COMPARATIVE STUDIES ON MONOCYTE-PLATELET INTERACTIONS IN DIABETIC AND HEALTHY INDIVIDUALS

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DECLARATION

I, DEEPA S, hereby declare that the thesis work entitiled '**Comparative studies on monocyte-platelet interactions in diabetic and healthy individuals**' was done by me under the direct guidance of **Dr. Anugya Bhatt, Scientist D,** Division of Thrombosis Research, Biomedical technology wing (BMT Wing), Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the thesis work entitiled '**Comparative studies on monocyte-platelet interactions in diabetic and healthy individuals**' submitted by Deepa S (2015/MPhil/04; 160014) in partial fulfillment for the Degree of Mater of Philosophy in Biomedical Technology was done under my supervision and guidance at Division of Thrombosis Research, Biomedical technology wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

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Abbreviations

%	Percentage
µl	Microlitre
µm	Micrometer
A	Ampere
ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention’s
CLEC-2	C type lectin receptor 2
CBBR-250	Coomassie Brilliant Blue R-250
DAB	3,3'-diaminobenzidine
dL	Decilitre
DM	Diabetes mellitus
DMS	Demarcation membrane system
ESEM	Environmental scanning electron microscope
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
g	Gravity
GMP-140	Granule membrane protein
GP	Glycoprotein complex
GPI	Glycosylphosphatidylinositol
HbA1c	Haemoglobin A1c
ICAM	Inter cellular adhesion molecule
IDA	International diabetic association
IL	Interleukin
kDa	Kilodalton
L	Litre
LLG	Leucine-Leucine-Glycine
LPS	Lipopolysaccharide

MCP	Monocyte chemoattractant protein
mg	Milligram
ml	Millilitre
MPA	Monocyte platelet aggregates
MS	Mass spectrometry
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF-kB	Nuclear factor kB
NIDDM	Non-insulin dependent diabetes mellitus
PADGEM	Platelet activation dependent granule external membrane
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBMNC’s	Peripheral blood mononuclear cells
PECAM-1	Platelet endothelial cell adhesion molecule1
PSGL	P-selectin glycol-ligand binding protein
PVDF	Polyvinylidine fluoride
RANTES	Regulated upon activation, normal T cell expressed and secreted
RGD	Arginine-Glycine-Aspartic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TEMED	Tetramethylethylenediamine
TLRs	Toll like receptors
TXA	Thromboxane
V	Volt
VWF	Von Willebrand factor
WHO	World health organization

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Synopsis

Platelets are being reported as hyperactive in diabetic subjects. These active platelets express p-selectin (CD62) on its surface which facilitates the binding of platelets to the monocytes via p-selectin glycol-ligand binding protein-1 (PSGL-1) which is present on the monocytes and form monocytes platelets aggregates (MPA). Recent studies have suggested the finding of MPA as an early detection of cardiac diseases in diabetic subjects. Diabetes has been shown to be an accelerating factor in the progression of atherosclerosis. The metabolic changes in diabetes contribute to modified platelet function and enhanced leukocyte–platelet aggregate formation. The attachment of activated platelets leads to the activation of leukocytes causing enhanced cytokine production and upregulation of surface adhesion molecules leading to the inflammation. Therefore, platelet–monocyte aggregates may be of great importance in the development of cardiovascular complications and other inflammatory diseases.

Monocyte-platelet aggregates (MPA) are heterotypic complexes detectable in the peripheral blood which form in response to platelet activation. Accordingly, circulating MPA level increases in patients with acute thrombotic events, such as myocardial infarction or stroke as well as in subjects with underlying atherothrombotic risk factors including hypertension and diabetes. The level of MPA reflects the degree of platelet hyperactivity thus providing a robust index of blood thrombogenicity. However, cross-talk between platelets and monocytes is now regarded as a crucial pathophysiological mechanism linking thrombosis and inflammation and is believed to mediate, at least in part, the pro-inflammatory action of activated platelets. Indeed, invitro studies have shown that contact with platelets

enhances cytokine and prostanoid production by monocytes, as well as their adhesiveness to the vascular endothelium. However, the importance of monocyte-platelet interaction in human inflammatory pathophysiology, as well as the precise mechanism by which such interaction modulates monocytic function remain unclear.

Circulating monocytes comprise different sub-populations with distinct infiltrative and migratory properties that can be distinguished on the basis of differential expression of the surface markers CD14 and CD16. We hypothesized that a mild systemic inflammatory stimulus will increase circulating MPA, thus inducing a pro-inflammatory change in monocyte phenotype. These proinflammatory monocytes will release inflammatory cytokines like IL-10, Monocytes Chemoattractant Protein -1 (MCP-1).

Thus this study is focused to understand the platelet monocytes interactions in the diabetic subjects and the phenotypic alteration in the circulating monocytes.

The dissertation consists of four chapters. Chapter I Introduction, II Materials and Methods, III Results and Discussion and IV Summary and Conclusion.

Chapter I includes introduction to the research topic, review of the work done so far related to the proposed research problem. Chapter describes a brief explanation of the work, diabetes mellitus, factors contributing to the platelet activation, various phenotypes of monocytes, role of monocytes and platelets in diabetes mellitus, inflammatory molecules produced during inflammation. Review of literature suggests the critical role of MPA in the disease progression serves as a sensitive marker for the platelet activation even better than the golden standard P-selectin. Based on this, gap area was identified and clear objectives were formed to

understand the levels of MPA in diabetic subjects as well as to analyze the phenotypic alteration of the monocytes from the classical subset to the proinflammatory subset.

Chapter II gives the details of materials used and methods employed. Platelets and monocytes were isolated from the blood of healthy and diabetic subjects. Flow cytometry was used as a tool to analyze the activated platelets, platelet monocyte interaction and phenotypic alteration in monocytes. Cell interaction was also studied by ESEM analysis with and without fibrinogen matrix. Western blot analysis was done to see the inflammatory markers.

Chapter III consists of results and discussions. Platelets and monocytes were isolated and characterized with specific markers. ESEM analysis revealed the interactions between monocytes and platelets. Monocytes platelet aggregates were clearly demonstrated in test group compared to the healthy controls. This was further confirmed by flow cytometry using CD62 and CD14. Phenotypic alterations in monocytes were studied using CD14 and CD16. A clear subset of CD16 were observed in test subjects. Further analysis of inflammatory markers revealed the inflammatory nature of monocytes.

Chapter IV consists of summary and conclusions of the study. What needs to be done further in this area has been discussed in future perspectives. Limitations of the study are also included which depicts the various parameters that were not considered in the study.

From the study it can be concluded that monocyte platelet interactions occur in diabetic subjects. These interactions lead to changes in phenotypic alterations in monocytes. The inflammatory molecules specific to monocytes are expressed by the monocytes confirming its proinflammatory nature.

CHAPTER 1

INTRODUCTION

1.1 Background

Platelets play a major role in the hemostatic process, and increased platelet activation and aggregation are the center of the pathophysiology of Diabetes mellitus (DM). Platelet activation occurs mainly because of increased levels of platelet-derived thromboxane and prostaglandin metabolites detected in patients with DM. Platelets have been recognized as having a major role in inflammation, hemostasis, and thrombosis, being the source of inflammatory mediators and being able to both produce and respond to chemoattractant cytokines.

The interactions between platelets and monocytes are increased in DM. These aggregates are formed when platelets are activated and degranulated after which they adhere to circulating monocytes forming MPA. MPA formation has proinflammatory effects, linking coagulation and development of atherosclerosis. Proatherogenic activity of monocytes and circulating levels of soluble cell adhesion molecules are more pronounced in DM.

It was demonstrated by three independent methods that circulating monocyte–platelet aggregates are more sensitive markers of in-vivo platelet activation compared with platelet surface P-selectin, which was (generally) considered to be the gold standard marker of platelet activation[1].

As per WHO, in 2012, an estimated 1.5 million deaths were directly caused by diabetic complications and in 2014 the global prevalence of diabetes was estimated to be 9% among adults aged 18+ years. WHO projects that diabetic complications

will be the 7th leading cause of death by 2030. The prevalence of diabetes is predicted to be 366 million in 2030[2]. Moreover if there is genetic predisposition to cardiovascular diseases, it may serve as an important factor in contributing to the difficulties developed during diabetes[3]. According to the U.S. Centers for Disease Control and Prevention's (CDC) National Diabetes Fact Sheet, nearly 26 million American adults and children have diabetes. About 79 million Americans aged 20 years and older have pre-diabetes, a condition that increases the risk for developing diabetes.

Type 2 diabetes usually begins gradually and progresses slowly. Symptoms in adults include: excessive thirst, increased urination, fatigue, blurred vision, weight loss. In women, vaginal yeast infections, fungal infections under the breasts or in the groin and in men erectile dysfunction are some of the symptoms. Severe gum problems, itching, unusual sensations, such as tingling or burning in the extremities are other symptoms.

NIDDM or the type 2 diabetes is mostly seen in aged adults usually above the age of 30-35 years. Type 2 diabetes also known as adult onset diabetes occurs when our body cannot make use of insulin properly. In most cases they remain asymptomatic for a long period of time. Certain complications like nephropathy, retinopathy, and cardiovascular problems may serve as initial markers of the disease. Type 2 may occur due to mutations in gene coding for the insulin receptor. Of all the types of diabetes, the most common is type2 diabetes mellitus. As per national library of medicine 90-95% of diabetic cases are caused by type 2 diabetes only. It mainly occurs in those who are above the age of 40years, overweight and also have a family history of diabetes.

Type 2 diabetes involves three stages. The first stage is insulin resistance. In the first stage insulin can attach to its receptors on the cells but is unable to transport glucose from blood to cells due to some unknown mechanisms. Most patients with type 2 diabetes produce variable, even normal or high, amounts of insulin. In the beginning, this amount is usually enough to overcome such resistance. As time advances, the pancreas becomes unable to produce enough insulin to overcome resistance thus proceeding to the second stage. In type 2 diabetes, the initial effect of this stage is usually an abnormal rise in blood sugar after a meal (called postprandial hyperglycemia). Gradually the glucose levels increases and this leads to the third stage. This is made evident by fasting hyperglycemia, in which glucose levels are high most of the time.

Many studies have shown that inflammation plays an important role in increasing the complications in diabetes as well as in diabetes there is an increase in the level of certain proinflammatory cytokines in blood[4]. Insulin plays an important role in controlling platelet reactivity. When insulin is present in the blood, it reduces the hyperactivity of platelets. Platelets has got receptors for insulin and binding of insulin to these receptors prevent the binding of platelet activators thus reducing or preventing the platelet activation[5]. Usually in inflammatory disease conditions the platelets are highly active and these hyperactive platelets have high tendency to form aggregates with leukocytes ultimately resulting in changes in their phenotype, may alter their normal functions and finally affecting the whole normal physiology[6]. Of the leukocyte most important ones are the monocytes. Monocytes are present in the form of different subsets of population based on the expression of their surface markers CD14and CD16. In normal conditions $CD14^{+}CD16^{-}$ which is the classical

subset population of monocytes are present. But during inflammatory disease conditions, $CD14^{+}CD16^{+}$ monocyte populations are reported[6]. Alteration in monocytes population in circulating blood of diabetic subjects and their interaction with platelets may be used as marker for the susceptibility towards inflammatory diseases.

1.2 Review of literature

1.2.1 Monocyte structure and function

Monocytes are the largest of leukocytes with a single large nucleus which makes them one among the mononuclear leukocytes and has numerous granules in the cytoplasm. They arise from a common progenitor cell in the bone marrow and then migrate to the other tissues like spleen, liver, lymph nodes etc. However the most important reservoir of monocytes is the spleen[7]. When appropriate stimulus of inflammation and migration arise, these monocytes migrate from their storage sites to infected tissues and get differentiated into macrophages. They make up to 2-7% of all the leukocytes in the body. Normal range of monocytes in blood is 0.2–$1.0\times10^{9}/L$[8].They have an approximate diameter of 8-9μm[9]. Monocytes develop in the bone marrow for 1-3 days and then circulate in the blood for one and half days approximately and then migrate to the tissues where they differentiate into phagocytic macrophages.

1.2.2 Monocyte phenotypes

Monocytes express distinct cell surface markers. The most important ones are the CD14 and CD16. The molecular weight of CD14 is around 55kDa. It is a glycoprotein with multiple leucine rich repeats and it is attached to the cell membrane

via a glycosylphosphatidylinositol (GPI) anchor, which is encoded on the X chromosome[10]. It serves as the receptor for lipopolysaccharide (LPS) secreted by the gram negative bacteria[10]. The number of CD14 receptors expressed by a single monocyte is approximately above 1,10,000 per cell[10,11]. CD16 is the other phenotypic marker which is expressed to a little extent in healthy individuals. It has an approximate molecular weight of 23kDa.

CD14+CD16+ monocytes are proinflammatory monocytes that are present during inflammatory conditions[12]. Based on the expression of cell surface marker there are three distinct subpopulations of monocytes. They are $CD14^{+}CD16^{-}$, $CD14^{high}CD16^{+}$ and $CD14^{low}CD16+$[13,14]. CD14+CD16- cells constitute 90% of the population and have a diameter of 18µm approximately, whereas $CD14^{low}CD16+$ comprise 10% of the population and have a diameter of approximately 14µm[7]. Compared to $CD14^{+}CD16^{-}$ the $CD14^{low}CD16^{+}$ shows less phagocytic activity, limited production of reactive oxygen species as well as less expression of certain receptors like monocyte chemoattractant protein (MCP-1)[15,16]. Certain studies show that there are only two subsets of monocyte population. One subset differentiates into macrophages during extravasation from peripheral circulation to tissues and the other subset differentiates into dendritic cells during inflammation[17].$CD14^{+}$ cells get differentiated to macrophages and are involved in tissue repair and $CD16^{+}$ cells get differentiated into dendritic cells[18,19]. All these changes depend upon the tissue response to pathogenic conditions. Monocytes exhibits the property of changing their phenotype based on specific environmental stimuli[15]. In addition to the above mentioned cell surface markers monocytes also express toll like receptors(TLRs) during inflammatory conditions[15].

1.2.3 Platelets structure and function

Platelets also known as thrombocytes are small, discoid cells without a nucleus that are involved in maintaining vascular integrity and regulating hemostasis[20]. Their diameter ranges from 2-3µm, 0.5µm in thickness and a mean volume of 6-10 femtolitres. They are also involved in inflammatory diseases and respond by expressing different markers on their surface. They are derived from megakaryocytes and each megakaryocyte can produce up to 5000-10000 platelets. Normal range of platelets is 150-400×10^3/µl of blood and they circulate in for about 5-9 days[20]. The most important role played by platelets is to form blood clots to prevent loss of blood by bleeding during an injury or disease condition. During clot formation there occurs a change in the shape of the cells as well as release of various cytokines that attract more and more platelets and finally form the clot.

Hematopoietic stem cells are the source for platelet formation. Hematopoietic stem cells give rise to the megakaryocytes which in turn leads to the formation of platelets by different ways. The three important ways that has been proposed for the development of platelets from megakaryocytes includes: (1) cytoplasmic fragmentation with the help of demarcation memebrane system (DMS); (2) platelet budding from the megakaryocyte surface; (3) proplatelet formation. The platelet structure comprises of three zones: (1) the peripheral zone which consists of the membranes and associated structures at the surface and lining channels of the surface connected open canalicular system. It serves as the site of adhesion and aggregation processes (2) sole gel zone consists of the fiber systems which helps in maintaining the altered shapes of the platelet depending on the condition. (3)

organelle zone consists of granules, dense electron bodies, mitochondria, glycogen particles etc.

The various types of receptors present on the surface of platelets are responsible for their reactivity or adhesion with other types of cells. Some of the receptors present on platelets are the glycoprotein complex (GP) Ib-IX-V, GPVI, CLEC-2, integrin αIIbβ3, thrombin receptors, platelet endothelial cell adhesion molecule1 (PECAM-1), P-selectin, and receptors for coagulation factors. One of the most important receptor is the platelet activating factor receptor (PAF) which is a lipid receptor, and serves as a mediator of inflammation. In humans, platelets approximately contain 300 PAF receptors on their surface. CD62 also known as platelet activation dependent granule external membrane/granule membrane protein (PADGEM/GMP-140) or P- selectin is one of the most important cell surface marker present on the platelet surface[21]. It is this CD62 that interacts with the monocytes.

1.2.4 Platelet activation

Usually platelets remain inactive unless there is a signal for them to become active. Platelet activation is caused by the binding of platelet activators or the thrombogenic substances like collagen, thrombin, components of atherosclerotic plaques etc. to the receptors present on the surface of platelets[22]. During platelet activation, they secrete ADP which activates P_2Y_{12} receptor thus causing enhanced aggregation of platelets[20]. Receptor binding triggers a series of events that include hydrolysis of membrane phospholipids, mobilization of intracellular calcium, and phosphorylation of important intracellular proteins[22]. This results in the release of arachidonic acid from the membrane phospholipids leading to the synthesis of thromboxane A_2 (TXA_2) which further contributes to the activation of platelets[22].

Activated platelets show the following features such as reduced membrane fluidity which may be due to changes in the lipid constitution of the membrane by the addition of sugar molecules to the membrane proteins followed by altered Ca2+ and Mg2+ homeostasis (increased intracellular Ca2+ mobilization and decreased intracellular Mg2+), decreased prostacyclin production, decreased NO production, decreased antioxidant levels, and increased expression of activation-dependent adhesion molecules (e.g., GpIIb-IIIa, P-selectin)[22]. This change in ionic concentration occurs due to failure of Na^+/K^+ ATPase activity and increased $Ca2^+$ ATPase activity[20]. Apart from this some of the other activities occurring during platelet activation are as follows:

(1) secretion occurs as a result of which adhesive receptors migrate to the surface of the platelets and release certain molecules that attract more platelets and leukocytes to the site.

(2) Activation of platelet surface integrin αIIbβ3(receptors for fibrinogen and Von Willebrand factor) occurs which are usually maintained in an inactive state in the circulating cells but upon activation change their conformation thus promoting the ligand binding.

(3) Upon platelet activation, P-selectin from the membrane of alpha granules get transported to the platelet surface[23].

(4) The number of fibrinogen receptors expressed on the surface of platelets gets increased upon activation. Around 40,000 receptors get expressed during platelet activation[24].

1.2.5 Monocytes platelet interaction and their role in diabetes

Platelets when become active interact with leukocytes including monocytes, neutrophils and lymphocytes to release certain immunomodulatory molecules. However the monocytes have shown to have increased reactivity towards the platelets when compared to other peripheral blood mononuclear cells and as well as the number of monocytes interacting with a single platelet is much more than other leukocytes[21]. Compared to all other peripheral blood mononuclear cells, monocytes have more affinity towards platelets and form heteroaggregates[25]. Platelets when interact with the monocytes they result in alteration in various functions of the both cell types[26]. Such heteroaggregates serve as markers of progressive cardiovascular diseases[25]. The platelets present in diabetic patient express various cell adhesion molecules as well as they tend to become larger in size when compared to healthy individuals[26]. The interaction studies between platelets and monocytes are of high importance in certain pathological conditions. It has been shown that such interactions results in changes in the functional aspects of cells. Platelets provide free cholesterol molecules to the monocytes and this helps the monocytes to synthesize cholesteryl ester. These monocytes play an important role in foam cell formation in atherosclerotic lesions[21]. P-selectin shows high affinity towards PSGL-1. Platelets when become active have the tendency to interact with leukocytes and form aggregates and this interaction mainly occurs between the P-selectin present on platelets and the PSGL-1(P-selectin glycoprotein ligand-1) present on the surface of leukocytes especially monocytes[27]. PSGL-1 is a highly O-glycosylated type-1 transmembrane protein with sulphated tyrosines in its N-terminal region and forms an important part of the binding site. P-selectin is a type 1 membrane protein

containing an N-terminal C-type lectin domain, an epidermal growth factor like motif, a cytoplasmic domain and a transmembrane domain. P-selectin is stored as an integral protein of the membrane of α-granules in platelets. When cell to cell contact occurs these granules fuse together with the cell membrane and expose P-selectin molecule. P-selectin binds to the amino terminal region of the PSGL-1 by recognizing a motif that contains the tyrosine sulfate residues.

The P-selectin and PSGL-1 interaction results in a signaling cascade that strengthens the adhesiveness between platelets and leukocytes. P-selectin induces a signal, which activates leukocytes through a series of molecular mechanisms, including tyrosine kinases belonging to the Srk family, PI3 kinases, actin and cytoskeleton proteins, finally inducing the activated form of the beta-2 integrin Mac-1. The receptor beta-2 integrin Mac-1 have a tendency to bind to the RGD-containing proteins (fibrinogen, vitronectin), LLG-containing proteins (VWF, ICAM-1), GpIbα and result into stable adhesions between cells. The P-selectin/beta-2 integrin cascade thus gives rise to heterotypic conjugates of platelets with leukocytes, which have been well characterized in washed cell system[27]. This interaction enhances the expression of nuclear factor kB (NF-kB) which stimulates transcription of various inflammatory molecules[27]. Increased levels of platelet-monocyte aggregates have been also seen in patients suffering from hypertension[28]. It has also been found that even non activated platelets that is those platelets that do not express CD62/GMP140 are also involved in binding to the monocytes but only to a very little extent[21].

1.2.6 Role of monocytes in inflammation

Inflammation plays an important role in type 2 diabetes by contributing to the development of insulin resistance. There are a large number of factors that contribute to insulin resistance. Some of them include lifestyle, obesity, diet etc which results in fluctuations in the normal levels of metabolites in the body leading to the activation of monocytes and thereby stimulating them to secrete various inflammatory cytokines[29]. These inflammatory cytokines released by the activated monocytes play a major role in developing insulin resistance. Such inflammations can pose the threat of developing cardiovascular diseases. CD14+CD16+ cells express large number of adhesion molecules thereby adhering to the endothelial cells and produces inflammatory cytokines[30,31]. The number of monocytes circulating in blood in diabetic patients has been found to be low compared to healthy individuals[32].Monocytes in diabetic patients have been found to have increased affinity for fibronectin and this property contributes to the development of atherosclerosis[33]. During diabetes mellitus monocytes express more number of mcp-1 receptors on their surface and the increased levels of mcp-1 in blood plasma makes the monocytes more attractant towards the arterial walls and makes them adherent to the endothelial cells[34].

When monocytes interact with the platelets, there occurs cell to cell adhesion. As a result of this adhesion changes occur at the genetic level. The p50-p65 heterodimer, commonly known as NF-kB, is located in the cytoplasm of monocytes. Upon platelet monocyte interaction the monocytes get stimulated and there occurs the translocation of p50-p65 (Rel A) into the nucleus. It is a component of NF-kB family of transcription factors that bind to the regulatory sequences of certain genes

that are coding for inflammatory molecules like monocyte chemoattractant protein-1, interleukin-10 etc[35,36]. RANTES (regulated upon activation, normal T cell expressed and secreted), is a chemokine secreted by activated platelets. RANTES plays an important role in the secretion of MCP-1 by monocytes[36]. The receptors for RANTES are present on the surface of monocytes. This shows that upon platelet activation, there occurs platelet monocyte interaction which inturn leads to the production of various chemokine factors. Such chemokines may serve as differential markers for identifying the inflammation and its associated disease conditions.

1.2.7 Summary

Diabetes mellitus is an inflammatory disease marked by hyperglycemia. The entire physiological processes taking place in the body gets disturbed due to increased blood glucose level. If appropriate measures are not taken the continuous increase in blood glucose level might result in the dysfunctioning of organs especially vital organs like heart, kidney, liver etc. The most important cells involved in inflammation are the platelets and monocytes. Platelets have important role in blood clotting and the monocytes play important role in immune responses by dealing with the pathogens that invade the body. However these two cells show increased activity at the time of inflammation. Platelets once become active expose various receptors and cell adhesion molecules to the surface. The study of monocyte platelet interaction becomes important because in many disease conditions which involve various inflammatory reactions, the monocyte platelet aggregates may play significant role in early detection of the disease. This occurs by examining various inflammatory molecules secreted by the cells during the period of disease development as well as by determining the phenotypic changes occurring in the

cells. During disease conditions they tend to become active and get involved in cell to cell contacts. They start expressing numerous cell adhesion molecules and receptors for a wide variety of molecules. There are various factors that contribute to the activation of platelets. These factors are present in the body during unfavourable physiological conditions. There is an up-regulation in the cell adhesion molecules and receptors. All these factors contribute to their interaction with other cell types thus facilitating the aggregate formation.

There are two types of aggregates formed: homotypic and heterotypic aggregates. That is either platelet platelet aggregates or platelet leukocyte aggregates. As a result of this interaction various intracellular and extracellular changes takes place. All these changes depend on the environment to which the cells are exposed. These changes also include the alterations in the phenotype of the monocytes that can serve as an important marker for analysis of the disease. Monocyte platelet interaction is directly or indirectly related to the production of inflammatory molecules in monocytes. Monocyte platelet aggregates play important role in destroying the endothelial cell integrity thus contributing to the blood vessel damage, leading to the formation of atherosclerotic plaques and thereby increasing the chances of atherosclerosis.

1.3 Gap area

Diabetes is the chronic metabolic disorder, where cell-cell interaction plays an important role. Though several studies have been done to understand the mechanism of diabetes, important phenomena of platelet monocyte interaction which may lead to the phenotypic changes in monocytes and could lead to the early disease marker is not being explored much.

1.4 Hypothesis

Platelet monocytes interactions may lead to the alterations of monocytes into proinflammatory phenotype and may result in altering proteomes of monocytes, which may be used as an early marker for the cardiac diseases.

1.5 Objectives of the study

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

- To determine the monocyte-platelet interactions using ESEM and flow cytometry.
- To determine phenotypic alterations in monocytes using flow cytometry.
- To determine the expression of inflammatory proteins in monocytes.
- To analyze the proteome change in monocytes by SDS PAGE.

CHAPTER-2

MATERIALS AND METHODS

2.1 Materials

Acrylamide (Sigma), Alexafluor 488 goat polyclonal antimouse antibody (Abcam), Ammonium persulphate (Sigma), Beta mercaptoethanol (Sigma), Bis-acrylamide (Sigma), Bovine serum albumin (Sigma), Bromophenol blue (Sigma), Citric acid (Merck), Coomassie Brilliant Blue R-250 (Sigma), Copper sulphate (Merck), Dextrose (Sigma), 3,3'-diaminobenzidine (DAB) (Sigma), Disodium hydrogen phosphate(Merck), Fibrinogen, Ficoll histopaque (Sigma), Folin-ciocalteu reagent (Nice), Formaldehyde (Merck), Glacial acetic acid (SDFCL), Glycerol (Sigma), Glycine (Sigma), Hydrogen peroxide (Sigma), Methanol (Merck), Nickel chloride (Sigma), Percoll gradient (GM Healthcare), Potassium chloride (Merck), Potassium dihydrogen phosphate (Merck), Protease inhibitor (PMSF) (Sigma), Protein marker (Biorad), Sodium carbonate (Merck), Sodium chloride (Sigma), Sodium dodecyl sulphate (Sigma), Sodium hydroxide (Merck), Sodium potassium tartarate (Sigma), TEMED (Sigma), Texas Red goat polyclonal antimouse antibody (Abcam), Trisodiumcitrate,Trizma base (Sigma), Trypsin, Tween twenty (Sigma), Antibodies: CD14 (Biolegend), CD16 (Biolegend), CD62, IL-10 (Biorbyt), MCP-1 (Biorbyt).

2.2 Study subjects

Blood samples from control and diabetic individuals were collected from BMT Wing campus, SCTIMST, Poojapura after getting the ethical committee approval. Five control and five diabetic samples were collected. SCT/IEC/818-2015.

Inclusive criteria: Diabetic patients (male) belonging to the age group 35- 60 were considered for the study. Diabetes was confirmed as per the IDA association recommendation of HbA1c and random blood glucose. HbA1c more than 6.5 and random sugar more than 150 mg/dL were considered as diabetic. Individuals having random sugar less than 120mg/dL and HbA1c less than 6.5 were considered as Control.

Exclusive criteria: Patients taking anticoagulant drugs were not included in the study.

2.3 Sample collection

8.5 ml blood was collected from five diabetic and five control individuals into 1.5 ml ACD taken into 15ml falcon tubes. Blood was gently mixed with acid citrate dextrose (ACD).

2.4 Platelet isolation

The collected blood was centrifuged at 600g for 10 minutes. The supernatant was centrifuged at 400g for 8 minutes. The supernatant was discarded. The obtained pellets were washed with PBS thrice at 400g for 5 minutes each.

2.5 Standardization for the monocytes Isolation

Two methods for the monocytes isolation were tried and best one was selected. The two methods tried were as follows: monocyte isolation from buffy coat method using histopaque density gradient and monocyte isolation from buffy coat method using histopaque-percoll density gradient.

2.5.1 Monocyte isolation from buffy coat by histopaque method

The collected blood was centrifuged at 600g for 10 minutes. The supernatant was discarded. Buffy coat layer formed at the interface between red blood cells and plasma was taken carefully and diluted with PBS in the ratio 1:4. This diluted mixture was gently layered on top of 4ml histopaque taken in another tube. Then it is centrifuged at 400g for 30 minutes. A white layer of cells is formed which is pipetted out and diluted with PBS (1:2). Then it is centrifuged at 400g for three times 6 minutes each to get the monocytes pellet.

2.5.2 Monocyte isolation by Histopaque-Percoll gradient method[37].

Buffy coat isolated from blood was mixed with PBS in the ratio 1:4. Into a separate falcon tube, 4ml histopaque was taken. The diluted buffy coat was then slowly added along the sides of the falcon tube so that it forms a separate layer on top of the histopaque. It is centrifuged at 400g for 30 minutes. After centrifugation a white layer can be seen that contains peripheral blood mononuclear cells (PBMNC's). Immediately the white layer diluted with PBS (1:1) was layered on isosmotic percoll density gradient and centrifuged at 400g for 30 minutes. The Percoll density gradient was made by first making the Percoll isosmotic with 1 molar NaCl. Percoll and NaCl are mixed in the ratio 9:1 and then this isosmotic Percoll and PBS was mixed in the ratio 1:1. No separate layer was formed and the supernatant was completely blank.

A slight modification of this method was tried in which the white layer isolated from histopaque gradient was layered on top of isosmotic Percoll gradient. This time isosmotic percoll was used as such without any dilution with PBS. A white layer of

cells was formed which was diluted with PBS and centrifuged thrice at 400g for 5 minutes each. To the cells 0.75µl of protease inhibitor was added and sonicated to isolate the proteins. The protein samples were stored at -80°C in PBS and protein estimation was carried out by Lowry's method[38].

2.6 Characterization of monocytes

2.6.1 Characterization of monocytes by immunostaining

Monocytes were isolated from buffy coat using histopaque gradient and double density gradient (histopaque and percoll gradient method). The isolated cells were washed with PBS and fixed in 3.7%formaldehyde for 20 minutes. Then the cells were blocked with 0.5% BSA solution. Pellets were washed at 400g for 5 minutes. The pellet was incubated with CD14-FITC antibody for 1 hour in dark at room temperature. Following incubation the cells were washed twice with PBS at 400g for 5 minutes each. The cells were observed under inverted fluorescent microscope Olympus lX71 using blue filter.

2.6.2 Characterization of monocytes by flow cytometry

Monocytes were isolated from buffy coat histopaque and Percoll gradient method. Cells obtained were washed thrice with PBS at 400g for 5 minutes each. Cells were fixed using 200µl of 3.7% formaldehyde solution for 20 minutes. After fixation cells were washed with PBS at 400g thrice for 5 minutes each. Cells were blocked using 0.5% BSA solution for 30 minutes. After blocking the cells were washed with PBS twice at 400g for 5 minutes each. Cells were strained through cell strainer (70µm). Cell count was taken using sysmex counter (Sysmex K-4500) and 1 $X10^5$cells/ml were incubated with primary antibody CD14 and for 1 hour at room

temperature. Cells were washed at 400g for 5 minutes. Then the cells were incubated with secondary antibody tagged with Texas red for 1 hour in dark at room temperature. Cells were washed at 400g for 5 minutes. Cells were analyzed using BD FACS Aria, and flowjo version 7.5 software. For flow cytometry 10,000 events were acquired. The compensation was done using single stained cells. Gating was done using unstained population. Experiment was done three times and percentage of cells positive for CD14 was calculated in control and test samples.

2.7 Phenotypic variation in monocytes

Monocytes from diabetic and control subjects were isolated as per the method described in section 2.5.2. Cells obtained were washed twice with PBS at 400g for 5 minutes each. Cells were fixed using 200µl of 3.7% formaldehyde solution for 20 minutes. After fixation cells were washed with PBS at 400g for 5 minutes. Cells were blocked using 0.5% BSA solution for 30 minutes. Cells were strained through cell strainer (70µm). Cell count was taken using sysmex counter (Sysmex K4500). Cells were incubated with primary antibody CD14 for 1 hour at room temperature. Cells were washed at 400g for 5 minutes three times each. The cells were incubated with secondary antibody tagged with texas red for 1 hour at room temperature in dark. Cells were washed at 400g for 5 minutes three times each. Then the cells were incubated with second primary antibody CD16 for 1 hour at room temperature. Washed the cells at 400g for 5 minutes three times each. Then the cells were incubated with secondary antibody tagged with alexafluor 488 for 1 hour at room temperature in dark. Cells were centrifuged at 400g for 5 minutes. Cells were gently resuspended in PBS and flow cytometric analysis was carried out using BD FACS ARIA and flowjo version 7.5 software.10,000 events were acquired. With stained

cells compensation was done and with unstained cells gating was done.Three times the experiment was repeated and percentage of cells positive for CD14 and CD16 was calculated in control and tests samples.

2.8 Activation status of platelets

Platelets from test and control subjects were isolated as per the described protocol in section 2.5.2. In brief isolated platelets ($1X10^5$cells/ml) from both the groups were fixed in 3.7% formaldehyde solution for 20 minutes and blocked in 0.5% BSA. The cells were incubated with CD62-PE antibody for 1hour in dark at room temperature. Cells were washed and gently resuspended in PBS and flow cytometric analysis was carried out using BD FACS Aria and flowjo version7.5. 10,000 events were acquired. Gating was done using unstained population. Compensation was done using single stained population. Experiment was repeated three times and percentage of cells positive for CD62 was calculated in control and test samples.

2.9 Platelet monocyte interaction

2.9.1 ESEM analysis

2.9.9.1 ESEM analysis with fibrinogen matrix coating

Monocytes and platelets were isolated. Isolated cells were fixed in 3.7% formaldehyde for 20 minutes. Cells were washed with PBS at 400g for three times five minutes each. The cells were processed. Cells were passed through ascending series of alcohol for dehydration. 30%-30 minutes, 50%-30 minutes, 70%-30 minutes, 90%-30 minutes and 100%-30 minutes. 50µl of fibrinogen (100mg/ml) was coated over the coverslips and allowed to air dry at room temperature. Once the coatings on the coverslips becomes dry, cells suspended in 100% alcohol were

gently layered on top of the fibrinogen coated coverslips. The coverslips were air dried again at room temperature. The ESEM analysis was carried out using 30kV Environmentally Scanning Electron Microscope (ESEM- Quanta 200, Germany).

2.9.9.1 ESEM analysis without fibrinogen matrix coating

Monocytes and platelets from both test and healthy individuals were isolated. Isolated cells were fixed in 3.7% formaldehyde for 20 minutes and layered on coverslips. Cells were washed with PBS at 400g for three times five minutes each. The cells on coverslips were processed. Cells were passed through ascending series of alcohol for dehydration. 30%-30 minutes, 50%-30 minutes, 70%-30 minutes, 90%-30 minutes and 100%-30 minutes. Cells suspended in 100% alcohol were used for ESEM analysis. The ESEM analysis was carried out using 30Kv Environmentally Scanning Electron Microscope (ESEM- Quanta 200, Germany).

2.9.2 Flow cytometry analysis of monocytes platelet aggregates

Isolated platelets and monocytes were washed twice with PBS at 400g for 5 minutes each. Cells were fixed using 200µl of 3.7% formaldehyde solution for 20 minutes followed by 3 times washing in PBS. Cells were blocked using 0.5% BSA solution for 30 minutes. Cells were strained through cell strainer (70µm). Cell count was taken using sysmex counter. $1X10^5$cells/ml were incubated with primary antibody CD14 for 1 hour at room temperature followed by the incubation with Texas red conjugated secondary antibody for 1hour in dark at room temperature. Cells were then incubated with CD62-PE for 1hour at room temperature in dark at room temperature. Cells were centrifuged at 400g for 5 minutes. Cells were gently resuspended in PBS and flow cytometric analysis was carried out using BD FACS

Aria and flowjo version7.5. For flow cytometry analysis 10,000 events were acquired. Unstained cells were used to gate the platelets monocyte population. Single stained cells were used to compensate the overlapping spectra. Experiment was done three times and percentage of cells dual positive for CD14/CD62 was calculated in control and diabetic samples.

2.10 Inflammatory proteome analysis of monocytes

2.10.1 Lowry's method of protein estimation

Protein measurement was carried out by Lowry's method[38]. 10µl, 20µl, 40µl, 60µl and 80µl of BSA working standard (1mg/ml) were taken into 5 eppendorf tubes. Each tube was made up to 100µl with distilled water. 100µl distilled water was taken as the blank solution. To each tube 1ml of alkaline reagent (2%sodium carbonate in 0.1N NaOH and 0.5%sodium potassium tartarate mixed in the ratio 50:1) was added and incubated for 10 minutes at room temperature. After incubation 100µl of Folin–Ciocalteu reagent was added into each tube and incubated at room temperature in dark for 30 minutes. Then optical density was measured at 600nm. A standard curve was plotted. For estimation of proteins in the collected samples, 10µl of sample was taken and made up to 100µl with distilled water. To the tube 1ml alkaline reagent was added and incubated for 10 minutes at room temperature. Then 100µl of Folin–Ciocalteu was added and incubated at room temperature in dark for 30 minutes. After incubation optical density was measured at 600nm and concentration of the protein in the samples were determined and a standard plot was generated. The absorbance was measured using UV-Visible spectrophotometer (Hewlett Packard Diode Array 8453, Germany).

2.10.2 SDS PAGE and Coomassie staining

20µg of protein was taken and mixed with equal amount of 1X SDS gel loading dye (gel loading dye and beta mercaptoethanol mixed in the ratio 80:20). Then the protein samples were placed in water bath at 95°C for 5 minutes. Then SDS PAGE was carried out using 12%resolving gel and 5%stacking gel. Control and diabetic samples were loaded into wells. The gel was run at 100volt till it finishes the stacking gel and then at 120volts throughout the resolving gel. The run is stopped when the dye front reaches the bottom of the gel. Then the gel was taken, gently washed with distilled water and was put in Coomassie Brilliant Blue R-250 stain (acetic acid, methanol and water mixed in the ratio 1:4:5; Coomassie brilliant blue R-250-0.1%) overnight at room temperature. After overnight staining, the gel was gently washed with distilled water and put in destaining solution (acetic acid, methanol and water mixed in the ratio 1:5:4) to remove the excess stain. Gels were documented using Alpha Imager Documentation System 2000.

2.10.3 Western blotting

Proteins were separated into bands by SDS PAGE. PVDF membrane soaked in methanol was placed on top of stacks of Whatmann filter papers soaked in transfer buffer. The gel was placed above the PVDF membrane. On top of this the filter papers were placed. The roller was gently rolled over the filter papers to remove any air bubbles present in between filter papers, membrane and gel that might interfere with the transfer. The whole set up was run at 25V, 0.1A for 1 hour at room temperature. The membrane was washed in PBST buffer for 2-3 times 5 minutes each and put in blocking solution (3% BSA) for 1 hour. Two different dilutions of antibodies were tried; they are as follows 1:500 and 1:1000. The membrane was

again washed in PBST buffer and incubated with primary antibodies IL-10 and MCP-1 at 4°C overnight. Following incubation, the membrane was washed with PBST thrice and incubated with secondary antibody for 2 hours at room temperature with continuous shaking. After 2 hours the membrane was washed with PBST and allowed to develop using the developer solution in dark. Once the specific band of required intensity was developed the development was stopped using distilled water.

2.11 Statistical analysis:

Statistical analysis was carried out for all the quantitative data. t-test was used to determine p value so that presence of significant difference can be found out. All the values given in tables are represented by Mean±SD.

CHAPTER-3

RESULTS AND DISCUSSION

3.1 Screening of subjects

The study was carried out for analyzing the platelet monocytes interaction in diabetic subjects and phenotypic alterations in the monocytes population leading to the expression of inflammatory markers. Diabetic and control samples were collected from BMT Wing staff clinic from the volunteers, after getting ethical committee approval.

The control and test samples (aged between 35-60 years) were grouped according to the recommendations of International Diabetic Association (IDA). According to IDA a person is diabetic, if random glucose level is higher than 140mg/dl and glycosylated haemoglobin (HbA1 c) level is greater than 6%. HbA1c is glycated haemoglobin which is formed when the blood haemoglobin gets glycated by the glucose present in the blood plasma. Glucose moiety gets linked to the β-chain of the haemoglobin. HbA1 c gives the average level of glucose over a period of 3 months. If a subject is having continued high levels of glucose in the blood it will be reflected in the HbA1 c concentrations[39]. As primary targets in the study was platelets, care was taken that these subjects were not on any antiplatelet therapy. The sample details regarding blood glucose and HbA1c levels of control and diabetic subjects taken after 2 hours of taking meals are provided in the table below.

	Control	Diabetic
Blood sugar (2 hours after meals)	80.2 ± 7.39	266.6 ± 87.72
HbA1c	5.22 ± 0.30	7.13 ± 1.01

Table 1: Blood sugar level and HbA1c levels in diabetic and healthy subjects. Values are given in Mean ± SD (n=5). P<0.05.

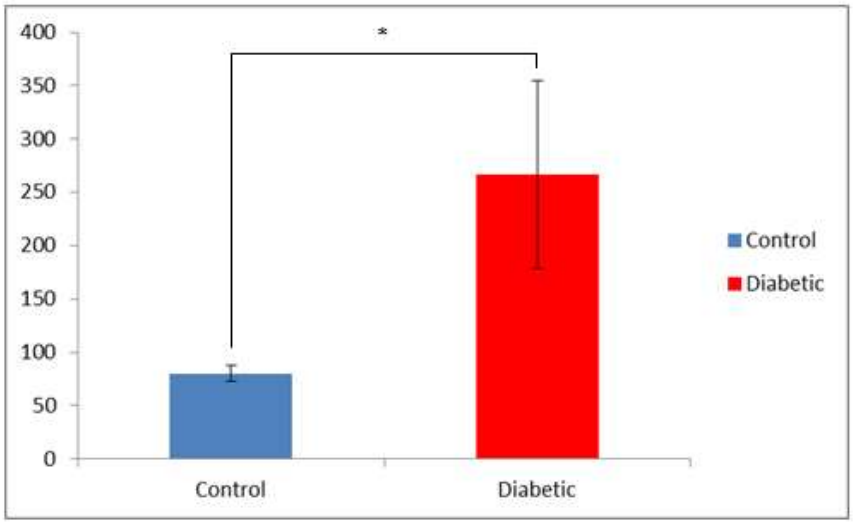


Figure1: Comparison of blood glucose levels in control and diabetic patients. Values are represented in Mean±SD (n=5). P-value<0.05.

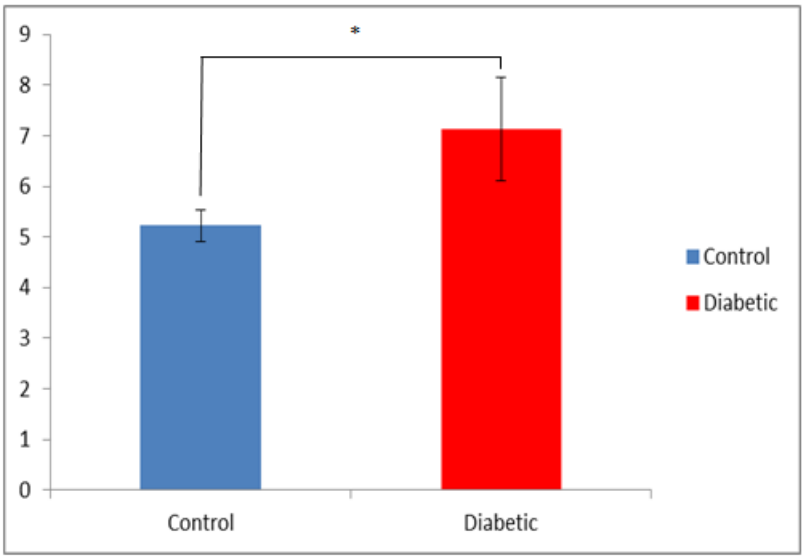


Figure 2: Comparison of HbA1c levels in control and diabetic subjects. Values are represented in Mean±SD (n=5). P<0.05.

P-value for blood glucose level was found to be 0.006 and P-value for HbA1c was found to be 0.028. Since P-value is less than 0.05, there exists significant variation in blood glucose level and HbA1c levels among control and test subjects.

3.2 Activation status of platelets by flow cytometry

Activated platelets secrete their granule contents into the circulation; as a result the expression of glycoprotein receptors on the platelet surface vary. P-selectin (CD62) is considered as a gold standard for the platelet activation marker. Results indicates that CD62 (P-selectin) expression increases in test subjects compared to control (figure 3). Results are represented as mean of three subjects.

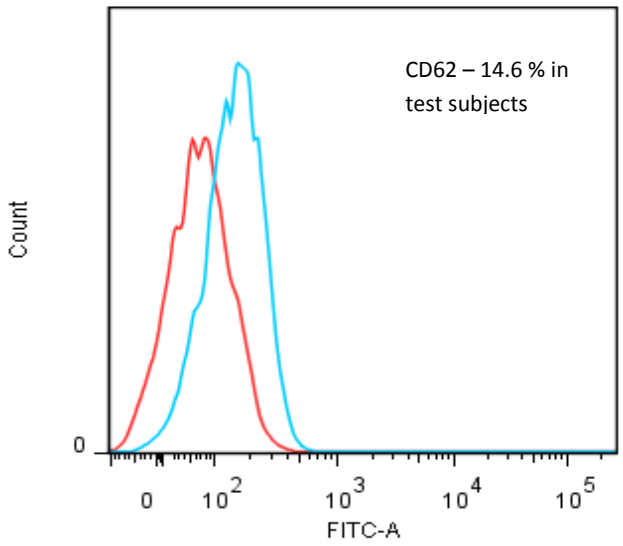


Figure 3: FACS analysis for platelet activation in control and test subjects. Red line denotes the control whereas blue denotes the test subjects.

	Control	Diabetic
Percentage of activated platelets.	6.28±5.7	8.2±7.6

Table 2: Comparison of platelet activation in control and test subjects.

Figure 3 is the representative figure for the flow cytometry analysis in platelets from control and test group. Here the graph represents fluorochrome on X-axis and count on Y-axis. X-axis represents the % of fluorochrome positive cells. The figure 3 clearly indicate that CD62 expression significantly increases in the test group.

P-selectin is a membrane marker which gets exposed even on mild activation of platelets. In the diabetic subjects as well as in the high risk groups like hypertension, and hypercholesterolemia, platelets are reported to be in their activated stage. P-selectin mediates rolling of monocytes on activated endothelium. In activated platelets, P- selectin is translocated from the alpha granules to surface of plasma membrane and mediates adhesion of platelet to neutrophils and monocytes.

It is clear from the data obtained in this study that test subjects have activated platelets in the circulation which may interact with monocytes to form monocytes platelet aggregates (MPA).

3.3 Monocytes Isolation and characterization:

Monocytes were isolated from the buffy coat by two methods and purity was analyzed by immunostainning and flowcytometry.

3.3.1Characterisation of monocytes by immunostaining

Figure 4 represents the CD14 stained monocytes isolated from blood using histopaque. Cells were stained with CD14-FITC and images were captured at 10X and 20 X magnification using inverted fluorescent microscope Olympus lX71.

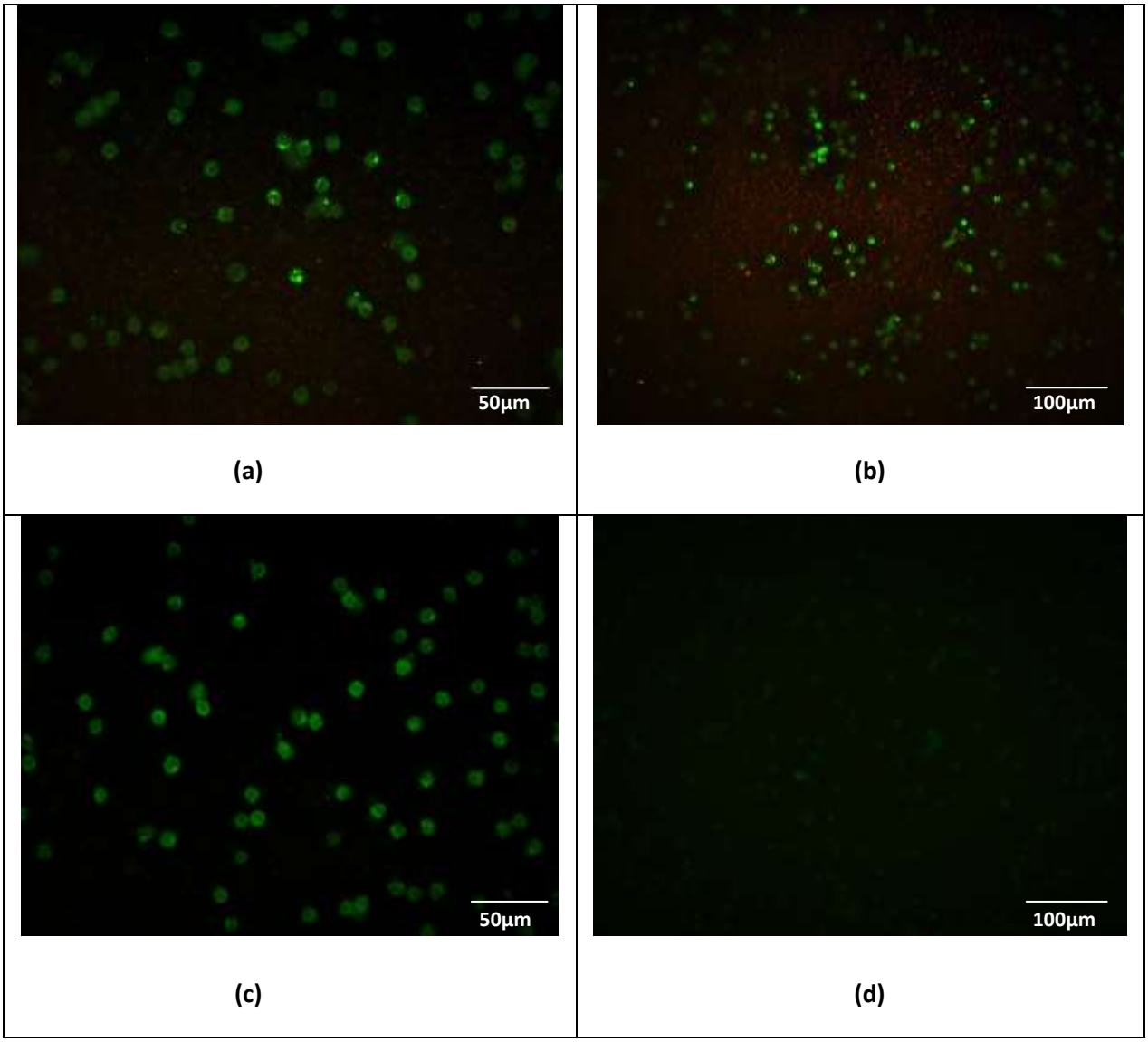


Figure 4: CD14-FITC immunostained images of cells isolated from control and test subjects by histopaque method. (a) and (b) shows the CD14-FITC stained monocyte population isolated from control subjects. (c) and (d) shows the CD14-FITC stained monocyte population isolated from test subjects.

Data clearly demonstrate that cells are monocytes. Another method was tried in order to improve the purity of cells and to get a more pure monocytes population. Percoll gradient method with a slight modification was tried and isolated cells were characterized. Representative images of cells isolated by histopaque-percoll density gradient method are shown in Figure 5.

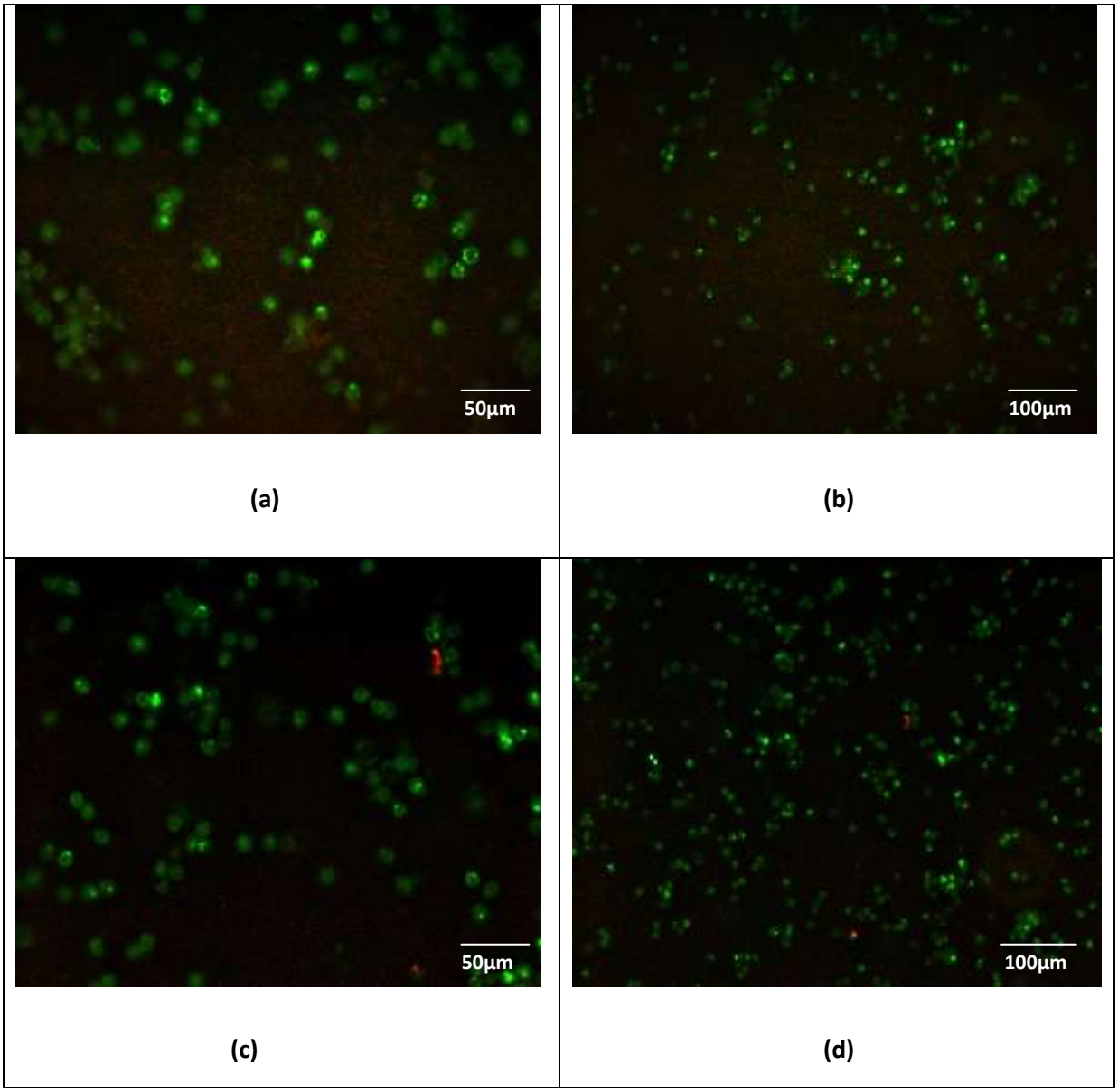


Figure 5: CD14-FITC immunostained images of monocytes obtained from buffy coat by histopaque Percoll density gradient method. (a) and (b) shows the CD14-FITC stained monocyte population isolated from control. (c) and (d) shows the CD14-FITC stained monocyte population isolated from diabetic subjects.

3.3.2 Characterisation of monocytes by flow cytometry

Purity of isolated Monocytes was further quantified by flow cytometry using CD14 antibody. CD14 is the monocyte specific cell surface marker expressed in normal conditions by the monocytes[40]. Representing FACS data is shown in figure 6. Here the individual graph represents fluorochrome on X-axis and count on Y-axis. X-axis represents the % of fluorochrome positive cells. More than 85% cells were

CD14 positive and experiment was repeated three times to ensure the repeatability of the protocol, which indicates the purity of the population and confirms the immunocytochemisrty data. The protocol was followed to isolate cells from control and test subjects and the cells were used to understand the monocytes platelet adhesion by ESEM as well as to study the inflammatory proteins released by test and control monocytes.

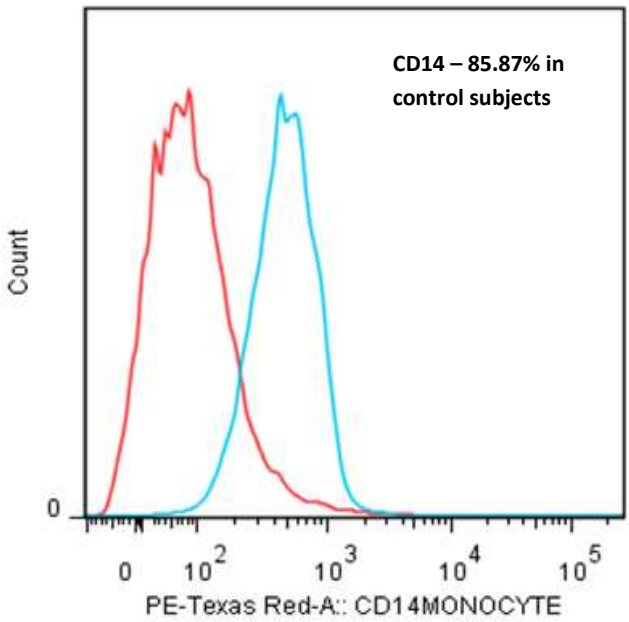


Figure 6: FACS analysis of CD14 positive monocytes. Red denotes unstained monocytes and blue denotes stained. Data are representative of 3 sets of experiments.

3.4 Platelet monocyte interactions

3.4.1 Platelet monocyte interaction analysis by ESEM

Platelets and monocytes isolated from control and test subjects were analyzed using Environmental Scanning Electron Microscope (ESEM- Quanta 200, Germany). Two approaches were used with fibrinogen matrix and without matrix. Images were captured at different magnifications.

3.4.1.1 ESEM analysis with fibrinogen coating

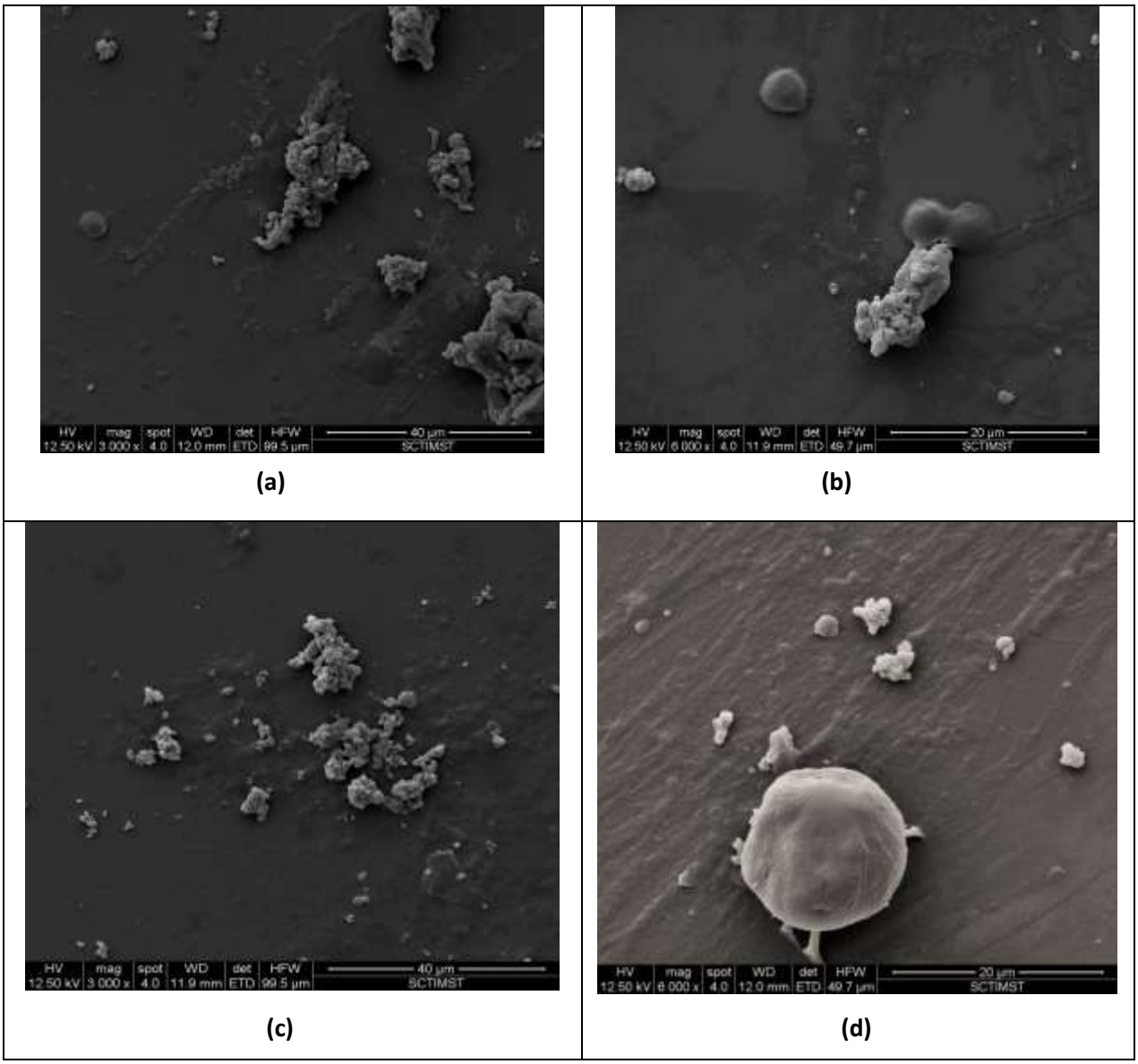


Figure 7: ESEM images of monocyte platelet interactions with fibrinogen coating. Monocytes and platelets on the surface of fibrinogen coated coverslips. (a) and (b) represent cells isolated from control subjects; (c) and (d) represent cells isolated from test subjects.

Figure 7 shows images of MPA on fibrinogen matrix at magnifications 3000X and 6000X. Not much of platelet and monocytes aggregates were observed from the test or control subjects. MPA were not observed so much on matrix, probably due to the adhesion of platelets to the fibrinogen with high affinity. To avoid that an approach without supporting matrix was tried and high MPA were observed in test

group compared to control, which confirms that MPA formation in test subjects are due to the activated platelets which is absent in the control subjects.

3.4.1.2 ESEM Analysis without fibrinogen coating

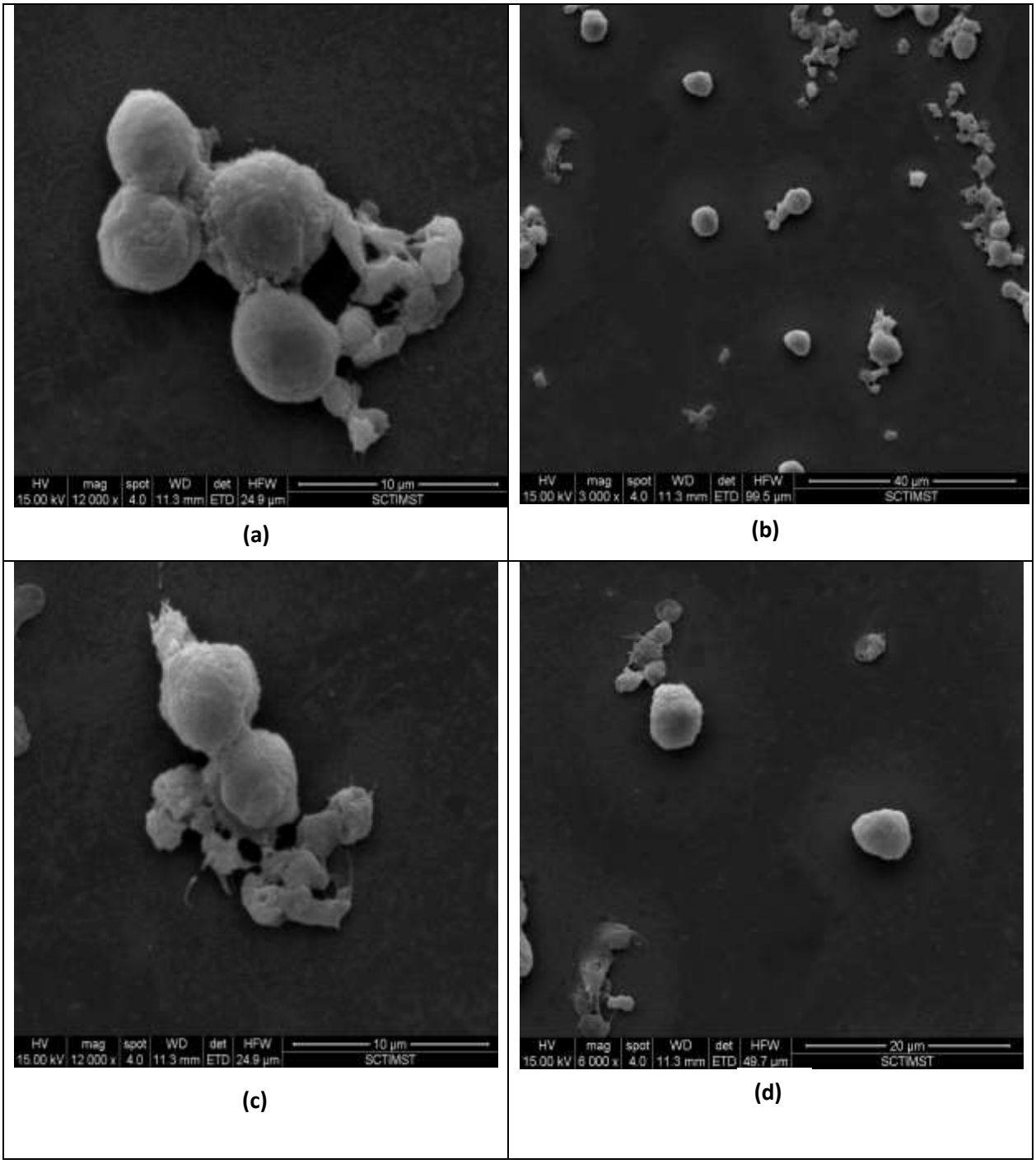


Figure 8: ESEM images of platelet-monocyte interactions in healthy individuals without fibrinogen coating. The interactions can be seen as platelet-monocyte aggregates. The monocyte-platelet aggregates are very few and most of the

monocytes and platelets can be seen as dispersed around. These images represent monocytes and platelets isolated from control subjects.

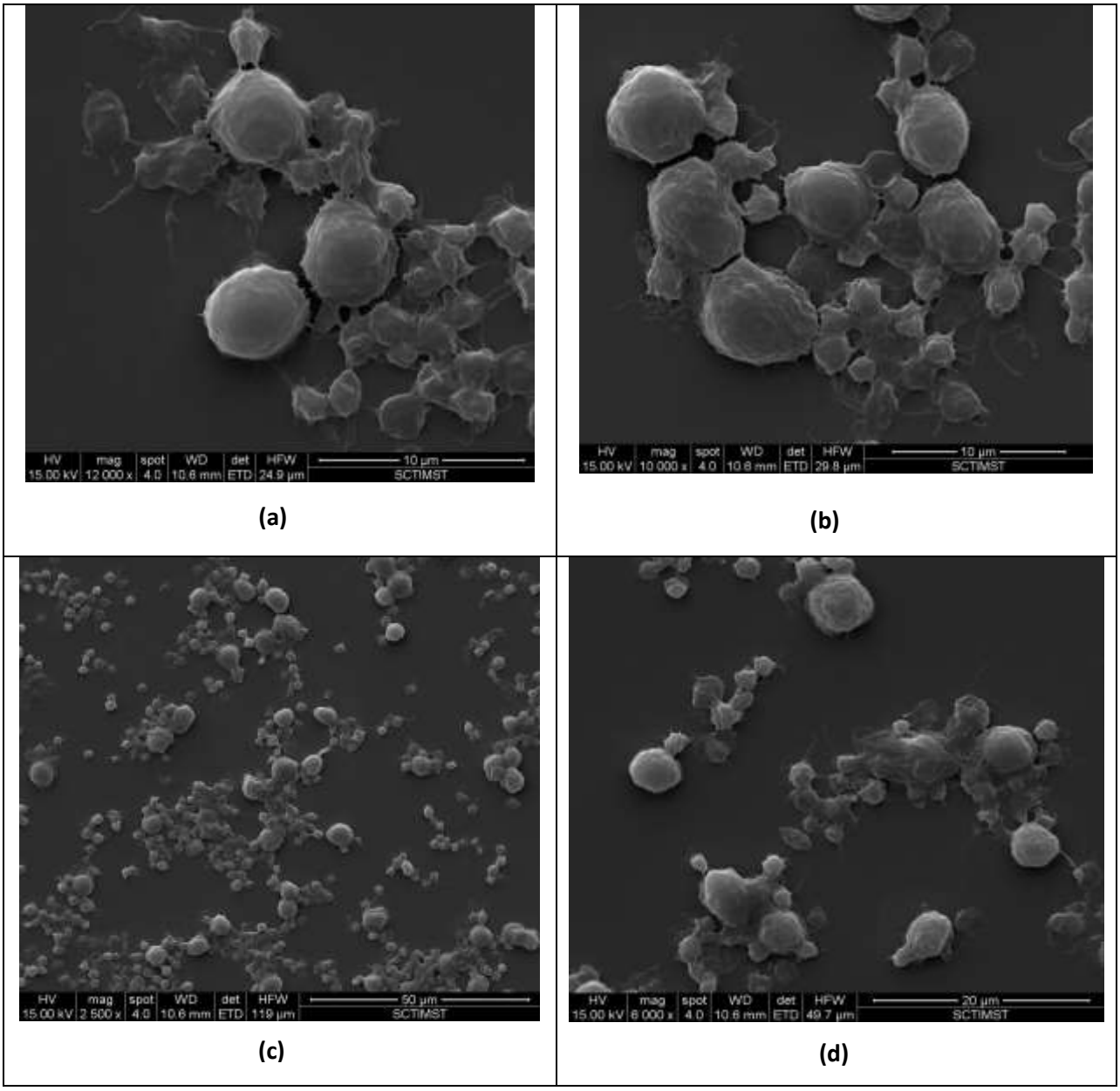


Figure 9: ESEM images showing platelet-monocyte interactions in diabetic individuals. Interactions can be seen in the form of monocyte-platelet aggregates. Smaller cells with extended pseudopodia like structures are the platelets which can be seen adhered to the surface of monocytes. Larger cells are the monocytes. (a), (b), (c) and (d) represent monocytes and platelets isolated from diabetic subjects.

Figures 8 & 9 shows the ESEM images from control and test group without any matrix support. It is evident that platelets spreads through the pseudopodia and

form aggregates with monocytes in test group while these aggregates were absent in the control group. Thus it is suggested that activated platelets has got high affinity towards monocytes and they form monocyte platelet aggregates with the help of various cell adhesion molecules that are expressed on the surface of both cells[25].

Activated platelets has got affinity towards monocytes. Platelets become active during inflammatory disease conditions like diabetes mellitus[41]. The levels of circulating monocyte platelet aggregates are also increased in diseases like acute myocardial infarction, hypertension etc[41]. The detection of monocyte platelet aggregates in patients can serve as a method for early detection of proinflammatory conditions. That is it can be used as a pathophysiological marker. The aggregate formation occurs via the various cell adhesion molecules expressed by both cell types. One such important cell adhesion molecule is the P-selectin molecule also known as CD62. This molecule is expressed by platelets on their surface when platelets become active. And this P-selectin molecule bind to the corresponding P-selectin binding ligand expressed on the surface of monocytes. Increased presence of monocyte platelet aggregates serve as an early detection marker of type 2 diabetes[25].It also depicts increased risk of atherosclerosis[25]. Platelet monocyte aggregates have also been found in acute coronary syndromes[42].

Activated platelets attract monocytes and form the aggregates. These aggregates attract more monocytes to the site and make the region inflammatory thereby contributing to the development of atherosclerosis. The monocyte platelet aggregates thus help in attachment of monocytes to endothelial cells of arterial blood vessels and contribute to atherosclerotic plaque formation[43].

3.4.2 Platelet monocyte interaction by flow cytometry

Flow cytometry analysis was done to determine monocyte platelet interactions occurring in control and diabetic subjects. The figure below shows the percentage of monocyte platelet interactions in control and diabetic subjects.

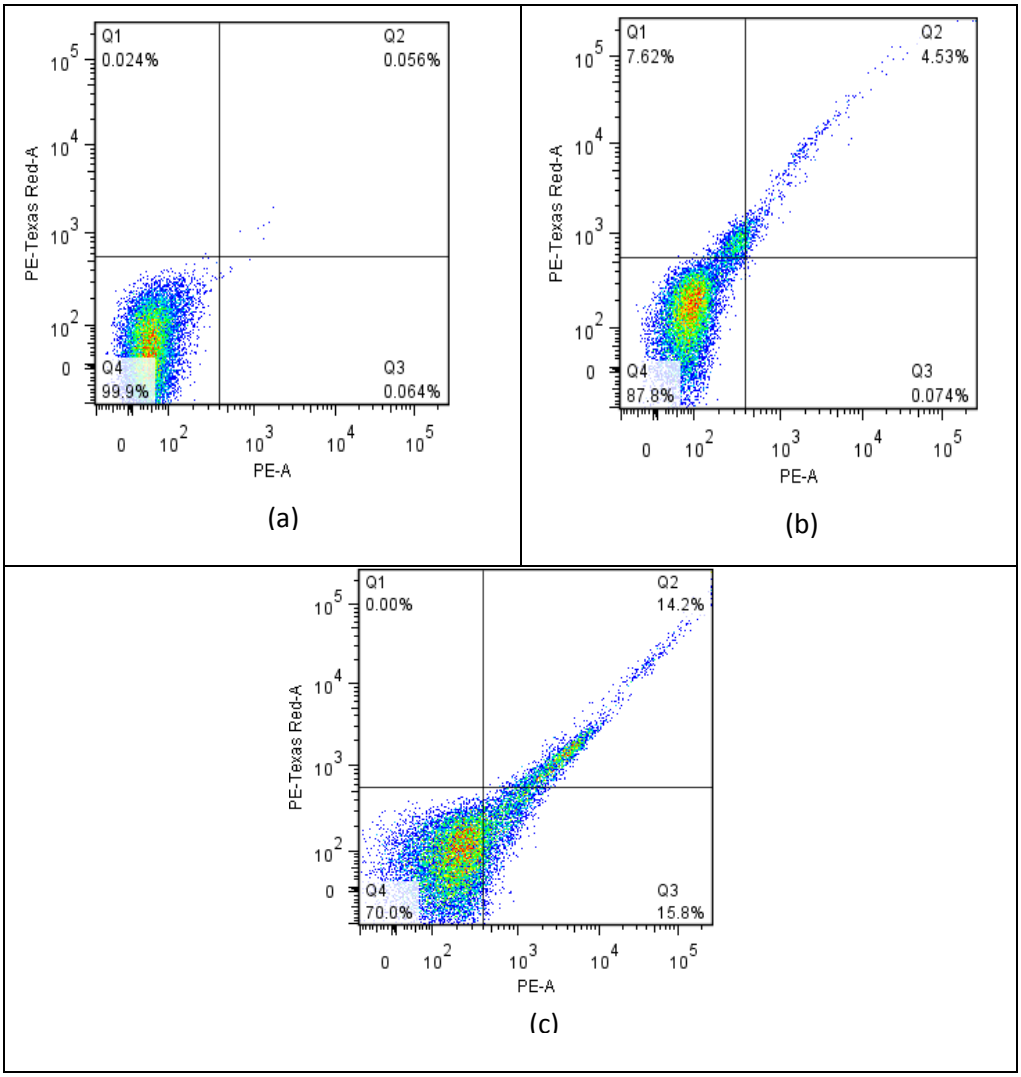


Figure 10: FACS analysis of monocyte platelet interactions. (a) unstained population of monocytes and platelets (b) stained monocyte platelet population from control subjects. (c) stained population of monocytes and platelets from diabetic subjects.

MPA formation was quantified using flowcytometry. Here the individual graph represents fluorochrome on X-axis and side scatter on Y-axis. Side scatter represents the granularity whereas X-axis represents the % of fluorochrome positive cells.

	Control	Diabetic
CD14/62	7.93±6.2	9.89±5.3

Table 3: Percentage of monocyte platelet aggregates in control and diabetic subjects. Values are represented in Mean±SD (n=3). P-value>0.05.

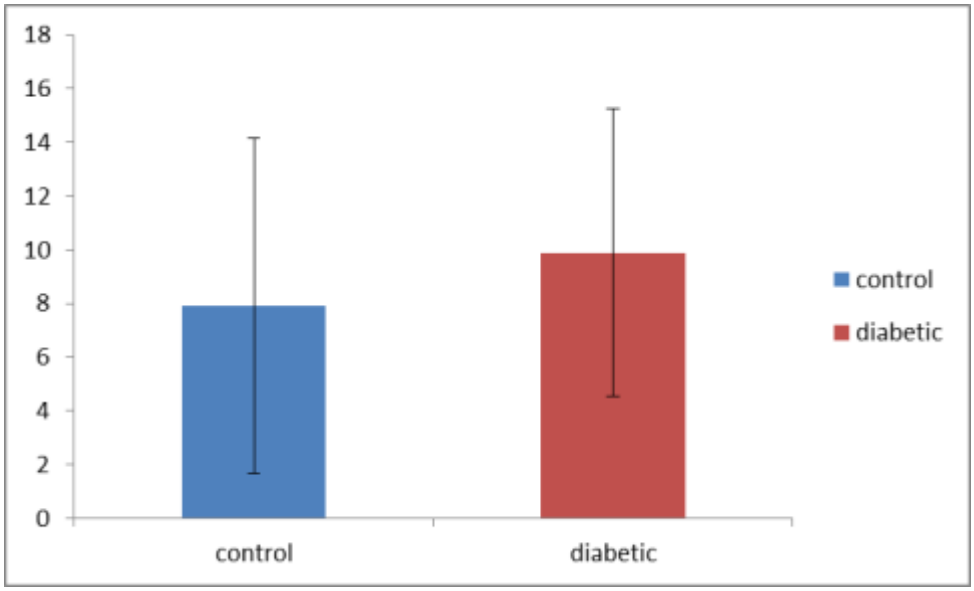


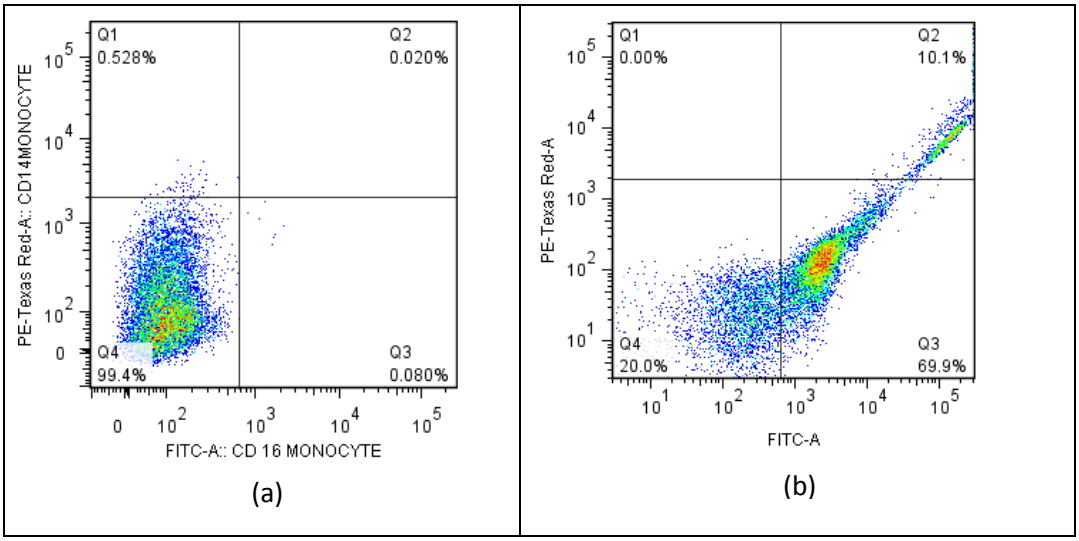
Figure 11: Comparison of monocyte platelet interaction in control and diabetic subjects. Values are represented in Mean±SD (n=3). P-value>0.05.

CD62 and CD14 antibodies were used to analyze the MPA formation. Figure 10 represents the FACS data, where dual positive cells ($CD62^{+}/CD14^{+}$) were considered as aggregates (MPA) and extent of formation of heteroaggregates is well correlated with the platelet activation. Higher the platelet activation, higher the percentage of MPA formed. These interaction are important as hyper activated platelets are reported in the inflammatory diseases and diabetic being an inflammatory disease, platelets tend to form aggregates with monocytes[42]. In our data though MPA were more in test group, but the changes were not significant, however ESEM images shown much higher MPA in test group compared to control.

Activated platelets and these interactions may leads to alteration of the monocytes phenotype to the proinflammatory $CD14^{+}/CD16^{+}$ phenotype, which may in-turn release the inflammatory cytokines and proteins which are primary responsible for the progression of inflammatory diseases and susceptibility of diabetes to the cardiac diseases.

3.5 Phenotypic variation in monocytes

For analyzing phenotypic alterations in monocytes, flow cytometry of monocytes in test and control subjects were performed. The figure below shows the percentages of alterations in the monocytes isolated from control and diabetic subjects. Here the individual graph represents fluorochrome on X-axis and side scatter on Y-axis. Side scatter represents the granularity whereas X-axis represents the % of fluorochrome positive cells. CD14 and CD16 were the two antibodies used for FACS analysis of monocyte phenotypic variations



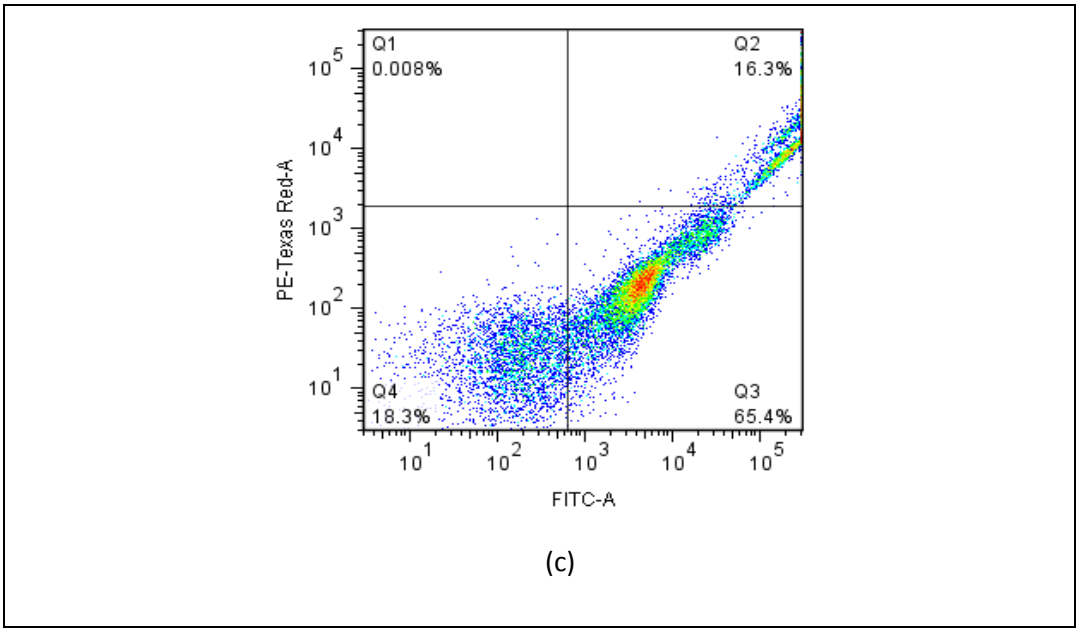


Figure 12: FACS analysis of phenotypic alteration in monocytes. (a) represents unstained monocytes. (b) represents CD14/CD16 stained monocytes from control subjects. (c) represents CD14/CD16 stained monocyte population from diabetic subjects.

	Control	Diabetic
CD14/16	9.44±3.22	12.21±3.65

Table 4: Percentage of double positive monocytes in control and diabetic subjects. Values are represented in Mean±SD (n=3). P value>0.05.

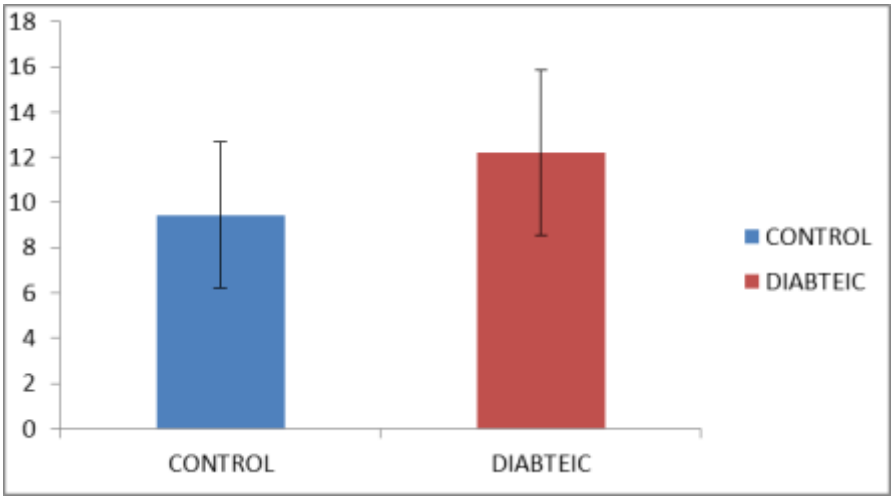


Figure 13: Comparison of degree of phenotypic alterations in control and diabetic subjects. Values are represented in Mean±SD (n=3). P value>0.05.

It was observed that CD14/CD16 positive population was high in test subjects compared to the healthy individuals. CD16 positive monocytes are considered to be proinflammatory in nature. There are three subsets of monocytes reported CD14+/CD16- the classical subset, CD14-/CD16+ and CD14+/CD16+ population. Monocytes are found as classical subset of $CD14^{+}$ /$CD16^{-}$ in normal physiological conditions, however in pathological conditions intermediate subset CD14-/CD16+ and proinflammatory subset CD14+/CD16+ increases, however not much is being reported about the presence of these subsets in diabetic subjects and their role in the inflammation in diabetes. Our study demonstrated high levels of CD14+/CD16+ subsets in diabetic subjects. Blood monocytes expressing both the CD14+ and CD16+ antigen constitute a proinflammatory subtype, which exhibit features of tissue macrophages. CD14+/CD16+ monocytes and CD14 were highly increased in patients with infectious and non-infectious inflammatory.There are a lot of reasons for such phenotypic alterations. One such most important cause for phenotypic alteration is the interaction of monocytes with platelets which might lead to alteration in phenotypes of monocytes. The change in phenotype of monocytes may results in the expression of proinflammatory markers on monocytes suggesting that inflammation plays an important role in phenotypic alteration of monocytes and there occurs a shift from classical monocyte population to non-classical monocyte population[44]. Thus in our study we explored the inflammatory markers secreted by monocytes to understand the impact of monocytes platelet interaction on inflammation, as inflammation is the key regulator for the diseases like diabetes and progression of cardiac diseases in diabetic subjects.

3.6 Analysis of inflammatory markers:

Monocytes proteins were isolated and quantified using Lowry's protein estimation. High concentration of protein was observed in the test subjects compared to the healthy individuals (Table 5), which indicates that synthesis or the secretion of proteins increase upon activation of the monocytes. Proteins were loaded on SDS-PAGE and western blot was performed for IL-10 and MCP-1.

	Control	Diabetic
Protein concentration (µg/10µl)	14.54±5.96	27.86±8.03

Table 5: Protein concentrations (in µg/10µl) in diabetic and healthy individuals**.**Values are represented in Mean±SD (n=5). P-value<0.05.

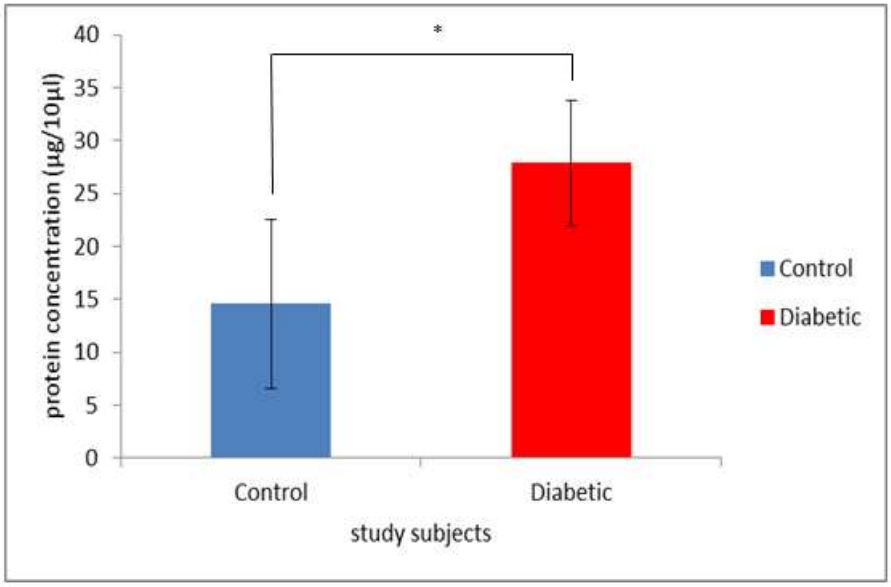


Figure 14: Comparison of protein concentrations in control and diabetic subjects. Values are represented in Mean±SD. P-value<0.05.

3.6.1 Monocyte proteome by SDS PAGE

Representative SDS-PAGE images from two replicates are shown here with the molecular weight marker (10-250kDA). Protein band intensity was determined

using Alpha Imager Documentation System 2000 and it was found to be high in test group compared to control.

SDS images also show more number of bands in the test group compared to the control. Band intensity profile of the bands in the low molecular weight also showed the presence of high intensity and more number of proteins in test group.

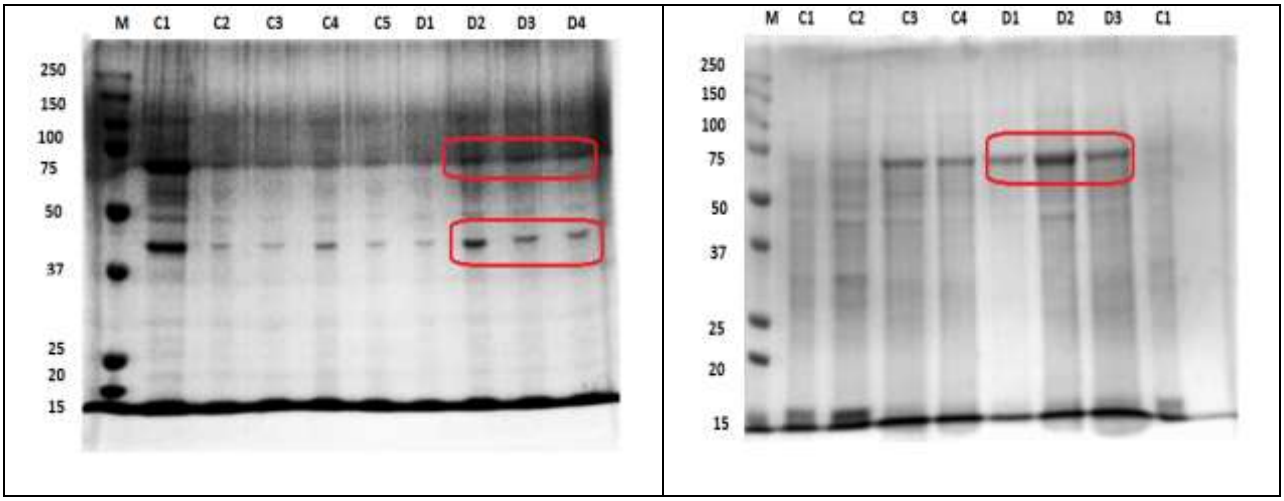


Figure 15: SDS PAGE images showing different protein bands of monocyte proteome isolated from control and diabetic subjects. Molecular weight range of protein marker (10-250kDa). C1-C5 represents monocyte proteins from control subjects. D1-D4 shows monocyte proteins from test subjects.

	Control	Diabetic
Integrated density value (IDV) of gel (a) of fig	59771.33 ± 5673.19	63011.33 ± 12623.12
Integrated density value (IDV) of gel (b) of fig	112437.3 ± 5053.58	119428 ± 10862.73

Table 6: comparison of band intensity of proteins isolated from control and diabetic subjects in terms of integrated density values. Values are given by Mean±SD (n=5). Pvalue >0.05.

3.6.2 Western blot analysis

Proteins were loaded on SDS-PAGE and western blot analysis was performed for IL-10 and MCP-1. Western blot analysis showed the presence of IL-10 and MCP-1 in test group while absent in control group. Figure 16 & 17 represents the blot for MCP-1 and IL-10 respectively from test and control group.

3.6.2.1 Western blot analysis of MCP-1

MCP-1 is one of the most important cytokine that plays an important role in recruiting monocytes to the sites of injury. Western blot of MCP-1 was tried in two different dilutions. 1:1000 and 1:500. The better one was 1:1000. Its expression was found to be in diabetic patients alone.

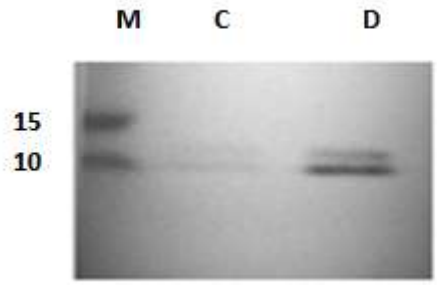


Figure 16: Western blot images for monocyte chemoattractant protein-1 (MCP-1). M-Protein marker (molecular weight range: 10kDa -250kDa), C-Control sample, D-Diabetic sample.

The molecular weight of MCP-1 ranges from 11-13kDa. The major function of MCP-1 is the recruitment of monocytes to the inflamed region. Apart from this another major function of MCP-1 is to regulate metabolism[45].Moreover in patients with acute myocardial infarction mcp-1 levels are found to be very high along with high glucose levels indicating that they are more prone to develop diabetes in future[46]. It also shows that increased levels of MCP-1 is an indicative of poor

regulation of blood glucose as previously discussed hyperglycemia contributes to increased levels of cytokines circulating in the blood. MCP-1 also plays an important role in nephropathy. The levels of MCP-1 in urine of diabetic patients was found to be significantly higher[47].This suggests that there occurs an upregulation of MCP-1 in diabetic patients with nephropathy and other inflammatory renal diseases such as glomerular lesions. So inhibitors to MCP-1 can act as important molecules that can reduce or down-regulate the inflammatory conditions. If MCP-1 molecule production can be reduced, it can cause reduced accumulation of macrophages at the inflammatory sites thereby decreasing the nephropathic conditions.

MCP-1 attracts monocytes to the inflammatory regions of the arterial walls of blood vessels and gets transformed into macrophage derived foam cells increasing the risk for atherosclerosis[48].When endothelial cells get exposed to higher glucose levels they cause increased expression of MCP-1 indicating that there occurs monocyte endothelial interactions which also may contribute to the increased risk for cardiovascular problems[48].

3.6.2.2 Western blot analysis of IL-10

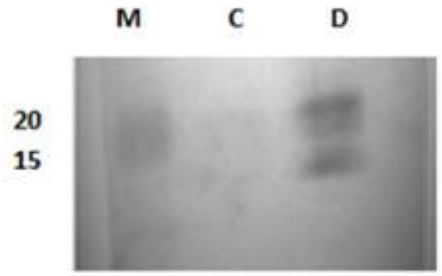


Figure 17: Western blot images for interleukin-10 (IL-10). M-Protein marker (molecular weight range: 10kDa -250kDa), C-Control sample, D-Diabetic sample.

Western blot of interleukin-10 (IL-10) was tried in two different dilutions. 1:1000 and 1:500. 1:500 was found to be better. IL-10 expression was found to be only in diabetic subjects. The molecular weight of IL-10 ranges from 18-20kDa. IL-10 also known as cytokine synthesis inhibitory factor (CSIF) is an anti-inflammatory molecule. It suppresses the secretion of other cytokines in order to reduce inflammation. During type 2 diabetes monocytes produce IL-10 so that other inflammatory cytokines are reduced in circulation[49].IL-10 also plays an important role in protection of endothelial cells from nitric oxide thus preventing the endothelial dysfunction[50]. IL-10 also indirectly involved in regulating the blood glucose levels by increasing insulin sensitivity[51]. IL-10 also plays an important role in regulation of immunological features by decreasing the expression of classII MHC molecules.

Chapter 4

Summary and Conclusion

4.1 Summary

Platelets are highly active in inflammatory conditions like diabetes mellitus. There are a lot of factors in plasma that contribute to platelet activation. Activated platelets interact with other cell types. The present study focused on studying the interaction between monocytes and platelets. Interaction of monocytes with platelets plays an important role in altering the phenotype and proteome of monocytes. During inflammation, platelets and monocytes begin to express more cell adhesion molecules which help in the formation of aggregates which can be heteroaggregates or homoaggregates.

Inflammation is marked by monocyte platelet interaction. As a result of this there are chances that some proteins in monocytes may get expressed in higher quantities in diabetic when compared to healthy individuals. The study was carried out to determine monocyte proteome variations and alteration in phenotype of monocytes in diabetic and healthy individuals. Monocytes exhibit different phenotypes which vary with the environmental stimuli to which they are exposed. Inflammation also results in the changes in the expression of inflammatory molecules. Monocyte platelet interaction may result in inflammatory molecule expression.

The objective of the study was to determine monocyte platelet interaction in diabetic and healthy individuals. As a result of this interaction whether any phenotypic changes are occurring in monocytes was also determined in the study. Monocyte proteins were isolated for studying whether there is any difference in the

protein expression between diabetic and healthy individuals. The study also focused on determining differences in inflammatory marker expression in monocytes. Various methods used include flow cytometry and ESEM analysis for determining monocyte platelet interaction, flow cytometry analysis for determining phenotypic alterations in monocytes, SDS PAGE for determining proteomic differences in monocyte proteome and western blot analysis for determining inflammatory molecule expression.

4.2 Conclusion

- Flow cytometry analysis using CD62 revealed that platelets in diabetic subjects are activated.
- The present study concludes that monocyte and platelets interact to form monocyte platelet aggregates during inflammatory conditions.
- Flow cytometry analysis (CD14/CD62) and ESEM analysis confirmed the interaction between monocytes and platelets and was found to be higher in diabetic subjects compared to control subjects.
- Alteration in phenotype of monocytes was confirmed by flow cytometry (CD14/CD16) and was observed to be higher in diabetic subjects.
- SDS PAGE showed that certain monocyte proteins are expressed at higher levels in diabetic than in control subjects.
- The study also concludes that monocytes show expression of inflammatory markers IL-10 and MCP-1 further confirming the intensity of inflammation in diabetic subjects.

4.3 Future aspects

The present study confirms the role of monocytes in inflammation. Monocytes can have deleterious effects on arterial endothelial cells. The effect of monocytes on endothelial cells can be studied to find out how the monocytes affect endothelial functioning. Specific proteins that are involved in disrupting the normal functioning of endothelial cells can be found out by MS-MS analysis.

4.4 Limitations of the study

Number of study subjects that were included for the study were minimum. All the diabetic subjects were on prescription drugs. The protein content measured was not correlated with the number of cells isolated.

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Appendix

Acid citrate dextrose (ACD-200ml)
Trisodium citrate – 4.4g
Citric acid - 1.6g
Dextrose – 5g
Store at 4^0C

Coomassie stain (100ml)
Acetic acid – 10ml
Methanol – 40ml
Distilled water – 50ml
Coomassie Brilliant Blue R250 – 0.1g

Destaining solution (100ml)
Acetic acid –10ml
Methanol – 50ml
Distilled water – 40ml

Developer solution (20ml)
Nickel chloride – 0.1g
DAB – 0.01g
Hydrogen peroxide - 40µl
PBS – 20ml

Lower tris (pH-8.8 50ml)
Tris base – 9.085g
10%SDS – 2ml

Phosphate buffered saline (PBS-1L) - pH-7.4
NaCl – 8g
KCl – 0.2g
Na_2HPO_4 – 1.44g
KH_2PO_4 – 0.24g
Store at room temperature

PBST buffer (100ml)
PBS – 100ml
Tween twenty - 100µl

Reagent A for Lowry (100ml)
2% Sodium carbonate – 2g
0.1N NaOH – 0.4g

Make up to 100ml with distilled water.

Reagent B for Lowry (100ml)
0.5% copper sulphate – 0.5g
1% Sodium potassium tartarate – 1g
Make up to 100ml with distilled water.

12% Resolving gel (6ml)
Distilled water – 1.92ml
30% acrylamide – 2.4ml
Lower tris – 1.56ml
10%SDS – 0.06ml
Ammonium persulphate – 0.06ml
TEMED – 0.0024ml

SDS sample loading buffer (4X- 10ml)
100Mm Tris (Ph6.8) – 2ml
4%SDS – 0.8g
0.2%BPB – 0.008g
100%Glycerol – 4ml
0.5M EDTA – 1ml

5% Stacking gel (2ml)
Distilled water – 1.4ml
30% acrylamide – 0.33ml
Lower tris – 0.25ml
10%SDS – 0.02ml
Ammonium persulphate – 0.02ml
TEMED – 0.002ml

Transfer buffer (100ml pH 8.2-8.5)
Tris – 0.58g
Glycine – 0.29g
SDS – 0.037g
Methanol – 20ml
Distilled water – 80ml

Tris glycine SDS buffer (10X – 1000ml)
Tris base - 30g
Glycine – 144g
SDS – 10g
Adjust the pH to 8.3.

Upper tris buffer (pH-6.8 50ml)
Tris base – 3.03g
10% SDS – 2ml