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BODY MASS INDEX, BLOOD PRESSURE, HEMATOLOGICAL PROFILES AND NON-ENZYMATIC ANTIOXIDANTS STATUS IN TYPE 2 DIABETIC PATIENTS: A CROSS SECTIONAL STUDY.

By

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This is to certify that the work described in this thesis entitled; Body mass index, blood pressure, hematological profiles and non-enzymatic antioxidants status in type 2 Diabetic Patients: A cross sectional study was carried out by Chala Olana at the federal police specialized hospital, Addis Ababa. It is submitted in partial fulfillment of the requirements for the degree of Master of Science in Medical Biochemistry complies with regulations of the Addis Ababa University and has not been submitted for any other degree.

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ABSTRACT

Background: Type 2 Diabetes Mellitus (T2DM) is commonly associated with vascular complications. In recent years, there has been renewed interest and increasing evidences that hematological abnormalities can be used as indicators of endothelial dysfunction and inflammation and thus, are responsible for both micro- and macro-vascular complications.

Objective: The aim of the present study was to investigate the average values of Body mass index, Blood Pressure, hematological profiles and non-enzymatic antioxidants status in Type 2 Diabetic Patients.

Methods: This study involved 70 patients with T2DM (male/females, 47/23) and 70 age and sex matched non diabetic healthy individuals (male/females, 46/24**).** The study was conducted from December 2016 to January 2017, which was a cross-sectional study. Anticoagulated Blood samples were collected from fasting individuals and analyzed in automated blood cell counter. Serum fasting blood sugar (FBS), uric acid (UA), and Total Bilirubin (TB) were analyzed using automated chemistry analyzer. The results were statistically analyzed by SPSS, V21 software. Two tailed significance level was determined by independent t-test. P values less than 0.05 ($P<0.05$) were considered as statistically significant and results presented as mean ± standard deviation.

Result: Male diabetics were characterized by significantly elevated levels of; body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), mean cell hemoglobin (MCH), red blood cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), FBS ($P<0.05$) and lower values of UA ($P<0.05$) as compared with the normal controls. Even though statistically not significant, mean values of hematocrit (HCT), mean cell hemoglobin concentration (MCHC), white blood cell count (WBC) and platelet crit (PCT) ($P>0.05$) were higher while mean values of red blood cell (RBC and TB ($P>0.05$) were lower in male patients. Similarly, female diabetics were characterized by significantly elevated levels of DBP, HCT, MCHC, RDW, WBC, PLT, MPV, PCT, and FBS ($P<0.05$). However, they had significantly lower values of DB and UA ($P<0.05$) than their corresponding female controls. Higher values of BMI, SBP, PDW and lower values of RBC were also observed in female patients but these differences were not statistically significant.

CONCLUSION: This study revealed remarkably elevated levels of anthropometric parameters such as BMI, SBP, DBP in male and DBP in female diabetics as compared with their corresponding healthy individuals. Hematological parameters like; RDW, PLT and MPV were considerably higher in both sexes of diabetic patients compared with their corresponding healthy individuals. MCH and PDW showed a significant elevation in male patients while HCT, MCHC, WBC and PCT were justifiably higher in female patients. This study also demonstrated significantly lower values of Non-enzymatic antioxidants like UA and TB in female and TB in male diabetic patients as compared with the healthy individuals.

Keywords: Diabetes, oxidative stress, hematological abnormalities, vascular complications.

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AGE	Advanced Glycation End Product
GSH	Gluthathione
GSH-Px	Glutathione Peroxidase
GSSG	Glutathione Disulfide
HbA1c	Glycated Hemoglobin
HCT	Hematocrit
HGB	Hemoglobin
MAPK	p38 mitogen-activated protein kinase,
MCH	Mean cell Hemoglobin
MPV	Mean Platelet Volume
NOS	Nitric Oxide Synthase
PI-3	Phosphoinositol-3 Kinase
PKA/B/C	Protein Kinase A/B/C,
PKC	Protein Kinase C
RAGE	AGE receptors,
RBC	Red Blood Cell
RDW	Red Blood Cell Distribution Width
ROS	Reactive Oxygen Species
T2DM	Type 2 Diabetes Mellitus
WBC	White Blood Cell
WBV	Whole Blood Viscosity

1. INTRODUCTION

1.1. Back Ground

Diabetes mellitus is a chronic metabolic syndrome characterized by abnormally high blood sugar resulting from defects in the secretion and action of insulin and impaired function in the metabolism of carbohydrates, lipids and proteins which leads to long term health complications (American Diabetes Association, 2010**;** Matough *et al*., 2012; Kahn *et al*., 2014**).** Type 2 diabetes is a combination of low amounts of insulin production from pancreatic β-cells and peripheral insulin resistance (Kasuga, 2006). Insulin resistance leads to the impairment of glucose transport into the muscle cells, subsequently leading to elevated hepatic glucose production. Further, it elevates the plasma level of free fatty acids and fat breakdown as well. For type 2 diabetes to develop, there must be insulin resistance and pancreatic β-cell dysfunction simultaneously (Al-Goblan *et al*., 2014).

Diabetes has taken place as one of the most important diseases worldwide, reaching epidemic proportions. Global estimates predict that the proportion of adult population with diabetes will increase 69% for the year 2030 (Shaw *et al*., 2010). Type 2 diabetes represents approximately 90% of the total diabetes cases, and it is characterized by impairment in insulin action and/or abnormal insulin secretion while, type 1 diabetes accounts for only 5-10% of the total cases of diabetes worldwide (American Diabetes Association, 2009).

Type 2 Diabetic Mellitus (T2DM) is the most common form of diabetes, and is associated with the development of a number of devastating microvascular complications, including diabetic retinopathy (DR), diabetic peripheral neuropathy (DPN), and diabetic nephropathy (DN) (Wang *et al*., 2013). These microvascular complications are the major causes of morbidity and mortality in patients with T2DM (Matough *et al*., 2012).

1.2. Classification and Etiology of Diabetes

The classification proposed by the American Diabetes Association in 1997 was based on the pathogenesis of the disease and comprises four categories: The most common forms of diabetes are Type 1 diabetes, Type 2 diabetes, gestational diabetes and other types. The classical type 1 diabetes is an autoimmune disease characterized by the targeted destruction of the insulin secreting β-cells within the pancreatic islet (Baumann *et al*., 2012). The precise etiology remains

uncertain; however, there is a general consensus that type 1diabetes is a T-cell mediated disorder that results from immune dysfunctions, with subsequent loss of tolerance to β cell antigens and destructive lymphocytic infiltration of the islets. This leads to insulin deficiency and hyperglycemia (Lernmark and Larsson, 2013).

Classic Type 2 diabetes is characterized by insulin resistance with significant complex metabolic disorder including obesity, impaired insulin function and secretion, and increased endogenous glucose output (Maiese *et al*., 2007). In Type 2 diabetes; insulin secretion is increased to overcome the resistance and characterized with insulin deficiency usually later in the course of the disease. There are various causes of type 2 diabetes. Most, but not all, patients with type 2 diabetes are overweight or obese. Obesity itself causes some degree of insulin resistance (American Diabetes Association, 2015). Insulin resistance refers to a problem with the cells that respond to insulin rather than a problem with insulin production. (Lin and Sun, 2010). Insulin resistance is a common condition in people who are overweight or obese, have excess abdominal (visceral) fat, and are not physically active. Muscle, fat, and liver cells stop responding properly to insulin, forcing the pancreas to compensate by producing extra insulin. As long as beta cells are able to produce enough insulin, blood glucose levels stay in the normal range. But when insulin production weakens because of beta cell destruction, glucose levels rise, leading to prediabetes or diabetes (Ferrannini, 1998).

Pre-diabetes is an intermediate state and includes impaired glucose fasting test or impaired glucose tolerance test. Studies have shown that 25% of patients with prediabetes will develop type 2 diabetes in 3 to 5 years, and it's more likely to happen in people with more risk factors such as those with obesity or a positive family history for diabetes (Nathan *et al*., 2007). Initially, in the face of insulin resistance, compensatory increases in pancreatic insulin secretion are able to maintain normal glucose concentrations. However, as the disease progresses, insulin production gradually diminish, leading to progressive stages of hyperglycemia. Insulin-resistant individuals frequently exhibit a constellation of other characteristics, including visceral obesity, dyslipidemia, hypertension, hyperinsulinemia, impaired fibrinolysis, endothelial dysfunction, hyperuricemia, vascular inflammation, and premature atherosclerosis (Stern, 1999).

Insulin resistance is also implicated at the adipocyte level, leading to unrestrained lipolysis and elevation of circulating free fatty acids. Increased free fatty acid, in turn, further alters the insulin response in skeletal muscle (Roden *et al*., 1996), while further impairing pancreatic insulin secretion as well as augmenting hepatic glucose production ("lipotoxicity") (Bergman and Ader, 2000). Consequently, type 2 diabetes results from co-existing defects at multiple organ sites: resistance to insulin action in muscle, augmented pancreatic insulin secretion, and unrestrained hepatic glucose production, all of which are worsened by defective insulin action in fat cells. These pathophysiological lesions are the primary causative agents' for the development and progression of hyperglycemia (Silvio, 2002).

1.2.1. Diagnosis and control of diabetes

The diagnostic tests for diabetes such as hemoglobin A1C, fasting blood glucose, and 2-hour postprandial plasma glucose after 75-g oral glucose tolerance test are used for diabetes screening and testing. These tests are also used to follow up the disease progression and control. Diabetes has several associated metabolic disturbances, diagnosis and management have historically relied on measures of circulating glucose in whole blood, plasma, or serum and, more recently, in capillary blood glucose or under some circumstances, for example in cutaneous insulin pumps, interstitial fluid. Chronic levels of hyperglycemia can be assessed with glycated protein assays, most often glycatedhemoglobin A1C (HbA1c). These assays reflect mean glucose levels integrated over the lifespan of the hemoglobin, which corresponds to the lifespan of erythrocytes (half-life about 120 days). The HbA1c assays are now standardized and are a reliable index of average glucose levels over the preceding 8 to 12 weeks (Nathan, 2015). The American Diabetes Association (ADA) for diagnosis of prediabetes and diabetes are summarized in the table below.

Table 1: Diagnosis of diabetic patients

	Glucose Measuring Method		
	Fasting	Oral Glucose Tolerance Test	Hemoglobin A1c
Diagnostic cut points			
Pre diabetes	110 - 125	140 - 199	5.7 -6.4
Diabetes	≥ 126	≥ 200	≥ 6.5

1.3. Pathogenesis of T2D

1.3.1. Obesity and Type 2 Diabetes

Obesity is probably the most important modifiable acquired risk factor in the pathogenesis of health disorders such as atherosclerosis and type 2 diabetes reported in most biochemical researches and cross-sectional studies. Obesity is a frequent co-morbid condition associated with excessive increase in weight (Innocent *et al.*, 2013). Early obesity and almost any weight gain after adolescence are risk factors for type 2 diabetes and the duration of obesity seems to be a significant risk factor for type 2 diabetes, independently of current degree of obesity. It has been indicated that, besides obesity, an increase in body weight of 3–20 kg is associated with an elevated risk of incident type 2 diabetes. From biochemical metabolic analysis, it has been established that fatty acids which constitute the body fat contents, can be synthesized from simple carbohydrates such as glucose. Increase in blood glucose levels have been associated with increase in lipid biosynthesis (lipogenesis) and hence, an increase in weight. Since BMI is proportional to weight from its standard formula weight/square height, it is expected that factors such as blood glucose which influence weight will ultimately affect BMI. Thus, increased blood glucose level can induce increase in BMI. Obesity is known to induce insulin resistance due to decrease in insulin-sensitive receptors as the weight increases (Schienkiewitz *et al.*, 2006).

As individuals become obese and their adipocytes enlarge, adipose tissue undergoes molecular and cellular alterations affecting systemic metabolism. Fasting whole body FFA and glycerol release from adipocytes is increased in obese women compared with lean women. Increased FFAs are well known to promote insulin resistance in tissues such as muscle (Al-Goblan *et al.*, 2014). One underlying cause for the increased release of FFAs is the alterations in perilipin expression. Perilipins are phosphoproteins found in adipocytes on the surface of triacylglycerol droplets that act as gatekeepers, preventing lipases from hydrolyzing triacylglycerol to facilitate the release of FFAs. Obese individuals have a deficiency of perilipins even if their fat cells are larger, hence their increased basal rate of lipolysis. On the other hand, several pro-inflammatory proteins including TNF-α, interleukin 6 (IL-6), transforming growth factor β1, procoagulant proteins such as plasminogen activator inhibitor type-1(PAI-1), and tissue factor (Greenberg & Obin, 2006).

Macrophage numbers in adipose tissue also increase with obesity, where they apparently function to scavenge moribund adipocytes, which increase dramatically with obesity. Macrophages are responsible for most of the cytokine production in obese adipose tissue (eg. almost all TNF-α and significant amounts of IL-6 expression). Thus, the chronological appearance of these inflammatory molecules before the development of insulin resistance, as well as their known ability to promote insulin resistance and other complications of obesity, strongly suggests adipose tissue inflammation as an important protagonist in the development of obesity-related complications (Greenberg & Obin, 2006)

1.3.2. Oxidative Stress and Type 2 Diabetes

The term "oxidative stress" has been used to define a state in which reactive oxygen species (ROS) and reactive nitrogen species (RNS) reach excessive levels, either by excess production or insufficient removal. Excess ROS and RNS have pathological effect and damages proteins, lipids and DNA. Consistent formation of ROS and RNS leads to oxidative stress that may result in physiological dysfunction, cell death, pathologies such as diabetes and cancer, and aging of the organism (Johansen *et al.*, 2005).

Increasing evidence suggests that oxidative stress plays a role in the pathogenesis of diabetes mellitus and its complications due to diminished antioxidant mechanisms in diabetic patients, which may further augment oxidative stress (Brownlee, 2001; Maritim *et al.*, 2003). Most of the studies have addressed the role of superoxide (•O2 -), nitric oxide (•NO), and peroxynitrite (ONOO-) in relation to diabetes and its complications. Molecular oxygen is the final electron acceptor and will be reduced to H2O in the electron transport chain (Giacco & Brownlee, 2010). However, between 0.4 and 4% of all oxygen consumed will be converted into superoxide anion. In hyperglycemia, electron transfer is inhibited at complex III, causing the electrons to go back to complex II where there are transferred to molecular oxygen prematurely and not to complex IV as it naturally occurs. Therefore, the end product of this transfer is superoxide (Lazo-de-la-Vega-Monroy & Fernandez-Mejia, 2013).

It has been proposed that, ROS produced due to hyperglycemia inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sheikh-Ali *et al.*, 2011) through the activation of enzyme poly-ADP-ribose polymerase-1 (PARP-1) that is involved in DNA repair and apoptotic pathways (Rains and Jain, 2011). This GAPDH inhibition results in increased

levels of all the glycolytic intermediates. Accumulation of glyceraldehydes-3-phosphate activates two major pathways involved in hyperglycemia-complications: a) It activates the AGE pathway; for the nonenzymatic synthesis of methylglyoxal from glyceraldehyde phosphate and dihydroxyacetone phosphate. b) Activates PKC pathway; by increasing production of diacylglycerol (Lazo-de-la-Vega-Monroy & Fernandez-Mejia, 2013).

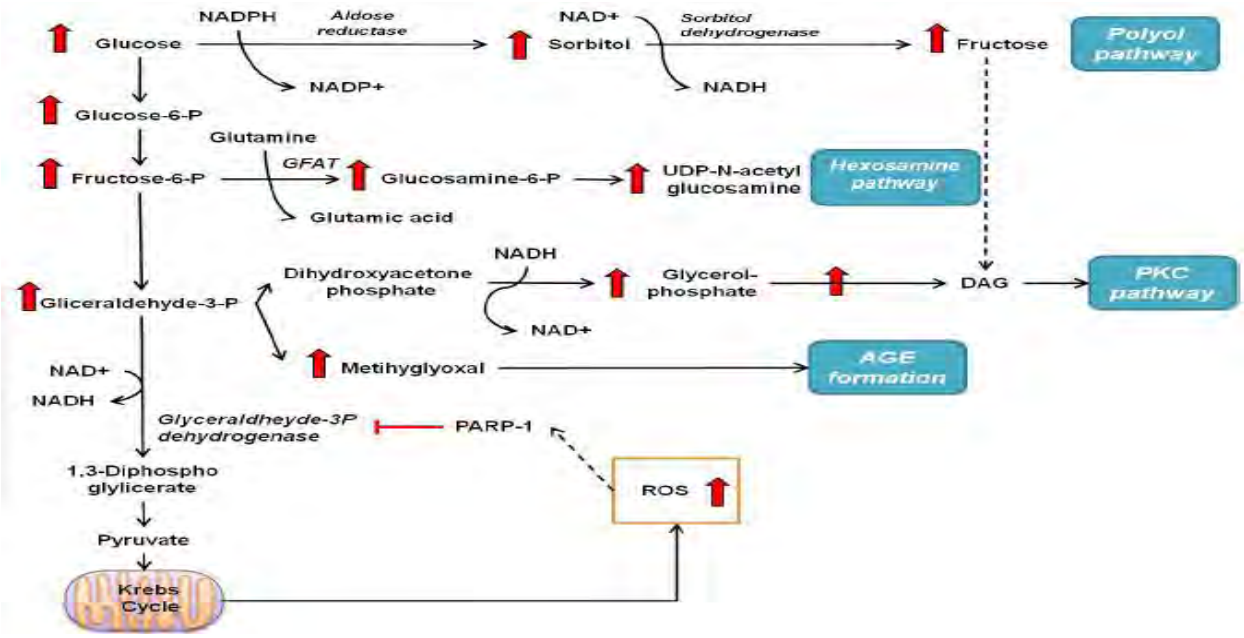

Figure 1 *Oxidative stress-related pathways derived from glucose metabolism (Lazo-de-la-Vega-Monroy & Fernandez-Mejia, 2013). Hyperglycemia increases ROS production that activates the poly-ADP-ribose polymerase-1 (PARP-1 enzyme which results in the inhibition of glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This increases levels of all the glycolytic intermediates resulting in the activation of many stress pathways.*

High level of fructose -6-phosphate increases flux through the hexosamine pathway, where it is converted to UDP–N-Acetyl glucosamine by the enzyme glutamine-fructose-6-phosphate amidotransferase (GFAT). Inhibition of GAPDH also favors the accumulation of the first glycolytic metabolite, glucose that increases its flux through the polyol pathway, consuming NADPH in the process (Giacco & Brownlee, 2010).

1.3.2.1. Oxidative Stress and the Thrombogenic State in Diabetes

Chronic hyperglycemia has been clearly identified as a causal factor for in vivo platelet activation and platelet hyper-reactivity in DM patients. Earlier studies demonstrated enhanced thromboxane (TX) biosynthesis in T2DM and provided evidence for its platelet origin. Tight metabolic control led to a reduction of TX levels in the same study. More recently, the same

authors demonstrated that the metabolic disorder rather than the attendant vascular disease appears to be responsible for persistent platelet activation (Ferroni *et al*., 2004). Hyperglycemia is responsible for nonenzymatic glycation of platelet membrane proteins that may cause changes in protein structure and conformation, as well as alterations of membrane lipid dynamics. This altered dynamic of platelet membrane may, in turn, result in enhanced expression of receptors which are crucial for platelet function, such as P-selectin and GpIIb/IIIa. These molecules are crucial for platelet function and their increased expression makes them much more susceptible for potential ligands leading to surface clotting factor activation and so directly enhance the thrombogenic state (Wang *et al*., 2007).

Increased oxidant stress in T2DM could also induce enhanced generation of a non-enzymatic oxidation product of circulating Low Density Lipoprotein (LDL) and Arachidonic acid called 8-iso-prostaglandin F2α (8-iso-PGF2α). This compound is widely recognized as a reliable marker of lipid peroxidation both in vitro and in vivo. It was shown that, the rate of formation of 8-iso-PGF2α correlated with the rate of TXA2 biosynthesis, implying that, 8-iso-PGF2α contributes to platelet activation. 8-iso-PGF2α induces vasoconstriction and may modify aspects of platelet function such as adhesive reactions and activation by low concentrations of other agonists. Thus, it was suggested that changes in the rate of arachidonate peroxidation to form biologically active iso-eicosanoids (8-iso-PGF2α), may represent an important biochemical link between altered glycemic control, oxidant stress and platelet activation in T2DM (Ferroni *et al*., 2004).

Moreover, glycated LDL may cause platelet dysfunction by an increase in intracellular Ca2+ concentration and platelet nitric oxide (NO) production, as well as inhibition of the platelet membrane Na+/K+-adenosine triphosphatase (Na+/K+-ATPase) activity. While the effects on intraplatelet calcium concentration are consistent with an enhanced sensitivity to aggregating agents, the higher NO production is expected to result in a reduction of platelet function and the effects of NO might be counteracted by the contemporaneous increase in platelet calcium (Creager and Lüscher, 2003). However, in diabetes NO and ROS production are not balanced. This may induce the reaction between NO and O2- producing a strong oxidant called peroxynitrite. Oxidized LDL has been shown to decrease nitric oxide synthase (NOS) protein expression in human platelets (Ferroni *et al*., 2004). Furthermore, the attack of LDL by ROS

may cause the release of bioactive isoprostanes, which, in turn, are responsible for enhanced agonist-induced platelet adhesion and aggregation causing cardiovascular diseases.

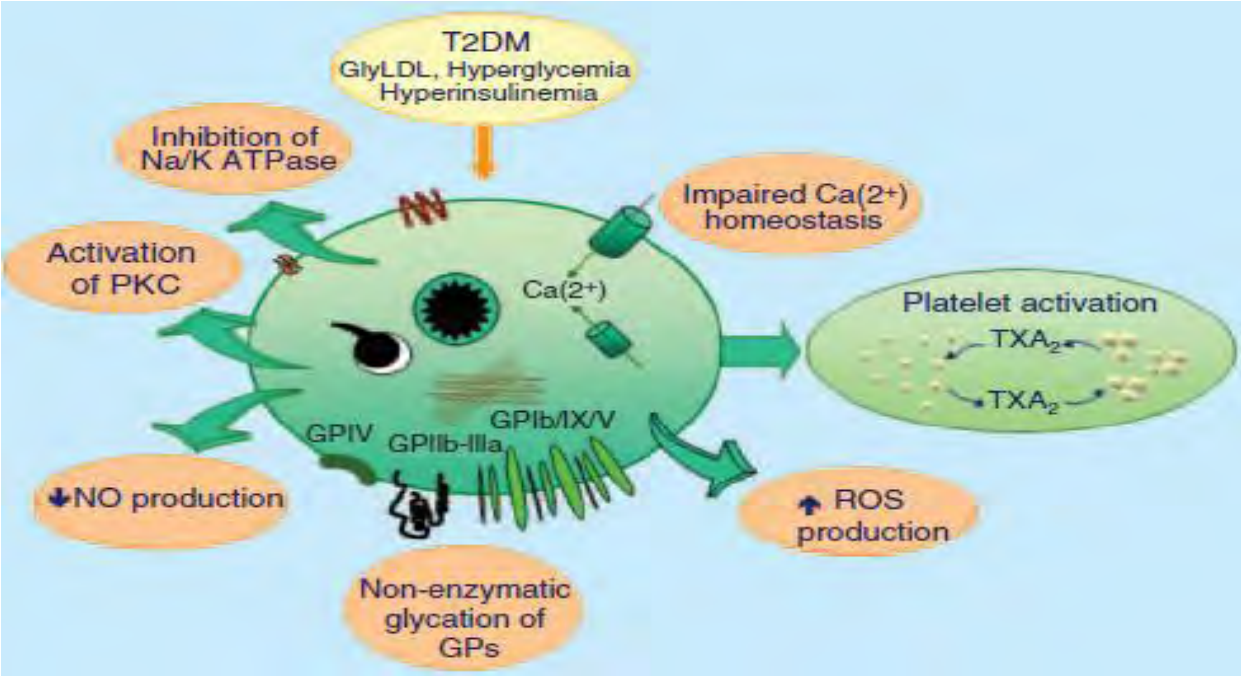

Figure 2: *Altered glycemic control impairs platelet function. As shown, elevated glucose levels as well as glycated low-density lipoproteins (GlyLDL) and hyper-insulinemia may led to impaired calcium homeostasis, inhibition of the platelet membrane Na+/K+-adenosine triphosphatase (Na+/K+-ATPase) activity, activation of protein kinase C (PKC), decreased production of nitric oxide (NO), increased formation of reactive oxygen species (ROS) and non-enzymatic glycation of platelet membrane glycoproteins (GPs). All these events can be ultimately responsible for activation of the arachidonic pathway resulting in increased TXA2 formation and platelet hyperfunction*

Thus, the increased intracellular calcium concentration and activation of protein kinase C (PKC), as well as the decreased NO bio-availability may be responsible for increased platelet sensitivity to agonists in diabetes. Similarly, generation of ROS by platelet enzymatic sources may lead to formation of lipid peroxides either from arachidonic acid or circulating low-density lipoprotein (LDL), which may cause a dose-dependent increase in calcium release from intracellular stores and platelet shape change, as well as enhanced aggregation response to sub-threshold concentrations of platelet agonists. Finally, the increased extent of glycosylation of platelet membrane GPs in diabetes appears to be related to reduced membrane fluidity and altered receptor availability, contributing to platelet hyper-function. All these events can be ultimately responsible for activation of the arachidonic pathway resulting in increased thromboxane A2 (TXA2) formation (Vinic *et al*., 2001; Ferroni *et al*., 2004; Kakouros *et al*., 2011).

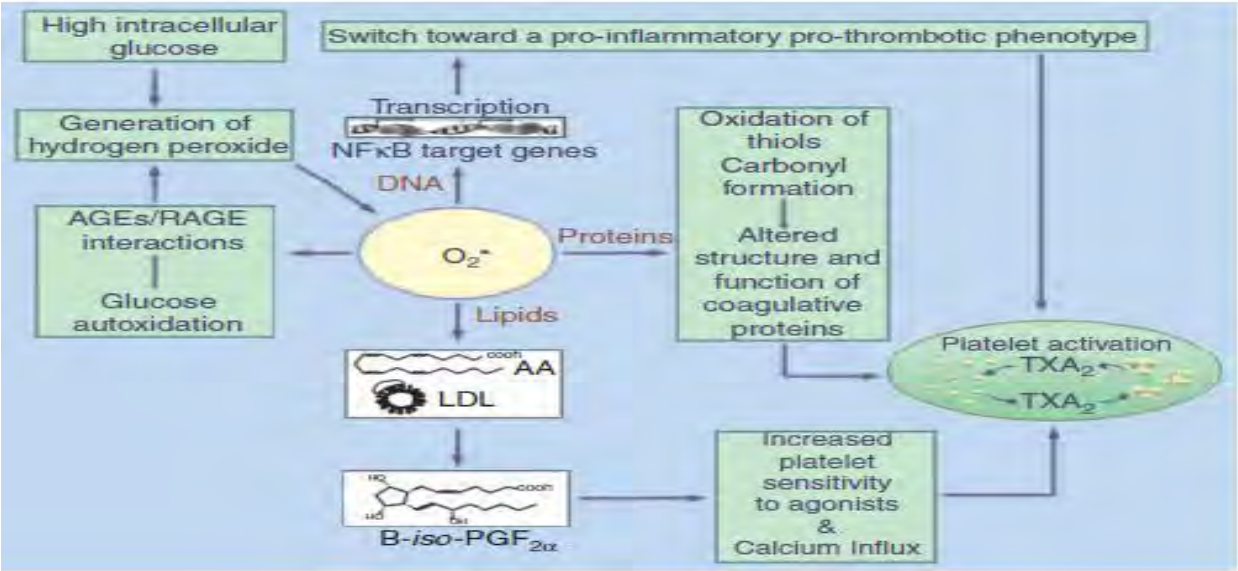

Figure 3: *oxidative stress in diabetes results in platelet activation associated (Ferroni et al., 2004). ROS may exert their effects on:(1) lipids resulting in the production of bioactive isoprostanes (i.e. 8-iso-prostaglandin-F2a-8-iso-PGF2a) from oxidation of arachidonic acid (AA) and low-density lipoproteins (LDL).(2) proteins through oxidation of thiols and carbonyl formation; (3) DNA, through activation of signaling molecules (i.e. nuclear transcription factor-kB – NF-kB) and subsequent transcription of genes encoding cytokines and adhesive proteins leading to a switch toward a prothrombotic phenotype of endothelial cells. All these phenomena may contribute to increased platelet sensitivity and activation of the AA pathway resulting in increased TXA2 formation.*

Another important idea is the availability of nitric oxide (NO) which is constitutively produced by endothelial NO synthase (eNOS) through a 5-electron oxidation of the guanidine-nitrogen terminal of L-arginine. The bioavailability of NO represents a key marker in vascular health. NO cause's vasodilatation by activating guanylyl cyclase on subjacent vascular smooth muscle cells. In addition, NO protects the blood vessel from endogenous injury (ie, atherosclerosis by mediating molecular signals that prevent platelet and leukocyte interaction with the vascular wall and inhibit vascular smooth muscle cell proliferation and migration (Creager and Lüscher, 2003). Conversely, the loss of endothelium-derived NO permits increased activity of the pro-inflammatory transcription factor nuclear factor kappa B (NF-kB), resulting in expression of leukocyte adhesion molecules and production of chemokines and cytokines. These actions promote monocyte and vascular smooth muscle cell migration into the intima and formation of macrophage foam cells, characterizing the initial morphological changes of atherosclerosis (Kakouros *et al*., 2011).

Hyperglycemia increases the production of superoxide anion and lipid second messenger diacylglycerol which causes the membrane translocation and activation of PKC. Peroxynitrite,

resulting from the interaction of NO and superoxide anion, oxidizes the NOS co-factor tetrahydrobiopterin. This uncouples NOS enzyme, which then preferentially increases superoxide anion production over NO production. Activation of PKC inhibits the phosphatidylinositol 3 kinase pathway, thereby limiting activation of Akt kinase and subsequently phosphorylates NOS, resulting in less NO production. Diminished endothelium-dependent relaxation of rabbit aorta exposed to elevated glucose levels is restored by PKC inhibition (Ferroni *et al*., 2004).

On the other hand, insulin which normally stimulates NO production from endothelial cells by increasing the activity of NOS via activation of phosphatidylinositol-3-kinase and Akt kinase in healthy individuals is impaired in T2D patients. This is due to insulin resistance and results in the impairment of NO-mediated endothelium dependent vasodilatation. It was indicated that, insulin mediated glucose disposal correlates inversely with severity of the impairment in endothelium dependent vasodilatation. Drug therapies that increase insulin sensitivity, such as metformin and the thiazolidinediones, improve endothelium-dependent vasodilatation. In insulin resistant individuals, insulin signals through mitogen activated protein kinase path way. It was indicated that, activation of this path way is associated with increased endothelin production and a greater level of inflammation and thrombosis (Vinic *et al*., 2001; Ferroni *et al*., 2004).

1.3.2.2. The Body's Defense Mechanism against ROS in diabetes

ROS are a byproduct in type 2 diabetes, generated during protein glycation and as a consequence of advanced glycation end products- receptor binding; they impair insulin signalling pathways and induce cytotoxicity in pancreatic beta cells. Neutralization of oxidants by increased antioxidant (enzymatic and non enzymatic) availability may mitigate these effects. So, the body has an integrated antioxidant system which acts synergistically to protect tissue against free radical attack and the onset of disease.

I. Enzymatic Antioxidants

The superoxide anion ($\bullet O_2^-$) formed is normally scavenged by the antioxidant system of the body like enzymes such as; Superoxide dismutase (SOD), Glutathione Peroxidase (GSH-Px) and catalase and by antioxidant vitamins such as; vitamin A, C, E and alpha-lipoic acid. However, in chronic hyperglycemia, the activity of these enzymes is insufficient (Maritim *et al*., 2003) and

$\bullet O_2^-$ undergo dismutation to H_2O_2 by SOD, which if not degraded by catalase or glutathione peroxidase, shifts to the Fenton reaction leading to the production of extremely reactive hydroxyl radicals(•OH) or ROS (Brownlee, 2001; Maritim *et al*., 2003).

SOD present in nearly all cells and is considered as a first-line defense against ROS. GSH peroxidase is found in mitochondria and catalyzes degradation of H_2O_2 by reduction, where two gluthathione (GSH) molecules are oxidized to glutathione disulfide (GSSG). Regeneration of GSH by GSH-reductase requires NADPH, which is oxidized to NADP+. On the other hand, catalase is localized primarily in peroxisomes, and so it detoxifies the H_2O_2 that diffuses from the mitochondria to the cytosol, by converting it into water and molecular oxygen (Lazo-de-la-Vega-Monroy & Fernandez-Mejia, 2013).

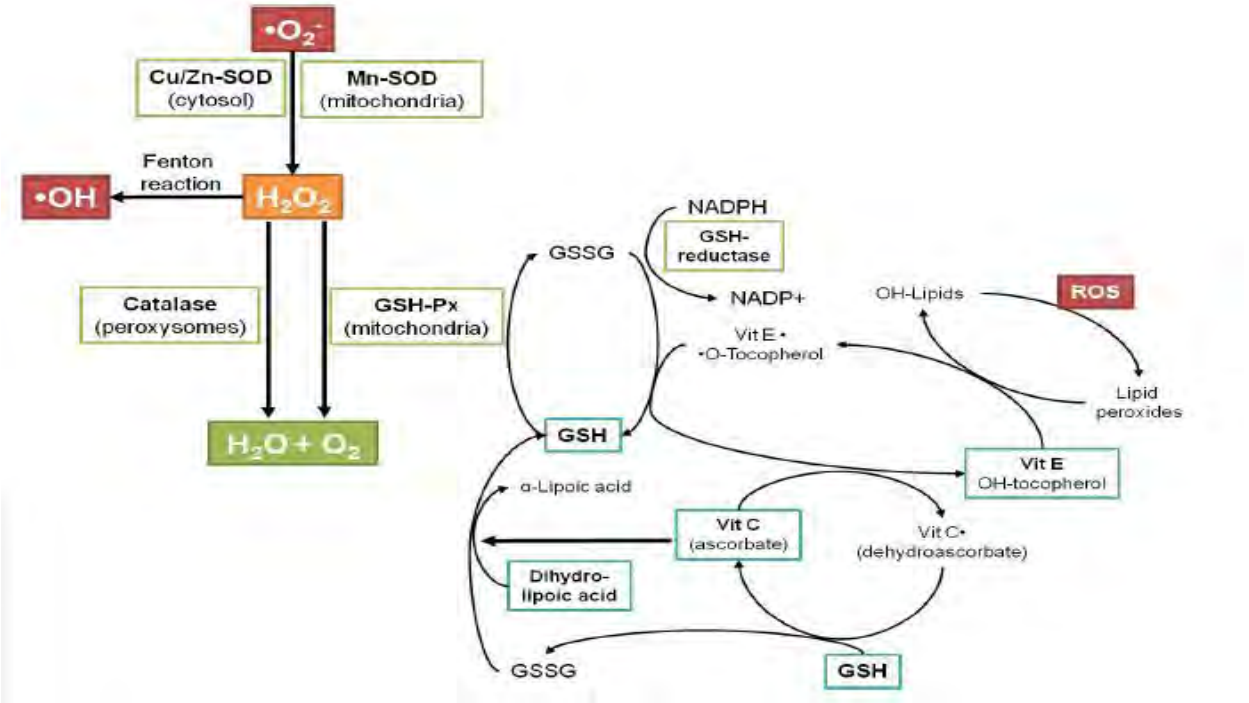

Figure 4: *Antioxidant defenses in the organism (Lazo-de-la-Vega-Monroy & Fernandez-Mejia, 2013).superoxide dismutase (SOD) converts superoxide anion (•O_2^-)to hydrogen peroxide (H2O2)which is then degraded either by glutathione peroxidase (GSH-Px) or catalase enzymes. GSH-Px degrades H2O2 by reduction where two molecules of glutathione (GSH) are oxidized to glutathione disulfide (GSSG).GSH then regenerated by glutathione reductase.*

II. Non Enzymatic Antioxidants

Levels of zinc, ascorbic acid, albumin, uric acid and bilirubin are often used as major non-enzymatic antioxidant biomarkers and they prevent free radical reaction by metal ion chelation in

plasma, providing the primary extracellular defense against oxidative stress **(**Hisalkar *et al*., 2012). Several studies have addressed the possible participation of dietary antioxidants, such as vitamins, in ameliorating the diabetic state and retarding the development of diabetes complications (Cuerda *et al*., 2011). Vitamins C, E, and A constitute the non enzymatic defense against oxidative stress, by regenerating endogenous antioxidants.

a. Vitamin C (Ascorbic acid):

Is an essential micronutrient required for normal metabolic functioning of the body because, human have no ability to synthesise vitamin C due to mutation in the gene coding for L-gukonolactone oxidase, an enzyme required for the biosynthesis of vitamin via the glucuronic acid pathway. It is an important water soluble antioxidant in biological fluids having a role in scavenging ROS and RNS by becoming oxidated itself **(**Hisalkar *et al*., 2012). The oxidized products of vitamin C, ascorbic radical and dehydroascorbic radical are regenerated by glutathione, NADH or NADPH. In addition, vitamin C can reduce the oxidized forms of vitamin E and glutathione (Lazo-de-la-Vega-Monroy & Fernandez-Mejia, 2013).

Several studies have reported that, plasma concentrations of ascorbic acid lowered in diabetics compared to healthy subjects. Low vitamin C status in diabetes may be due to a higher turnover rate of ascorbic acid, with increased oxidation to the oxidized form dehydroascorbate. Another possible mechanism for the impaired ascorbate status is a competitive inhibition between glucose and ascorbic acid, which both share a close structural homology and possibly occupy common membrane transport sites. Supplementation with ascorbic acid may have a beneficial effect (Hisalkar *et al*., 2012).

b. Uric Acid

Like ascorbic acid, uric acid is a strong reducing agent (electron donor) and a potent antioxidant. In humans, over half of the antioxidant capacity of blood plasma is derived from uric acid. The plasma concentration of uric acid is almost 10- fold higher than other non enzymatic antioxidants, such as vitamin C and E **(**Becker, 1993). Moreover; uric acid has much higher antioxidant capacity, Urate (the soluble form of uric acid in the blood) can scaverge super oxide, hydroxyl radical, singlet oxygen and can chelate transition metals. Uric acid can also block a particular

reaction between super oxide anion with nitric oxide to produce toxic product called peroxynitrite that can injure cells by nitrosylating the tyrosine residues (nitro tyrosine formation) of proteins (Ceriello, 2003).

It was observed that, serum uric acid level significantly decreases in type 2 diabetic patients as compared to non diabetics. Oxidative stress causes reduction of the antioxidant status of the body including serum uric acid. The higher serum glucose concentration may increase fractional excretion of urate caused by an effect of glucose at the renal tubule resulting in decreased uric acid (Hisalkar *et al*., 2012).

c. Blirubin and Albumin

Bilirubin, a bile pigment and metabolite of hemoglobin also plays a role as an antioxidant by scavenging peroxyl radicals. The protective role of bilirubin includes inhibition of oxidative modification of plasma proteins and formation of protein carbonyl groups. Bilirubin in circulation is mainly found bound to albumin. Localization of bilirubin and albumin protects albumin from oxidation as well as the albumin-bound linoleic acid from peroxyl radical induced oxidation. Serum albumin and total bilirubin level significantly lower in type 2 diabetic patients (Hisalkar *et al*., 2012).

1.4. Pathophysiology of Diabetes

1.4.1. High Serum Triglyceride Associated With Diabetic Endothelial Dysfunction

T2D patients are characterized by abnormalities of the lipid metabolism particularly, hypertryglycidemia and low level of high density lipoprotein (HDL). Elevated triglyceride concentrations lower HDL by promoting cholesterol transport from HDL to very-low density lipoprotein (VLDL). This result in triglyceride rich VLDL that potentiates platelet activity, an effect mediated partly through apolipoprotein E and an interaction with the platelet LDL receptor. These abnormalities change LDL morphology, increasing the amount of the more atherogenic, small, dense LDL. Both hypertriglyceridemia and low HDL have been associated with endothelial dysfunction (Creager and Lüscher, 2003; Schneider, 2009 Kakouros *et al*., 2011).

1.4.2. Succinate a Citric Acid Cycle Intermediate Increases Systemic blood pressure

G-protein coupled receptor-91 (GPR91) is a metabolic receptor for the citric acid cycle intermediate succinate and is highly expressed in the kidney. This receptor can lead to renin-dependent activation of Renin-Angiotensin System (RAS) and increased systemic blood pressure (He *et al*., 2004). In the liver and kidneys, succinate triggers paracrine signaling through GPR91 leading to (patho) physiological alterations in organ function. In kidney it has long been known to cause renin release from the juxta)glomerular apparatus (JGA) (Hebert, 2004; Correa *et al*., 2007). New data has demonstrated that, localized succinate accumulation occurs in the intact diabetic kidney as well as in the dissected, in *vitro* microperfused JGA preparation acutely subjected to high glucose levels. High glucose and succinate-induced GPR91 activation trig ger paracrine signaling from the (juxta)glomerular endothelium to the adjacent renin-producing JG cells to increase renin synthesis and release (Toma *et al*., 2008), the rate-limiting step of RAS activation.

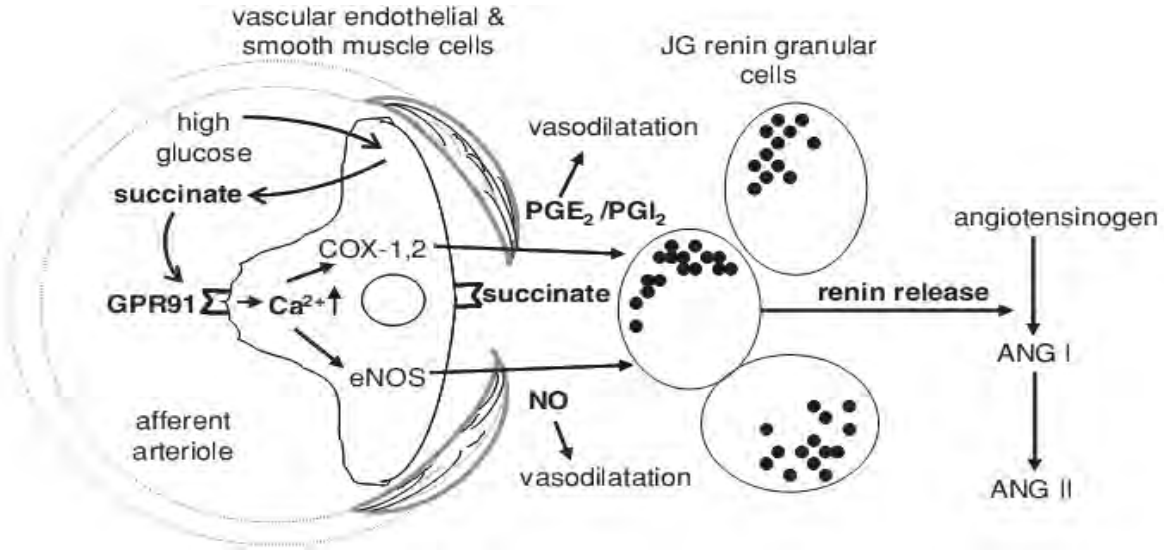

Figure 5: *Elevated blood glucose can result in the increased systemic blood pressure (Peti-Peterdi et al., 2008). Elevated blood glucose results in the activation of GPR91 through accumulation of metabolic intermediate succinate in the plasma and local interstitium, increases endothelial cytosolic calcium, nitric oxide (NO) and prostaglandin (PGE2) production and release from endothelium. PG act on renin-producing JG cells resulting in renin release that converts angiotensinogen to angiotensin-I leading to angiotensin-II synthesis and RAS activation. ANG-II is apotent vasoconstrictor that may result in the increased systemic blood pressure.*

Elements of the signal transduction cascade involve succinate and GPR91-dependent elevations in vascular endothelial [Ca2+]i as well as the synthesis and release of NO and PGE2,classic mediators of rennin release**.** Endothelial NO and prostaglandin production also directly causes

vasodilatation of the afferent arteriole, which may be important in the development of glomerular hyper-filtration. Thus GPR91-mediated paracrine signaling pathway provides an alternative to the 'tubular hypothesis' and offers a direct mechanism for the development of both hallmarks of diabetes: glomerular hyper-filtration and JGA rennin activation (Peti-Peterdi *et al.*, 2008).

1.5. Hematological profiles in T2D patients.

1.5.1. Red Blood Cell (RBC) indices

1.5.1.1.Abnormalities of Red Blood Cell Count (RBC) in Type 2 Diabetic patients

The consequent elevation of glucose in the blood plasma affects primarily RBCs and the vascular endothelial cells, including the walls of capillaries. An earlier study showed that as the degree of hyperglycemia increased, so did the incidence of complications, with the "poor" control group having the greatest incidence of retinopathy, nephropathy, and neuropathy (Pirat, 1978). It was indicated that, lower RBC count is an independent predictor of the risk of micro-vascular complications in patients with T2DM partly due to effect of decreased RBC count on RBC function, after controlling for conventional risk factors, including smoking, diabetes duration, BMI, blood pressure, lipids, and glucose. Chronic hyperglycemia causes non-enzymatic glycosylation of RBC membrane proteins and decreased RBC count that reduces the negative surface charge of RBCs leading to accelerated aging of RBCs (Wang *et al.*, 2013).

The decreased cell surface charge is thought to firm adhesion between surfaces resulting in electrostatic repulsion between the cells leading to diminished RBC aggregation and then to low shear-rate viscosity yielding stress of blood (Rogers *et al.*, 1992). Therefore, a decrease in the negative charge of RBCs leads to a collinear decrease of membrane deformability and an increase in microviscosity, aggregation, and adhesiveness of RBCs resulting in low RBC velocity and/or complete standstill when moving through capillary segments (Huang *et al.*, 2011; Wang *et al.*, 2013). In normal mammalian cells, phosphatidylserine containing negative charge is exclusively confined to the inner layer of the plasma membrane. However, in hyperglycemia, increased intracellular oxidative stress can impair maintenance of phospholipid asymmetry, resulting in phosphatidylserine externalization. Loss of this asymmetric distribution and exposure of phosphatidylserine on the external surface generate a thrombogenic surface and signal macrophages to remove cells by phagocytosis (Wang *et al.*, 2013).

Hyperglycemia reduces RBC life span, leading to high variability of the RBC volumes. It has been demonstrated that, increased extracellular oxidative stress can be responsible for erythrocyte caspase-3 activation in type 2 diabetes. Activated caspase-3 impairs the maintenance of erythrocyte shape and function, thus contributing to the shortened life span of RBCs. studies indicated that, a modest but consistent increase in erythrocyte half-life after the establishment of tight glycemic control compared with the same patients studied in poor control (Nada, 2015).

The erythrocyte aggregation is an important hemorheological parameter because it directly affects WBV. Enhanced erythrocyte aggregation is the principal cause of vascular complications in DM since RBC aggregates cannot pass through the capillaries. RBC aggregation is considered to be a primary cause of elevated WBV at low shear rates with respect to higher shear rates. (Kim *et al*., 2006). Blood viscosity depends both on macrorheological parameters, namely, hematocrit and serum proteins (fibrinogen and globulins), and on microrheological parameters, namely, the degree of RBC aggregation and deformability. Hematocrit is one of the most important variables affecting the overall WBV specimen; however, the magnitude of low shear viscosity is primarily determined by erythrocyte aggregation, whereas differences in high shear viscosity are robustly influenced by the erythrocyte deformability. Accordingly, low shear viscosity has a close correlation with the plasma fibrinogen and individual globulin concentrations (Cho *et al*., 2008).

Multiple pathophysiological disorders have been involved in the development of diabetic microangiopathy, including rheological disorders of red blood cells (RBCs) and decreased RBC deformability. The abnormalities in hematological parameters like hematocrit, plasma proteins, erythrocyte aggregation, and erythrocyte deformability are associated with markedly increased both plasma and whole blood viscosity (WBV) (Wang *et al*., 2013). Abnormally high blood viscosity play a role in further aggravating myocardial ischemia, because oxygen delivery is already diminished due to atherosclerotic plaque at the coronary artery and it may also increases injurious forces at the endothelial wall, thus adversely impacting endothelial function and thereby contributing to the inflammatory process (Cho *et al*., 2008).

The erythrocyte deformability can have a profound impact on microcirculation, because the size of red cells is typically approximately 8 µm. It is crucial to perfusion for the red cells to pass

through the capillaries in order to supply oxygen to the surrounding tissues (Cho *et al*., 2008). Additionally, it has been suggested that the impaired perfusion at the tissue level observed as, a complication of diabetes mellitus and is primarily due to the reduced erythrocyte deformability (Zimny *et al*., 2001). Therefore, Wang *et al,* have concluded that a decreased RBC count is associated with micro-vascular complications in Chinese patients with T2DM indicating that, lower RBC counts may be related to RBC membrane protein alterations, a decrease in hemoglobin levels, and erythropoietin deficiency (Wang *et al*., 2013)

1.5.1.2.Abnormalities of the Hematocrit (HCT) value in T2DM patients

HCT in a given individual may not remain constant but rather is a dynamic parameter that may change rapidly and significantly as a part of physiological, pathophysiological, and even psychosomatic processes. An acute rise in HCT might be the result of a relative increment of RBC mass in the circulatory system because of a reduction of intravascular volume. The primary cause of this volume reduction may be fluid loss by various means or may result from constriction of the circulatory system that shifts the balance of forces governing the fluid exchange at the tissue level. A fluid shift from the vascular space to the interstitial area then results in the higher level of HCT value in the vasculature even if there is not an absolute increase in RBC mass (Baskurt & Meiselman, 2003). In hyperglycemia, increased glucose level increases blood osmolarity and capillary permeability. These may result in the increased HCT value and subsequently the blood viscosity. Thus, in T2D patients, hyperglycemia may lower plasma volume and increase HCT value causing an osmotic diuresis. Widespread increased microvascular permeability might lead to reduced plasma volume and hence increased HCT. Increased HCT is associated with slowed retinal circulation. Since HCT is the major determinant of whole blood viscosity, increased HCT increases blood viscosity. Both hematocrit and blood viscosity decreased after institution of good diabetic control. In summary, diabetes patients had higher blood viscosity than healthy people partly due to increased HCT value (Cho *et al*., 2008)

1.5.1.3.Abnormalities of Red blood cell distribution width (RDW) in T2DM patients

RDW is defined as the quotient of standard deviation of RBC volume & its mean volume & is expressed as a percentage. It is a quantitative measure of the heterogeneity of the volume of red blood cells (RBCs) with higher values reflecting greater heterogeneity in cell sizes

(anisocytosis). It is originally used together with the mean corpuscular volume (MCV) in clinical practice to differentiate between causes of anemia. RDW has been shown to independently predict overall and cardiovascular mortality in the general population and various high-risk populations**.** It is also a strong predictor of mortality in many conditions such as obesity, malignancies, and chronic kidney diseases. Chronic inflammation and increased level of oxidative stress in hyperglycemia, elevates the values of RDW that reflect greater variation in distribution of RBC volumes. This is related to the impairment of erythropoiesis and degradation of erythrocytes by fragmentation or agglutination (Nada, 2015).

Higher RDW values are associated with increased risk for cardiovascular disease and nephropathy in adult patients with diabetes. Increased RDW was associated with decreased RBC deformability, which can impair blood flow through the microcirculation. RBC deformability plays a role, as elevated RDW is associated with increased inflammation and decreased levels of antioxidants. Moreover RDW is elevated in conditions of ineffective red cell production (eg, iron deficiency, anemia of chronic disease, B12 or folate deficiency) and increased red cell destruction (eg, hemolysis) (Wang *et al*., 2013).

1.5.1.4.Abnormalities in Hemoglobin (HGB) level

Hyperglycemia causes the abnormal glycation, that can adversely affect hemoglobin and membrane proteins in erythrocytes and it has been shown to correlate with reduced membrane fluidity (Cho *et al*., 2008). High values of glycosylated hemoglobin have also been found to correlate with decreased deformability of erythrocytes separately (Bauersachs *et al*., 1989). Some hemoglobin molecules of aging RBCs are aggregated and attach to the inside of the cell membrane that can lead to a reduction in membrane flexibility and influence the oxygenation of hemoglobin A low RBC negative charge in patients with T2DM is also associated with Diabetic Retinopathy (DR) and changes in the properties of the retinal basement membrane (Huang *et al*.,2011; Wang *et al*., 2013).

It has been indicated that, RBC count has a positive correlation with the serum hemoglobin level. So that, decrease in RBC count in diabetics leads to a decreased hemoglobin level contributing to the occurrence of microvascular complications. Anemia causes tissue hypoxia that can contribute to renal vasoconstriction by stimulating the sympathetic nervous system and intrarenal renin–

angiotensin system. The renal vasoconstriction may initiate nephropathy. Anemia in early Diabetic Neuropathy (DN) is also induced by a poor response of erythropoietin to low hemoglobin levels as a result of impaired sensing mechanisms associated with autonomic DN (Wang *et al*., 2013).

1.5.2. Abnormalities in White Blood Cell (WBC) count in T2DM Patients

Several epidemiologic studies have indicated a close relationship between WBC count plus RBC count and components of metabolic syndrome (Chen *et al*., 2006). Insulin resistance is observed in a number of pathologic conditions; including obesity, T2DM, cardiovascular diseases, and chronic inflammations (Chen *et al*., 2006). In Insulin resistance conditions, IL-6, a cytokine generated from adipose tissue, is significantly elevated and thereby stimulates (elevates) WBC (Mohamed-Ali *et al*., 1997). Activated (elevated) WBC enhances atherosclerotic process and vascular injury. Furthermore, insulin resistance and/ or hyperinsulinemia also enhance erythropoiesis independent of erythropoietin (Chen *et al*., 2006). Elevated WBC count is also linked to the risk of T2DM complications (myocardial infarction, coronary mortality, and ischemic stroke) (Shimakawa & Bild, 1993).

1.5.3. Abnormalities in Platelets (PLT) parameters

1.5.3.1.Abnormalities in Platelet Count

In T2DM, insulin resistance increases platelet aggregation. As insulin resistance or glycemic status progresses, platelet count also increases (Chen *et al*., 2006). So, hyperactivity of platelet function and overconsumption of platelets resulting from the underlying prothrombotic condition are common in insulin-resistant or diabetic subjects (Vinik *et al*., 2001). It is indicated that, increased platelet count may independently predict insulin resistance among non-obese Japanese type 2 DM patients (Taniguchi *et al*., 2003). Hyperglycemia can increase platelet reactivity by inducing nonenzymatic glycation of proteins on the surface of the platelet, by the osmotic effect of glucose and activation of protein kinase C. Such glycation decreases membrane fluidity and increases the propensity of platelets to activate (Kakouros *et al*., 2011).

In inflammation, superoxide increases intraplatelet release of calcium after their activation and thus enhancing platelet reactivity (Kodiatte *et al*., 2012) but decreases the biologic activity of nitric oxide (NO) because the oxidative stress impairs endothelial function that reduces

production of NO and prostacyclin bringing about increased platelet reactivity. The increased platelet activity is emphasized to play a role in the development of vascular complications of DM (Kodiatte *et al*., 2012). In response to stimuli generated by the endothelium of blood vessels, platelets change shape, adhere to sub-endothelial surfaces, secrete the contents of intracellular organelles, and aggregate to form a thrombus. These pro-aggregatory stimuli include thrombin, collagen, epinephrine, ADP (dense storage granules), and thromboxane A2 (activated platelets) (Kodiatte *et al*., 2012) Thus, platelets may assume an important role in signaling of the development of advanced atherosclerosis in diabetes (Colwell & Nesto., 2003).

1.5.3.2.Abnormalities in Mean Platelet Volume (MPV)

Platelet volume, a marker of the platelet function and activation, is measured as mean platelet volume (MPV). Diabetic patients have an increased risk of developing micro- and macrovascular disease, and platelets may be involved as a causative agent with respect to altered platelet morphology and function (Hekimsoy *et al*., 2004). MPV is an indicator of the average size and activity of platelets. Larger platelets are younger, more reactive and aggregable. Hence, they contain denser granules, secrete more serotonin and β-thromboglobulin, and produce more thromboxane A2 than smaller platelets (Chang *et al*., 2010). All these can produce a pro-coagulant effect and cause thrombotic vascular complications. This suggests a relationship between the platelet function especially MPV and diabetic vascular complications thus indicating changes in MPV reflect the state of thrombogenesis. Thus, DM has been considered as a "prothrombotic state" with increased platelet reactivity (Kodiatte *et al*., 2012).

Therefore, in diabetes mellitus, platelets become more reactive and aggregable and their mean volume (MPV) is increased. The increased platelet size (count) may be one factor in the increased risk of atherosclerosis associated with diabetes mellitus and vascular complications. Hence, MPV would be a useful prognostic marker of cardio-vascular complications in diabetes. (Kodiatte *et al*., 2012).

1.6. Statement of the Problem

Diabetes Mellitus (DM) is a complex metabolic syndrome characterized by chronic hyperglycemia resulting in complications affecting the peripheral nerves, kidneys, eyes, and micro- and macrovascular structures. The prevalence of all types of diagnosed diabetes in most western societies is 3–7% (Kodiatte *et al*., 2012). Thirty five (35) out of 219 countries (16% of the total) has very high prevalence of diabetes of 12% or higher. These countries are located mainly in Western Pacific, and Middle East and North Africa regions. Africa is the region with the lower prevalence of diabetes (4.9%), having Reunion (15.4%), Seychelles (12.1%) and Gabon (10.7%) as the top three countries with higher prevalence and 10 out of 48 countries with prevalence of diabetes higher than the upper quartile (6.3%) prevalence. DM occurs throughout the world, but is more common in the more developed countries. Globally, in 2010, an estimated 285 million people had diabetes, with type 2 making up about 90% of the cases. In 2013, according to International Diabetes Federation, it is estimated that almost 382 million people suffer from diabetes for a prevalence of 8.3% and by the year 2030, this number is estimated to almost double with the greatest increase in prevalence is expected to occur in Asia and Africa (more than 80% of the 382 million diabetic patients will be in developing countries) (Wild *et al*., 2004). The prevalence of diabetic microvascular complications is higher in people with poor glycemic control, longer duration of DM, associated hypertension, and obesity. Prevalence of DM and its vascular burdens are increasing day by day. This leads to increased morbidities and mortalities in DM patients. Diabetes and its vascular complications can cause a financial havoc, become a burden to a country's national economy and dent (suppress) its growth. India, having the highest number of diabetics, faces such issues (Kodiatte *et al*., 2012). According to the International Diabetes Federation (IDF) report of 2011, the number of adults living with diabetes in Ethiopia was 3.5% an increased to 4.36% in 2013; implying that, the prevalence of DM is significantly increasing in Ethiopia.

1.7. Significance of the study

This study aimed at determining and comparing the hematological profiles and non enzymatic antioxidants levels of diabetic individuals with health controls in federal police referral hospital, Addis Ababa, Ethiopia, which is a rapidly growing and urbanizing city. Diabetes is a global health problem with T2DM more prevalent accounting about 90% of the total diabetes cases. In Ethiopia, the prevalence was 3.5% in 2011 and grown to 4.36% in 2013 according to IDF report. The disease is commonly associated with cardiovascular diseases of both micro- and macro-vascular complications. These complications are the leading cause of disability, high morbidity and premature mortality in which the primary cause of premature mortality is the macro-vascular complications and micro-vascular complication is the primary cause for disability and morbidity. Diabetes and its vascular complications dent (suppress) the growth, can cause a financial havoc and become burden to individual's, family's and country's national economy. Hematological abnormalities are responsible for both micro- and macro-vascular complications in diabetes. Thus, the study can provide information and understanding of the importance and potential role of hematological profiles in diabetes pathology and help to distinguish defects of the parameters as an underappreciated culprit in diabetes. Generally this study may aid to open up a way to the poorly understood relevance of hematological abnormalities in diabetics and further initiate researchers to participate in other studies to be done in the general population of diabetics in Ethiopia. Thus early diagnoses of hematological abnormalities are very essential to prevent micro- and macro-vascular complications thereby preventing disability, morbidity and premature mortality.

2. OBJECTIVES OF THE STUDY

2.1. General Objective

To investigate the Body Mass Index, Blood Pressure, Hematological Profiles and Non-enzymatic Antioxidants Status in Type 2 Diabetic Patients.

2.2. Specific Objectives

- ✓ To find out the mean blood glucose level variation between the diabetic and non-diabetic healthy individuals.
- ✓ To compare hematological profiles between diabetic and non-diabetic healthy individuals.
- ✓ To investigate non enzymatic antioxidants (Blirubin and Uric acid) level between diabetic and non diabetic healthy individuals
- ✓ To compare the average levels of BMI, systolic and Diastolic Blood Pressures among diabetic and non diabetic healthy individuals.

3. MATERIALS AND METHODS

3.1. Study Design

Hospital based, cross sectional study with comparative nature was conducted on T2DM patients in Federal police Specialized Hospital.

3.2. Study Area

Study area was Addis Ababa city, Federal police Specialized Hospital, Diabetic centre. The hospital is the only referral hospital designed to serve all Ethiopian police members. The hospital is located in the capital city of Ethiopia, Addis Ababa and it has been giving service for all patients of police members from every corner of the regions.

3.3. Study Population

All adult diabetic out patients (>18 years of age) attending the diabetic clinic of federal police specialized hospital (FPSH) during the study period, Addis Ababa.

3.4. Study Subjects

All adult diabetic out patients (>18 years of age) attending Diabetic clinic of the hospital at the time of data collection period fulfilling the inclusion criteria to be selected as per the sampling procedure were the study subjects. The study was designed to include 70 type 2 diabetic patients (male and female) and 70 healthy controls (male and female) who were willing to give informed consent and fulfill inclusion criteria.

3.5. Study period

Data collection period was from December 2016 - January 2017.

3.6. Inclusion and Exclusion Criteria

3.6.1. Inclusion Criteria

- Patients with different duration of diabetes, different age group and sex and those with different blood pressure, body mass index (BMI), fasting blood glucose and blood cell counts were included in the study.
- Patients taking antihypertensive and anti-diabetic medications were included in the study.

3.6.2. Exclusion Criteria

- Patients with a history of known hematologic diseases like; hemolytic anemias, post-hemorrhagic anemia and renal anemia that may affect hematological profiles were excluded from the study.
- Patients with a history of severe arthritis, liver cirrhosis, pregnancy, inflammatory bowel diseases, and serious infections
- Hematologic diseases with WBC count > 15,000/mm^3 and platelet count > 500,000/mm^3 were excluded from the study.
- Individuals with micro- and macro-vascular complications were excluded, since these complications affect both hematological and biochemical profiles.
- Patients having habits of smoking and alcohol consumption were also excluded from the study, since smoking and alcohol consumption negatively affects the anti-oxidant levels of an individual.

3.7. Non-diabetic health control characteristics

The non-diabetic control group consisted of randomly selected healthy individuals from the police members at the Federal Police Specialized Hospital (FPSH). They were healthy and homogenous with patients (in gender and age) and fulfilled the inclusion criteria of the study. The proportion of healthy controls per diabetic was 1:1. Participants were asked to fast for 8 to 14 hours; if they reported fasting for fewer than 8 or greater than 14 hour, the visit was excluded. Their fasting blood glucose levels were recorded.

3.8. Sample Size Determination

The required sample size was determined using single population formula for estimating single population proportion. The formula for calculating the sample size (n) would be:

$$\mathbf{n} = \frac{\left(\frac{Za}{2}\right)^2 \mathbf{P(1-p)}}{\mathbf{d^2}}$$, considering the following assumptions:

- P= assumed the highest population proportion prevalence of diabetes mellitus in Ethiopian adults 4.36%, (IDF-2013)
- Level of significance = 0.05, and Non-response rate= 10%

Where:

- n= sample size
- Z(a/2) = Z-score at 95% confidence interval = 1.96;
- P= 4.36% = 0.0436 (positive prevalence); 1-P=Q= 0.9564 (negative prevalence)
- d= marginal error=0.05 (5%)

Therefore **n** becomes: $$\mathbf{n} = \frac{(1.96)^2 0.0436(1-0.0436)}{0.05^2}$$

$$\mathbf{n} = \frac{(3.8416)\text{x}0.0436\text{x}(0.9564)}{0.0025} = 64.08$$

To avoid error in non response rate, 10% of the calculated sample size was added. Therefore, the total sample was; **n** = 64.08+10% (64.08) = 64.08+6.408 ≈ **70.**

3.9. Sampling Technique

Conventional non-probability sampling technique was used in which All adult diabetic out patients (>18 years of age) available at the diabetic clinic of the study hospital were included during sampling process. The total sample size of the study was 140, including 70 diabetic patients and 70 non –diabetic health controls.

3.10. Variables

3.10.1.Dependent variables

- Biochemical tests like; fasting blood sugar (FBS), Uric acid (UA), Total bilirubin (TB) were the dependent variables of the present study.
- Hematological profiles like; Red blood cell count (RBC), Hemoglobin (HGB), Hematocrit (HCT), Mean cell hemoglobin (MCH), Mean cell hemoglobin concentration (MCHC), Red blood cell distribution width (RDW), White blood cell count (WBC), Platelet count (PLT), Mean platelet volume (MPV), Platelet distribution width (PDW) and Platelet Crit (PCT) were the independent variable of this study.

3.10.2.Independent variables

Age, Sex, Body mass index (BMI), systolic blood pressure (SBP), Diastolic blood pressure (DBP) and duration of diabetes were the independent variables of the study.

3.11. Data Collection Method

3.11.1.Questionnaire

Patients were interviewed using structured questionnaires that was prepared in English and translated into Amharic and then back to English to check its consistency. The questionnaire was pre- tested to ensure that it was clear for respondents and correction was done accordingly. The contents of questionnaire include: socio demographic characteristics of clients, duration of diabetes, diabetic complications, alcohol consumption, smoking habits, age, sex, anti-diabetic, antihypertensive and anti-lipidemic drugs. Any sicknesses in the past and present other than T2DM, a history of hematological abnormalities were also included in the questionnaire.

3.11.2.Anthropometric Data and Clinical Information

- Height and weight of patients were measured and then the body mass index for each individual was calculated by using Quetlet index with weight in Kg divided by square of height in meter (Kg/m^2) formula.
- Systolic and diastolic blood pressures were measured from the arms after at least 5 minutes of rest in sitting position.

3.11.3.Specimen Collection and Handling Method

Clinicians (physicians) in the diabetic center identified the subjects of interest, take the medical history and perform a physical examination for each subject. The questionnaire Data's were collected by professional nurses working in diabetic clinic and blood samples were collected by Laboratory Technologists who are assigned in the clinical laboratories together with the primary investigator. Measurement of the sample was performed side by side collection of sample using automated full blood counter, automated chemistry machine. Then data were recorded on a standardized recording book.

Blood Specimen: A5ml of venous blood were collected directly in an ethylene-diamine-tetra acetic acid (EDTA) vacutainer in sitting position from antecubital vein. The specimen then transported to laboratory and immediately analyzed to prevent whole blood hemolysis.

3.12. Data Entry and Statistical Analysis

Collected data were verified prior to computerized data entry. The statistical package for social sciences (Version 21.0; IBM Corporation, Armonk, NY, USA) was used for the statistical analysis of data. Descriptive statistics like mean and standard deviation were applied. Two tailed significance level was determined by independent t-test. The P values less than 0.05 **($P<0.05$)** were considered as statistically significant and results were presented as mean ± standard deviation.

3.13. Data Quality Assurance

The data quality starts from the questionnaire that was pre-tested before the actual data collection. Questionnaire was prepared by local language (Amharic). Data collectors were

instructed to check the completeness of each questionnaire at the end of each interview. The Supervisors and primary investigator were rechecked the completeness of the questionnaire immediately after interview at the spot.

Blood specimen was collected by venous puncture with appropriate procedure and sterile syringe. The syringe had to be disposable so that, blood transmitted diseases could be avoided. Then collected blood was transported to the laboratory for analysis using icebox. The samples were made free from any contamination. Collected results were checked for completeness on daily basis by the immediate laboratory supervisor and principal investigator. Attention was given to insertion of data to SPSS statistical software on computer. The completed results were rechecked repeatedly to maintain the quality of data.

3.14. Ethical Considerations

Prior to the commencement of the study ethical approval was taken from the Department of Biochemistry Research and Ethical Review Committee (DRERC) that was approved on meeting No. DRERC: 03/15 with protocol number of M.Sc. Thesis 07/15 and Ref. No. SOM/BCHM/29/2007. The nature and purpose of the study was explained to subjects, and a written informed consent was obtained from each subject. Any activity of this study would not harm the study subjects and it would be made clear to each subject that he/she had aright to hold information, decline cooperation and withdraw herself/himself from the study. So, subjects who were volunteers to give a written informed consent were only participated in the study. To maintain confidentiality, any information related to the study subjects were identified by using codes and analyses of data were done using these codes. Finally, sample collection and laboratory analysis were performed by trained laboratory technologists along with the principal investigator according to ethical steps and procedures.

3.15. Dissemination of Results

This study could be used as a reference material to researchers, experts or policy makers for intervention. To reach these bodies the finalized paper was submitted to Addis Ababa University, School of Medicine. So it can serve as a reference in the library. In addition, the results will be disseminated through publication in peer reviewed local and international journals.

3.16. Laboratory Assays

3.16.1.Analysis of the biochemical tests

3.16.1.1. Determination of Fasting Serum Glucose

Fasting serum glucose levels was determined by enzymatic glucose oxidase method using commercial reagents kit.

Principle: In the Trinder reaction the glucose is oxidized to D-gluconate by the glucose oxidase (GOD) with the formation of hydrogen peroxide. A colorless mixture of phenol and 4 aminoantpyrine(4-AA) is then oxidized by hydrogen peroxide in the presence of the enzyme, peroxidase, to form a red quinoneimine dye product. The concentration of the coloured product, determined from the optical density change at 540 nm in the reaction, is proportional to the concentration of glucose in the original serum sample.

$$\text{B-D - Glucose} + H_2O \xrightarrow{GOD} \text{D - Gluconate} + H_2O_2$$

$$\text{4-AA} + \text{phenol} \xrightarrow[POD]{H_2O_2} \text{Quinoneimine dye} + H_2O$$

Reagent composition

R1 Monoreagent. Phosphate buffer 100 mmol/L pH 7.5, glucose oxidase > 10KU/L, peroxidase >2 KU/L, 4-aminoantipyrine 0.5 mmol/L.

Glucose standard: Glucose 100 mg/dl organic mixture based primary standard.

Procedure: Ten microliters of serum was mixed in a cuvette with 1ml of glucose reagent R1, and then incubated at room temperature for 10 minutes. Then the optical density was read at 540nm, against a reagent blank. Standard glucose concentrations were used to determine the specific concentration of glucose in serum samples, according to the Lambert-Beer law.

3.16.2.Analysis of the hematological profiles

The automated cell-dyne 1800 hematological analyzer was used to determine hematological profiles. This auto analyzer uses two independent measurement methods. These are;

- ✓ Electrical impedance method for determining WBC, RBC and PLT

- ✓ Modified Methemoglobin method for determining HGB

Principle: - approximately 30µL of whole blood was aspirated from collection tube through sample aspiration probe. Then samples were transferred to the premixing cup. 7.5ml of diluents was added to the pre-mixing cup to achieve a dilution ratio of 1: 251. The diluted sample was then divided into two (one for RBC/PLT determination and other for WBC/HGB determination.

3.16.2.1. Determination of RBC and PLT count

100 µL of the diluted sample was aspirated and mixed with additional 5ml of diluents in the RBC/PLT mixing chamber to create a dilution ratio of 1:12801. This dilution was pulled through the aperture of the transducer bath where electrical impedance was used to count RBC and PLT.

3.16.2.2. Determination of WBC

The remainder of 1:251 sample dilution for WBC/HGB determination was mixed with 1ml of lyse reagent in the WBC mixing chamber. This lysed dilution was pulled through the aperture of the von Bohrens WBC transducer. As each cell was drawn through the aperture, achange in electrical resistance occurs generating an equivalent voltage pulse. The number of pulses sensed during each cycle corresponds to the white cells counted.

3.16.2.3. Determination of HGB level

After the WBCs have been counted and sized, the remainder of the lysed dilution was transferred to the Hemoglobin (Hgb) Flow cell Assembly. The ability of the lysed dilution to absorb light at a wavelength of 540nm (nanometers) was the HGB level.

3.16.2.4. MCV, HCT and RDW Determination

The CELL-DYN 1800 determines the mean cell volume (MCV) from the RBC size –distribution data. Hematocrit (HCT) result was calculated from the RBC count and the MCV value.

$$HCT = \frac{RBC \times MCV}{10}$$

RBC Distribution Width (RDW) is the coefficient of variation of RBC heterogeneity determined from the RBC size- distribution data.

3.16.2.5. MCH and MCHC determination

Mean Cell Hemoglobin (MCH) and Mean cell Hemoglobin concentration (MCHC) values were calculated automatically.

MCH = (HGB/RBC) x 10

MCHC = (HGB/HCT) x 100

3.16.2.6. MPV, PCT, PDE determination

- An algorithm is used to analyze the PLT histogram to obtain Mean Platelet Volume (MPV). Results for the plateletcrit(PCT) are calculated from the PLT count and MPV.

$$PCT = \frac{PLT \times MPV}{1000}$$

- PLT Distribution Width (PDW) is the geometric standard deviation (GSD) of the PLT size-distribution.

4. RESULTS

A total of 140 individuals participated in this present study among which 70 were type 2 diabetic patients (male/female, 47/23) and 70 were non-diabetic health controls (male/female, 46/24). In this study, male patients were compared with male controls and female patients were compared with female controls. The mean age of male/female patients was 53.17±11.64/49.7±9.89 years and for male/female controls it was 53.07±10.17/47.88±8.38 years indicating that, the data is homogenous regarding age.

4.1. Anthropometric and clinical characteristics

The anthropometric and clinical characteristics of the patients and controls were illustrated in **Table 2**. The study showed statistically higher values of body mass index (BMI, P=0.001), systolic blood pressure (SBP, P=0.002) and diastolic blood pressure (DBP, P=0.019) with no difference in the mean age (P>0.05) of male T2D patients. However, there were no significant difference in the mean values of age, BMI and SBP (P>0.05) but higher values of DBP (P=0.008) was observed in female patients.

Table 2 Comparison of the Anthropometric and Clinical Characteristics of Male/Female Patients with Male/Female healthy individuals.

Variable name	Males Mean ± SD		p-value	Females Mean ± SD		P-value
	Patient N=47	Control N=46		Patient N=23	Control N=24	
Age(years)	53.17±11.64	53.07±10.17	0.96	49.7±9.89	47.88±8.38	0.499
BMI(Kg/m^2)	25.47±2.64	23.43±2.88	**0.001**	27.52±3.95	25.38±4.567	0.092
SBP(mmHg)	125.74±11.56	119.13±8.39	**0.002**	124.78±10.39	120.83±8.3	0.156
DBP(mmHg)	82.23±8.65	78.26±7.4	**0.019**	83.48±7.141	77.92±6.58	**0.008**
DMD (years)	8.21±7.45	-		7.30±4.46	-	0.592

Results are presented as mean ± standard deviation and P<0.05 is statistically significant (indicated in bold). ***Abbreviations:*** *SBP - Systolic Blood Pressure, DBP - Diastolic Blood Pressure, BMI - Body mass Index, DMD - Duration of Diabetes.*

4.2. Biochemical Parameters.

As indicated in **Table 3** male patients had remarkably: lower values of Uric Acid (U.A, P=0.001) and higher values of fasting blood glucose (FBS, P=0.001) with only a numerical difference in direct bilirubin (TB, P=0.429). Similarly, female patients had statistically decreased levels of direct bilirubin (TB, P=0.001), Uric Acid (U.A, P=0.002) and elevated values of FBS (P=0.001).

Table 3 Comparisons of the Biochemical indices in Male/Female Patients with Male/Female healthy individuals.

Variable name	Males Mean ± SD		P-Value	Females Mean ± SD		P-Value
	Patient N=47	Control N=46		Patient N=23	Control N=24	
TB(mg/dl)	0.191±0.157	0.213±0.101	0.429	0.117±0.065	0.212±0.089	**0.001**
U.A(mg/dl)	3.94±1.169	5.41±0.717	**0.001**	3.83±1.029	4.75±0.847	**0.002**
FBS(mg/dl)	152.79±68.48	97.67±12.90	**0.001**	165.13±53.51	95.29±7.937	**0.001**

Results are presented as mean ± standard deviation and P<0.05 is statistically significant (indicated in bold). ***Abbreviations;*** *TB – Total Bilirubin, U.A – Uric Acid, FBS – Fasting Blood Sugar, mg/dl - milligram per deciliter*

4.3. Platelet indices

From analysis of the platelet indices the present study demonstrated that male patients had statistically higher values of platelet count (PLT, P=0.024), mean platelet volume (MPV, P=0.000) and platelet distribution width (PDW, P=0.001) with no change in the mean values of platelet crit (PCT, P=0.603). Similarly, female patients had remarkably elevated values of platelet indices like PLT count (P=0.000), MPV (P=0.000) and PCT (P=0.005) with no difference values in PDW (P=0.154) **Table 4.**

Table 4 Comparisons of the Platelet indices in Male/Female Patients with Male/Female Healthy individuals.

Variable name	Males Mean ± SD		P-Value	Females Mean ± SD		P-value
	Patient N=47	Control N=46		Patient N=23	Control N=24	
PLT($x10^3$/L)	310.64±160.72	251.46±69.26	**0.024**	336.43±64.49	247.04±64.82	**0.001**
MPV(fL)	10.87±1.69	9.47±1.34	**0.001**	10.6±1.36	9.08±1.122	**0.001**
PDW(10(GSD))	16.93±1.27	16.21±0.54	**0.001**	16.51±0.59	16.21±0.81	0.154
PCT (%)	0.26±0.05	0.25±0.06	0.603	0.29±0.07	0.24±0.06	**0.005**

Results are presented as mean ± standard deviation and P<0.05 is statistically significant (indicated in bold). ***Abbreviations;*** *PLT - Platelet count, MPV - Mean Platelet volume, PDW - Platelet distribution Width, PCT – Platelet Crit, fL-femtolitre, GSD-Geometric standard deviation.*

4.4. RBC indices

The mean values of the RBC indices indicated in **Table 5**. As indicated, comparison of the male subjects showed that, the mean values of red blood cell distribution width (RDW, P=0.001), mean cell volume (MCV, P=0.007) and mean cell hemoglobin (MCH, P=0.03) were notably elevated. In addition, male patients had numerically higher values of hematocrit (HCT, P=0.329), mean cell hemoglobin concentration (MCHC, P=0.523) and hemoglobin (HGB, P=0.413). In contrast, red blood cell count (RBC, P=0.167) were insignificantly lowered in patients. On the other hand, comparison of female subjects revealed that, HCT (P=0.005), RDW (P=0.012) and MCHC (P=0.015) were remarkably elevated. Similarly, numerically higher values of HGB (P=0.057) and MCV (P=0.271) but lower RBC count (P=0.863) was observed in female patients.

Table 5 Comparisons of the RBC indices in Male/Female patients with Male/Female Healthy individuals

Variable name	Males Mean ± SD		P-Value	Females Mean ± SD		P-value
	Patient N=47	Control N=46		Patient N=23	Control N=24	
RBC($x10^6$/L)	5.64±0.568	5.78±0.417	0.167	5.39±0.499	5.42±0.504	0.863
HGB(g/dL)	16.55±1.417	16.35±0.924	0.413	15.17±1.267	14.54±0.932	0.057
HCT (%)	51.21±3.967	50.52±2.681	0.329	47.70±3.430	45.29±1.899	**0.005**
MCH(pg)	29.72±2.534	28.78±1.413	**0.03**	28.30±2.285	28.46±1.179	0.771
MCHC(g/dL)	32.33±0.967	32.19±1.056	0.523	31.61±0.583	32.21±0.977	**0.015**
RDW (%)	14.28±1.862	12.76±0.993	**0.001**	14.04±1.397	13.08±1.10	**0.012**

*Results are presented as mean ± standard deviation and P<0.05 is statistically significant (indicated in bold).**Abbreviations:** RBC-red blood cell count, HGB-hemoglobin, HCT- hematocrit, MCH- mean cell hemoglobin, MCHC- mean cell hemoglobin concentration, RDW- red blood cell distribution width, g/dl – gram per deciliter, pg - picogrem*

4.5. White blood Cell (WBC) indices

As indicated in **Table 6** the results of the present study showed that, there were no significant differences observed in the average values of WBC count (P=0.09), Neutrophil (Neu, P=1.532), Lymphocyte (Lymph, P=0.329) and mixed cells (MID, P=0.088) in male patients. Female patients had significantly higher average levels of WBC count (P=0.000) with no statistical difference in the mean values of Neu, Lymph and MID (P>0.05).

Table 6 Comparisons of the WBC and Platelet indices in Male/Female patients with Male/Female Healthy individuals.

Variable name	Males Mean ± SD		P-Value	Females Mean ± SD		P-value
	Patient N=47	Control N=46		Patient N=23	Control N=24	
WBC($x10^3$/L)	6.77±1.844	6.20±1.31	0.09	7.48±1.620	5.83±1.007	**0.001**
Neu(%)	58.04±8.645	60.83±4.281	1.532	60.13±7.665	60.63±3.797	0.779
Lymph(%)	33.28±8.428	31.22±3.514	0.329	32.04±7.119	31.63±2.716	0.79
MID(%)	8.49±1.53	7.96±1.445	0.088	7.74±1.544	7.83±1.736	0.845

Results are presented as mean ± standard deviation and P<0.05 is statistically significant (indicated in bold). ***Abbreviations:*** *WBC- white blood cell count, Neu- Neutrophil, Lymph- Lymphocyte, MID- Mixed cell.*

4.6. Correlation Analysis

As indicated in table below, SBP, DBP, UA and RBCs were negatively correlated with MPV and this correlation was not statistically significant. In similar way, the values of RDW and TB were positively correlated with MPV but this was not statistically significant. However, PDW was positively and significantly (P=0.001) correlated with MPV.

Table 7 Pearson correlations of MPV with various parameters in diabetic individuals.

Variable name	**Diabetic Patients** N=70	
	Correlation **(r)**	**P- value**
DBP	-0.001	0.995
SBP	-0.008	0.946
RBC	-0.137	0.258
RDW	0.129	0.286
PDW	0.373	**0.001**
TB	0.022	0.858
UA	-0.108	0.374

Results are presented as mean ± standard deviation and P<0.05 is statistically significant (indicated in bold).

5. DISCUSSION

5.1. Anthropometric characteristics

This study enrolled both male and female subjects in which male T2D patients were compared with healthy male controls while female T2D patients were compared with healthy female controls. The present study revealed, statistically higher values of BMI in male patients and only a numerical difference in female individuals. This is in consonance with; Belay *et al*., 2014; Ganz *et al*, 2014; Bukhari *et al*., 2015, Who reported higher values in both male and female patients. Similarly, Sarah *et al.*, 2014, demonstrated significant values of BMI in T2DM patients. Ganz *et al*(2014) suggested that, BMI is strongly and independently associated with the risk of T2D and the magnitude of this association is larger for higher BMI values (Ganz *et al*, 2014).

Increased BMI may have a role in the pathogenesis of T2D. Biochemically, this may be explained as increase in BMI can result in the alteration of perilipin expression. Perilipins are phoshoproteins found on the surface of triacylglycerol droplets in adipose tissue and they serve as gatekeepers protecting triacylglycerols from hydrolytic activity of lipase enzymes that facilitate the release of FFAs. Zhang *et al* (2003) demonstrated that perilipin expression decreased with increasing BMI and obesity resulting in the increased release of FFAs (Zhang *et al.,* 2003). Al-Goblan *et al* (2014) suggested that increased release of FFAs observed in type 2 diabetes and obesity that is associated with insulin (Al-Goblan *et al*., 2014). On the other hand, *Greenberg & Obin* (2006) stated as, the number of macrophages in adipose tissue increased with increasing BMI and obesity. These macrophages are responsible for most of pro-inflammatory cytokine production including Tumor Necrosis factor- α (TNF-α), interleukin-6 (IL-6), transforming growth factor-β (TGF-β) and procoagulant proteins such as plasminogen activator inhibitor type-1(PAI-1) and tissue factors. These will result in the development of thrombotic state and atherosclerosis (Greenberg & Obin, 2006).

Our finding of the blood pressure shows that, SBP was significantly higher in male patients but not in female patients. However, DBP was significantly higher in both male and female patients compared to the corresponding values in their respective healthy control individuals. This result is in agreement with the findings of Alao *et al*., 2009; Bukhari *et al*., 2015, who reported significantly higher values of both SBP and DBP in both sexes of T2D patients.

Hyperglycemia causes microvascular complications in many organs including diabetic nephropathy the leading cause of end-stage renal disease (ESRD) in developed countries (Ritz & Dikow, 2006). In addition, *Gurley & Coffman* (2007) suggested that diabetes may lead to other vascular complications, including systemic hypertension through activation of intra renal rennin angiotensin system (RAS) (Gurley & Coffman, 2007). As reported by He *et al* (2004), the metabolic receptor G-protein coupled receptor 91 (GPR91) is highly expressed in the kidney and activated by the citric acid cycle intermediate succinate because succinate is locally accumulated in the intact diabetic kidney (He *et al*., 2004). This is a new discovery that directly links high glucose levels and rennin release from the juxta-glomerular apparatus (JGA) in the kidney. *Toma and his colleagues* (2008) suggested that, high glucose and succinate-induced GPR91 activation trigger paracrine signaling from the (juxta) glomerular endothelium to the adjacent rennin producing JG cells to increase rennin synthesis and release the rate-limiting step of RAS activation (Toma *et al*., 2008)**.** Once released Rennin starts to convert Angiotensinogen to angiotensin-I. This is then converted to and increases the concentration of a potent vasoconstrictor angiotensin-II leading to increased systemic blood pressure (Peti- Peterdi *et al.,* 2008). This may explain elevated values of Systolic (SBP) and diastolic (DBP) blood pressures in the present study.

On the other hand, increased SBP and DBP in patients of this study may be explained in terms of advanced glycation end products (AGE). It has been suggested that AGE and its cell surface receptor (RAGE) are the typical molecular consequence of diabetes (Brownlee, 2001). But Mayer *et al* (2015) reported that the soluble iso-form of the receptor (sRAGE) decreased in diabetes which is normally used as a decoy for capturing, preventing circulating AGEs from binding to the RAGE and act as physiological defense against AGEs (Mayer *et al.,* 2015). AGE have a pathophysiological role in the progressive stiffening of large arteries. According to, Tan *et al* (2002), binding of AGEs to RAGE has been shown to enhance oxidant stress and induce a state of endothelial cell activation, stimulate cell adhesion molecule expression, and induce migration of macrophages and T-cells into the intima (Tan *et al*., 2002). AGEs also quench NO (nitric oxide) in vitro and may reduce NO-dependent vasodilatation. It also induced the production of the vasoconstrictor endothelin-1 by endothelial cells through nuclear factor-kB activation (Mayer *et al.,* 2015). Therefore, since endothelin-1 is a potent vasoconstrictor, it increases the systemic blood pressure.

5.2. Biochemical indices

Both Bilirubin and Uric Acids are used as a protective agent as they scavenge free radicals. In the present study, Uric Acid levels were significantly lowered in both male and female patients while total bilirubin levels were remarkably lowered in female patients but showed only a numerical difference in male individuals with lower values in patients. This was in agreement with; Hisalkar *et al.*, 2012, who reported lowered values of the parameters in diabetic patients.

It was observed that, uric acid may function as anon enzymatic antioxidant molecule in the plasma. According to *Becker* (1993) the concentration of this parameter is almost 10-fold higher than other antioxidants like ascorbic acid and α-tocopherol (Becker, 1993). Similarly, *Ceriello* (2003) suggested that, uric acid has much higher antioxidant capacity in that, urate the soluble form of uric acid in the blood can scavenge superoxide anion, hydroxyl radical and singlet oxygen and can chelate transition metals (Ceriello,2003). In addition, it can also block the formation of reactive nitrogen species by blocking the reaction between nitric oxide and super-oxide anion thereby inhibiting the formation of toxic product called peroxynitrite (Ceriello, 2003). However, in T2D patients, elevated level of glucose poses an effect on the renal tubule to increase the fractional excretion of the soluble form of uric acid urate (Hisalkar *et al.*, 2012). This may result in decreased plasma uric acid level that is unable to perform its antioxidant activity.

On the other hand, Ziberna *et al*(2016) suggested that bilirubin has antioxidant and anti-inflammatory activity and is inversely correlated with cardiovascular diseases risks such as ischemic heart dises, hypertension, type 2 diabetes, metabolic syndrome and obesity among others (Ziberna *et al.*, 2016)). Accrding to Minetti *et al (*1998) **i**ts protective role is through inhibition of oxidative modification of plasma proteins and formation of protein carbonyl groups. In circulation bilirubin is mainly found bound to albumin. This protects albumin from oxidation and also albumin bound linoleic acid from peroxyl radical induced oxidation (Minetti *et al.*, 1998). Diabetes may be associated with oxidative stress and increased free radical formation that may leads to a reduction of the antioxidant level of the body including uric acid and bilirubin. Thus, reduced level of serum uric acid and total bilirubin in the present study suggests that, these parameters are also considered as one of the total antioxidant molecules present in the body.

Regarding fasting blood sugar (FBS) the present study showed that it was justifiably elevated in diabetic patients of both gender. This is similar with the findings of (Hisalkar *et al*., 2012; Alhadas *et al*., 2016). This may be explained in terms of insulin resistance in which cells are impaired to take glucose resulting in high glucose level. Elevated blood glucose may affect the erythrocyte deformability and aggregation while blood becomes more viscous compared to healthy individuals. Increased blood viscosity affects micro circulation in diabetic patients which may lead to micro-angiopathy.

5.3. Platelet indices

In this study, analysis of the platelet indices demonstrated that, mean platelet volume and platelet counts were significantly higher among diabetic patients of both sex. This is in corroboration with the studies conducted by, Demirtunc *et al*., 2009; Kodiatte *et al*., 2012; Demirtas *et al*., 2015; Alhadas *et al*., 2016. In contrast, Hekimsoy *et al*., 2004 reported remarkably low level of platelet count in diabetic patients. This may be due to the fact that, platelet count could be dependent on several variables like; mean platelet survival, platelet production rate and turnover rate in T2D patients (Kodiatte *et al*., 2012). Similar to our study, Akinsegun *et al*., 2014, observed significantly higher values of platelet count, however, in contrary to our observation, they reported remarkably lower values of MPV in diabetic individuals. The discordant of their study result may be due to the fact that, the majority of diabetics enrolled in their study had been taking anti-platelet medications. This suggests that anti-platelet medication may reduce the thrombotic potential without causing a reduction in the absolute platelet count**.**

The MPV and platelet counts are indicators of thrombotic potential and risk factors for micro-vascular complications in diabetes (Zuberi *et al*., 2008; Akinsegun *et al*., 2014). According to Chen *et al* (2006) increased insulin resistance and glycemic status increases platelet count in hyperglycemia. Taniguchi *et al* (2003), has been indicated that, increased platelet count may independently predict insulin resistance among non-obese Japanese type 2 DM patients (Taniguchi *et al*., 2003). Platelet size is another aspect that deserves attention because it seems to be related to their function. It has been demonstrated that, platelets with greater volume (larger platelets) are younger, more reactive and aggregatable. Hence, they contain denser granules, secrete more serotonin and β-thromboglobulin, and produce more thromboxane A2 leading to increased thrombotic potential when compared with smaller and less active platelets (Demirtunc

et al., 2009; Chang *et al*., 2010; Kodiatte *et al*., 2012; Alhadas *et al*., 2016). Thus, large circulating platelets are reflected by increase in MPV which is the indicator of the average size, a marker of platelet function and activity **(**Alhadas *et al*., 2016**).** Elevated MPV is considered as an independent risk factor for thromboembolism, stroke and acute myocardial infarction (Jabeen *et al*., 2013; Demirtas *et al*., 2015; Alhadas *et al*., 2016). In diabetic patients, higher level of MPV could predict an increased risk factor for thrombosis and chronic complications (Jabeen *et al*., 2013; Alhadas *et al*., 2016). Platelet hyperreactivity and increased baseline activation in patients with diabetes is multifactorial and associated with biochemical factors such as hyperglycemia and hyperlipidemia, insulin resistance, and an inflammatory and oxidant state (Kakouros *et al*., 2011) discussed below.

One possible explanation for increased platelet activity in DM may be the osmotic swelling of platelets due to raised blood glucose and its metabolites. According to Ulutas *et al* and Shetty *et al,* Glucose entry into platelets is mediated through insulin independent mechanism resulting in the enlargement of platelets which are functionally active and are measured as mean platelet volume (MPV) (Ulutas *et al*., 2014; Shetty *et al*., 2014). This suggests that, exposure of platelets to hyperosmolar solutions causes increased reactivity, implying that hyperglycemia may have a direct osmotic effect (Demirtas *et al*., 2015). Shetty *et al* (2014) suggested the short life span of platelets, higher platelet turn over rate and younger platelets in diabetes contributes to increased platelet activity. This fact has been proved as MPV correlates with percentage of reticulated platelets and megakaryocytic ploidy, in diabetics**.** Platelets from patients with diabetes also have dysregulated signaling pathways that lead to an increased tendency to activate and aggregate in response to a given stimulus (Shetty *et al*., 2014). Thus enlarged platelets (MPV) are an indicator of thrombotic potential and risk factor for microvascular complications in diabetes.

Insulin resistance and insulin deficiency may increase platelet activation in diabetes. In pre-diabetic stage of T2D, insulin resistance leads to enhanced insulin production by pancreatic β-cells to compensate insulin resistance and fasting glucose level. After T2D is manifested, pancreatic β-cells undergo apoptosis leading to a reduction in β-cell mass (Kakouros *et al*., 2011). This will result in absolute insulin deficiency. In healthy individuals, insulin antagonizes the effect of platelet agonists such as collagen, ADP, epinephrine, and platelet-activating factor and then inhibits platelet activation (Schneider, 2009). This antagonism is mediated through

binding of insulin to its receptor (IR). Insulin-IR complex activates insulin receptor substrate-1(IRS-1) and initiates the association of IRS-1 to Giα subunit. This reduces the activity of Gi that impairs adenylate cyclase (AC) enzyme activity and leads to the increased production of cAMP (Kakouros *et al*., 2011). Increased levels of cAMP lead to platelet inhibition through cAMP-dependent protein kinase (PKA) which inhibits signaling though the mitogen-activated protein kinases pathway (MAPK), receptor activation, thromboxane A2 formation, and activation of key enzymes such as protein kinase C. However, insulin resistance in diabetes causes, impairment of insulin signaling through IRS-1 pathway leading to inactivation of AC and low level of cAMP molecule (Vaidyula *et al*., 2006). Insulin then signals through MAPK pathway resulting in the increased intraplatelet calcium concentration, increased monocyte-platelet aggregates and platelet hyper-reactivity that can leads to increased thrombotic state and atherosclerosis. This may explain elevated level of MPV in diabetic patients.

Inactivation of endothelial nitric oxide synthase enzyme (eNOS) increases platelet activation that may explain the increased levels of MPV in patients of the present study. Nishikawa *et al(2000)* suggested that, Hyperglycemia increases mitochondrial superoxide anion (O_2^-) production which results in the increased production of intracellular AGEs (Nishikawa *et al*., 2000) and activation of hexosamine pathway which diminishes NOS activation by inhibiting protein kinase Akt (Du *et al*., 2001). *Laursen and his colleagues(2001)* suggested that, high level of O_2^- interacts and diminishes NO producing Peroxynitrite that oxidizes the co-factor of NOS called tetrahydrobiopterin leading to eNOS inactivation (Laursen *et al*., 2001). Similarly, *Creager and Lüscher* (2003) suggested elevated levels of O_2^-, diacylglycerol and FFAs due to increased mobilization of fatty acids may result in the activation of PKC in diabetes (Creager & Lüscher, 2003). Activated PKC decrease insulin receptor substrate-1–associated phosphatidylinositol-3-kinase (PI3K) there by inhibiting the PI3K pathway and Akt kinase activity. Activated PKC also phosphorylates and inactivate NOS leading to less production and availability of NO. This will leads to increased production of vasoconstrictive substances like endothelin-1 (ET-1) instead of NO resulting in the diminished endothelium dependent vasodilatation (Kakouros *et al*., 2011). On the other hand, *Creager & Lüscher*, (2003) reported that, increased O_2^- increases ROS that activates transcription factors like nuclear factor kappa B (NF-kB) and Activator protein (AP-1) (Creager & Lüscher, 2003). NF-kB and AP-1 stimulates the expression of pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α. These cytokines induces the phonotypical change in

endothelial cells and/or monocytes leading to the increased production of prothrombotic factors such as tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) (Kakouros *et al*., 2011). The consequence of increased TF and PAI-1 is the increased platelet activation, granule release and aggregation leading to increased thrombogenic state and atherosclerotic plaques. This may describe the higher values of MPV in the diabetic patients of the present study.

Elevated levels of MPV in diabetic patients of the present study may also be explained in terms of oxidative stress. Increased ROS in diabetes induces nonenzymatic glycation of proteins on the surface of the platelet (Schneider, 2009). Such glycation leads to over accumulation of advanced glycation end products (AGEs) (Nishikawa *et al*., 2000; Wautier and Schmidt, 2004). Some of these AGE cause externalization of platelet membrane phosphatidylserine (Wang *et al*., 2007) that may cause changes in protein structure (conformation) and alterations of membrane lipid dynamics (Ferroni *et al*., 2004). Altered lipid membrane also causes the release of arachidonic acid. Increased release of arachidonic acid from platelet membrane increases the activation of cyclooxygenase-1 (COX-1) enzyme. This enzyme is the key enzyme in the conversion of arachidonic acid into thromboxane A_2 (TXA_2) (Kakouros *et al*., 2011). This TXA2 creates a local positive feedback thereby amplifying the activation response of platelets to most agonists and activating the bystander quiescent platelets. Increased TXA2 is the implication of activated platelets, increased aggregation response and thrombogenic state that leads to atherosclerosis.

Other biochemical clue that may explain increased values of MPV in diabetic patients may be the increased platelet membrane receptors. Wang *et al* (2007) said increased AGE in diabetes alters the platelet membrane lipid dynamic that may result in enhanced expression of megakaryocyte production of receptors such as P-selectin, glycoprotein GpIb, GpIIb/IIIa complex (Wang *et al*., 2007). Expression of these molecules on platelet membrane increases the thrombogenic state. As *Collwell & Nesto* (2003) reported, increased glycosylation of LDL and arachidonic acids results in increased intraplatelet calcium (Ca^{+2}) concentration in diabetes **(**Collwell & Nesto, 2003). Intraplatelet Ca^{+2} are critical for regulating the secretion of ADP and several proteins from platelet granules including von Willebrand factor (vWF) (Schneider, 2009). According to *Collwell & Nesto* (2003), ADP binds to platelet purinergic receptors and triggers a conformational change in glycoproteins IIb and IIIa on the platelet surface. This causes the formation of a functional heterodimeric platelet glycoprotein (Gp) IIb-IIIa receptor complex

(Kakouros *et al*., 2011). So, people with diabetes exhibits increased expression of the surface GPIb and IIb/IIIa receptor complex**.** GPIIb/IIIa complex binds to fibrinogen and links adjacent platelets as part of the platelet aggregation process (Collwell & Nesto, 2003). GPIb mediates platelet binding to von Will-brand factor and maintains the attachment of platelets to vascular surface leading to the amplification of platelet activation and aggregation **(**Creager & Lüscher, 2003). This may also explain the increased values of MPV in patients of the present study.

Regarding PDW, the present study showed that, there were no significant difference between female patients and health individuals. However, we observed that, male patients had significantly elevated values of PDW. This is inconsonance with, Dalamaga *et al*., 2010; Alhadas *et al*., 2016. According to Vagdatli *et al*, activated platelets undergo structural change from discoid to spherical shape and produces pseudopodia leading to a change in the PDW (Vagdatli *et al.,* 2010). Due to this reason, activated platelets may be different in size from non-activated platelets. PDW have also been reported as significantly elevated in diabetic patients with complications when compared with diabetic patients without complication (Jindal *et al*., 2011; Alhadas *et al*., 2016). Thus, different sizes of platelets can be found, a consequence of which was the enlarged histogram plotting of PDW and increased levels of PDW (Vagdatli *et al*., 2010; Alhadas *et al*., 2016).

Plateletcrit (PCT) is the other platelet parameter which has no significant difference in male individuals, however, the significant elevation was observed in female patients of the present study. This is in agreement with, Alhadas *et al*., 2016. In normal individuals, when platelet volume is increased, platelet count tends to decrease in order to maintain the values of PCT within normal ranges. Thus, platelet mass or PCT must be kept at constant levels**.** However, in diabetic patients platelets become larger and more reactive through different mechanisms leading to the increased platelet mass thereby increasing PCT. According to Alhadas *et al*., 2016, this parameter was significantly elevated when there is chronic complication. In general Platelet indices (PLT, PCT, MPV and PDW) are determinants of platelet functionality, among which MPV and PDW stand out due to their involvement in the development of thromboembolic complications (Jabeen *et al*., 2013; Alhadas *et al*., 2016).

5.4. RBC indices

Our data demonstrated that, RBC count was lowered in diabetic patients of both gender but this difference was not statistically significant. Wang *et al* (2013) suggested that, decreased RBC count is an independent predictor of the risk of microvascular complications in patients with T2DM and this is mediated partly through an effect of decreased RBC count on RBC function (Wang *et al.,* 2013).

Decreased RBC count in this study may be explained in terms oxidative stress which can result in the mechanical alterations of RBC membrane protein. Altered membrane proteins are associated with the development of microvascular complications in diabetes. The biconcave discoid shape of RBC is maintained by the membrane cytoskeleton which is known to be the major determinant of the cells dynamic behavior. Normal RBCs tend to orient themselves with flow streamlines under high shear (deforming) forces implying that, these cells are highly deformable bodies. They also behave as elastic bodies because the shape change is reversible when deforming forces are removed (Baskurt & Meiselman, 2003). The most important component of the RBC membrane cytoskeleton network (network of proteins lying beneath the cell membrane) is a protein called spectrin. However, in diabetes chronic hyperglycemia causes a non-enzymatical glycosylation of spectrin network for further oxidation leading to erythrocyte membrane abnormalities and accelerated aging of RBCs (Moussa, 2007; Wang *et al*., 2013). This might be responsible for increased impairment of RBC deformability among diabetics.

The altered fluid-electrolyte balance of the RBCs may also be the other mechanism that reduces RBC count. Availability of the metabolic energy in the form of adenosine triphosphate (ATP) is essential for the maintenance of RBC deformability. The cat-ion pumps in the RBC membrane (Na+/K+ ATPase and Ca+2-ATPase) requires ATP to regulate intracellular cat-ion and water content, thereby maintaining cell volume and then cell surface to volume ratio. The source of ATP in RBCs is the anaerobic Glycolysis. Because RBCs do not store glucose and their metabolism depends on the availability of glucose in their microenvironment, glucose supply to the RBC is critical for the maintenance of this mechanism (Baskurt & Meiselman, 2003). However, in T2D patients' glucose entry into RBC is impaired due to insulin resistance. This will leads to the impairment of the cat-ion pumps resulting in the increased cytosolic Ca+2. It has been demonstrated that an increased Ca+2 levels rigidifies the cytoskeletal network most likely

through a calmodulin dependent mechanism. Thus, increased cytosolic Ca+2 may increases the rigidity of RBC membrane and decrease its deformability, thereby reduce RBC count by accelerating the RBC aging.

The other possible mechanism of reduced RBC count may be due to reduced surface negative electric charge. Chronic hyperglycemia causes nonenzymatic glycosylation of sialic acid moieties of RBC membrane glycoproteins. Sialic acid moieties of RBC are the principal determinants of the negative charge on the surface. Glycosylation of sialic acid moiety then reduces the negative surface electric charge; leading to accelerated aging of RBCs. A decrease in the negative charge of RBC may increase microviscosity, aggregation and adhesiveness of RBCs and also leads to reduced membrane deformability or increased membrane rigidity. Thus, a reduction in the net surface charge of RBCs causes a slowed down velocity of RBCs due to complete standstill of RBCs when entering and moving through capillary segments (Huang *et al*., 2011). Reduced RBC count can further decrease the negative charge of RBCs. On the other hand, oxidative stress in diabetes can impair maintenance of the phospholipid asymmetry and causes externalization of the phosphatidylserine. In normal RBCs phosphatidylserine is restricted to the inner layer of plasma membrane. Thus, disturbed phospholipid asymmetry and exposure of the phosphatidylserine on the external surface causes a thrombogenic surface and calls macrophages to remove cells by phagocytosis (Wang *et al*., 2013).

Hyperglycemia increase generation of superoxide anion that may cause several structural and functional modifications of the RBCs. One of the major modifications in this context is the aggregation and attachment of hemoglobin to the inside of the RBC membrane which is a cytoskeletal spectrine protein network (Cho *et al*., 2008). This may result in the alteration of the cell shape and mechanical properties of RBCs. hemoglobin attachment to spectrin network also increases the intracellular or cytosolic viscosity of the erythrocytes which is related to the mean cell hemoglobin concentration (MCHC) (Baskurt & Meiselman, 2003; Wang *et al*., 2013). In this present study, MCHC was elevated in diabetic patients and the difference was statistically significant in female patients and insignificant in male patients. Increased MCHC may result in the reduction of RBCs membrane flexibility (deformability) and increase the membrane rigidity (Wang *et al*., 2013). Reduced deformability may result in the complete standstill of RBCs

moving through capillary segments that may lead to increased thrombogenic state and then atherosclerosis.

The concentration and sites of super oxide anion generation (intracellular or extracellular) may have different effects on RBCs properties. It was experimentally indicated that extracellular oxidative stress affects the RBCs aggregablity than deformability (Baskurt & Meiselman, 2003). It has been shown that, extracellular oxidative stress activates the erythrocyte caspase-3 in T2D. Activated caspase-3 is responsible to impair the maintenance of erythrocyte shape and function. This contributes to the shortened lifespan of RBCs (Nada, 2015) that may result in the decreased RBC count. Intracellular generated oxidative stress deteriorates RBCs deformability with slight effect on RBCs aggregation. This may be due glycosylation of membrane proteins.

Regarding RDW our data revealed that the parameter was remarkably elevated in diabetic patients of both gender corroborating the results of Nada, 2015. RDW is a quantitative measure of the red blood cell volume (RBCV) heterogeneity. Thus the higher the values of RDW are the greater heterogeneity in cell sizes (anisocytosis) (Wang *et al*., 2013). Chronic inflammation and increased oxidative stress in diabetes causes the impairment of erythropoiesis and degradation of RBCs by fragmentation or agglutination related to anisocytosis (Ferrucci *et al*., 2005). This may shorten the RBCs lifespan leading to decreased RBC count (Nada, 2015) which may also explain the result of the present study. It has been shown that, increased RDW is an independent predictor of the overall and cardiovascular mortality in the general population and in those with various high risks (Nada, 2015). Diabetes is thus, a known disorder that reduces the life span of RBCs resulting in the increased variability of the RBC volume (RDW). Increased RDW causes impairment on RBCs deformability and negatively affects blood flow through microcirculation because of the complete standstill movement of RBCs (Wang *et al*., 2013; Nada, 2015). This may result in the increased thrombogenic state and atherosclerosis.

Our data showed a numerical difference of hematocrit (HCT) value in male patients and a significant elevation in female patients. The possible explanation for this is that, increased sugar level increases the blood osmolarity and capillary permeability in diabetes. This may result in the increased hematocrit value and subsequently blood viscosity. Cho *et al* (2008) suggested that hyperglycemia may cause an osmotic diuresis and hence may lower plasma volume leading to increased hematocrit (Cho *et al.,* 2008). HCT is one of the major determinants of whole blood

viscosity (WBV) because the level of HCT is positively associated with the level of WBV. Thus, HCT increases blood viscosity (Baskurt & Meiselman, 2003). Abnormally high blood viscosity is known to play a role in aggravating myocardial ischemia by diminishing oxygen delivery due to atherosclerotic plaque at the coronary artery. Elevated blood viscosity also increases injurious forces at the endothelial wall and adversely affects the endothelial function. Increased HCT through mechanism of increasing blood viscosity contributes to the inflammatory process (Hisalkar *et al.*, 2012) and may increase the thrombogenic states. Thus, increased HCT value in the present study may suggests that, HCT is also considered as one of the hematological profiles which may contributes to cause atherosclerosis when elevated.

6. CONCLUSION

In this study, an increase in BMI was observed in patients. This may strengthen the idea that, BMI plays a role in the pathogenesis of T2DM. Elevation of DBP and SBP were also observed in diabetic patients which may suggests that, Hyperglycemia causes microvascular complications including systemic hypertension through activation of intra renal rennin angiotensin system (RAS) and damaging endothelial cells by increasing formation of AGE. The current study also revealed the lower levels of total Bilirubin and Uric Acids. This may support the justification that, these molecules have a protective role from oxidative stress and lowered along with other antioxidants with increasing formation of ROS in diabetes. Since, analysis of Bilirubin and Uric acid are simple and cost effective diagnostic tool, it might be used as a prognostic marker of oxidative stress in T2D patients without arthritis. In addition, the present study demonstrated higher values of hematological parameters in diabetic patients than health controls. The significant elevation of Platelet parameters, RDW and WBC may strengthen the notion that the parameters are considered as inflammatory markers. This suggests that, hematological parameters might be a useful prognostic marker of cardiovascular complications and thus used in control of T2D disease progression. Therefore, they may contribute to the early detection of complications and may have a role in potential reduction of morbidity and mortality in diabetic patients. This study in Ethiopian population can be considered as an initial one that necessitates further studies to define the relation between hematological parameters with its prognostic value, different diabetic complications, other antioxidants and different diabetic medications. Further investigations may be required to define the specific hematological aberrations observed in the present study and to consider it for prediction as a specific risk factor in diabetic patients.

7. RECOMMENDATIONS

- Even though this Cross sectional study demonstrated a very good results further prospective studies needs to be conducted to clearly address the cause-effect relationship regarding hematological abnormalities in Ethiopian diabetic patients.

- The moderate sample size of the present study did not explore the general population of Ethiopia other than Addis Ababa. Increasing sample size and study area may provide additional information on current study. Further studies need to be attempted at different population levels in Ethiopia.

- Due to limitation of the availability of resources, the hemoglobin A1c test was not performed on the study subjects. There is a need for measurement of HbA1c, as fasting blood glucose only tells one time status of sugar level and may not accurately explain how well diabetes is controlled.

- Further research needed on different anti-diabetic drug use and its effect on hematological parameters levels before and after treatment.

8. LIMITATIONS OF THE STUDY

- It was very difficult to convince non-diabetic healthy individuals to participate in the study as a result data collection from healthy individuals took long time.
- Even though consent form was filled by the study participants, the study hospital refused to give the print out of the laboratory results, this also takes long process to convince them and collect results.
- The hemoglobin A1c test was not performed on the study individuals due to the limitation in the availability of resources.

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Annex I: English Version Questionnaire

Addis Ababa University

School of Medicine Department of Biochemistry

Information Sheet.

Name of the primary investigator: Chala Olana

Advisors Name: Gnana S (PhD), Daniel S (PhD) and Mennon M (PhD).

Name of the collaborating organization: Federal Police Specialized Hospital (FPSH).

Name of the sponsor: **Addis Ababa University College of Health Science.**

This Information Sheet was prepared by the Primary Investigator For a project that aims to assess the Hematological Profiles and Non Enzymatic Antioxidants Level in T2DM Patients.

i. **Aims of the Study:**

This study aims to investigate the hematological abnormalities and the levels of non enzymatic antioxidants that may be altered in T2DM patients leading to further complications.

ii. Study Design and Procedure

If you agree to take part in the study, the principal investigator or health workers will give you verbal and/or written information about the study and you will be given the consent form to sign. The physician or health professional will ask you some questions about your general health and perform a complete medical examination to assess whether you fulfill the inclusion criteria of this study. If you fulfill the inclusion criteria for the study, urine and blood (5-10ml) samples will be collected for Blood cell counts, Ascorbic acid, Uric acid, Blirubin and Albumin tests.

iii. Risk and Discomfort

Participating in this project will not cause any harm/discomfort than is expected and no extra sample will be taken. If you have any discomfort, you can contact the investigator of this project. The amount of blood that will be taken from each volunteer throughout the study period is 5-10ml, which will not affect your health.

iv. Benefits and incentives

The results of the laboratory findings will be reported to your physician for use in the management of the problem. You will have a chance to know your general health status from the medical examination and if it reveals any incidental health problems that need immediate treatment, you will be referred to an appropriate health facility. The study can benefit T2DM patients whose hematological profiles and non enzymatic antioxidants levels are altered.

v. Confidentiality

All information about the subjects will be kept confidential. Log books used in the laboratory will have no names but codes.

vi. Right to Refuse or Withdraw

You have full right to refuse or withdraw from participating in this study at any time before and after consent without explaining the reason. Your decision will not affect your right to get health services you are supposed to get.

vii. Whom to contact

This study protocol is reviewed by Addis Ababa University, department of biochemistry and medical faculty ethical research committee. The purpose of the review by these committees is to make sure that research participants has to be protected from harm. To know more information about the study, you can contact any of the following individuals.

1) Mr. Chala Olana E-mail: olanachala29@gmail.com Tel: +251 913 31 22 66
2) Dr. Gnana (mob: - 09 24 38 41 89).
3) Dr. Daniel (mob:- 09 11 23 27 54)
4) Dr. Mennon (mob:-)

Consent Form

I have read the information sheet above and clearly understood the purpose and anticipated benefit of the research. I hereby need to assure with my signature below that I, without any coercion or forceful act by the research team, have decided to voluntarily participate in the study to contribute my part.

Name of the study subject_____ Code No. _____

Signature _____ Date_____

Interviewer's name_____Signature_____

Date_____

Date of interview_____Time started_____ Time finished_____

Supervisor's Name_____ Signature_____

I thank you for your cooperation

Questionnaire Form

1. Personal identification

a. Full name of the subject_____.

b. Identification number_____.

2. demographic information

i. Age_____.

ii. Birth place

Region _____Zone _____Woreda _____Town/Kebele

iii. Place of Residence

Region _____Zone _____Woreda _____Town/Kebele

iv. For how long have you lived in Addis Ababa?_____.

v. Ethnic group_____.

3. Anthropometrical and Clinical Information

a. Height _____Weight_____.

b. Blood Pressure; Systolic/Diastolic_____/_____.

c. Heart Beat_____/minute

d. Body Temperature_____^{0}C

e. Sickness in the past history;

Hematological Disease_____Cardiovascular Disease_____.

Renal Disease_____ Liver Disease_____Other_____.

f. Pregnancy_____.

g. What type of antidiabetic drug you are taking_____.

h. Duration of your diabetes_____.

4. Habits;

Alcohol Drinking Habits_____.

Smoking Habits_____.

Annex II: Amharic version questionnaire

Addis Ababa University School of Medicine

ባዮኬሚስትሪ ትምርት ክፍል

የመረጃ ቅፅ (Information Sheet)

ይህ መጠይቅ በስኳር በሽታ ታማሚዎች ላይ ልታዩ የሚችሉ የደም ሴሎች መጠን መዛባት እንድሁም እንዛይም ያልሆኑና በሰዉነታችን ዉስጥ የሚፈጠሩ ፍሪ ራድካሎችን የሚጠርጉትን መጠን ለማጥናት የተዘጋጀ ነዉ፡፡

- **የዋና ተመራማሪ ስም ፡** <u>አቶ ጫላ አላና ሙሊታ</u>
- **የአማካሪዎች ስም ፡** <u>ዶክተር ግናና፣ዶክተር ዳንኤል እና ዶክተር መኖን</u>
- **ለምርምሩ አጋዝ የሆኑ ድርጅቶች ፡** አድስ አበባ ዩኒቨርሲቲ ስፔሻላይዝድ ቲችንግ ሆስፕታል የስኩዋር በሽተኞች ማህበር፣የፌደራል ፖሊስ ሆስፒታል፡፡
- **የጥናቱ ስፖንሰር ፡** _____.

i. **የጥናቱ አላማ**

የዚህ ጥናት አላማ በስኩዋር በሽታ ታማሚዎች ላይ ልከሰቱ የሚችሉ እና ለተባባሴ ተጉዋዳኝ በሽታዎች ምክንያት የሚሆኑ የደም ሴሎች ችግሮችን ማጥናት ነዉ፡፡

ii. **በጥናቱ ዉስጥ የተሳትፎ ሁኔታ**

በጥናቱ ለመሳተፍ ከተስማሙ ሐኪሙ ወይም የጤና ባለሙያዉ ጤነኛ መሆንዎን ለማረጋገጥ የጤና ምርመራ ያደርግልዎታል፡፡ ከዝያም መሳተፍ እንደሚችሉና እንደማይችሉ ይነግሮታል፡፡ በጥናቱ መሳተፍ ከቻሉ ጥናቱ መሠረት ያደረገዉን የሽንት እና የደም (5-10ml) ናሙናዎች ተወስዶ የደምሴሎች ቁጥር ፡ ብሊሩብን፡ አልቡምን፡አስኮርብክ አሲድ እና ዩሪክ አሲድ መጠን ይመረመራል፡፡ ሌላ ተጨማሪ ናሙና መስጠት አይጠበቅብዎትም፡፡

iii. **ሊከሰቱ ስለሚችሉ ስጋቶች እና የምቾት መጉዋደል**

በዝህ ፕሮጀክት ዉስጥ ተሳታፊ መሆንዎ የተለየ ጉዳት ወይም የምቾት መጉዋደል አያስከትልብዎትም ፡፡ናሙና አወሳሰድና ሌሎች ምርመራዎች የሚከናወኑት በሐኪም ወይም በሰለጠኑ ባለሙያዎች እና የሕክምና ደንብ በሚፈቅደዉ የንፅህና አጠባበቅ በመሆኑ ይህ ነዉ የሚባል ስጋት አይኖርም እንድሁም ይደም ናሙና የሚወሰደዉ ከ 5-10ml በመሆኑ በጤንነትዎ ላይ ስጋት አይፈጥርም፡፡

iv. **ጥቅሞችና ማካካሻ**

ከላቦራቶሪ ምርመራ የተገኘዉ ወጤት ለሐኪም ሪፖርት ይደረጋል ችግር እንዳለ የሚያሳይ ከሆነ ችግሩን ለመቆጣጠር ጥቅም ላይ ልዉል ይችላል፡፡ አጠቃላይ የጤና ምርመራ ይደረግልዎታል፡፡ በዝህ ምርመራ የተለየ ወይንም ያልተጠበቀ ዉጤት ቢታይ አስፈላጊ ህክምና የሚያገኙበት ሁኔታ ይመቻችሎታል፡፡ በዝህ ጥናት ተሳተፊ በመሆንዎ የተለየ ጥቅም አያገኙም ነገር ግን ከጥናቱ ጋር በተያያዜ ለሚደረግልዎ አጠቃላይ ምርመራ ወጪ በፕሮግክቱ ይሸፈናል፡፡

v. **ሚስጥር ስለመጠበቅ**

የሚትሰጡን መልሶች ሁሉ በሚስጢር የተጠበቁ ይሆናሉ፡፡ በቤተ ሙከራ የሚቀመጠዉ መዝገብ ምንም አይነት የተሳታፊ ስም አይኖረዉም፡፡ የተሰበሰበዉ ናሙና በምንም አይነት ሁኔታ ከጥናቱ አላማ ዉጪ ለሌላ አላማ አይዉልም፡፡ የጥናቱ ሪፖርት ይፋ በሚሆንበት ጊዜ የእርስዎ ስም አይገለጽም፡፡

vi. **በጥናቱ ያለመሳተፍና እራስን የማግለል መብት**

በጥናቱ ያለመሳተፍ ሙሉ መብት አለዎት፡፡ጥናቱ ከተጀመረ በኃላ በማንኛዉም ሰአት ራስዎን ከጥናቱ ማግለል ይችላሉ፡፡ ይህን በማድረግዎ ምንም አይነት የእንክብካቤ መጉዋደል አያስከትልብዎትም፡፡

vii. **መረጃ ስለማግኘት**

ይህ ጥናት ከአድስ አበባ ሜድካል ፋኩልቲ ባየኬሚስትሪ ትምርት ክፍል እና ከሜድካል ፋኩልቲ የምርምር ሥነ ምግባር ኮሚቴ ተገምግሞ ድጋፍ አግኝተዋል፡፡ የእነዝህ ኮሚቴዎች ዋና አላማ የጥናቱ ተሳታፊዎች ከጉዳት መጠበቃቸዉን ለማረጋፈጥ ነዉ፡፡ በማንኛዉም ጊዜ ጥያቄዎችን መጠየቅ ከፈለጉ ከዝህ በታች ከተጠቀሱት ሰዎች አንዱን ማነጋገር ይችላሉ፡፡

1. **አቶ ጫላ ኦላና**……(Tel: +251 913 31 22 66)
2. **ዶክተር እና**……..(Mob: - 09 24 38 41 89).
3. **ዶክተር ዳንኤል**……(mob: - 09 11 23 27 54).
4. **ዶክተር መኖን**……..(mob:-)

የቃል ስምምነት (Consent Form)

ከላይ የተíፈውን የመረí kî አንብ¾ የØናቱን Þላማና Økም በግልî ተረድቻለሁ፡፡ በዚህም መሰረት Áለ Øናት ቡድኑ Þባላት ተîእኖ በሙሉ ፈiደኝነት በዚሁ Øናት ውስØ በመሳተፍ የሚጠበkብኝን አስተዋîኦ ለማበርከት መወሰኔን በፊርማዬ አረጋግጣለሁ፡፡

የታካሚዉ ስም_____ የታካሚው መለያ ቁጥር _____

ፊርማ _____ቀን_____

የመረጃ ሰብሳቢ ሥም _____ፊርማ _____
ቀን_____

መረጃ የተሰበሰበበት ቀን_____የተጀመረበት ሠዓት _____ለቀበት ሰዓት _____

የተቆጣጣሪ ሥም _____ ፊርማ _____
ቀን_____

ትብብር ስላደረጉልን እናመሰግናለን

የቃለ መጠይቅ ፎርም (questionnaire form)

1. የተሳታፊዉ ሙሉ ስም_____.
2. የተሳታፊዉ መለያ ቁጥር _____ዕድሜ_____.
3. የትዉልድ ቦታ

   ክልል _____ዞን _____ወረዳ _____ቀበሌ_____.
4. ለምን ያህል ግዜ አድስ አበባ ዉስጥ ኖረሃል ወይም ኖረሻል_____.
5. የመኖርያ ቦታ

   ክልል _____ዞን _____ወረዳ _____ቀበሌ_____.
6. ቁመት _____ክብደት_____.
7. የደምግፊት_____.
8. የልብ ምት _____የሰዉነት ትኩሳት_____.
9. ከዝህ በፊት ሌላ ህመም ነበረብዎት_____.

   የኩላሊት _____የልብ _____የጉበት_____.

   የደም ማነስ/ የደም ሴሎች ችግር_____.

   የምትወስደዉ የመድሃኒት አይነት_____.

10. ሱስ አለብዎት

    ስጋራ የማጨስ_____.

    አልኮል የመጠጣት_____.

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university, and all sources of material used for the thesis have been duly acknowledged.

Name	**Signature**
_____	_____