Chapter One

Introduction & Literature Review
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1.1 Introduction

1.1.1 Diabetes Mellitus

1.1.1.1. Definition
Diabetes mellitus is not a single disease entity, but rather a group of metabolic disorders sharing the common underlying feature of hyperglycemia (Mitchell et al., 2006).

1.1.1.2 Incidence of Diabetes Mellitus
Worldwide, over 140 million people suffer from diabetes making this one of the most common diseases. In the Western population the prevalence of DM has been estimated to be 3-5% and the incidence is rapidly growing up and will be more than doubled within 15 years. Type II DM accounts for more than 80% cases of DM and is slow-onset, heterogeneous disorder, resulting from interactions between environmental factors and polygenetic inheritance (Ostenson et al., 2001).

1.1.1.3 Classifications of Diabetes Mellitus
The World Health Organization guidelines recommend the following categories of diabetes: type 1, type 2, and gestational diabetes, and other specific types of diabetes. Type 1 or juvenile, diabetes mellitus mainly affects children and adolescents and is due to absolute or severe shortage of insulin. It is usually due autoimmune destruction of pancreatic beta cells which produce insulin. This makes it necessary for the patient to depend on insulin therapy- hence the name insulin dependent diabetes mellitus (IDDM). Type 2 or maturity onset, is caused by a combination of peripheral resistance to insulin action and inadequate compensatory response to insulin secretion" relative insulin deficiency". The patient may require insulin or may not need if the pancreas can be stimulated by
oral hypoglycemic agents- hence the name non-insulin dependent diabetes mellitus (NIDDM). Gestational diabetes mellitus is any degree of glucose intolerance with onset or first recognition during pregnancy. GDM is carbohydrate intolerance with onset or first recognition in pregnancy. Up to 70% of affected women will manifest type 2 diabetes mellitus within 10 years thereafter. The causes of GDM include metabolic and hormonal changes patients with GDM frequently return to normal postpartum. The other specific types associated with certain conditions (secondary) including genetic defects of beta cell functions or insulin action, pancreatic disease, diseases of endocrine origin, drug or chemical induced insulin receptor abnormalities, and certain genetic syndromes (Bishop et al., 2000).

1.1.1.4 Complications of diabetes mellitus

Long-standing diabetes mellitus inevitably leads to the development of vascular, renal and other pathologies. Some common long-term effects of DM include vascular complications (such as atherosclerosis, ischemic heart disease, ischemia and gangrene of the foot and microangiopathy), diabetic nephropathy (glomerular damage and renal failure), eye change (diabetic retinopathy and cataract), and nervous defects (Schneider et al., 2009).

1.1.1.5 Diabetes Mellitus and Vascular Diseases

Diabetes is associated with accelerated rates of thrombosis, circulatory dysfunction, and atherosclerosis. Most of the morbidity and mortality seen in patients with diabetes mellitus, especially in type II ('non-insulin dependent') diabetes, is the result of micro- and macro-vascular occlusive disease in which thrombosis plays an important part (Colwell et al., 2003).
1.1.2 Platelet.

1.1.2.1 Platelet Production (Megakaryopoiesis and Thrombopoiesis)
Platelets are produced in the bone marrow by fragmentation of the cytoplasm of megakaryocytes. The precursor of the megakaryocyte the megakaryoblast arises by a process of differentiation from haemopoietic stem cells. The megakaryocytes mature by endomitotic synchronous nuclear replication, enlarging cytoplasm volume as the number of nuclear lobes increases in multiples of two. At variable stages in development most commonly at eight nucleus stage the cytoplasm become granular and platelets are liberated. Platelet production follows formation of micro-vesicles in the cytoplasm of the cell which coalesce to form platelet demarcation membrane. Each megakaryocyte is responsible for the production of about 4000 platelets (Hoffbrand et al., 2001).

1.1.2.2 Circulating platelets and their life span
In steady state, when platelet production equals destruction, platelet turnover has been estimated at 1.2 to 1.5 $\times$ 10$^{11}$ cells per day (Hartge et al., 2007). The sites for platelet removal appear to be the spleen, the liver, and the bone marrow. The platelet count varies among the healthy population (1.5 to 3.5 $\times$ 10$^5$/μl) but remains within a fairly narrow range in any given individual (Zarbock et al., 2007).

1.1.2.3 Platelet structural and Functional Anatomy
In describing detailed platelet anatomy, most information is derived from transmission electron microscopy, and platelet structure is classified into four general areas:

1. The platelet surface.
2. The membranous structure.
3. The cytoskeleton (sol-gel-zone).
4. The granules (Greer et al., 2004).
1.1.2.4 Platelet Surface

The platelet surface includes the plasma membrane that separates intra- from extra cellular regions, it is exceptionally complex in composition, distribution and function, incorporating a number of glycoproteins and lipids into is phospholipids bilayer and integrating a variety of extra- and intra-platelet events such as permeability, agonist stimulation, and platelet adhesion, activation\secretion, and aggregation; and the glycocolyx that is a fuzzy layer of lipids, sugars, and proteins, coats the outside surface of the platelet plasma membrane, including the surface-connected tubular system, and interacts with both the plasma and the cellular components of the blood and blood vessels (Greer et al., 2004).

Platelets possess different types of surface molecules which interact with corresponding molecules on platelets and other cells such as intercellular adhesion molecule (ICAM), Von Willebrand factor (vWF), fibrinogen (FG), lymphocyte function-associated antigen-1 (LFA-1), and P-selectin glycoprotein ligand-1 (PSGL-1). The Glycoprotein IIb/IIIa integrin constitutes the most abundant platelet adhesion receptor. The GIIb/IIIa receptor is an important molecule for the aggregation of platelets and platelet-neutrophil-interaction. Upon activation, platelet GPIIb/IIIa binds soluble extracellular adhesion molecules, such as vWF, fibrinogen, fibronectin, and thrombospondin. The absence of GPIIa/IIIb in Glanzmannthrombasthenia is associated with a severe bleeding due to defective platelet aggregation and clot retraction (Zarbock et al., 2007).
Table 1.1 Contents of the three different granule subpopulations (Zarbock *et al.*, 2007)

<table>
<thead>
<tr>
<th>Granules</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dense granules</td>
<td>Nucleotides (ATP, ADP, GTP, GDP)</td>
</tr>
<tr>
<td></td>
<td>Amines (Serotonin, Histamine)</td>
</tr>
<tr>
<td></td>
<td>Bivalent cations</td>
</tr>
<tr>
<td>a-granules</td>
<td><strong>Adhesion molecules</strong></td>
</tr>
<tr>
<td></td>
<td>P-selectin (CD62P)</td>
</tr>
<tr>
<td></td>
<td>Platelet endothelial cell adhesion molecule- (PECAM 1)</td>
</tr>
<tr>
<td></td>
<td>Glycoprotein IIb/IIIa (GPIIb/IIIa, αIIbβ3 integrin)</td>
</tr>
<tr>
<td></td>
<td>von Willebrand factor (vWF)</td>
</tr>
<tr>
<td></td>
<td>Thrombospondin-1 (TSP1)</td>
</tr>
<tr>
<td></td>
<td><strong>Mitogenic factors</strong></td>
</tr>
<tr>
<td></td>
<td>Platelet-derived growth factor (PDGF)</td>
</tr>
<tr>
<td></td>
<td>Vascular endothelial growth factor (VEGF)</td>
</tr>
<tr>
<td></td>
<td><strong>Coagulation factors</strong></td>
</tr>
<tr>
<td></td>
<td>Fibrinogen, Kininogens, Factors V, VII, XI, XIII</td>
</tr>
<tr>
<td></td>
<td><strong>Protease inhibitors</strong></td>
</tr>
<tr>
<td></td>
<td>Cl inhibitor</td>
</tr>
<tr>
<td></td>
<td>Plasminogen activator inhibitor-1 (PAI-1)</td>
</tr>
<tr>
<td></td>
<td>Tissue factor pathway inhibitor (TFPI)</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>Glycosidases, Proteases, Cationic proteins</td>
</tr>
</tbody>
</table>

1.1.2.5 Platelet Granules

Considering platelet granules and organelles, platelet process secretary granules and mechanisms that serve these purpose by releasing additional stimulatory materials previously sequestered within the resting platelet, into the environment for developing a haemostatic or thrombotic mass. Two main secretary granules, the alpha granules and dense bodies, appear to be the main effectors with their highly reactive and readily available contents. The role of these other platelet granules (lysosomes,
peroxisomes) and organelles such as mitochondria is less dramatic than those of the alpha granules and dense bodies (Greer et al., 2004).

1.1.2.6 Platelet Structure
Under light microscopy, platelets appear as small, a nucleated fragments with occasional reddish granules, measuring approximately 2 μm in diameter with a volume of approximately 8 fl. By scanning electron microscopy, circulating platelets appear as flat discs with smooth contours, rare spiny filopodia, and random openings of a channel system, the surface-connected canalicular system (SCCS), which invaginates throughout the platelet and is the conduit by which granule contents exocytose after stimulation. Platelets are roughly discoid shape and contain cytoplasmic organelles, cytoskeletal elements, invaginating open-canalicular membrane systems, and platelet-specific granules called alpha and dense granules. Platelets have numerous intrinsic glycoproteins attached to the outer surface of their plasma membrane that are receptors for such ligands as fibrinogen, collagen, thrombin, and thrombospondin to von Willebrand factor and fibronectin (Randriamboavonjy and Fleming 2010).

1.1.2.7 Platelet function
The main function of platelets is the formation of mechanical plugs during the normal haemostatic response to vascular injury. In the absence of platelets spontaneous leakage of blood through small vessels may occur. Central to their function are platelets activation, adhesion, secretion, aggregation, fusion and procoagulant activity. Other functions for platelets are also known: stimulation of leucocytes to accumulate around the platelet plug; that is, they may release chemotactic substances, release of some vasoactive amines and transportation of serotonin from sites of synthesis to other sites of function (Wekseler and Coupal 1973).
Platelet activation

Physiologic stimuli that can activate platelets both in vivo and in vitro are amazingly diverse. Substances shown to produce this reaction include materials as diverse as proteolytic and structural proteins (such as thrombin and collagen), vasoactive material (such as serotonin and epinephrine), nucleotides (such as ADP), polypeptides hormones (such as vasopressin), and non-biologic surfaces (such as glass and latex particles). Other stimuli arise under pathologic conditions. Among these are antigen-antibody complexes or aggregated gamma globulins that react with an Fc receptor on human platelets and a complement receptor on rabbit platelets. The responses to these stimuli are initiated when the agonists bind to specific receptors on the plasma membrane (Randriamboavonjy and Fleming 2010).

Platelets respond to a variety of substances by changing their shape, and then become sticky so that they aggregate if brought into contact by stirring, and finally secrete the contents (Holmsen et al., 2002).

Platelet shape change

Most stimuli cause a change in shape and this change involves first the formation of very fine pseudopodia (i.e., filopodia) from the rim of the disc, followed by a general "rounding up" of the platelet so that it
becomes a spiny sphere, often with much broader pseudopodia (Majumder B et al., 2012). Platelet shape change can be measured in vitro by flow cytometry or electron microscopy. Shape change with or without secretion causes the microtubule bundle that lies beneath the rim of the disk to become centralized and surround the platelet granules, which are consequently concentrated toward the center of the platelet (Randriamboavonjy and Fleming 2010).

1.1.2.10 Platelet Adhesion
The adhesion of platelets to the collagen exposed on endothelial cell surfaces is mediated by Von Willebrand factor (Tracy et al., 2001). The function of vWF is to act as a bridge between a specific glycoprotein on the surface of platelets (GPIb/IX) and collagen fibrils. In addition to its role as a bridge between platelets and exposed collagen on endothelial surfaces. Adhesion to this surface is associated with loss of granules (i.e., secretion), which is not inhibited by non-steroidal anti-inflammatory (NSAI) agents (Michiels et al., 2005).

1.1.2.11 Platelet release reaction
Collagen exposure or thrombin action results in the secretion of platelet granule content. The initial activation of platelets is induced by thrombin binding to specific receptors on the surface of platelets, thereby initiating a signal transduction cascade. The thrombin receptor is coupled to a G-protein that, in turn, activates phospholipase C-g (PLC-g). PLC-g hydrolyzes phosphatidylinositol-4, 5-bisphosphate (PIP2) leading to the formation of inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces the release of intracellular Ca2+ stores, and DAG activates protein kinase C (PKC) (Majumder B et al., 2012). The collagen to which platelets adhere as well as the release of intracellular Ca2+ leads to the activation of phospholipase A2 (PLA2), which then hydrolyzes membrane phospholipids, leading to liberation of Arachidonic acid (Theroux et al.
Arachidonic acid release leads to an increase in the production and subsequent release of thromboxane A$_2$ (TXA$_2$). TXA$_2$ is a potent vasoconstrictor and inducer of platelet aggregation by lowering platelet cAMP. The release reaction is inhibited by substances which increase the levels of cAMP. One such substances, is the prostaglandin prostacyclin (PGI$_2$) which is synthesized by vascular endothelial cells. It is a potent inhibitor of platelet aggregation and prevents their deposition on normal vascular endothelium (Newby et al., 2001).

1.1.2.12 Platelet aggregation

ADP, thrombin, serotonin, vasopressin, and epinephrine initiate aggregation within a few seconds. Fluid-phase calcium or magnesium is necessary for aggregation. In addition, fibrinogen is also required. With ADP or epinephrine, this protein must be present in the suspension medium, whereas with thrombin or collagen it is secreted from the alpha granules (Hou et al. 2015).

Another enzyme activated by the released intracellular Ca$^{2+}$ stores is myosin light chain kinase (MLCK). Activated MLCK phosphorylates the light chain of myosin which then interacts with actin, resulting in altered platelet morphology and motility. One of the many effects of PKC is the phosphorylation and activation of a specific 47,000-Dalton platelet protein. This activated protein induces the release of platelet granule contents; one of which is ADP. ADP further stimulates platelets increasing the overall activation cascade; it also modifies the platelet membrane in such a way as to allow fibrinogen to adhere to two platelet surface glycoproteins, GPIIb and GPIIIa, resulting in fibrinogen-induced platelet aggregation (Newby et al., 2001).
1.1.2.13 Platelet procoagulant activity

Once platelet aggregates are formed, there is a tendency for the fibrin threads to be laid on them to form a clot. This process is facilitated by the platelets, possibly via more than one mechanism (Kamath et al., 2001). Phospholipids on the platelet membrane support the intrinsic pathway of coagulation, which results in the formation of thrombin from prothrombin by activated factor X. The platelet surface also prevents active coagulation factors from inactivation by their natural inhibitors. Considering platelet release reactions, platelet factor 4 looks to possess anti heparin activity, and fibrinogen that is released from platelet granules may potentially contribute further to the formation of the thrombus. Also, P-selectin expression could result in platelet–leukocyte interaction making fibrin deposition by the leukocytes to form thrombus (Palabrica et al., 1992). In summary the procoagualt activity of platelet called by platelets; the coagulation process is a complex series of enzymatic reactions involving the proteolytic activation of circulating coagulation factors (zymogens) and activity of cofactors (V, VIII), leading to production of thrombin which converts soluble plasma fibrinogen into fibrin. The fibrin enmeshes the platelet plug, forming a stable thrombus which prevents further blood loss from the damaged vessel (Cheesbrough et al., 2000).

1.1.3 Platelet Indices

Circulating platelets are very different in size, metabolism, and functional activity. Automated counters provide platelet counts and generate the MPV and a measure of their size variability (PDW). The great dispersion of platelet volumes (log-normal distribution) depends on the process of platelet production, by fragmentation of cytoplasm of megakaryocytes and pro platelet formation (Buttarello et al., 2008). Several investigators have used a series of platelet indices measured by hematology analyzers,
given the fact that platelet activation causes morphologic changes of platelets. Recently, platelet indices; MPV and PDW have been investigated as prospective platelet activation markers (Boos et al., 2007). The present effort for finding simple and widely used platelet activation indices focused on the fact that platelet activation causes morphologic changes of platelets, including both the spherical shape and pseudopodia formation. Platelets with increased number and size of pseudopodia differ in size, possibly affecting PDW. MPV and especially PDW increase during platelet activation, as depicted by automated hematology analyzers. PDW seems to be a more specific indicator of platelet activation than MPV, since it was not elevated during single platelet distention caused by platelet swelling. The combined use of MPV and PDW could predict activation of coagulation more efficiently (Vagdatli et al., 2010).

1.1.3.1 Mean Platelet Volume (MPV)

1.1.3.1.1 Definition

The mean platelet volume is an indication of platelet size. Normal MPV ranges are approximately 7 to 11 fl (Corcoran and Marchant, 2002).

1.1.3.1.2 Clinical Value of MPV

Thromboembolic diseases are among the major cause of mortality in developed countries. Early diagnosis of progressive activation of coagulation can help manage these diseases successfully. A significant list of reliable markers have been investigated recently, concerning activation of coagulation, such as prothrombin fragment, thrombin-antithrombin complex (TAT), and platelet activation, such as β-thromboglobulin (β-TG) or soluble platelet P-selectin1. However, laboratory measurement of these indices is laborious and expensive. Additionally, the above mentioned indices cannot be included in routine laboratory tests (Vagdatli et al., 2010).
The MPV can be an indication of platelet turnover because younger platelets tend to be larger. A spectrum of platelet sizes is seen in patients with rapid turnover (Corcoran and Marchant 2002). The Largest platelets are more reactive and release a greater quantity of thrombogenic factors as they contain more dense granules and produce more thromboxane A2. Increased MPV has been associated with greater in vitro aggregation in response to ADP and collagen. Platelet volume seems to be correlated with megakaryocyte ploidy as, the increase of MPV in conditions with increased platelet turnover is probably mediated by several cytokines (interleukins 6 and 11 and thrombopoietin) that affect megakaryocyte ploidy and result in the production of larger and more reactive platelets (Hou et al. 2015). Several experimental and clinical studies have demonstrated that platelet size and function correlate since large platelets are hemostatically more reactive than platelets of normal size. Elevated MPV levels have been identified as an independent risk factor for thrombotic diseases (Buttarello et al., 2007).

1.1.3.1.3 Causes of raised MPV

MPV is significantly increased in:
1. Idiopathic thrombocytopenic purpura.
2. Bernard- Soulier disease.
3. May-Hegglin anomaly.

1.1.3.1.4 Causes of low MPV

MPV is significantly decreased in:
1. Aplastic anemia.
2. Wiskott-Aldrich syndrome.
3. Thrombocytopenia-absent radii (TAR).
4. Storage Pool disease (Hou et al. 2015)
1.1.3.1.5 Influences of drugs on MPV
Little is known about effects of various drugs on platelet size. Previous in vitro studies found no effect of aspirin on platelet size. Clinical data on a possible association of MPV with various platelet inhibitors in patients do not exist (Greisenegge et al., 2004).

1.1.3.2 Platelet distribution width (PDW)
The PDW can also be useful in differentiating reactive thrombocytosis from the essential type, especially when it is combined mathematically with the MPV and platelet count to obtain a discriminate function (Osselaer et al., 1997). PDW is also potentially useful marker for the early diagnosis of thromboembolic diseases and an increase in PDW due to platelet activation, resulting from platelet swelling and pseudopodia formation was hypothesized, however PDW is reported to be a more specific marker of platelet activation than MPV, since it does not increase during simple platelet swelling (Vagdatli et al., 2010).

1.1.3.3 Platelet indices measurement
The widespread availability of particle counters in the clinical laboratory now permits the accurate measurements of MPV, on a routine practice. MPV is included among other haematological parameters in some automated blood counters in which the blood cells are counted in systems based on either Aperture impedance (voltage – pulse) or light – scattering technology (electro–optical counting). The aperture impedance technology depends on the fact that red cells are poor conductors of electricity while certain diluents are good conductors, this difference form the basis of the counting system used in Beckman –coulter, Sysmex, Abbott, Roche and a number of other instruments (Greer et al., 2004).
1.1.3.3.1 Detection principle
Blood sample is aspirated, measured to a predetermine volume, diluted at the specified ratio, then fed into each transducer. The transducer chamber has a minute hole called the aperture. On both side of the aperture, there are the electrodes between which flows direct current. Blood cells suspended in the diluted sample pass through the aperture, causing direct current resistance to change between the electrodes. As direct current resistance changes, the blood cell size is detected as electric pulses. Blood cell count is calculated by counting the pulses, and a histogram of blood cell sizes is plotted by determining the pulse sizes. Also, analysing a histogram makes it possible to obtain various analysis data (Greer et al., 2004).

1.1.3.3.2 Platelets discriminator
As to platelet lower discriminator and upper discriminator, the optimum position 2 -6 fl and 12-30 fl, respectively, are automatically determined by microcomputer. Platelets are calculated from the particle counts between this lower discriminator and upper discriminator (approximately 2-30 fl) (Majumder B et al., 2012)

1.1.3.3.3 Platelet Histogram
Platelet histogram is analysed using three discriminators: two discriminators (LD) and (UD) – determined automatically between 2 – 6 fL and between 12 – 30 fL, respectively and fixed discriminator at 12 fL. Regarding platelet histogram, check is made to see that there are no relative frequency errors at discriminators (LD) and (UD), distribution width error, and there is a single peak. (Majumder B et al., 2012)
1.1.3.3.2.2 Platelet distribution width DW

PDW is the distribution width on 20% frequency level with the peak taken as 100%. The unit applied is fL (femto-liter = 10^{-15}).

![Figure 1.2 Histogram of platelet size distribution and the definition of platelet size deviation width (PDW), and platelet-large cell ratio (P-LCR).](image)

**Figure 1.2 Histogram of platelet size distribution and the definition of platelet size deviation width (PDW), and platelet-large cell ratio (P-LCR).** (Sysmex, 1998)

1.1.3.3.2.3 Mean Platelet Volume

MPV is calculated by the following formula:

\[
\text{MPV (fL)} = \frac{\text{PCT} \, (\%)}{\text{PLT} \times 10^3/\mu \text{L}} \times 1000
\]

Where PCT (%) represents the value weighted with PLT frequency and is called platelet-crit or platelet volume ratio. It is calculated by PLT pulse height detection method.

1.1.4 Normal Values of Platelets Indices

Reference intervals (normal population reference ranges) were developed for the KX-21N using normal individuals the ranges for each parameter -- PDW, MPV, P-LCR were determined and displayed in Table 1.2(Sysmex 1998).
Table 1.2: Normal Population Reference Ranges (Sysmex 1998)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range for females</th>
<th>Range for males</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDW (fL)</td>
<td>9.4 – 18.1</td>
<td>9.8 – 18.0</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>8.5 – 12.4</td>
<td>8.1 – 12.4</td>
</tr>
<tr>
<td>Plts (X 10³/μL)</td>
<td>150-450</td>
<td>150-450</td>
</tr>
</tbody>
</table>

1.1.5 Platelets as accelerators of atherothrombosis

Abundant recent data support the concept of atherosclerosis as a chronic inflammatory disease. Vascular lesions leading to the exposure of ECM proteins such as vWF and collagen to the blood and platelet adhesion to the exposed matrix is considered to be the initial step in thrombus formation. Platelets adhere to vWF via the membrane adhesion receptor glycoprotein Ib/IX/V (GPIb/IX/V) and to collagen via GPVI (Nieswandt and Watson 2003).

Platelets contribution to the process of atherosclerosis is explained by:

- Platelets adhere to the arterial wall in vivo even in the absence of EC denudation.
- Platelets adhere to predilection sites of atherosclerosis before lesions are detectable.

Circulating activated platelets and platelet–leukocyte/monocyte aggregates promote formation of atherosclerotic lesions (Huo et al., 2003).

Platelet activation has been reported for a variety of atherosclerotic diseases; such as coronary artery disease and transplant vasculopathy. Enhanced systemic platelet activation correlates with progression of intima media thickness of the carotid artery in type 2 diabetes (Fateh et al., 2005).
1.1.6 -Endothelial dysfunction mediates CVD in type II DM
In patients with type II DM, the major cause of mortality and morbidity is CVD and in the increased CVD risk found in persons with diabetes, dysfunction of the vascular endothelium plays an important role. Alterations in the vascular endothelium linked to diabetes that contribute to endothelial dysfunction include elevated expression and plasma levels of vasoconstrictors such as angiotensin II and endothelin-1, increased expression of adhesion molecules and associated enhanced adhesion of platelets and monocytes to vascular endothelium, plus impairment of NO release and reduced NO responsiveness (Sowers et al., 2001).

1.1.7 Platelet alterations in diabetes mellitus
The abnormal metabolic state that accompanies diabetes renders arteries susceptible to atherosclerosis by altering the functional properties of multiple cell types, including endothelium and platelets (Eibl et al., 2004).

1.1.7.1 Increased reactivity and adhesion
Increased platelet reactivity and adhesion are reported in DM and induction of hyperglycemia and hyper-insulinemia in healthy subjects without diabetes increases platelet reactivity. Also, improved glycemic control has been associated with decreased platelet reactivity (Eibl et al., 2004).

Hyperglycemia can increase platelet reactivity by inducing non enzymatic glycation of proteins on the surface of the platelet and such glycation decreases membrane fluidity and increases the propensity of platelets to activation (Schneider et al., 2009).

Osmotic effect of glucose is a second mechanism by which hyperglycemia can increase platelet reactivity. People with diabetes exhibit increased expression of the surface glycoproteins Ib and IIb/IIIa (Schneider et al., 2009).
1.1.7.2 **Amplified agonist-receptor coupling**

“Primed” diabetic platelets respond more frequently even to sub threshold stimuli, sooner become exhausted, and thus contributing to accelerated thrombopoiesis and release of 'fresh' hyperactive platelets. In diabetes disturbed carbohydrate and lipid metabolism may lead to physicochemical changes in cell membrane dynamics, and consequently result in altered exposure of surface membrane receptors. Also, spontaneous aggregation (i.e., the formation of small-sized aggregates in the absence of agonist stimulation) occurs at a high rate in platelets from NIDDM patients (Schneider et al., 2009).

1.1.7.3 **Increased capacity for prostanoid generation**

Platelets obtained from diabetic patients possess increased activity of the prostaglandin syntheses system, and this characteristic may be related to the increased platelet aggregation associated with the disease (Kiritoshi et al. 2003).

1.1.7.4 **Decreased capacity for nitric oxide (NO-) generation**

Nitric oxide (NO) has been shown to have important anti-platelet actions as well as vascular mediator. By activating guanylyl cyclase, inhibiting phosphoinositide 3-kinase, impairing capacitive calcium influx, and inhibiting cyclooxygenase-1, endothelial NO limits platelet activation, adhesion, and aggregation. Platelets are also an important source of NO, and this platelet-derived NO pool limits recruitment of platelets to the platelet-rich thrombus. A deficiency of bioactive NO is associated with arterial thrombosis in animal models, individuals with endothelial dysfunction, and patients with a deficiency of the extracellular antioxidant enzyme glutathione peroxidase-3. This enzyme catalyzes the reduction of hydrogen and lipid peroxides, which limits the availability of these reactive oxygen species to react with and inactivate NO. The complex biochemical reactions that underlie the function and inactivation
of NO in the vasculature represent an important set of targets for therapeutic intervention for the prevention and treatment of arterial thrombotic disorders (Bitar et al., 2005).

1.1.7.5 Enhanced generation of reactive oxygen species
Reactive oxygen species (ROS) influence many physiological processes including host defense, hormone biosynthesis, fertilization, and cellular signaling. Increased ROS production (termed “oxidative stress”) has been implicated in various pathologies, such as diabetes. ROS plays a physiological role in the regulation of endothelial function and vascular tone and a pathophysiological role in endothelial dysfunction, inflammation, hypertrophy, apoptosis, migration, fibrosis, angiogenesis, and rarefaction, important processes underlying cardiovascular and renal remodeling in hypertension and diabetes (Kiritoshi et al. 2003).

1.1.7.6 Resistance to NO- and prostacyclin
Loss of sensitivity to the normal restraints exercised by prostacyclin (PGI2) and nitric oxide (NO) generated by the vascular endothelium presents as the major defect in platelet function. Insulin is a natural antagonist of platelet hyperactivity. It sensitizes the platelet to PGI2 and enhances endothelial generation of PGI2 and NO. Thus, the defects in insulin action in diabetes create a milieu of disordered platelet activity conducive to macrovascular and microvascular events (Gödecke et al., 2002).

1.1.7.7 Increased cytosolic calcium mobilization
Diabetes and is associated with abnormal platelet calcium homeostasis and this may be due to an attenuated negative direct or indirect feedback control by nitric oxide, due to its reduced production in DM (Bitar et al., 2005).
1.1.7.8 Increased α-granule content with concomitantly increased release
Both platelet factor 4 and beta-thromboglobulin are elevated in DM and this deviation could be regarded as indicator of an additional risk for the development of vascular complications in DM (Suslova et al. 2014).

1.1.7.9 Increased platelet volume
Increased functional properties of diabetic platelets might be already conditioned during thrombopoiesis in the stem cell system as a consistent, significant shift of the volume distribution to larger platelets was found in diabetics (Suslova et al. 2014).

1.1.7.10 Increased numbers of glycoprotein receptors GPIb and GPIIb/IIIa
The number of glycoprotein (GP) GPIB and GPIIB/IIIa molecules that could be determined on resting, peripheral platelets by means of flowcytometry after immune-staining with monoclonal antibodies are elevated in diabetic patients (Suslova et al. 2014).

1.1.7.11 Increased membrane protein glycation
Hyperglycemia contributes to greater platelet reactivity through direct effects and by promoting glycation of platelet proteins. The relationship of non-enzymaticglycation of platelet proteins to altered platelet function was shown in diabetic patients. Platelets isolated from diabetic patients were glycated to a greater extent than those isolated from non-diabetic controls (Schneider et al., 2009).

1.1.7.12 Altered membrane fluidity
Platelets from diabetic patients showed an increased fluidity and micro heterogeneity of the platelet membrane, a higher Ca2+-ATPase activity, and a reduced Na+-K+-ATPase activity in comparison with platelets from healthy subjects (Bitar et al., 2005).
1.1.7.13 Increased binding of adhesive RGD-protein ligands (e.g. fibrinogen)
Platelets from diabetics are known to be more sensitive to aggregating agents and to produce more prostaglandins and thromboxane than platelets from normal subjects. The increased fibrinogen binding and aggregation of platelets from diabetic subjects in response to ADP or collagen is mediated by increased formation of prostaglandin Endoperoxide or thromboxane A2, or both (Kiritoshi et al. 2003).

1.1.7.14 Increased content and release of plasminogen activator inhibitor-1
Platelet plasminogen activator inhibitor 1 (PAI-1) concentration is higher in type II diabetic patients and it may contribute to enhanced thrombosis in type II diabetes (Schneider et al., 2009).
1.2. Previous Studies

(Kodiatte et al., 2012) determined the MPV in diabetics compared to non-diabetics, to see if there is a difference in MPV between diabetics with and without vascular complications, and to determine the correlation of MPV with glycosylated hemoglobin (HbA1c) and duration of diabetes in the diabetic patients. They found that the mean platelet counts and MPV were higher in diabetics compared to the non-diabetic subjects [277.46 ± 81 X 10^9/l vs. 269.79 ± 78 X 10^9/l (P= 0.256)], 8.29 ± 0.74 fl versus 7.47 ± 0.73 fl (P= 0.001), respectively. MPV showed a strong positive correlation with fasting blood glucose (FBG) and HbA1C levels (P=0.001). They concluded that elevated MPV could be either the cause for or due to the effect of the vascular complications. Hence, platelets may play a role and MPV can be used as a simple parameter to assess the vascular events in diabetes.

(Papanas et al., 2004) investigated MPV in type 2 DM and evaluated its associations to diabetic complications. They found that MPV was significantly higher (P = 0.01) in diabetics (14.2 +/- 2.2 fl) than in non-diabetics (7.1 +/- 1.2 fl). Among diabetic they found that no association was found between MPV and age, gender, duration of diabetes and HbA1c. Among type 2 diabetic patients MPV is higher in those who have micro-vascular complications.

(Akinsegun et al., 2014) in Nigeria conducted an unmatched case-control study involving 200 participants consisting of 100 diabetics and 100 non-diabetic controls. Four and half milliliters of blood were collected from diabetics and non-diabetic controls into EDTA anticoagulant tubes. Full blood count was performed using the Sysmex KN-21N, (manufactured by Sysmex corporation Kobe, Japan) a three-part auto analyzer able to run 19 parameters per sample including platelet counts and mean platelet volume. The mean fasting blood sugar for the diabetics was
147.85±72.54 mg/dl and the controls 95.20±30.10 mg/dl. The mean platelet count for the diabetics was 235.29±76.81 X 10^9/L and controls, 211.32±66.44 X 10^9/L. The mean platelet volume, for the diabetics was 8.69±0.67 fl and the controls, 8.91±0.80 fl. There was a statistically significant difference in platelet counts of diabetics and healthy controls (p=0.038) while none existed between the mean platelet volume in diabetics and healthy controls (p = 0.593). This study revealed a higher mean platelet count for diabetics on treatment than for non-diabetic controls while mean platelet volume was lower in cases than controls. However, both parameters in diabetics on treatment were within the normal reference range for healthy individuals.

Sharpe and Trinick, (1993) measured MPV in patients with diabetes mellitus, compared with MPV in non-diabetic control subjects. Mean MPV was significantly increased in the diabetic subjects (8.9 +/- 0.07 fl, mean +/- SEM) compared with non-diabetic subjects (8.0 +/- 0.05) (p < 0.001). They reported that since platelet size is a determinant of platelet function, with larger platelets being more reactive per unit volume, they believe platelets may play a part in the micro- and macro-vascular complications of diabetes mellitus.

In Sudan Hamza et al.,(2013) determine plasma fibrinogen level, platelet count and mean platelet volume (MPV) of diabetic patients (type 2) with retinopathy, 32 were males and 18 were females 150 were enrolled in the study with age ranged between 40-69 years, 50 of them were diabetic patients with retinopathy, mean age 52.7 ± 7.5 years, 50 were type 2 diabetes mellitus patients with mean age of 54.8 ± 8.5 years and 50 were non diabetic apparently healthy subjects with mean age was and 53.3 ± 7.9 years. Five ml venous blood was collected from each participant. They found that Plasma fibrinogen level and MPV were elevated in diabetic patients with retinopathy compared to diabetic
patients and control subjects. Elevated fibrinogen plasma level and MPV reflects hypercoagulability associated with diabetic retinopathy irrespective of diabetes mellitus onset. Further studies are needed to confirm the current
1.3 Justification

Type 2 Diabetes mellitus is a global public health crisis that threatens the economies of all nations, particularly developing countries. CVDs are the major causes of mortality in persons with diabetes. Platelets hyperactivation in type II DM is believed to play an important role in the development of CVD in Type 2 Diabetes mellitus. Recent advances in diagnostics have established MPV and PDW are considered as platelet activation markers. In this study we aimed to study platelets status in Type 2 Diabetes mellitus using platelet indices as markers of activation. The study correlates of platelets activation with the duration of Type 2 Diabetes mellitus, diabetes control status and the presence of CVD. The results may have practical clinical applications in risk stratification and targeting of prevention and management therapy for patients with Type 2 Diabetes mellitus.
1.4 Objectives of the study

1.4.1 General objective
To assess Platelet Indices of Sudanese Patients with Type II Diabetes Mellitus

1.4.2 Specific objectives
1. To correlate platelet indices (platelet count, MPV and PDW) in type II diabetic patients according to the duration of type II DM compared to control
2. To determinate possible association between platelet indices with presence of CVD and Diabetic duration
3. To correlate platelet indices with the control status in type II diabetic patients
4. To correlate platelet indices of diabetic patients with age and gender.
Chapter Two

Materials and Methods
CHAPTER TWO

Materials and Methods

2.1 Study design
This was a prospective and analytical case-control study.

2.2 Study area
This study was conducted in Khartoum Sudan, during the period from May to November 2015, in Fedail Specialized hospital.

2.3 Study population
100 Sudanese patients with Type2 DM diagnosed according to WHO criteria, 50 of them have cardiovascular complications (neuropathy, myopathy and nephropathy) their age ranged between 40 and 70 and gender- matched 100 healthy individuals were included in this study.

2.3.1 Inclusion criteria:
Sudanese patients with Type2 DM. Both males and females were enrolled.
Age ranged between 40 and 70 years.

2.3.2 Exclusion criteria
- Subjects below 40 years and those above 70 years old.
- Patients with autoimmune disorders. Patients on medication that affect platelet functions
- None of the patients of was diagnosed as type 1 DM and participant with no history for a drug interfering with platelet activation.
2.4 Sampling

2.4.1 Data collection

The data was collected using a direct interviewing questionnaire. Medical information was collected from the patients with help of the physicians. The questionnaire was used to collect data regarding name, age, gender, residence, duration of diabetes, presence of CVD complications, history of systemic diseases and medication (Appendix I).

2.4.2 Collection of blood sample

Five ml venous blood was collected from each enrolled subject each blood sample was divided into three parts: one part was collected in EDTA anti-coagulated container for full blood count parameters and HbA1c. Gentle mixing of the anti-coagulated specimens was achieved to avoid hemolysis, clotting or platelet aggregation. A thin blood film was made immediately from EDTA container after collection.

2.5 Tests Applied

2.5.1 Measurement of platelet indices and platelet count

MPV and platelet count were measured using a Sysmex KX N21 autoanalyzer that uses aperture-impedance technology to size platelets on the red blood cell/platelet channel that produces the following parameters: PLT (109/L), MPV (fL), and PDW [calculated as (σ × 100) (fL)/MPV (fL) after log transformation of the MPV]. In addition to this, cells are hydrodynamically focused through a small aperture, and a voltage pulse is generated that is proportional in size to the volume of the cell. Mobile "auto discriminators" distinguishes between machine noise at the lower end and red blood cells at the upper end of each individual platelet volume distribution. MPV is calculated by the following formula: MPV (fL) =platelet crit (Pct) (%) x1000÷Plt (x103/µL), where platelet is the platelet count and is the number of particles between the upper and
lower discriminators, Pct is calculated electronically from the raw histogram data.

Complete blood count including platelet indices was measured within 1 hour of collection to minimize variations due to sample aging. The complete blood count including platelet indices was performed following manufacturer instructions as follow:

The whole blood mode (WB) was selected to analyze the whole blood sample without pre-dilution. The sample number was entered before each sample. This procedure was followed:

- A well-mixed anti-coagulated sample was set to the sample probe, and the start switch was pressed till the aspirating process was finished. (Volume aspirated approx. 50mL).
- The sample was removed straight down and the sample probe was automatically cleaned.
- The aspirated sample was then automatically suspended into the different detector blocks and different parameters were measured.
- The results of parameters were then viewed on the screen and subsequently printed out.

2.5.2 Microscopic Examination of Thin Blood Film

The well spread thin blood films that were made immediately after collection was stained as follows:

- Films were left to dry.
- After drying well they were covered by Leishman stain for 3 minutes.
- Then double volume of Leishman buffer (pH = 6.8) was add and left for further 7 minutes.
- The slides were washed under tap water.
- The films were then wiped clean on the other side, and left to dry.
- Lastly the well –spread, stained and dried blood films were examined microscopically using X 100 objective lens. Any specimen showed
fragment of RBCs or other blood cells or aggregated platelet was excluded.

2.5.3 HbA1c:
HbA1c was measured using chromatography methods (Appendix IV). The diabetic patients was divided into two groups according to the HbA1c levels into well controlled group (HbA1c< 6.5%) and insufficiently controlled group (HbA1c> 6.5%) (Appendix IV):
- 20 µl of well mixed blood was added to 1 ml of hemolysis solution.
- Then the test was programmed and performed by automated procedure in Hitachi (Roche-Germany).

2.6 Ethical clearance
- This study was ethically approved by the committee of the College of Medical Laboratory Sciences, Sudan University of Science and Technology. Verbal consent was obtained from each participant before blood sample collection.

2.6 Statistical analysis
The statistical analysis of the results was performed by using the Statistical Package for Social Sciences (SPSS) version 15.0 (SPSS Inc., Chicago, IL, USA) for windows version 10 using T-test for testing difference significance and Pearson correlation test (r value as the coefficient). P value < 0.05 was considered statistically significant.
Chapter Three

Results
Chapter three

Results

3.1 The baseline characteristics of study subjects

This study included 100 patients with type II DM and 100 control subjects.

The diabetic group was composed of 52 (52%) males and 48 (48%) females and the control group was composed of 55 (55%) males and 45 (45%) females. The mean age of diabetic patients was 52.9 ± 11.2 (years) and the mean age of the control group was 48.3 ± 13.3 (years). This study showed that 50 (50%) diabetic patients had CVD. Diabetic patients were divided into three groups according to the duration of DM > 10 years: 30 (30%), 10-20 years: 38 (38%) and > 20: 32 (32%). According to control status according to HbA1c values, 57 (57%) were well controlled patients while 43 (43%) were controlled poorly controlled (Table 3.1)

Table 3.1 Demographic data of cases and control

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study Group (n=200)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (n =100) Mean ± SD</td>
<td>Control (n =100) Mean ± SD</td>
<td>P. Value</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>52.9 ± 11.2</td>
<td>48.3 ± 13.3</td>
<td>0.84</td>
</tr>
<tr>
<td>gender</td>
<td>Male 52, female 48</td>
<td>Male 55, female 45</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>CVD 50%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Well controlled</td>
<td>57%</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Platelet indices were compared between diabetics and non-diabetics as well as between CVD complicated versus CVD non-complicated diabetics. The associations of Platelet indices with duration of DM and with control status judged by HbA1c were also studied.

The platelet counts in diabetics were insignificant than in non-diabetics. This difference was statistically insignificant (were 292.0 X 10^3/µL and 280 X 10^3/µL, P = 0.113). The mean values of MPV and PDW were significantly higher in diabetics compared to non-diabetics, (10.2 vs. 9.3 (fl), \( P= 0.005 \)) (11.9 vs. 10.5 (%), \( P= 0.009 \)), respectively. Table 3.2.

**Table 3.2 Comparison between Platelet Indices in case and control groups**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study Group (n=200)</th>
<th></th>
<th></th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases (n =100) Mean ± SD</td>
<td>Control (n =100) Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>10.2 ± SD</td>
<td>9.3± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDW (%)</td>
<td>11.9 ± SD</td>
<td>10.5± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plts (X 10^3/µL)</td>
<td>292.0± SD</td>
<td>280± SD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MPV and PDW values were significantly higher in patients with CVD complicated diabetics versus diabetics without CVD, (10.8 (fl) vs. 9.6 (fl), \( P = 0.002 \)) (12.1(%) vs. 11.5 (%), \( P=0.020 \)), respectively (Table 3.3).
No significant difference was seen in values of platelet count in CVD complicated diabetics versus diabetics without CVD (mean 296.0 X10³/µL vs. 288.0 X10³/µL, $P = 0.119$). The platelet indices values were significantly raised with longer duration of Type 2DM.

**Table 3.3 Associations of platelet indices with cardiovascular disease among the diabetic group.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>With CVD</th>
<th>Without CVD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPV (fl)</td>
<td>10.8± SD</td>
<td>9.6± SD</td>
<td>0.002</td>
</tr>
<tr>
<td>PDW (%)</td>
<td>12.1± SD</td>
<td>11.5± SD</td>
<td>0.020</td>
</tr>
<tr>
<td>Plts (X 10³/µL)</td>
<td>296.0± SD</td>
<td>288.0± SD</td>
<td>0.119</td>
</tr>
</tbody>
</table>

Control status of diabetes was not associated with platelet count (296.0 X 10³/µL vs. 288.0 X 10³/µL, $P = 1.21$). There is no difference in mean values of MPV and PDW between patients with HbA1c< 6.5% and those with HbA1c> 6.5%, (10.0 (fl) vs.10.4 (fl), ($P = 0.79$) (11.7 (%) vs. 12.1 (%), ($P =0.51$) respectively. Table 3.4

**Table 3.4 Associations of platelet indices with control status according to HbA1c among the diabetic group.**

<table>
<thead>
<tr>
<th>Patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well controlled</td>
<td>Poorly controlled</td>
</tr>
<tr>
<td>(HbA1c&lt; 6.5%)</td>
<td>(HbA1c&gt; 6.5%)</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>10.0± SD</td>
</tr>
<tr>
<td>PDW (%)</td>
<td>11.7± SD</td>
</tr>
<tr>
<td>Plts (X 10³/µL)</td>
<td>288.0± SD</td>
</tr>
</tbody>
</table>
The platelets, MPV and PDW were significantly raised with longer duration of type II DM. The p. value for platelet count MPV and PDW, ($P=0.070$), ($P=0.002$) and ($P=0.020$) respectively, table 3.5.

**Table 3.5 Associations of platelet indices with duration of type II DM among the case group**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>&lt; 10 years</th>
<th>10-20 years</th>
<th>&lt;20 years</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPV (fl)</td>
<td>9.3± SD</td>
<td>10.3± SD</td>
<td>11± SD</td>
<td>0.002</td>
</tr>
<tr>
<td>PDW (%)</td>
<td>10.7± SD</td>
<td>11.3± SD</td>
<td>12.7± SD</td>
<td>0.020</td>
</tr>
<tr>
<td>Plts</td>
<td>278 ± SD</td>
<td>284± SD</td>
<td>294± SD</td>
<td>0.070</td>
</tr>
</tbody>
</table>

There was no significant difference between platelet count, MPV and PDW count in all studied males compared to females. ($P = 0.979$) and ($P = 0.882$) respectively.

Table 3.6 showed no association was between gender and platelet count ($P = 1.11$), MPV ($P = 0.95$) and PDW ($P = 0.99$).

**Table 3.6 Platelet Indices of type II diabetic patients according to gender**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study Group (n=200)</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>9.8± SD</td>
<td>9.7± SD</td>
</tr>
<tr>
<td>PDW (%)</td>
<td>11.0 ± SD</td>
<td>11.4± SD</td>
</tr>
<tr>
<td>Plts (X 10³/µL)</td>
<td>286± SD</td>
<td>284± SD</td>
</tr>
</tbody>
</table>
Chapter Four

Discussion, Conclusion and Recommendations
CHAPTER FOUR

Discussion, conclusion and recommendation

4.1 Discussion

In this study, higher significant levels of MPV and PDW but not platelet count were also associated with the presence of CVD in patients with type II DM. This finding is in agreement with previous by (Papanas et al., 2004, Kodiatte et al., 2012).

Significant higher levels of MPV and PDW but not for platelet count were found among diabetic patients with CVD compared to CVD non complicated diabetics and this is consistent with the previous studies (Sharpe and Trinick). MPV is positively associated with the indicators of platelet activity, thrombocyte aggregation, and the release of thromboxane A2, platelet factor 4, and β-Thromboglobulin (Sharp et al., 1995).

No association between platelet count, MPV or PDW with poor or sufficient diabetic controls. This is in agreement with a previous study (Papanas et al., 2004). Previous study found elevation of MPV with poor control (Demirtunc et al., 2009).

Significant increase in MPV and PDW levels were found to be associated with Type 2DM; however there is no statistically significant difference between platelet count in diabetics and non-diabetic subjects. These results are in agreement with the results of previous studies (Jindal et
In this study, the longer duration (those with duration more than 20 years have highest platelet count which is significant MPV and PDW, those between 20 and 10 years have higher values than those with duration below 10 years) of diabetes was also found to be a significant factor for platelet activation. Longer DM duration more than 10 year because of the effect of glycemic control and duration of diabetes on platelet parameter was associated with more elevation of platelet count, MPV and PDW. Longer DM duration gives more chance for CVD to occur. This is in agreement with the previous study (Kodieatte et al., 2012, Unubol et al., 2012).

No associations were found between age and gender with platelet indices and this is in accordance with previous study. This is in accordance with previous studies (Kodieatte et al., 2012; Papanas et al., 2004).
4.2 Conclusions

1. CVD complicated type II DM is associated with platelet hyperactivity. This not related to reduced or sufficient glycemic control.

2. Platelets in type II DM are activated as evidenced by platelet indices.

3. Duration of type II DM was directly correlated to platelet hyperactivation in type II DM.

4. There was no association between gender and platelet indices in type II DM.
4.3 Recommendations

1. The values of platelet indices should be reviewed as routine to prevent development of CVD.

2. Platelet function using standards methods should be used platelet induced aggregation tests and flocytometry method with platelet indices should be investigated among type II DM.

3. Large studies needed to determine the contribution of platelet hyper activation to each type of CVD, e.g. nephropathy, retinopathy, and neuropathy is needed and using large sample size.
Reference
References


Appendix
Appendices
Appendix I
Questionnaire

Date:…………………………

1. Name:-------------------------------------------------------------------------
2. Number:-----------------------------------------------------------------------
3. Residence:---------------------------------------------------------------------
4. Tel:--------------------------------------------------------------------------------

5. Age: YY/MM  ☐  ☐
6. Gender:  ☐  ☐
   7.1. Male  7.2. Female

8. Medications ....................................................................................................

9. Duration of DM type II: Years/Month  ☐  ☐

10. Retinopathy complications:  ☐
   14.1 No
   14.2 Yes

11. Nephropathy:  ☐
   14.1 No
   14.2 Yes

12. Neuropathy:  ☐
   14.1 No
   14.2 Yes

13. Ischemic heart disease(IHD):  ☐
   14.1 No
   14.2 Yes
Appendix II
HbA1c Estimation

Specimen Collection and Preparation
Special preparation of the patient is unnecessary. Fasting specimens are not required. No special additives or preservatives other than anticoagulants are required. Collect venous blood with EDTA using aseptic technique. All human specimens should be regarded as potentially biohazardous. Therefore, universal precautions should be used in specimen handling (gloves, lab garments, avoid aerosol production, etc.).

To determine HbA1c, a hemolyzate must be prepared for each sample.
1. Dispense 3 ml Hemolysis Reagent into tubes labeled Control, Patients, etc.
2. Place 20 ml of whole blood into the appropriately labeled lysed reagent tube, mix.
3. Allow to stand for 5 minutes or until complete lysis is evident. Hemolyzates may be stored up to 10 days at 2-8°C.

Storage and Stability
1. All reagents are stable to the expiration date stated on the labels. Do not use the reagents past their expiration date.
2. R1 and R2 are stable for at least one month after opening stored at 2-8°C.
3. Hemoglobin A1c in whole blood collected with EDTA is stable for one week at 2-8°C.

Interferences
1. Below 1.0 mgic/L, ascorbic acid to 50 mgic/L, triglycercides to 2000 mgic/L, carboxylated HB to 7.5 mgic/L, and acetylated HB to 5.0 mgic/L, do not interfere in this assay.
2. It has been reported that results may be inconsistent in patients who have the following conditions: cigarette addiction, lead-poisoning, alcoholism, ingestion, large dosages of exogenous corticosteroids.
3. It has been reported that elevated levels of AST may lead to underestimation of HbA1c.
4. It has been determined that Hemoglobin variants HbA2, HbC, and HbS do not interfere with this method.
5. Other very rare variants of hemoglobin (e.g., HbE) have not been assessed.

Materials Provided
Refer to "Reagents"

Materials Required but not Provided
1. Pipettes to dispense 20 ul and 1.0 ml and Test Tubes to hold 1.0 ml.
2. Hemoglobin A1c calibrator set, control set, and Hemozys mg/L for 120 ml kits.

Procedure (automated-Hitachi 717)

<table>
<thead>
<tr>
<th>TEST NAME</th>
<th>HbA1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASSAY CODE</td>
<td>[1-POINT][50-60]</td>
</tr>
<tr>
<td>SAMPLE VOLUME</td>
<td>[5-10]</td>
</tr>
<tr>
<td>R1 VOLUME</td>
<td>[1.0-1.2]</td>
</tr>
<tr>
<td>R2 VOLUME</td>
<td>[1.0-1.2]</td>
</tr>
<tr>
<td>WAVELENGTH</td>
<td>[530]</td>
</tr>
<tr>
<td>CALIBRATION</td>
<td>[NONLINEAR][4][8]</td>
</tr>
<tr>
<td>STD (1) CONC-POS</td>
<td>[50]</td>
</tr>
<tr>
<td>STD (2) CONC-POS</td>
<td>[50]</td>
</tr>
<tr>
<td>STD (3) CONC-POS</td>
<td>[50]</td>
</tr>
<tr>
<td>STD (4) CONC-POS</td>
<td>[50]</td>
</tr>
<tr>
<td>STD (5) CONC-POS</td>
<td>[50]</td>
</tr>
<tr>
<td>STD (6) CONC-POS</td>
<td>[50]</td>
</tr>
<tr>
<td>DUPLICATE LIMIT</td>
<td>[999]</td>
</tr>
<tr>
<td>SENSITIVITY LIMIT</td>
<td>[1]</td>
</tr>
</tbody>
</table>
Hemoglobin A1c
Reagent Set

Limitations
1. This assay should not be used for the diagnosis of diabetes mellitus.
2. Patient specimens should always be assayed using a calibration curve.
3. It has been reported that results may be inconsistent in patients who have the following conditions: acute infection, lead poisoning, alcoholism, ingestion of large doses of aspirin. Therefore, it is recommended that these conditions be minimized.
4. It has been reported that elevated levels of Hf may lead to overestimation of HbA1c and, that vitamin D interferes with HbA1c determination by immunoassay. Therefore, it is recommended that these conditions be minimized.
5. It has been determined that Hemoglobin variants HbA, HbC and HbS do not interfere with this method.
6. Other very rare variants of hemoglobin (e.g. HbE) have not been assessed.

Quality Control
The reliability of test results should be monitored whenever patient samples are assayed using a standard and quality control materials analyzed in the same manner employed for the unknowns. We suggest the use of commercially available Hemoglobin A1c controls with an assigned range. If controls do not fall into the assigned range, patient values from that run should not be reported. The run should be repeated, making sure that all mixing and handling instructions are strictly followed.

Linearity of the assay should be verified with a commercial linearity check set, or dilutions of a high specimen, at least every six months.

Calculations / Results
HbA1c results for the unknowns and controls are determined using the prepared calibration curve. An example curve is illustrated below:

![Graph of HbA1c vs %](image)

Expected Values
Recommended values: less than 6% for a non-diabetic, less than 7% for a diabetic control of a person with diabetes.

Each laboratory should establish its own expected values. In using Hemoglobin A1c to monitor diabetic patients, results should be interpreted individually. That is, the patient should be monitored against him or herself. There is a 3-4 week time lag before Hemoglobin A1c reflects changes in blood glucose level.

Performance
1. Linearity: The Hemoglobin A1c assay range is 0.0%-16.0%.
2. Comparison: A study using 40 human specimens between this Hemoglobin A1c procedure and an automated HbA1c procedure (Dextran T500) yielded a correlation coefficient of 0.983 and a linear regression equation of y = 1.09x + 0.481, (Sxy = 0.32).
3. Precision:
   - Within Run: The within run precision was established by assaying two blood samples following NCELS protocol EPS on a Hitachi 917.
     - Level: Mean Std. Dev. % CV
     - Low: 5.48 0.078 1.43
     - High: 10.28 0.175 1.72
   - Day to Day: Between day precision was established by assaying two blood samples following NCELS protocol EPS on a Hitachi 917.
     - Level: Mean Std. Dev. % CV
     - Low: 5.48 0.162 2.77
     - High: 10.28 0.275 2.68
4. Sensitivity: Sensitivity was investigated by reading the change in absorbance at 600nm for a saline sample and a whole blood sample with a known concentration. Ten replicates of each sample were performed. The results of this determination indicated the, on the analyzer used (Hitachi 717), the HbA1c reagent showed little or no drift on the zero sample. Under the reaction conditions described, a 0.07% absorbance change is approximately equivalent to 1.0% HbA1c.

References