 30% ethanol precipitated plasma

To further analyse the differences seen in protein profile of control and diabetic plasma as seen in SDS-PAGE, 2-D gel electrophoresis of 30% ethanol precipitated samples was carried out. 4-7pI IPG strips were used based on previous experiments. Focusing conditions and protein concentration was optimized. Different protein concentration was tried (ranging from 200 µg to 400 µg) and strips were focused from 20000VHr^{-1} to 40000VHr^{-1}(figure 10). Second dimension was carried out in 10% polyacrylamide gel. It was found that 200 µg of protein concentration (after ionic and detergent clean up) and focusing conditions till 40000VHr^{-1} (Step 1- 250V- Rapid- 15 minutes, Step 2- 4000V- Gradual- 1 hour, Step 3- 6000V- Rapid- 40,000 volt hr, Step 4- 500V- Hold) is optimum for resolving the circulating platelet proteins on IPG (figure 11).

After optimising the focussing conditions and protein concentration for 2-D gel electrophoresis, the experiment was carried out in triplicates for both the groups. Gels were analyzed by using Image Master 2D Platinum gel analysis software version 7.0. Images were analyzed for spot intensity, overlapping and difference in spot match, total no of proteins spot in each gel. Results are shown in figure 12. It is clearly demonstrated by gel analysis that there are significant differences between the groups.

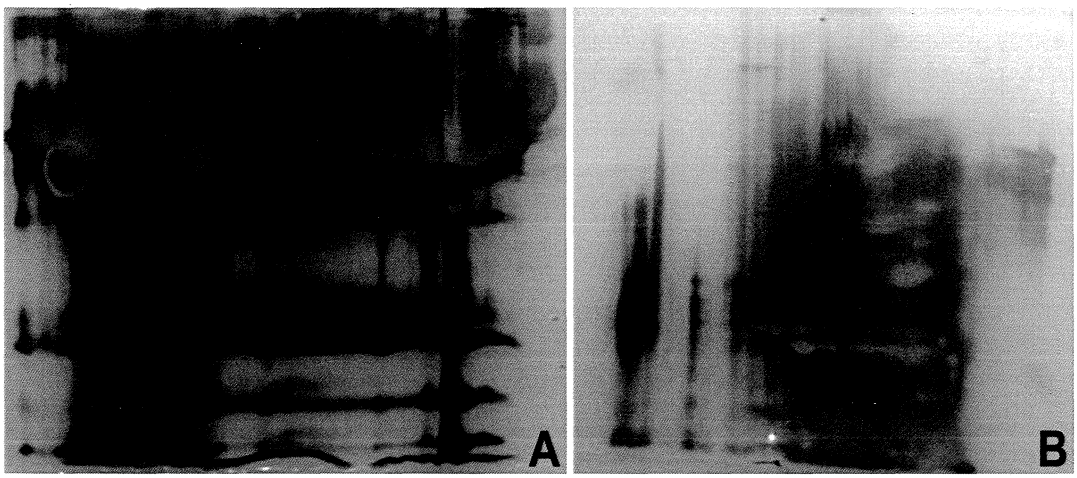

Figure 10: 2-D gel electrophoresis of 30% ethanol precipitated control plasma. Panel A- concentration of protein loaded is 400µg. Panel B- concentration of protein loaded is 300µg.Focussing conditions for both samples: Step 1- 250V- Rapid- 15 minutes, Step 2- 4000V- Gradual- 1 hour, Step 3- 6000V- Rapid- 20,000 VHr^{-1}, Step 4- 500V- Hold.

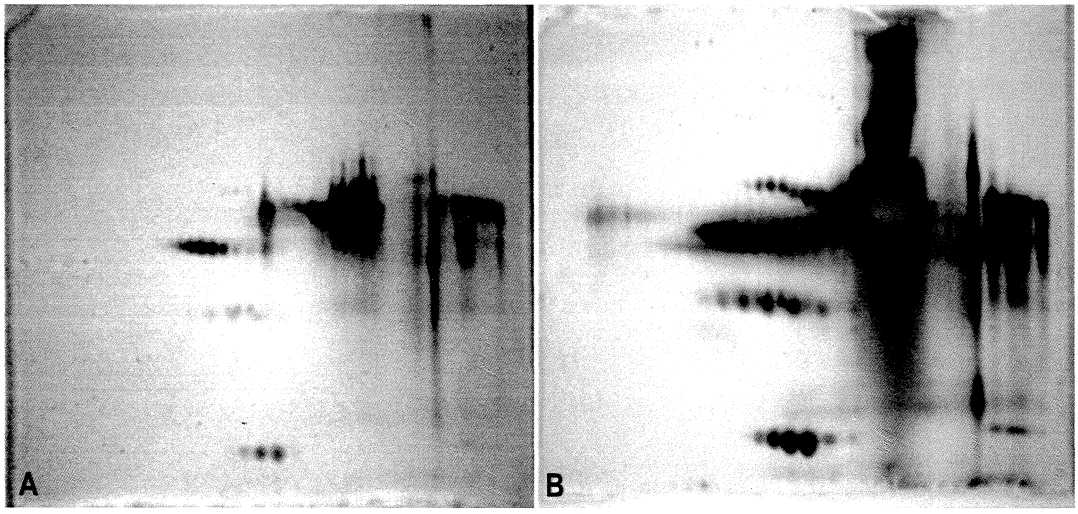

Figure 11: 2-D gel electrophoresis of plasma protein with 200µg after protein clean-up. A- Control plasma sample, B- Diabetic plasma sample. Focussing conditions for both gels: Step 1- 250V- Rapid- 15 minutes, Step 2- 4000V- Gradual- 1 hour, Step 3- 6000V- Rapid- 40,000 VHr^{-1}, Step 4- 500V- Hold.

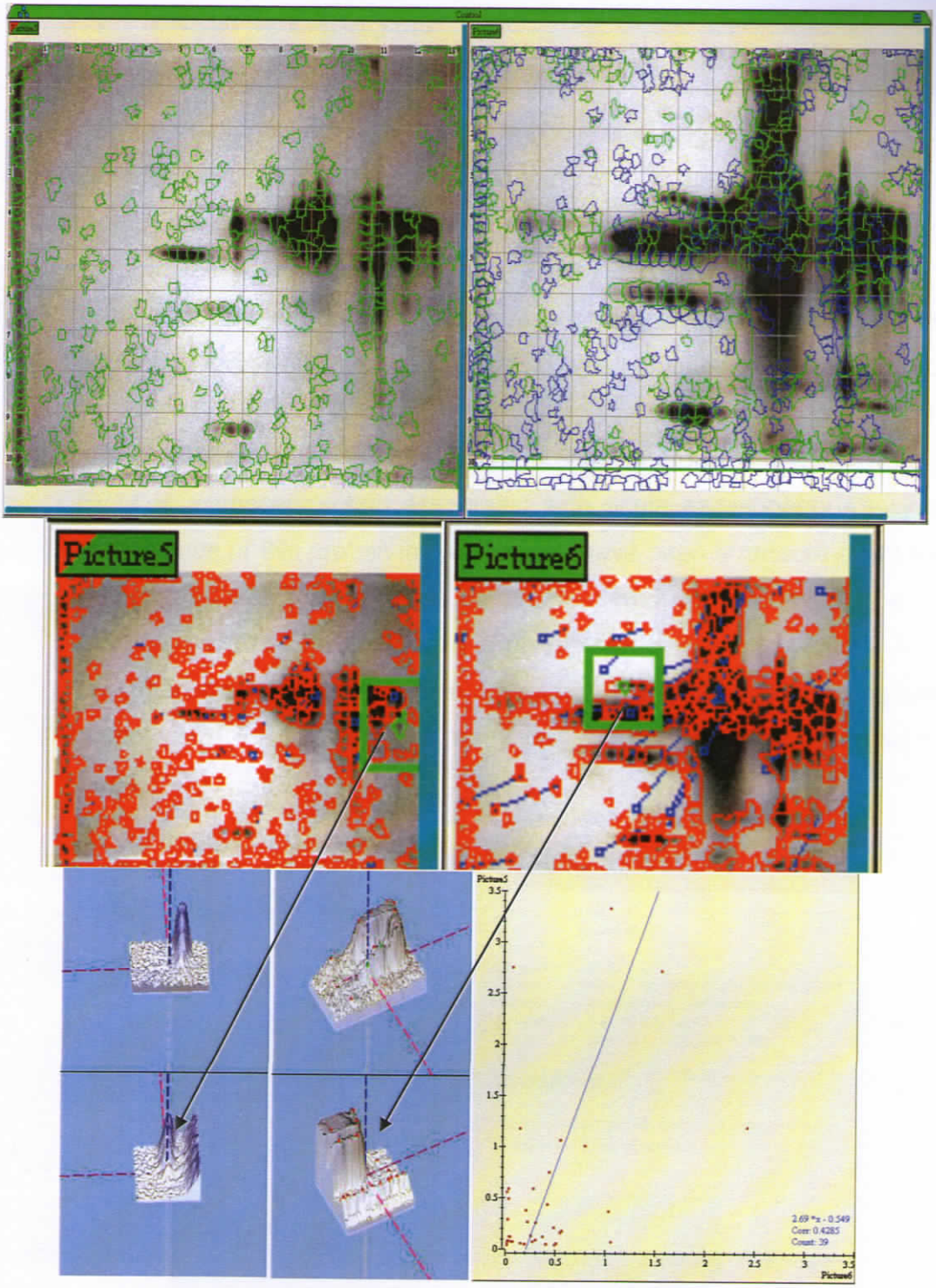

Figure 12: Comparative analysis of 2-D gels of 30% ethanol precipitated plasma from control and diabetic group. 3-D analysis of the gels shows difference in protein profile between control and diabetic groups. 686 spots were identified in both gels out of which 55 spots were matched. Spots indicated in green are matched spots and spots indicated in blue are unmatched spots.

3.5 MALDI-Tof Analysis

MALDI-Tof mass spectroscopy analysis was carried out for two gel spots randomly picked from control and diabetic platelet proteome 2-D gels (figure 8). Both spots were picked under the criteria that it was absent in other group. The protein samples were analysed using MALDI/TOF/TOF mass spectrometer (UltrafleXtreme, Bruker Daltonics). The data obtained was matched with protein database using Mascot search engine (Matrix Science). The protein spot from the control sample was identified as a globulin protein and did not yield any positive result. From the diabetic sample, a proteins score of 59 was obtained for CEV14 protein of Homo sapiens (figure 13). CEV14 is a cancer protein associated with acute myyelogenous leukemia. The presence of this protein in platelet may have been a random event as the sample was taken from a single donor. Hence conclusions cannot be made regarding the presence of CEV14 in platelets of diabetic individuals. A more comprehensive study of proteome difference between the two groups can be obtained with MS/MS analysis of whole platelet proteome from both the groups.

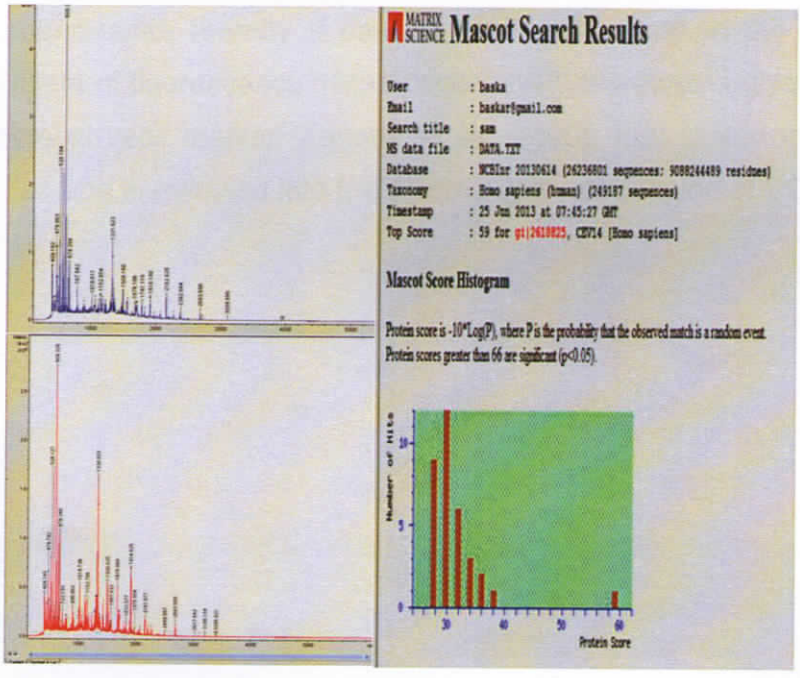

Figure 13: MALDI-Tof data

Chapter IV

Result and Discussion:

Effect of Circulating Platelet Proteins on Endothelial cells

4.1 Isolation and characterization of endothelial cells

To study the effects of circulating platelet proteins on endothelial cells, the human umbilical vein endothelial cells (HUVEC) were isolated from the umbilical cord by method described by Prasad *et al.,* 2005. The cells were characterized as endothelial cells using the Ac LDL Uptake assay and von Willibrand Factor (vWF) (figure 14 and 15).

AcLDL is taken up by macrophages and EC's via "Scavenger cell pathway" of LDL metabolism [Vyota *et al.*, 1984]. AcLDL labelled with a fluorescent probe 1, 1'- dioctadecyl- 3, 3, 3', 3'- tetramethyl-indocarbocyanine- perchlorate (DiI AcLDL) was used for the assay. Once DiI AcLDL enters ECs it is degraded by the lysosomes and it accumulates in the lysosomal membranes thereby it can be easily visualised in the standard rhodamine filters of fluorescence microscopes. vWF, the plasma glycoproteins is an endothelial cells marker [Zanetta *et al.*, 2000]. It is stored in Wiebel-Palade bodies and is released into the plasma upon stimulation of ECs.

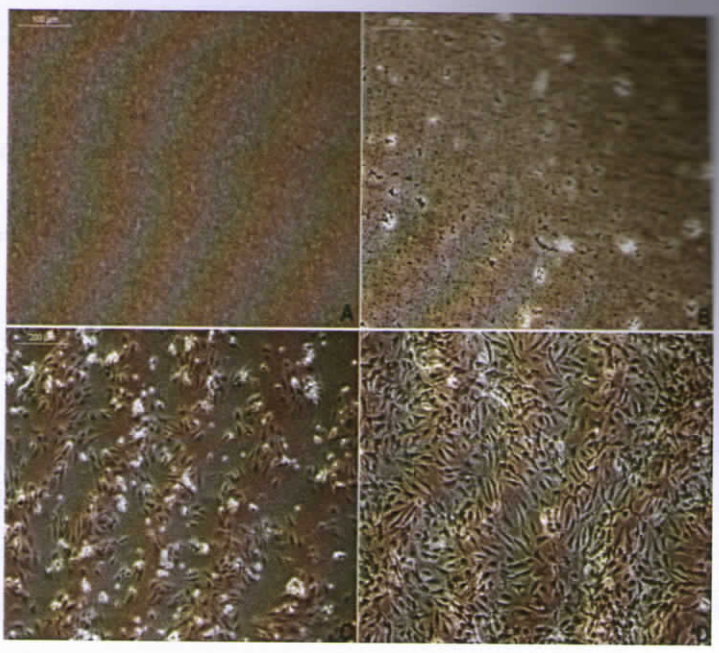

Figure 14: Cell culture dish coating and HUVEC isolation. A- Bare tissue culture dish B- Fibrin growth factor composite coated tissue culture dish C- HUVEC after 12 hours of isolation. D- HUVEC after 24 hours of isolation. Upper panel images taken at 20X magnification and lower panel images taken at 10X magnification

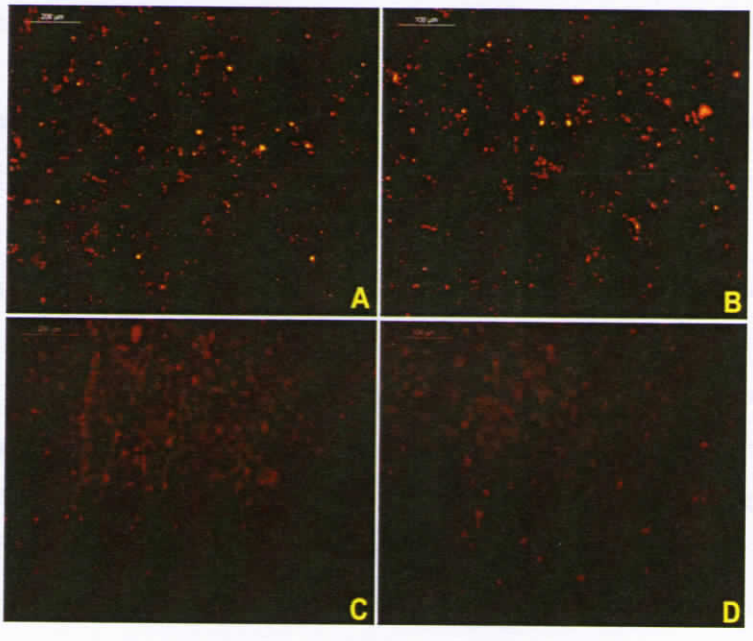

Figure 15: Characterisation of HUVEC. Images in the upper panel shows AcLDL uptake by HUVEC. Images in the lower panel shows the expression of vWF in HUVEC. A & C- Cells under 10X magnification B & D- Cells under 20X magnification.

4.2 Effect of circulating platelet proteins on endothelial cells

Endothelial dysfunction has been proposed as a fundamental component in the pathophysiology of diabetes. To study the effect of circulating platelet proteins in impairing the function of ECs, HUVEC was exposed to 100µg of the circulating platelet protein in *in-vitro* cell culture system. Hundred µg of protein concentration was chosen from the previous study done in the same laboratory (unpublished data). In the first phase of the study apoptotic assay was carried out and in the second phase difference in endothelial gene expression in the presence of control and diabetic plasma was studied.

4.2.1 Apoptosis Assay

To study the effects of circulating platelet proteins, the platelet proteins (100µg) isolated from the diabetic subjects and control were incorporated in the fibronectin based matrix and cells from 2-4th passage were seeded on to the matrix. Apoptosis assay was carried out using commercially available kit (Annexin-PI) by flow cytometry. Morphological analysis of HUVEC after 48 hours of culture showed considerable change in diabetic group as compared to the reference HUVEC (figure 16). Such changes were not very prominent in HUVEC cultured with plasma from the control group. The observation in culture co-related with the FACS data since the percentage of apoptotic cells in HUVEC exposed to diabetic plasma was more when compared to HUVEC cultured in control plasma matrix. Representative FACS data is shown in figure 17. A comparative data analysis from both the groups is shown in figure 18. The effect of these circulating platelet proteins were analysed by coating it on the cell culture dish because this strategy helps in studying the effect through slow release. Slow release of the proteins into the cell culture medium mimics the body system since platelets release the proteins into circulation in a gradual process.

The vascular endothelium is an indispensable organ in the regulation of tonicity and vascular homeostasis. Endothelial dysfunction is a marker of

atherosclerosis and contributes to the atherogenic process and the development of atherothrombotic complications. The endothelial functional alteration and atherosclerosis are commonly found in association with diabetes. ECs are highly sensitive to the changes in the environment which result in functional impairment of these cells. Reports indicate that the development of atherosclerosis can involve apoptotic process in the vasculature [Tricot *et al.*, 2000]. Apoptotic cells were also found in the neo-intima of atherosclerotic vessels and it is being suggested that apoptosis is also a possible mechanism by which the remodelling of the vessel wall takes place during atherogenesis [Han *et al.*, 1995]. From the study conducted it is evident that platelet proteins present in plasma of diabetic subjects can induce apoptosis in endothelial cells which can predispose these vessel walls to initiate plaque development.

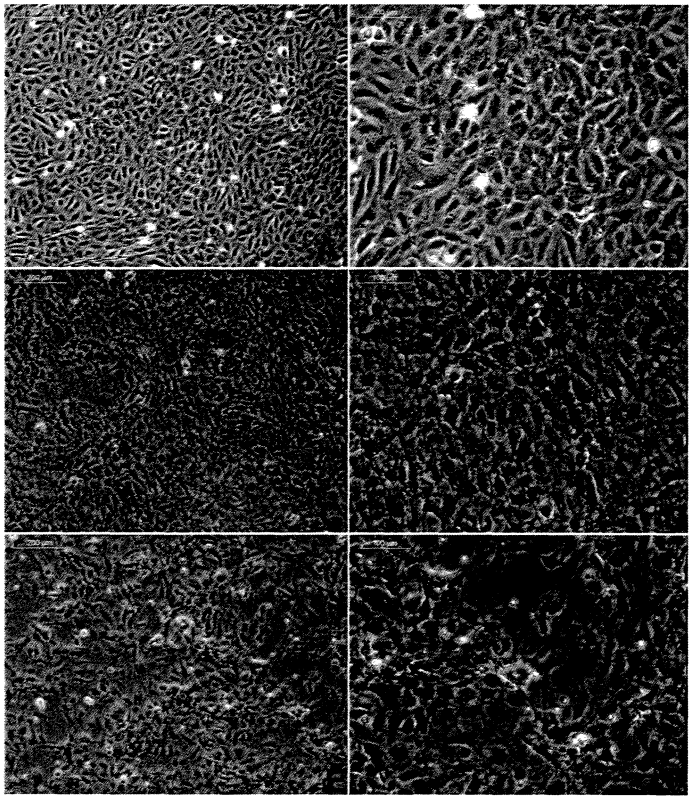

Figure 16: Effect of circulating platelet proteins in HUVEC. A & B- Control HUVEC. C & D- HUVEC cultured in control plasma coated dishes. E & F- HUVEC cultured in diabetic plasma coated dishes. Images on the left panel are 10X magnification and images on the right panel are 20X magnification.

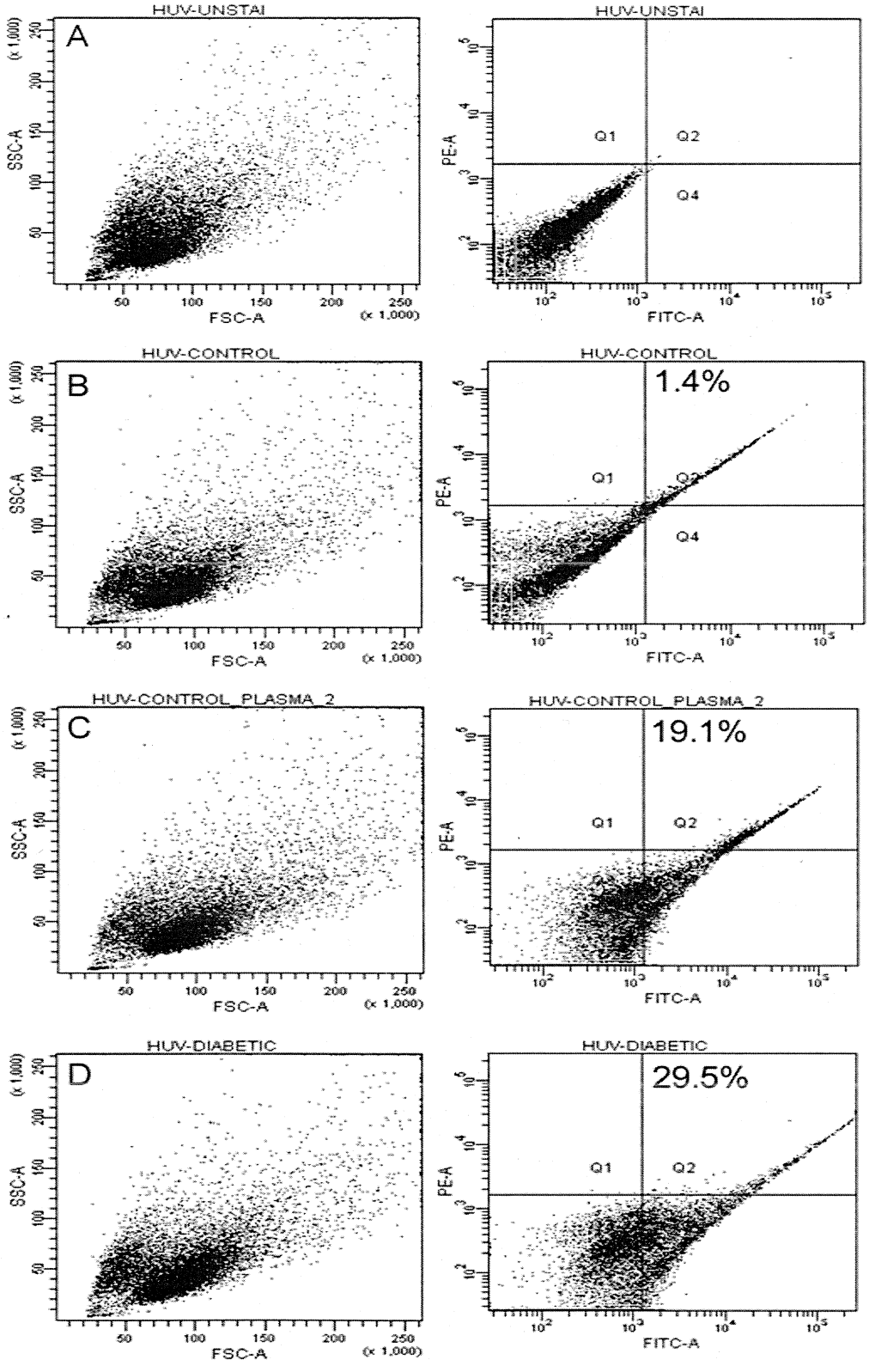

Figure 17: FACS analysis of apoptosis. A- Unstained HUVEC, B- Control HUVEC, C- Control plasma treated HUVEC D- Diabetic plasma treated HUVEC

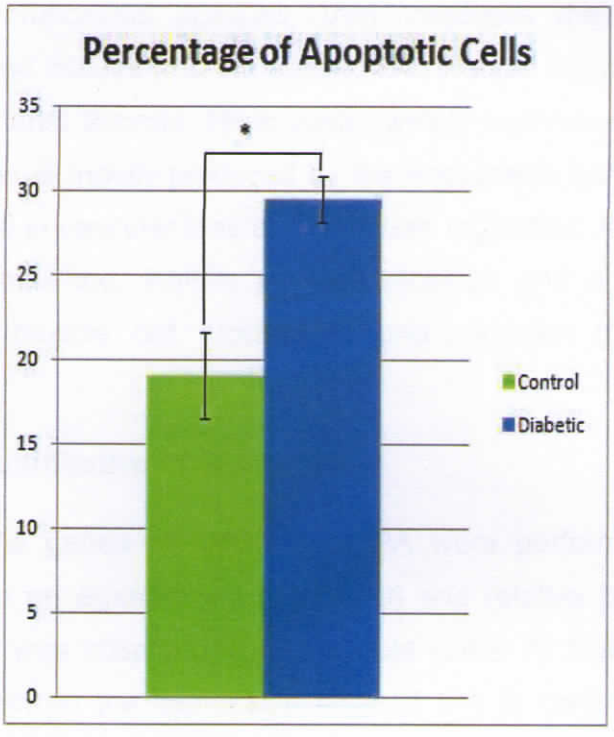

Figure 18: Comparison percentage apoptotic cells in control and diabetic group. Values are given as Mean ± SD (n=3). P<0.05

4.2.2 Gene Expression Studies

PCR analysis of gene expression was carried out in order to study the effect of circulating platelet proteins in causing endothelial dysfunction. The genes selected for the study were tPA, vWF and eNOS. GAPDH was taken as the house keeping gene. tPA is a serine protease which converts plasminogen to plasmin to degrade fibrin clot [Oliver *et al.*, 2005]. ECs in culture constitutively secrete tPA. The rate of synthesis is affected by many components like thrombin, histamine, plasmin etc [Hans and Collen, 1987]. vWF is an important marker for endothelial dysfunction [Meigs *et al.*, 2004]. Increased plasma levels of vWF is an already extablished biomarker for CVD [Ray *et al.*, 2005]. vWF being secreted by ECs would also be over-expressed by these cells during endothelial dysfunction. vWF is a prothrombotic adhesive glycoprotein synthesised by ECs and megakaryocytes. vWF affects platelet adhesion and aggregation, blood coagulation and fibrinolysis.

Through multiple functional domains, VWF mediates the attachment of platelets to exposed tissues and the subsequent platelet aggregation leading to formation of arterial thrombi. Nitric oxide, widely expressed in virtually all vascular cell types, is mostly produced by the endothelial isoform (eNOS). It plays a crucial role in vascular tone and structure regulation. It also exerts an anti-inflammtory influence, inhibits platelet adhesion and aggregation and prevents smooth muscle cell proliferation and migration [Desjardins and Balligand, 2000].

4.2.2.1 Semi-quantitative PCR Analysis

PCR for the genes of eNOS and tPA were performed. The PCR products were run on agarose gel (figure 19) and relative band intensities were analysed. It was observed from the data (table 7) that there was no significant difference in the expression level of tPA in control and diabetic plasma treated group. Down-regulation of tPA would indicate the prothrombotic nature of ECs. On contrary to what was expected the level of tPA expression did not show significant change in diabetic group, when compared to control group. The expression level of eNOS showed variation in control and diabetic group compared to reference HUVEC. There was up-regulation of eNOS in cells cultured on diabetic plasma as compared to control plasma.

In the second phase of semi-quantitative PCR analysis, HUVEC was exposed to 30% ethanol precipitated plasma of individual donors from both control and diabetic groups for 48 hours in culture. The expression levels of vWF, eNOS and tPA was studied (figure 20). The band intensities were analysed for each gene (table 8). It was observed from the band intensities that there was significant difference in the expression level of vWF and eNOS (figure 21). The expression level of vWF was upregulated in HUVEC upon exposure to circulating platelet proteins from diabetic. This indicates that ECs were shifting to prothrombotic phenotype. This result correlates with reports

which indicates that prothrombotic endothelium up-regulates the expression of vWF (Ray *et al.,* 2005).

The expression level of both tPA and eNOS was also found to upregulated with eNOS showing significant upregulation in diabetic plasma treated HUVEC as compared to control group. It is reported that eNOS gets upregulated upon stimulation [Duran *et al,* 2010]. In this study these proteins may stimulate HUVEC to synthesize/produce more eNOS. However, these results are in contrary to the reported literature, that there is diminished capacity of eNOS generation in ECs on exposure to in vitro diabetic environment [Hattori *et al*, 1991].In this study ECs were not exposed to high glucose conditions, thus eNOS expression resulted is only due to the stimulating effect of proteins. The culture conditions were provided for 48 hours and this may not be enough to bring about appreciable change in diabetic treated group. It is possible that this deviation in gene expression may be the result of alteration response of cells to stress. Change in gene expression may occur through long term exposure of these proteins on endothelial cells.

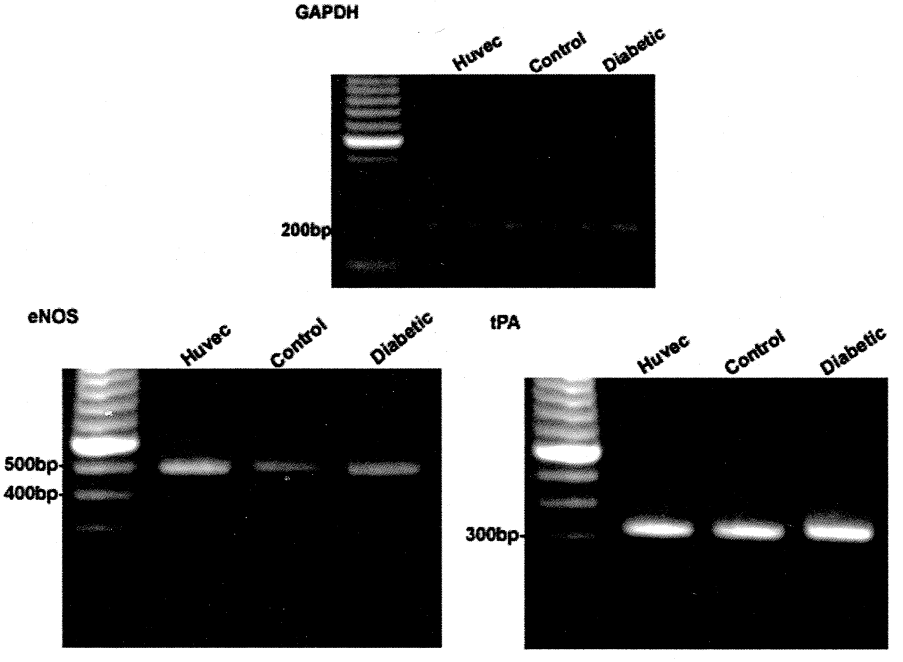

Figure 19: Semi-quantitative PCR analysis (1)

Gene	HUVEC	Control Plasma	Diabetic Plasma
eNOS	32.2%	25.9%	41.9%
tPA	32%	32%	36%
GAPDH	33.8%	33.4%	32.8%

Table 7: Relative band intensity difference of PCR products

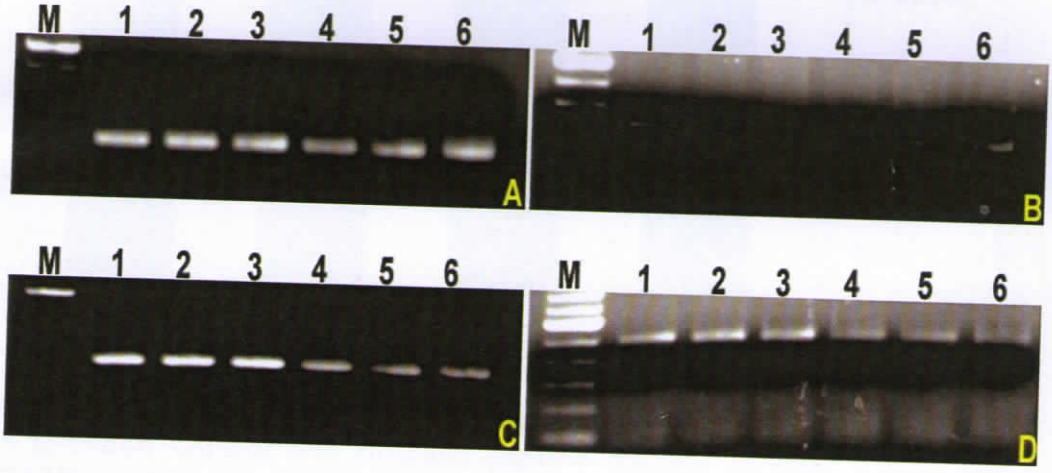

Figure 20: Semi-quantitative PCR analysis (2). Panel A- GAPDH, Panel B- tPA, Panel C- vWF, Panel D- eNOS. Loading order in all gels- M: DNA Ladder, 1: Diabetic 1, 2: Diabetic 2, 3: Diabetic 3, 4: Control 1, 5: Control 2, 6: Control 3.

Gene	Control Plasma	Diabetic Plasma
GAPDH	17.07±0.586	16.27±1.662
vWF	11.63±1.550	21.73±0.513*
tPA	15.33±2.316	17.97±4.618
eNOS	14.77±0.379	18.57±1.168*

Table 8: Relative band intenstity difference of PCR products. Values are given as Mean ± SD (n=3). $P<0.05$

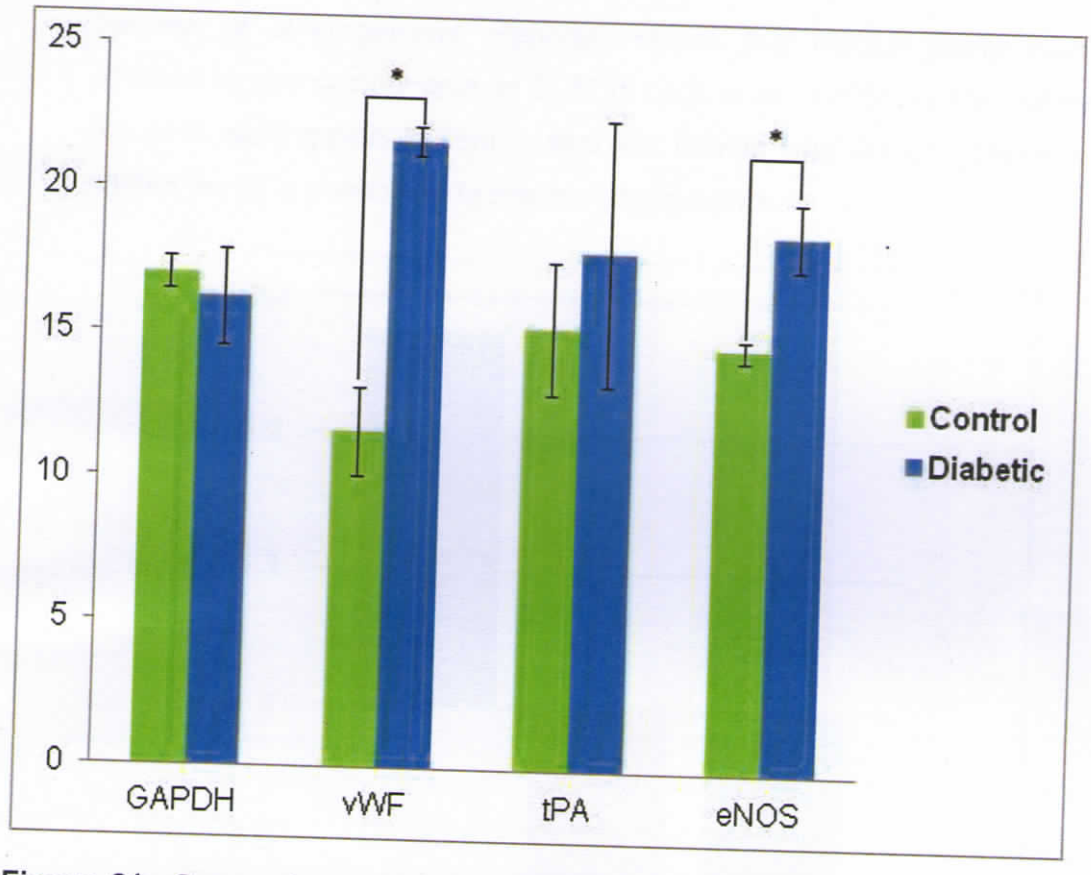

Figure 21: Comparison of band intensities of GAPDH, vWF, tPA and eNOS between control and diabetic plasma treated groups. Values are given as Mean ± SD (n=3). $P<0.05$

4.2.2.2 Real-Time PCR

In order to analyze the quantitative expression of eNOs, tPA and vWF RT-PCR was carried out. GAPDH was used to normalise the expression levels of all the genes. Cells were exposed to plasma proteins from both the groups for 72 hours in cultures. It is clear from the data that eNOS and tPA are down-regulated and vWF gets upregulated in the presence of diabetic platelet proteins when compared to control platelet proteins (figure 22). It is clear from the data that balance between the prothrombotic and antithrombotic genes expression is altered and is more favourable to the prothrombotic nature of ECs. The data is significant because upregulation of vWF makes ECs prothrombotic which predisposes the diabetic individual to

the risk of atherogenesis. Reports indicate that cellular phenotypes are affected by the components of ECM (Storck *et al.*, 1996). In the experiment the cells were grown in fibrin composite coated with diabetic plasma. This shifted the ECs phenotype to prothrombotic nature.

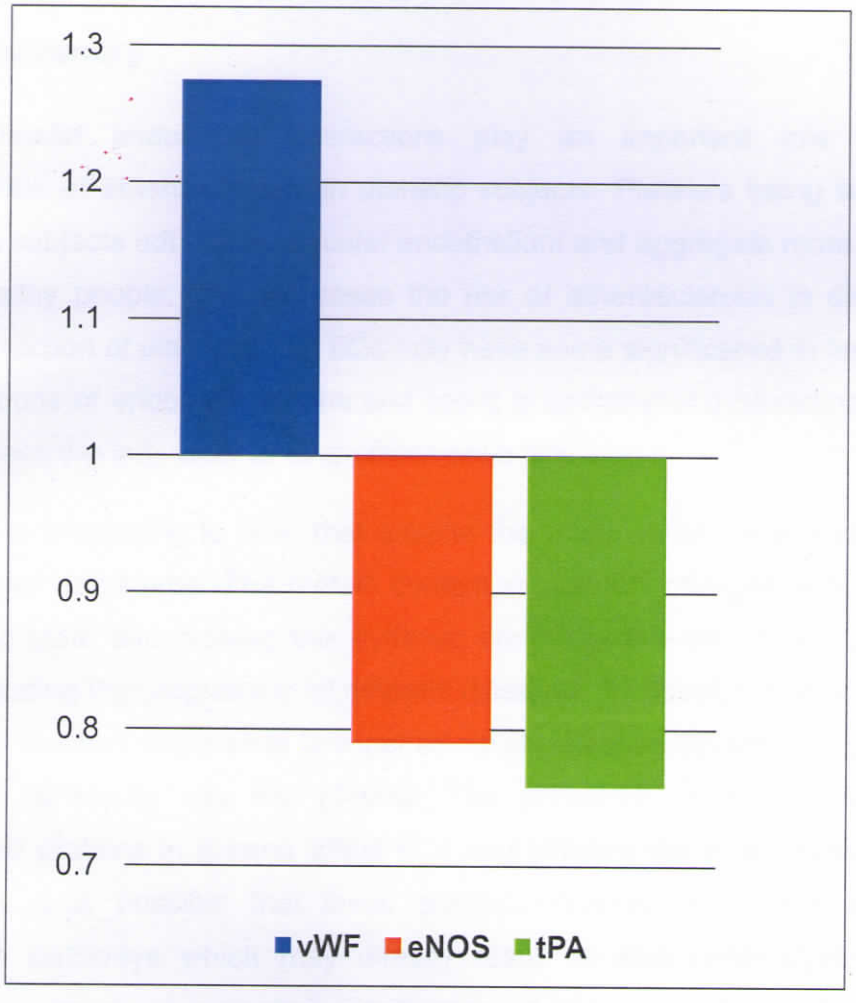

Figure 22: Fold-change in expression of vWF, eNOS and tPA

Chapter V

Summary and Conclusion

5.1 Summary

Platelet endothelial interactions play an important role in the progression of atherosclerosis in diabetic subjects. Platelets being active in diabetes subjects adhere to vascular endothelium and aggregate more readily than healthy people. This increases the risk of atherosclerosis in diabetes. This interaction of platelets with ECs may have some significance in impairing the functions of endothelium. The end result is endothelial dysfunction which predisposes the individual to atherothrombotic risk.

It is interesting to note that despite the absence of nucleus platelets have a vast secretome. The protein content in platelets changes with altered metabolic state and probing this dynamic protein profile will prove useful in understanding the progression of different diseases. In diabetic conditions, the secretory function of platelets is impaired which releases several of it granule contents constantly into the plasma. The presence of these differently expressed proteins in plasma affect ECs and initiates the modulations in its functions. It is possible that these platelet proteins can initiate several signalling pathways which may directly result in endothelial dysfunction. Despite so much of biological significance of these proteins in the body system, not many studies are focussed in this direction to probe this difference.

The objective of this study was to isolate and characterise whole platelet proteins and circulating platelet proteins in plasma. The project also aimed to study the effect of the circulating platelet proteins in diabetic on ECs. In the initial step of the study, samples were screened to confirm that all subjects were diabetic and were only on diet control. Plasma and platelet

proteins preparations were prepared from diabetic and healthy control groups for comparative analysis.

Plasma being abundantly rich with several proteins, an effective method was required for depletion in order to study the low abundant platelet proteins. Proteins precipitation using ethanol was the strategy adopted for this purpose and plasma was depleted using different concentrations of ethanol. It was observed that 30% ethanol was the most effective concentration to deplete the plasma. Once the conditions were standardised, the depleted plasma samples from both groups were compared in SDS-PAGE and 2-D gel electrophoresis. Results of the study demonstrated clear difference between protein profiles of two groups. In order to study if the difference observed in plasma proteins between the two groups were from platelet source, whole platelet proteome comparison was also carried out. It was found in the study that there was significant difference in the platelet proteome from both groups.

In the next phase of the study the effect of the isolated platelet proteins in plasma source on ECs were analysed through apoptotic assay and gene expression studies. It was observed in the study that ECs turned apoptotic in the presence of diabetic plasma as compared to control plasma. The gene expression studies also co-related with this data since it was found that there was upregulation of vWF expression and down-regulation of tPA and eNOS in endothelial cells in the presence of diabetic circulating platelet proteins. This shift in expression clearly indicates that ECs became prothrombotic when exposed to diabetic plasma.

5.2 Conclusion

- The study concludes that the platelet proteome of diabetic individuals vary significantly from that of healthy controls.
- Abundant proteins in plasma can be effectively depleted by 30% ethanol precipitation in order to study the low abundant proteins in plasma.

- The 2-D analysis of plasma protein from diabetic and control group was found to significantly different.
- The differently expressed proteins in plasma of diabetic individuals are from platelet source.
- Apoptosis is induced in endothelial cells on exposure to diabetic plasma.
- The change in EC phenotype was clearly demonstrated in culture after 72 hours of exposure to diabetic circulating platelet proteins.
- ECs shifted to prothrombotic phenotype as evidenced by upregulation of vWF gene and down-regulation in the gene expression of tPA and eNOS.

5.3 Future prospects

In this study several proteins were found to be differently expressed in platelets. Only through the complete analysis of the platelet proteome from both groups one can understand the full length of proteome difference in diabetes. Such an analysis will help to identify the culprit proteins in plasma which mediates endothelial dysfunction in diabetic conditions. The effect induced by circulating platelet proteins in ECs is a gradual process and hence only through long term culture conditions one can elucidate the damage these proteins can cause in ECs. So in future study focus shall be:

- MS-MS analysis of whole platelet proteome from diabetic and control group.
- Long term exposure of diabetic plasma in culture conditions to track changes in gene expression pattern.
- To study the effect of circulating platelet proteins on the expression of inflammatory marker IL-8 of ECs.

5.4 Limitation of the study

Blood sample collection criteria for the diabetic group was that the patients were strictly on diet control and not on any prescription drugs. Finding samples suitable for the study proved difficult as diabetic patients on diet control within the age group of the study was hard to come by and majority of the individuals who turn up at hospitals were already on prescription drugs.

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Appendix

Acid citrate dextrose (200mL)

Trisodium citrate	-	4.4g
Citric acid	-	1.6g
Dextrose	-	5g

Solution is filtered and stored at 4°C

Tyrode's buffer (500mL) pH 7.4

Dextrose	-	500mg
$MgCl_2$	-	99.5g
KCl	-	201mg
NaCl	-	4.06g
Tris base	-	878mg

Solution is filtered and stored at room temperature.

PBS (1000mL) pH 7.4

NaCl	-	8g
KCl	-	0.2g
Na_2HPO_4	-	1.44g
KH_2PO_4	-	0.24g

Solution is filtered and stored at room temperature.

30% Acrylamide (100mL)

Acrylamide	-	29.2g
Bisacrylamide	-	800mg

Solution is filtered and stored in amber bottle at 4°C

Upper tris (50mL) pH 6.8

Tris base - 3.03g

10% SDS - 2mL

Filter the solution and store at 4°C

Lower tris (50mL) pH 8.8

Tris base - 9.085g

10% SDS - 2mL

Filter the solution and store at 4°C

SDS Gel loading buffer (2X)

100mM Tris pH 6.8

SDS 4%

BPB 0.2%

Glycerol 20%

85µL of buffer is mixed with 15µL of β-mercaptoethanol to make 100µL of working 2X buffer fresh before use. The stock is stored at 4°C.

Tris-Glycine SDS buffer (8X- 500mL)

Tris base - 12g

Glycine - 57.6g

pH of the solution is adjusted to 8.3 and stored at room temperature after filtration

Tris-Glycine SDS buffer (1X- 400mL)

8X TGS buffer - 50mL

10% SDS - 4mL

Coomassie Staining Solution

Glacial acetic acid - 10%

Methanol - 40%

Distilled water - 50%

CBB- R 250 - 0.1g

Destaining solution

Glacial acetic acid - 10%

Methanol - 40%

Distilled water - 50%

Reagents for Lowry's protein estimation

Reagent A : 2% Na_2Co_3 in 0.1N NaOH

Reagent B : 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartarate

Reagent C : 50mL reagent A:1mL reagent B

Reagent D: 1mL Folin's reagent:1mL deionised water

Reagents for Silver staining with glutaraldehyde as fixative

Solution 1: Distilled water: methanol: acetic acid in the ratio 4:5:1 containing 0.25% glycerol

Solution 2: 10% glutaraldehyde

Solution 3: 0.1% silver nitrate

Solution 4: 0.01% DTT

Developer: 3% sodium carbonate containing formaldehyde

Stopper: 6% citric acid

Reagents for silver staining compatible with mass spectroscopy

Fix 1: 50% methanol: 10% acetic acid

Fix 2: 5% methanol: 1% acetic acid

Fix 3: 50% methanol

Stopper: 6% acetic acid

Sensitizer: 0.02% sodium thiosulphate

Stain: 0.2% silver nitrate

Developer: 6% sodium carbonate containing 2mL sensitizer and 50µL formaldehyde per 100mL of solution

2-D rehydration buffer (2mL)

Urea	-	8M
CHAPS	-	2%
DTT	-	50mM
3/10 Ampholytes	-	0.2%
Bromophenol blue	-	0.002%

2-D equilibration buffer I

Urea	-	6M
SDS	-	2%
Tris-HCl (pH 8.8)	-	0.375M
Glycerol	-	20%
DTT	-	2%

2-D equilibration buffer II

Urea	-	6M
SDS	-	2%
Tris-HCl (pH 8.8)	-	0.375M
Glycerol	-	20%
Iodoacetamide	-	2%

Fibrin Composite

Thrombin	-	5IU/mL
Fibrinogen	-	10mg/mL
Gelatin	-	0.2%
VEGF	-	50μg/mL

20% IMDM (50mL)

IMDM	-	40mL
FBS	-	10mL
Sodium bicarbonate	-	50mg

Antibiotics (10X)	-	500µL
VEGF	-	50µg/mL

10% IMDM

IMDM	-	45mL
FBS	-	5mL
Sodium bicarbonate	-	50mg
Antibiotics (10X)	-	500µL
VEGF	-	50µg/mL

SFM

IMDM	-	50mL
Sodium bicarbonate	-	50mg
Antibiotics (10X)	-	500µL

HBSS (1000mL) pH- 7.4

KCl	-	0.4g
KH_2PO_4	-	0.06g
NaCl	-	8g
Na_2PO_4	-	0.0482g

The solution was filtered autoclaved and stored at 4°C

DNA Gel loading dye – 6X

Glycerol	-	6mL
0.5M EDTA	-	1.2mL
Sterile Water	-	2.8mL
BPB	-	2mg
Xylene cyanol	-	2mg

10X TAE buffer (200mL

Tris	-	9.68g
0.5M EDTA (pH 8)	-	4mL
Glacial Acetic Acid	-	2.28mL