CHAPTER ONE

1.0 BACKGROUND

Diabetes mellitus is defined as a “metabolic disorder caused by different factors characterized by a chronic high level of blood glucose with disturbances to carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both” (Mario et al., 2008). Diabetes Mellitus is divided into type 1 diabetes mellitus or insulin dependent diabetes mellitus also known as juvenile onset diabetes and type 2 diabetes mellitus (T2DM) or noninsulin dependent diabetes mellitus also known as adult onset diabetes. Others include Gestational diabetes mellitus (GDM), which is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Type 1 diabetes mellitus occur as a result of the autoimmune destruction of insulin producing pancreatic β cells (Bluestone et al. 2010). The pathogenesis of type 2 diabetes mellitus is complicated by several factors. Patients present with a combination of varying degrees of insulin resistance and relative insulin deficiency, and it is likely that both contribute to T2DM (Robertson, 2009). Furthermore, each of the clinical features can arise through genetic or environmental influences, making it difficult to determine the exact cause in an individual patient. Moreover, hyperglycemia itself can impair pancreatic beta cell function and exacerbate insulin resistance, leading to a vicious cycle of hyperglycemia causing a worsening metabolic state (Li et al. 2010).

Diabetes Mellitus is associated with evolving atherosclerosis and with a high prevalence of micro- and macro-vascular diseases, which together are the main cause of morbidity and mortality in diabetic patients. T2DM is characterized by fluctuating hyperglycemia, and as a consequence the development to varying degrees of protein glycation. Some of the established effects of hyperglycemia such as oxidative stress, endothelial cell dysfunction,
extracellular matrix formation and apoptosis are considered to play an important role in diabetes mellitus-related vascular damage (Badescu et al., 2000).

Abnormalities in blood flow, hypercoagulability of the blood, and injury to the vessel wall are causally related to thrombus formation (Fig 1.1). Reduced venous blood flow during, for instance, immobilization, prolonged bed rest, limb paresis, long-distance travel, or in obese or pregnant individuals, has been shown to increase the risk of deep vein thrombosis (DVT) (Kyrle and Eichinger 2005). In keeping with Virchow's concept, alterations of the coagulation system that induce a hypercoagulable state also confer an increased risk of DVT. For example, patients with high clotting factor levels have an increased risk of a first and recurrent DVT (Weltermann et al., 2003). Alterations of the vessel wall that lead to plaque formation and plaque rupture play a key role in the development and progression of arterial occlusive disease.

![Virchow's triad](image)

Fig 1.1 Virchow's triad Source : (Kumar and Abbas., 2009)

This simplified view is now modified by the recognition that the process of thrombus formation (thrombogenesis) requires complex interactions involving injury to the vascular
endothelium, platelet adherence, aggregation and release, and clotting factor activation; this process eventually leads to thrombin generation and fibrin formation.

1.1 Statement of the problem

The incidence of type 2 diabetes mellitus (T2DM) is rapidly growing in the world. In 1985, an estimated 30 million people suffered from this chronic disease, which, by the end of 2006, had increased to 230 million, representing 6% of the world population. Of this number, 80% are found in the developing world (Roglic et al., 2005). An increase of as high as 146% is predicted to occur in developing countries, while the increase would only be 47% in developed countries. This means that developing countries will contribute more than 80% of the total number of diabetic patients in the world by the year 2030 (Mario et al., 2008). This rapidly growing prevalence among developing countries is primarily as a result of demographic and epidemiological transition occurring in these countries as a consequence of urbanization, industrialization and globalization. (Frank et al., 2011).

A population based survey conducted in Zambia found the prevalence of T2DM to be 2.1% among males and 3.0% among females and the prevalence is expected to increase in subsequent years (Nsakashalo-Senkwe et al., 2011).

Patients with diabetes mellitus have a high risk of atherothrombotic events. Eighty percent of patients with diabetes mellitus die due to thrombosis, and 75% of these deaths are due to cardiovascular complications (Carr et al., 2001). At Ndola Central Hospital more than 50% of patients admitted for cardiovascular complications, have T2DM (NCH Hospital records).

The risk of hypercoagulability among type 2 diabetic patients is on an increase. Carr et al., (2001) reported a 20% prevalence of hypercoagulability worldwide while Pandolfi et al., (2007) reported a prevalence of 30% indicating that there is an increase in T2DM patients at risk of thrombosis.
1.2 Study Justification

The study examined the haemostatic profile of T2DM at Ndola Central Hospital. This Hospital was chosen because of it being a provincial hospital and therefore has a large catchment area and also because of its close proximity to Ndola College of Biomedical Sciences Haematology and Clinical chemistry laboratories with state of the art equipment which were used in the study.

Currently in Zambia, haemostatic profile tests are not part of tests done in the management of T2DM patients. Furthermore the prognosis is not good when diabetic patients suffer from cardiovascular or cerebrovascular thrombotic diseases. Early detection of the prothrombotic state is therefore critical for the administration of preventive therapy in T2DM patients. The results from this study would be used as a basis to advocate for inclusion of haemostatic profile laboratory tests in the general management of T2DM patients at Ndola Central and other hospitals under the Ministry of Health. This will result in commencement of early treatment in those patients suspected to be in hypercoagulable state and hence reduction of deaths occurring as a result of thrombosis. Soltani et al., (2011) and Lip et al., (2010), have shown von Willebrands factor (vWF) to be predisposing factor for thrombotic development in T2DM patients as endothelial dysfunction occurring as a result of hyperglycemia in T2DM patients lead to the disturbances in blood flow and the accumulation of vWF plasma concentration. High plasma concentration of vWF has been shown to increase the concentration of factor VIII and increased adherence of platelets to the sub-endothelial layer and hence promoting hypercoagulability. Several studies have also reported increased fibrinogen levels in T2DM patients than in healthy non T2DM individuals and consequently higher fibrinogen levels have been associated with increased risk of thrombosis due to increased blood viscosity, increased fiber density of the fibrin clot and increased resistance to fibrinolysis (Kafle and Shresta, 2010).
It is for this reason that vWF and fibrinogen were used as biomarkers for hypercoagulability in T2DM patients.

PT and APTT are inversely related to vWF and fibrinogen concentration. In hypercoagulation, both PT and APTT tend to reduce where as vWF and fibrinogen plasma concentrations tend to increase above the reference value. However most assays designed to estimate plasma vWF and fibrinogen concentration are very expensive and tedious to perform and hence cannot be done routinely in most laboratories in Zambia. The total cost of analyzing a sample for vonwillebrands factor is about 300 Zambian Kwacha (ZMK), while the cost of PT and APTT test is less than 20 ZMK per sample. It was therefore cardinal that a study was done to determine suitability of PT-APTT as markers for hypercoagulability in T2DM using vWF and fibrinogen as biomarkers of hypercoagulability. This is very vital; especially that PT and APTT can easily be done in most laboratories as these tests are cheaper to perform and reagents are readily available. Measurement of PT and APTT may be a safe, noninvasive, reproducible and inexpensive way of assisting in clinical diagnosis of hypercoagulability or as an indicator of disease progression in T2DM patients. Risk factors and patient attributes that may predispose T2DM patients to be in hypercoagulable state were also investigated. These included age, gender duration of diabetes, glycaemic control, Body mass index (BMI) and knowledge about T2DM risk factors and its complications. It was very cardinal to investigate these factors so as to enable clinicians to identify type 2 diabetic patients at risk of developing thrombosis so as to intensify the use of antithrombotic medication in these patients.
1.3 Hypothesis
Ho: There is no difference in hypercoagulability between male and female T2DM patients.

1.4 General objective
To determine hypercoagulability state of T2DM patients by measuring the haemostatic profiles.

1.4.1 Specific Objectives
i. To compare the haemostatic profiles of T2DM patients and non-diabetic healthy individuals
ii. To compare the haemostatic profiles of male and female T2DM patients.
iii. To establish risk factors associated with hypercoagulable state in T2DM patients.
iv. To determine suitability of PT-APTT as markers for hypercoagulable state in T2DM patients.
CHAPTER TWO

2.0 Literature review

Atherothrombosis is the leading cause of morbidity and mortality in patients with diabetes mellitus. The pathogenesis of the atherosclerosis in diabetes mellitus is not entirely clear and conventional risk factors such as smoking, obesity, blood pressure and serum lipids fail to explain fully this excess risk. Fibrin deposition is an invariable feature in atherosclerotic lesions. Therefore, disturbances of haemostasis leading to accelerated fibrin formation (hypercoagulability) and delayed fibrin removal (impaired fibrinolysis) may contribute to the development of atherosclerosis.

Several mechanisms contribute to the diabetic prothrombotic state, such as endothelial dysfunction, coagulative activation and platelet hyper-reactivity. In particular, diabetic platelets are characterized by dysregulation of several signaling pathways leading to enhanced adhesion, activation and aggregation (Vazzana et al., 2011). These alterations result from the interaction between hyperglycemia, insulin resistance, inflammation and oxidative stress. Many studies have shown a variety of diabetes mellitus-related abnormalities in hemostasis and thrombosis. Venous thrombosis has also been found to occur more frequently in diabetics (Pandolfi et al., 2007). There is no data available regarding haemostatic profile and prevalence of hypercoagulability among T2DM patients in Zambia. Many of the previous studies on haemostatic changes in diabetic patients have been conducted in Europe and Asia.

Pandolfi et al., (2007) reported an increase in the number of T2DM patients in hypercoagulable state, indicating that there is an increase in T2DM patients at risk of thrombosis. The reason for this increase in hypercoagulability among T2DM patients is not known but may be multifactorial. It could be due to poor glycaemic control, aging, obesity, unhealthy diet and sedentary life styles among T2DM patients.
2.1 Risk factors for hypercoagulability in T2DM patients

The development of hypercoagulability in T2DM patients is caused by a combination of lifestyle and other factors. While some are under personal control, such as diet and glycaemic control, increasing age and duration of the disease are not (Soltani et al., 2011).

2.1.1 Age

Age is the main non-modifiable risk factor of T2DM and hypercoagulability in Africa (Jamison et al., 2006). Glucose intolerance in Sub-Saharan Africa, as in other regions of the world, increases with age in both men and women. However, published studies lack uniformity on the age range in which T2DM and hypercoagulability are most prevalence. Nsakashalo-Senkwe et al., (2011) have shown that Diabetes Mellitus is associated with older age groups in Lusaka, Zambia. However no study has been done specifically to determine the age range in which hypercoagulability is most prominent among T2DM patients in Zambia. Bruno et al., (2000) and Soltani et al., (2011) reported a significant correlation between age and hypercoagulability in T2DM patients. Similar results were reported by Zhaolan et al., (2010), who reported a significant association between age and hypercoagulation in T2DM patients. Khattaba et al., (2010) reported that older T2DM patients were more likely to be hypercoagulable than the younger patients. The risk of hypercoagulability with increasing age could be attributed to the changes that occur to the vascular system as a result of aging thus tilting the scale to hypercoagulability in older patients.

2.1.2 Social Economic Status

Socio-Economic Status (SES) and its constituent elements are accepted as being determinants of health. There is considerable evidence to show that poverty is associated with shorter life expectancies and increased mortality, particularly cardiovascular mortality (Rabbi et al., 2006). Significant socio-economic gradients have been shown in the prevalence of several cardiovascular disease risk factors, including diabetes mellitus (DM).
Residence seems to be a major determinant in the prevalence of Diabetes Mellitus in Sub-Saharan Africa, since urban residents have 1.5 to 4.0 times higher prevalence of Diabetes Mellitus than their rural counterparts. This is attributable to lifestyle changes associated with urbanization and Westernization. Urban lifestyle in Africa is characterized by changes in dietary habits involving an increase in the consumption of refined sugars and saturated fat and a reduction in fiber intake (Mennen et al. 2000). However T2DM patients based in rural areas are more at risk of hypercoagulability than those in urban areas (Al-Nozha et al., 2005). Rural based T2DM are less likely to have adequate access to care or to receive high-quality clinical and prevention care services. This therefore may lead to poor management of these patients and hence may have increased complications of T2DM (Khattaba et al., (2010).

Education and literacy are among the major determinants of health and development. Education equips people with knowledge and skills for problem solving, ability to access and understand information on health, such as T2DM (ZDHS, 2007). Mohammed et al., (2012) reported that lack of education was significantly associated with hyperglycaemia and hence increased risk of hypercoagulability.

2.1.3 Glycaemic control

Poor glycaemic control may lead to hypercoagulability in diabetic patients because of accumulation of advanced glycation end products (AGEs), which directly damages the endothelium (Santilli et al., 2009). Chronic hyperglycemia has also been clearly identified as a causal factor for in vivo platelet activation through the impairment of calcium homeostasis in platelets (Davi et al., 2007). This may increase the risk of thrombosis in T2DM patients with poor glycaemic control.

Hyperglycemia is also a risk factor for impaired fibrinolysis. A significant correlation between glucose concentration and Plasminogen Activator Inhibitor (PAI-1) has been observed in T2DM patients. Glucose can directly increase PAI-1 production in human
endothelial cells. PAI-1 binds and rapidly inhibits both single- and two-chain tissue plasminogen activator (t-PA) and urokinase. This predisposes diabetic patients to thrombotic events and progression of atherosclerotic lesions as a result of reduced fibrinolysis (Lemkes et al., 2010)

2.1.4 Duration of T2DM and hypercoagulability

Chantal et al., (2010) reported a significant correlation between hypercoagulability and duration of T2DM. Similar results were found by Barbic et al., (2010), who reported an increase in hypercoagulability among T2DM with a longer duration of the disease in comparison to those patients with a shorter duration.

Zhaolan et al., (2010) also reported a correlation between duration of T2DM and its chronic complications, including hypercoagulability. Long term high glucose levels damage the endothelium by accelerating glycosylation of proteins and lipids to generate advanced glycation end products (AGEs) (Santilli et al., 2009). AGEs accumulate in the vessel wall, where they may directly disturb cell structure and function. Morgan et al., (2000) observed an increase in diabetic complications as the duration of the disease increased. Similar results were reported by Roman et al., (2008).

2.1.5 Hypercholesterolemia, Obesity and Hypertension in T2DM patients

Both hypercholesterolemia and obesity have been implicated in the development of T2DM and both are also associated with hypercoagulation. Studies have shown that raised plasma insulin levels with insulin resistance appear to be an atherogenic factor. Insulin stimulates cholesterol synthesis in smooth muscle cells and macrophages of the arterial walls and also stimulates the proliferation and migration of smooth muscle cells. (Fein et al., 2002).

correlation between total cholesterol and glycated haemoglobin (HbA1C) in T2DM patients and hypercoagulability was higher in patients who had elevated HbA1C than those whose glycaemic control was good thus proving the importance of better metabolic control for these patients in order to avoid hypercoagulation.

Obesity is an independent risk factor for the development of cardiovascular atherosclerosis (Hubert et al., 2003). An individual with a Body Mass Index (BMI) of greater 30 kg/m² is four times more likely to suffer from cardiovascular disease than an individual with a BMI of 25 kg/m² or less (Manson et al., 2005). In the Swedish Obese Subjects Study, Sjostrom et al., (2007) observed that diabetes was present in 13–16% of obese subjects at baseline.

Hypercoagulation in obesity is thought to be caused primarily by the synthesis of factors activating coagulation and inhibiting fibrinolysis (for example factor VII activator and the fibrinolytic inhibitor PAI-1) in adipose tissue (Kozek et al., 2004).

Watt et al., (2009) discovered that fat cells release a novel protein called PEDF (pigment epithelium-derived factor), which triggers a chain of events and interactions that lead to development of Type 2 diabetes. When PEDF is released into the bloodstream, it causes the muscle and liver to become desensitized to insulin. The pancreas then produces more insulin to counteract these negative effects. This insulin release causes the pancreas to become overworked, eventually slowing insulin release from the pancreas, leading to T2DM. Therefore with prolonged obesity there is an increased risk of persistent hyperglycaemia which may eventually lead to endothelial dysfunction and consequently hypercoagulability. Haemostatic abnormalities may also result from the synthesis in adipose tissue of cytokines that are mediators of inflammation and insulin resistance, such as interleukin 6 and TNF-alpha. In addition to this direct effect, the metabolic and lipid alterations that accompany obesity and T2DM are likely to indirectly influence coagulation properties in these patients (Sakkinen et al., 2009).
Diabetes and high blood pressure are closely related diseases. They occur together so frequently that they are officially considered to be “comorbidities” (diseases likely to be present in the same patient). Unfortunately, diabetes makes high blood pressure more difficult to treat, and high blood pressure makes diabetes even more dangerous (Epstein et al., 2002). Overall, when averaged across diabetes type and age range, about 35% of all people with diabetes have high blood pressure. Adler et al., (2006) found a close association between systolic blood pressure and hypercoagulation in T2DM patients with more female patients affected than male patients. Development of hypertension in T2DM patients is mediated by activation of the tissue-based renin-angiotensin-aldosterone axis, volume expansion secondary to hyperglycemia, reduced baroreceptor response, endothelial dysfunction and vascular oxidative stress (Adler et al., 2006).
2.2 Mechanism of hypercoagulability in T2DM patients

2.2.1 Endothelial Dysfunction

Endothelial dysfunction is a common feature of T2DM, and is directly associated with increased cardiovascular risk. Several mechanisms contribute to the endothelial dysfunctional phenotype in T2DM, including altered glucose metabolism, impaired insulin signaling, low grade inflammatory state, and increased reactive oxidant species (ROS) generation (Vazzana et al., 2011). Diabetic hyperglycemia is a prominent causal candidate for induction of endothelium damage by accelerating glycosylation or sorbitol pathways therefore long term hyperglycemia in T2DM patients could be used as proof of the prediction of accelerated endothelial damage (Li et al., 2003). In addition, systemic arterial hypertension is frequently
observed in T2DM patients, and therefore contributes to endothelial dysfunction (Ferroni et al., 2006). Enhanced oxidative stress in the hyperglycaemic milieu accelerates the glycoxidation of proteins and lipids to generate advanced glycation end products (AGEs) (Santilli et al., 2009). AGEs accumulate in the vessel wall, where they may directly disturb cell structure and function. Furthermore, the receptor for AGEs (RAGE) activation on endothelial cells inhibits nitric oxide (NO) biosynthesis by endothelial NO synthase (eNOS) down regulation, with increased generation of reactive oxidant species (ROS). ROS directly quench NO by forming the highly oxidant peroxynitrite ion, which in turn uncouples eNOS to produce superoxide anion and asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS. NO is important in haemostasis because it inhibits platelet aggregation (Devangelio et al., 2007). Consistent with this hypothesis, poor glycaemic control and hypercholesterolemia have been associated with enhanced lipid peroxidation (Devangelio et al., 2007).

Besides its metabolic actions, insulin has relevant vascular actions (Kim et al., 2006). In particular, insulin-signaling pathways regulate endothelial generation of NO and endothelin-1, as well as the expression of adhesion molecules. Insulin resistance is a major determinant of endothelial dysfunction, even in prediabetic states (Kim et al., 2006). In fact, insulin-resistant obese individuals with normal glucose tolerance have the same degree of endothelial dysfunction as patients with overt T2DM.
2.2.2 Coagulative activation

T2DM is associated with a prothrombotic state characterized by a number of changes in thrombotic and fibrinolytic coagulation factor level/activity, which together increase the risk of thrombus formation. Both glucose and insulin are involved in the pathogenesis of this prothrombotic state (Fig. 2.2). In vitro induced thrombin generation is increased in platelet-rich plasma from diabetics compared with that from healthy subjects and a significant elevation of thrombin levels can be demonstrated in T2DM patients in poor metabolic control when compared with well controlled patients (Lemkes et al., 2010). Enhanced levels of prothrombin fragment 1+2 (F1+2) and thrombin-antithrombin complexes (TAT) have been shown in T2DM patients in comparison with healthy subjects. Tissue factor (TF) is a
prothrombotic transmembrane protein, expressed by both vascular and non-vascular cells, including monocytes, and platelets. TF expression by endothelial cells is up-regulated in the presence of low-grade inflammation, frequently found associated with T2DM. In contrast, vascular smooth muscle cells (VSMC), which become exposed after plaque rupture, constitutively express TF and this expression is further enhanced after stimulation with cytokines. Patients with T2DM have higher levels of circulating TF, as well as increased monocyte TF mRNA, which are directly modulated by both glucose and insulin, with an additive effect (Vaidyula et al., 2006). In fact, increasing glucose and insulin levels together results in a much larger rise of TF procoagulant activity, associated with increases in TAT and F1+2. TF levels may also be influenced through the formation of AGEs, which activate NF-kB leading to TF production. Improving glycemic control with metformin or sulphonylurea results in TF levels reduction, whereas induction of hyperglycemia in healthy volunteers increases TF procoagulant activity (Vaidyula et al., 2006). These observations indicate that both insulin and glycemic levels have a direct role in the pathogenesis of the prothrombotic state of T2DM.

Increased levels of TF in T2DM patients activate factors VII and change it to VIIa. Activated factor VII (VIIa), triggers the extrinsic pathway of coagulation cascade via converting factor X to Xa. High levels of TF could be used as criteria for the presence of endothelium damage. Activated factor VIII (VIIIa) is another threatening factor for cardiovascular disease. Factor VIIIa, in the intrinsic pathway of coagulation cascade catalyzes the activation of factor IX to IXa. Factor VIII circulates in the plasma as complex with von Willebrand factor (vWF) to protect FVIII from proteolysis and prolongs its half life. Abnormally higher levels of factor VIII were reported in T2DM patients with cardiovascular disease (Kucharska-Newto et al., 2009). Several studies have shown that elevated plasma levels of factor VIII are associated with an increased risk of venous thrombosis (Kamphuisen et al., 2001 and Mettinger, 2005)
and the risk of recurrent venous thromboembolism (Kyrle et al., 2009). Like TF and fibrinogen, vWF is also known as a marker for endothelium damage and in turn is increased in T2DM patients (Frankel et al., 2008; Bonnetti et al., 2003 Meigs et al., 2006). The theory that vWF plays a role in thrombosis is supported by the demonstration that the largest multimeric forms of the glycoprotein aggregate platelets in vitro under conditions of high shear stress such as those occurring in stenotic arteries(Moake et al.,2005).

Jansson et al., (2003) demonstrated that in individuals with cardiovascular disease, high plasma levels of vWF predict the subsequent occurrence of major clinical events such as death and myocardial infarction. It is often believed that the value of vWF in the prediction of cardiovascular events is related to the fact that plasma levels of the protein signal the extent of damage in the vascular endothelium (Guardado et al., 2009). High fibrinogen levels predict progression of coronary artery calcification in Type 1 Diabetes Mellitus, and levels of this protein may predict silent myocardial ischemia in T2DM (Guardado et al., 2009).

Increased fibrinogen levels in diabetes may be related to the associated low-grade inflammation. Interleukin (IL)-6 levels are elevated in diabetes and this cytokine is able to stimulate hepatocytes to produce fibrinogen, representing an important link between inflammation and hypercoagulation. In T2DM, insulin resistance is associated with increased hepatic fibrinogen production in response to insulin, in contrast with T2DM and healthy controls (Alzahrani et al., 2010). A correlation between plasma glucose and fibrinogen levels has been found, directly implicating glycemia in modulating fibrinogen levels, although improvement in glycemic control does not necessarily result in reduction in protein levels. Accordingly, one study supports that induction and progression of vascular damage leads to inflammation and increased Tissue Factor (TF) production and is concomitant with hyperfibrinogenemia (Bots et al., 2002). In hyperglycemic environment, fibrinogen can become hyperglycosylated and when this abnormal fibrinogen clots, the resulting fibrin
structure is composed of small diameter fibers that are markedly resistant to degradation by plasmin hence increase in hypercoagulability (Zhao et al., 2011).

2.2.3 Platelet activation
Platelet activation may occur early in the diabetic state as suggested by animal studies showing that enhanced platelet aggregation in response to several agonists occurs well before vessel wall changes develop (Nicholaos et al., 2011). Indeed, enhanced platelet aggregation and thromboxane A2 synthesis are detectable within days of making rats diabetic with streptozotocin. Platelets of patients with diabetes mellitus are characterized by dysregulation of several signaling pathways, leading to a hyperreactive phenotype with enhanced adhesion, aggregation, and activation. Hyperglycemia, resulting from defects in insulin secretion, insulin action, or both, may play a causal role in the platelet hyper-reactivity in diabetes mellitus patients. In fact, hyperglycemia is responsible for impairment of calcium homeostasis in platelets, with increased calcium mobilisation from intracellular storage pools, resulting in increased intracellular calcium levels. Hence, its effects on intraplatelet calcium concentration are consistent with enhanced sensitivity to aggregating agents. Platelets from diabetes mellitus patients have diminished sensitivity to natural anti aggregating agents, such as nitric oxide (NO) and prostacyclin (PGI2) (Vazzana, 2011). Chronic hyperglycemia has been clearly identified as a causal factor for in vivo platelet activation (Davi et al., 2007).

Acute hyperglycemia may potentially contribute to precipitating arterial thrombotic occlusion at stenotic sites, inducing increased activation of platelets exposed to high shear stress conditions both in vitro and in vivo. This is documented by elevated levels of surface adhesion molecules such as P-selectin (Gresele et al., 2003) and soluble markers of platelet activation (e.g. soluble CD40 ligand [sCD40L]). Moreover, HbA1C and fasting plasma glucose are related to P-selectin expression in T2DM patients undergoing coronary
angioplasty (Yngen et al., 2006), suggesting once again that improving metabolic control may reduce platelet activation.

Platelets of patients with diabetes mellitus type 2 are hypersensitive to agonists, which cause platelet aggregation, such as ADP and arachidonic acid. A number of mechanisms could contribute to this hypersensitivity. Increased presence of glycoprotein receptors GPIb and GPIIb/IIIa for agonists and adhesive proteins on the platelet surface is one of them. Increased fibrinogen binding was also observed in diabetic patients but platelets did not show increased receptor numbers. (Davi, 2007).

Activated platelets release multiple chemical substances and proteins from their dense and alpha granules. Levels of some of these products serve as markers of in vivo platelet activation. Various studies have found high levels of thromboxane A2, β-thromboglobulin, platelet factor 4 and fibronectin in patients with diabetes (Nicholaos et al., 2011).

2.2.4 Disturbances of fibrinolysis

The fibrinolytic system is natural defence against thrombosis. A balance exists between plasminogen activators and inhibitors, and impairment of this balance can be caused either by diminished release of tissue plasminogen activator (t-PA) or increased levels of plasminogen activator inhibitor 1 (PAI-1). PAI-1 is a serine protease inhibitor and evidence suggests that it is the major regulator of the fibrinolytic system. It binds and rapidly inhibits both single- and two-chain t-PA and urokinase. t-PA and PAI-1 rapidly form an inactive irreversible complex (Lemkes et al., 2010). Abnormalities of the fibrinolytic system have been described in both Type 1 and T2DM. Impaired fibrinolysis, as described in T2DM, is commonly accompanied by an increased plasma levels of PAI-1 and by increased concentration of t-PA antigen, which reflects predominantly t-PA/PAI-1 complexes (Grant et al., 2007).

Impaired fibrinolysis not only predisposes to thrombotic events but also plays a role in the formation and progression of atherosclerotic lesions. Hyperglycemia is an additional risk
factor for impaired fibrinolysis. Glucose can directly increase PAI-1 production in human endothelial cells. In T2DM patients a significant correlation between glucose concentration and PAI-1 and has been observed (Panhaloo et al., 2001).

Hypofibrinolysis is well established in T2DM and characterized by elevated levels of plasminogen activator inhibitor-1 (PAI-1) as well as prolonged clot lysis time. The higher PAI-1 levels can be observed in the more poor controlled T2DM patients (Lemkes et al., 2010). Hyperglycemia is a risk factor for impaired fibrinolysis. Glucose can directly increase PAI-1 production in human endothelial cells. In patients with T2DM a significant correlation between glucose concentration and PAI-1 and has been observed. Glucose lowering treatment obtained by either glipizide or metformin comparably decreased PAI-1. In patients with T2DM, a highly significant correlation was demonstrated between mean HbA1c over the course of 18 years and impaired fibrinolysis as shown by elevated PAI-1 and decreased tissue plasminogen activator (tPA) , implicating glycemia in modulating fibrinolysis potential. Besides hyperglycemia, hyperinsulinemia increases PAI-1 levels (Lemkes et al., 2010), which explains elevated levels of this protein in insulin resistant states.

It has been proposed that insulin resistance or hyperinsulinemia could influence the synthesis of PAI-1 via effects on lipid metabolism (Carr 2001). In patients with diabetes mellitus, dyslipidaemia, in particular high triglyceride and low high-density lipoprotein level, is common. Studies in vitro have demonstrated the effect of various lipoproteins on PAI-1 synthesis. Very-low-density lipoproteins from hypertriglyceridaemic patients increase endothelial cell production of PAI-1 to a greater degree than that from normo-triglyceridaemic subjects. Oxidized low-density lipoproteins also stimulate endothelial cell PAI-1 synthesis as does lipoprotein (α). Lipoprotein (α), low-density lipoprotein, and high-density lipoproteins also suppress t-PA secretion from human endothelial cells in dose dependent manner (Panahloo et al., 2005).
2.3 Haemostatic profile of T2DM patients

2.3.1 Fibrinogen and Vonwillebrands factor

Fibrinogen is a soluble plasma glycoprotein, synthesized by the liver and is converted by thrombin into fibrin during blood coagulation. This is achieved through processes in the coagulation cascade that activate the zymogen prothrombin to the serine protease thrombin, which is responsible for converting fibrinogen into fibrin. Fibrin is then cross linked by factor XIII to form a clot. FXIIIa stabilizes fibrin further by incorporation of the fibrinolysis inhibitors alpha-2-antiplasmin and TAFI (thrombin activatable fibrinolysis inhibitor, procarboxypeptidase B), and binding to several adhesive proteins of various cells (Muszbek et al., 2008) Both the activation of Factor XIII by thrombin and tissue plasminogen activator (t-PA) are catalyzed by fibrin (Muszbek et al., 2008). Fibrin specifically binds the activated coagulation factors Xa and thrombin and entraps them in the network of fibers, thus functioning as a temporary inhibitor of these enzymes, which stay active and can be released during fibrinolysis.

Fibrinogen plasma levels are elevated in both Type 1 and T2DM subjects compared with age-matched healthy controls (Rodriguez et al., 2010). Marks (2001) reported higher fibrinogen levels in T2DM patients than in control participants and indicated that elevated fibrinogen concentration may be one of the risk factors for atherosclerosis among diabetics. High fibrinogen levels predict progression of coronary artery calcification in Type 1 Diabetes Mellitus, and levels of this protein may predict silent myocardial ischemia in T2DM (Guardado et al., 2009).

Machlus et al (2011) investigated the effects of high fibrinogen levels using a combination of murine models of thrombosis and in vitro studies and reported that high fibrinogen levels lead to increased formation of thrombi that are more resistant to proteolytic degradation and hence causes sustained thrombosis. There are clearly a number of mechanisms by which higher
levels of fibrinogen could cause thrombosis, including increased blood viscosity, increased fiber density of the fibrin clot, increased resistance of the fibrin clot to fibrinolysis, and altered mechanical properties of the fibrin clot (Figure 2.3).

![Figure 2.3 Potential mechanisms by which the risk for thrombosis is increased with elevated fibrinogen. Source (Kafle and Shresta, 2010)](image)

Increased fibrinogen levels in T2DM may also be related to the associated low-grade inflammation. Interleukin (IL)-6 levels are elevated in diabetes and this cytokine is able to stimulate hepatocytes to produce fibrinogen, representing an important link between inflammation and hypercoagulation. In T2DM, insulin resistance is associated with increased hepatic fibrinogen production in response to insulin, in contrast with T2DM and healthy controls (Alzahrani et al., 2010). Increased concentration of fibrinogen (hyperfibrinogenemia) in uncontrolled T2DM patients is suspected to take part in vascular damage induction (Zachary and Bloomgraden, 2011). Instead, in well controlled T2DM patients there is no report showing hyperfibrinogenemia (Borissoff et al., 2010). Increased fibrinogen synthesis has also been shown postprandially in T2DM, but not in healthy controls, further suggesting hepatic dysregulation of fibrinogen synthesis (Alzahrani et al., 2010). Bots et al., (2002) supported that induction and progression of vascular damage leads to inflammation and
increased Tissue Factor (TF) production and is concomitant with hyperfibrinogenemia. Zhao et al., (2011) reported increased Fibrinogen concentrations among Chinese T2DM patients. Kannel et al., (2005) reported that fibrinogen is often elevated in T2DM patients and this elevation is associated with poor glycaemic control. Chan and Pan (2009) reported increased Fibrinogen levels among female Chinese T2DM than male patients. Similar results were obtained by Bruno et al., (2000) who reported higher fibrinogen levels in female T2DM patients than male patients. This was further supported by Soedama et al., (2008), who reported significantly higher fibrinogen levels in female T2DM patients than male patients. However, Soltani et al., (2011) found no statistical evidence of any difference in fibrinogen levels between male and female type diabetes mellitus patients. Plasma fibrinogen is an important component of the coagulation cascade, as well as a major determinant of blood viscosity and blood flow. Increasing evidence from epidemiological studies suggests that elevated plasma fibrinogen levels are associated with an increased risk of cardiovascular disorders, including ischaemic heart disease (IHD), stroke and other thromboembolism. (Meade TW, 1986). The risk of developing a cardiovascular event such as IHD or stroke is 1.8 to 4.1 times higher in subjects with fibrinogen levels than in those with lower levels. Preliminary evidence also suggests that reducing fibrinogen levels in patients with high baseline levels and coronary disease may be beneficial (Ernst et al., 1993). In the Framingham Study, the risk of developing cardiovascular disease was significantly related to plasma fibrinogen levels. In both sexes, cardiovascular and stroke risk increased progressively in relation to antecedent fibrinogen values over the 1.8–4.5 g/l range (Kannel et al., 2005). This increase in plasma fibrinogen levels may promote a prothrombotic or hypercoagulable state, and may in part explain the risk of stroke and thromboembolism in conditions such as atrial fibrillation (AF) (Ernst et al., 1993).
Von Willebrand factor (vWF), is a large glycoprotein encoded by a gene on chromosome 12, it is synthesized by vascular endothelial cells. In plasma, vWF forms a non covalent complex with coagulation factor VIII (Sadler et al., 2000). This molecular complex is essential for normal survival of factor VIII, which is stabilized in the circulation, potentiated in its cofactor activity in clot formation, and protected from proteolytic inactivation (Sadler et al., 2000).

The other important function of vWF in physiological hemostasis is in the formation of platelet plugs at sites of endothelial damage, in which the protein binds to the exposed subendothelium and forms a bridge between this surface and platelets. These functions are facilitated by the peculiar structure of vWF, which is arranged in multimers of increasing size up to $2 \times 10^7$ Da built up from a subunit of $2.5 \times 10^7$ Daltons (Da), and by its exposure on the platelet membrane to the glycoprotein complexes Ib/IX/V and IIb/IIIa, which function as receptors for vWF (Sadler et al., 2000). VWF-mediated platelet adhesion to the injured endothelium is the first step in thrombus formation. The theory that vWF plays a role in thrombosis is supported by the demonstration that the largest multimeric forms of the glycoprotein aggregate platelets in vitro under conditions of high shear stress such as those occurring in stenotic arteries (Moake et al., 2005). vWF is produced by endothelial cells and megakaryocytes throughout the body (Blann et al., 2009).

vWF is also known as a marker for endothelium damage and in turn is increased in T2DM patients (Frankel et al., 2008; Bonnetti et al., 2003 Meigs et al., 2006). This accords the results obtained by Mohamed et al., (2005), who found increased vWF levels in T2DM patients than control participants. Alzahrani et al., (2010), reported elevated vWF concentrations in T2DM patients as compared to non-diabetic control participants. These findings were supported by Soltani et al., (2011), who reported elevated vWF in T2DM patients than the control subjects. Chan and Pan (2009), obtained elevated vonwillebands factor among female Chinese T2DM than male patients but conflicting results were reported
by Soltani et al., (2011), who found no statistical evidence of any difference in vWF levels between male and female T2DM. Frankel et al., (2008) found higher levels of vWF to be associated with risk of CVD in people with T2DM or insulin resistance, which suggests that vWF may be a risk factor unique to these populations. Therefore vWF can be a biomarker for endothelial dysfunction and hence hypercoagulability. As a biomarker, elevated levels of vWF represent both endothelial dysfunction and hemostatic imbalance. Increased vWF concentrations were found in plasma from patients with acute myocardial infarction (AMI) compared to control subjects (Goto et al., 2007). Furthermore, detection of vWF in fresh, human coronary thrombi suggests a causative role of vWF in platelet thrombus growth and hence increased thrombosis (Yamashita et al., 2006). Raised vWF levels have been associated with increased thromboembolic events, for example, in patients with deep venous thrombosis (Wahlberg et al., 2000). The prospective value of vWF in venous thromboembolism was shown in a study of patients after major abdominal surgery where high preoperative concentrations of vWF were associated with an increased risk of postoperative deep venous thrombosis (Nilsson et al., 2009). The importance of vWF in human thrombotic disease can be explained by the association of low vWF levels with a bleeding tendency (Nichols et al., 2007), and elevated levels are associated with thrombotic risk (Kyrle et al., 2009), plasma vWF levels must be regulated by a balance between vWF secretion and vWF clearance. The importance of this balance is underscored by the occurrence of the bleeding disorder vonWillebrand disease, with low vWF levels because of mutations causing defective vWF secretion (Allen et al. 2000). At the other extreme is the thrombotic disorder, thrombotic thrombocytopenic purpura (TTP ) caused by deficiency or acquired inhibition of the vWF cleaving protease a disintegrin-like and metalloprotease domain with thrombospondin type motifs (ADAMTS-13), which prolongs the survival of vWF multimers and leads to microvascular thrombi and end-organ failure (Zheng et al., 2009). A number of studies have
shown that elevated VWF correlates very well with increased factor VIII and is a risk factor for both venous and arterial thrombosis (Lip et al., 2010 and Paramo et al., 2005). Nossent and his colleagues (2006) demonstrated increased vWF secretion to be a biomarker of thrombosis risk. They reported a dose–response relationship between vWF and thrombosis risk, with vWF being significantly higher in thrombosis subjects than in controls.

2.3.2 Prothrombin (PT) and Activated Partial Thromboplastin Time (APTT)

PT and APTT tests are standard screening tests for function of the coagulation system and their utility in monitoring therapeutic anticoagulation is widely accepted (Boekel et al., 2002). The APTT assay is traditionally used for identifying abnormalities in the contact (factor XII, prekallikrein, and high-molecular-weight kininogen), intrinsic (factors XI, VIII, IX) and common (factors X, V and II and fibrinogen) pathways of coagulation (Tripodi et al., 2004). Prolonged APTT values have clinical relevance as an indicator of factor deficiency or the presence of coagulation inhibitors. However, there is mounting evidence that shortened APTT values in some cases may reflect a hypercoagulable state, which is potentially associated with increased thrombotic risk and adverse cardiovascular events (Ng et al., 2009 and Lippi et al., 2009). Shortened APTTs may result from an accumulation of circulating activated coagulation factors in plasma caused by enhanced coagulation activation in vivo (Ng et al., 2009 and Lippi et al., 2009). The prothrombin time (PT) is the screening test for the coagulation pathway initiated by tissue factor. Acang et al., (2005) observed that there were significantly shortened PT and APTT values, in diabetic patients, especially in patients with long-term diabetes with chronic complications. Similar results were obtained by Lippi et al., (2009), who reported shortened PT and APTT in T2DM patients than in non-diabetes control subjects. Tripodi et al. (2004) found that hypercoagulability detected by shortened APTT values was independently associated with venous thromboembolism (VTE) and hypothesized that shortened APTT could be considered as a risk marker for VTE.
Hypercoagulability due to high coagulation factors XI, VIII, IX, II, and fibrinogen is recognized as a risk factor of venous thromboembolism (VTE). These factors are cumulatively explored by the activated partial thromboplastin time (APTT). APTT may reflect the procoagulant imbalance consequent to increased levels of coagulation factors and might be associated with an increased risk of venous thromboembolism (VTE). The first prospective clinical trial to evaluate the presence of a short APTT as a possible risk factor for hypercoagulability was done by McKenna et al., (1977) who evaluated the incidence of thromboembolic events in medical and surgical hospitalised patients with a short APTT. These investigators found the presence of a short APTT at any time during the study period to be related to a 10-fold increased incidence of thromboembolic events. Tripod et al., (2004) in a case-control study reported that patients with an APTT lower than 30 seconds have a 2- to 3-fold increased relative risk of VTE, independently of inherited thrombophilic abnormalities. These results are biologically plausible because shortened APTTs have been associated with high levels of biochemical markers of thrombin generation and fibrin deposition such as prothrombin fragment 1 +2, thrombin-antithrombin complex, and D-dimer and thus could be helpful for detecting subpopulations of patients at higher risk for thrombosis (Korte et al., 2008). Furthermore, the study done by Landi et al., (2003) suggested that a short APTT was associated with an increased risk of thrombosis in specific clinical situations. In a multicentre prospective study Legnani et al., (2006) reported that short APTT values were a risk factor of VTE recurrence in patients with a first unprovoked VTE episode. In acute arterial thrombosis such as coronary artery thrombosis, similar finding of shortened APTT was also described as seen in venous thrombosis and a significant negative correlation between APTT and FVIII, was reported. (Abdullah et al., 2010).

Chan and Pan (2009) and Ukaejiofo et al., (2007) reported shorter PT and APTT among T2DM patients than non-diabetic patients with the female T2DM patients having both
parameters shorter as compared to the male T2DM patients. Though Soltani et al., (2011) had reported shorter PT and APTT in T2DM patients than in control participants, there was no statistical difference in PT and APTT between male and female diabetic patients.

From the literature searched so far no studies were found that have explicitly evaluated PT and APTT as markers of hypercoagulation in terms of Sensitivity, Specificity, Positive and Negativity predictive values. This will be the first study to evaluate PT and APTT as markers of hypercoagulation in T2DM patients.
CHAPTER THREE

3.0 Research design and methodology

3.1 Research site

The study was conducted at Ndola Central Hospital, a third level referral hospital for Copperbelt and Northern part of Zambia. It is located in Ndola, the provincial headquarters of the Copperbelt Province. The hospital has a bed capacity of 851.

3.2 Study population and design

The study was a cross-sectional analytical study involving T2DM patients attending Ndola Central Hospital Out-patient Department (OPD) Diabetic clinic and healthy adult male and female participants visiting OPD for medical examinations between November 2012 to May 2013. The latter group included individuals coming for pre-employment medical examinations and first year students from various colleges attending medicals as part of the requirement for registration purposes. The study included only consenting adult males and females above the age of 18 years who met the inclusion criteria.

3.3 Inclusion criteria for T2DM patients

- Type 2 diabetic patients
- Male or female above the age of 18 years
- Willing to consent

3.3.1 Inclusion criteria for control participants

- Healthy non diabetic male or female individuals above the age of 18 years
- Willing to consent

3.4 Exclusion criteria

- Thrombocytosis
- A history of venous thromboembolism or known inherited coagulation disorders
- Cancer and Hyperthyroidism
• Pregnancy
• Recent surgery
• Patients taking standard anticoagulant treatment
• Less than 18 years of age
• Those not willing to consent

3.5 Sample size

Since we were comparing the proportion of T2DM patients with haemostatic prothrombotic state and normal control participants the formula which was used for sample size calculation was:

\[ N = \frac{u \sqrt{\pi_1(1-\pi_1) + \pi_0(1-\pi_0)} + v \sqrt{2\pi(1-\pi)}}{(\pi_0-\pi_1)^2} \]

Where \( N \) = Sample size of each group
\( \pi_0, \pi_1 \) = Proportions
\( \pi \) = Average of the proportions
\( U = 0.84 \) for 80% Power
\( v = Z \) statistic = 1.96 if \( \alpha = 0.05 \)

It is expected that:
- Based on Soltani et al., (2011) who reported a 3% prevalence of hypercoagulability in healthy non-diabetic individuals, it will be anticipated that 3% of the general population in Ndola will be in hypercoagulable state.
- Based on Pandolfi et al., (2007) who reported a proportion of 30% T2DM patients to be in hypercoagulable state, it will be expected that 30% of T2DM patients at NCH will be in hypercoagulable state.
The calculated sample size 155 was increased by 10% to 170 for T2DM patients and control participants to cater for non response and missing data.

Based on an expected T2DM prevalence of 3% in Zambia and 3% prothrombotic haemostatic profile in individuals without T2DM, a minimum of 340 participants were to be enrolled in order to have 80% power to detect 27% difference in prothrombotic haemostatic profile (30%) in T2DM patients, using $\alpha = 0.05$.

The actual total number of participants recruited in this study was 385. This included 213 T2DM patients and 172 non-diabetic healthy adult individuals as control participants. The sample size was increased as most T2DM patients were willing to participate into the study and also to improve the statistical power of the study and this was done after getting approval from UNZA-BREC.

### 3.6 Sampling

Convenience sampling was used to recruit 213 T2DM patients and 172 non-diabetic healthy controls attending medical examinations at Ndola Central Hospital.

### 3.7 Variables and indicators of measurements

For the purpose of this study individuals were considered to have type 2 diabetes mellitus (T2DM) when fasting plasma glucose was $> 7.5$ mmol/l and random blood glucose of $> 11.5$ mmol/l on more than two occasions and with classic symptoms of diabetes, including polydipsia, polyuria, polyphagia and weight loss. Patients on treatment with glucose lowering medication where also considered to have T2DM.

The plasma von willebrands and fibrinogen concentration were used as proxy markers for hypercoagulability states and these were the dependent variables. A Hypercoagulable state is
the medical term for a condition in which there is an abnormal increased tendency toward blood clotting. T2DM Patients were considered to be at risk of hypercoagulability if the vWF and fibrinogen concentration were above 2.6 IU/mL and 3.2g/l respectively.

Potential confounding variables for the study included, hypercholesterolema, cancer, pregnancy and Immobility.

Hypercholesterolema and obesity were controlled during statistical analysis of the results. Cancer, pregnancy and immobility were controlled by excluding individuals with any of these conditions from the study. T2DM patients were considered to have hypercholesterolema if the plasma Cholesterol concentration is above 5.2 mmol/l.
Table 1.1: Study variables and their cut-off points or unit of measurements

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variables</th>
<th>Cut-off point</th>
<th>Indicator/Unit of measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Willebrands Factor</td>
<td>Gender</td>
<td>Male</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>(2)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Employment status</td>
<td>Employed</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Unemployed</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td>Hypertensive</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NO</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Level of awareness about risk factors, complications and management of T2DM</td>
<td>Adequate knowledge</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inadequate Knowledge</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Duration of the disease</td>
<td>&gt; 5 years</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-10 years</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Above 10 years</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>Years</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycated haemoglobin</td>
<td>≤ 7%</td>
<td>Good glycaemic control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≥ 7%</td>
<td>Poor glycaemic control</td>
</tr>
<tr>
<td></td>
<td>Body Mass Index</td>
<td>Kg/m²</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Random Blood Glucose</td>
<td>mmol/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prothrombin Time</td>
<td>Seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*APTT</td>
<td>Seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>mmol/l</td>
<td></td>
</tr>
</tbody>
</table>

*APTT - Activated partial thromboplastin time
3.8 Ethical considerations
This study was performed under a protocol that was reviewed and approved by the University of Zambia-Biomedical Research Ethics Committee (UNZA-BREC). Written permission was obtained from the Permanent Secretary of the Ministry of Health as well as from the Senior Medical Superintendent of Ndola Central Hospital. All the prospective participants in this study were informed about the study, privileges and right to participation. The purpose of the study was thoroughly explained to the participants and those that declined to participate in the study were not forced, but were assured of their protected privileges and rights to treatment. Privacy and confidentiality was maintained. The names of the respondents did not appear anywhere on the forms instead codes were used on the forms. The forms were kept in lockable cabinets and no one apart from the researcher had access to the cabinets. Data on the computer was pass-word protected such that access was limited to only the researcher. The respondents were thus assured of utmost confidentiality.
Only qualified medical professionals such as nurses and laboratory staff were involved in the collection of venous blood samples from study participants. The total number of blood containers needed for all the laboratory tests was four (4). Three (3) ml of venous blood was collected in each container using the evacuated blood collection system which was very ideal and safe for multiple blood sample collection as blood was delivered directly into the containers. In total twelve (12) ml of venous blood was collected from each research participant. Consenting patients and control participants were made to sign the consent form before being enrolled into the study.
3.9 Data collection

3.9.1 Questionnaire and Laboratory request forms

A structured questionnaire was used to collect information from the participants. The questionnaire was divided into the following sections: Demographic information (Age, Gender, place of residence, level of education and Employment status), Physical measurements (Height, Weight and Blood pressure), Levels of awareness about T2DM risk factors, management, complications and management. Part 4 of the questionnaire was used to capture information pertaining to the duration of T2DM disease for each participant.

The questionnaires were piloted on ten participants. All questions which were not clear were revised.

Each participant’s sample was accompanied by a laboratory request form when being taken for analysis. Clinical Chemistry request form was used for Glucose and random cholesterol tests and Haematology request forms were used for fibrinogen, Prothrombin, Activated Partial Thromboplastin, von Willebrands factor and Glycated Haemoglobin tests.

3.9.2 Physical Measurements

The WHO STEPs surveillance training and practical guide recommends that physical measurements be taken in the following order: Height, Weight and Blood Pressure. Therefore these measurements were taken in that order (Nsakashalo-Senkwe et al. 2011).

3.9.2 (i) Height and Weight

The seca Brand 214 Portable stadiometer was used to measure the heights of the participants. Height was measured without the participants wearing foot or head gear and it was recorded in meters.

Participants’ weight was measured using the Heine Portable Professional Adult Scale 737 and was recorded in kilograms.
Body Mass Index (BMI) was calculated by dividing the participants’ weight in kilograms by the square of height in meters.

### 3.9.2 (ii) Blood Pressure

Blood pressure readings were taken from each participant and those who had more than 140 and 90 mmHg for either systolic or diastolic blood pressure respectively were regarded as being hypertensive. Participants who were on blood pressure medication were also regarded as being hypertensive despite the blood pressure readings being within the normal range.

### 3.10 Laboratory Tests

Good Laboratory Practice (GLP) principles according to the Ministry of Health laboratory quality manual was observed to ensure uniformity, consistency, reliability and reproducibility of all the laboratory test results that have been produced in this study. Venous blood collection was done by using the evacuated blood collection system. 5 ml of venous blood was collected for each test.

#### 3.10.1 Random Blood Glucose

Venous blood for glucose estimation was collected in Sodium Fluoride containers. The samples were immediately centrifuged and plasma separated and frozen at -30°C. Random Blood Glucose was determined by using the Humalyser 2000 semi-automated Chemistry analyser. Glucose liquicolor reagents manufactured by Human Gesellschaft of Germany was adapted for use with the humalyser 2000. The method used was the Glucose oxidase (GOD)-4 aminophenazone (PAP) method which is based on the original method invented by Trinder in 1972 (Human Gesellshaft 2011). Detailed principle and procedure of the method used for glucose estimation was as provided by the reagent manufacturer as shown in appendix 9, page 105.

Random glucose levels were grouped into low glucose level (< 3.3 mmol/l), normal (3.9 - 7.8 mmol/l), and high glucose level (>11.0 mmol/l).
3.10.2 Total Cholesterol

Venous blood for total cholesterol estimation was collected in plain containers. The samples were centrifuged within 4 hours of blood collection and serum was separated from the red cells and frozen at -30°C for subsequent analysis. Total Cholesterol was determined using the Humalyser 2000 semi-automated clinical chemistry analyser. The cholesterol liquicolor reagents manufactured by Human Gesellschaft of Germany were adapted for use on the Humalyser 2000 analyser. The method used was the Cholesterol Oxidase—Phenol-4-aminophenazone (CHO-PAP) method. The principle of this method is based on Flegg and Richmond (Human Gesellshaft, 2011). Detailed procedure for the method used for cholesterol estimation was as provided by the reagent manufacturer as shown in appendix 10. Cholesterol levels were classified as normal if less than or equal to 5.2 mmol/l and raised if greater than 5.2 mmol/l.

3.10.3 Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT)

Three (3) ml of venous blood for PT and APTT was collected from each of the study participants in sodium citrate containers and centrifuged at 1500g for 15 minutes. Plasma was then separated and transferred into siliconized glass tubes and stored at 4°C in a fridge until analysis. The reagents used for PT and APTT were sourced from SPINREACT Diagnostics Company of Spain.

PT and APTT results were reported in seconds. The reference ranges for PT and APTT was 11-16 seconds and 30-40 seconds respectively. Detailed principle and procedure of PT and APTT method is as provided by the reagent manufactures as shown in appendix 11 and 12 respectively.

Any participants’ PT and APTT results which were below 11 seconds and 30 seconds respectively were considered as being hypercoagulable state (Spinreact Diagnostics 2010 and Korte et al. 2008).
3.10.4 Plasma fibrinogen determination

Blood for fibrinogen determination was collected from the study participants in sodium citrate containers and centrifuged at 1500g for 15 minutes. Plasma was then separated and transferred into plastic or siliconized glass tubes and stored at -20°C until analysis. The reagents for this test were sourced from SPINREACT Diagnostics Company of Spain. The kit used utilizes the thrombin clotting time assay based on the method originally described by Clauss. (Burtis et al., 2000). The detailed principle and procedure used for determination of fibrinogen is shown in appendix 13.

The reference range for fibrinogen concentration was taken as 1.6 to 3.2 g/l. Any participants’ fibrinogen result above 3.2 g/l concentration will regarded as hypercoagulable state.

3.10.5 von Willebrands Factor estimation

The same participants’ plasma samples for PT and APTT analysis as prepared in section 3.10.4 was also used for vWF analysis. vWF was determined by the Human ELISA kit manufactured by Abnova of USA. The detailed procedure for vWF estimation was as provided by the manufacturer of the kit as shown in appendix 14.

Plasma vWF concentration results were reported in International units/ml (IU/ml).The reference range for plasma concentration of vWF is 0.6 to 2.6 IU/mL. Any result above 2.6 IU/mL was regarded as being in hypercoagulable state as this increase indicates enhanced endothelial damage and hence predisposition to thrombosis.

3.10.6 Glycated Haemoglobin (HbA1C) estimation

Venous blood collected in EDTA containers was used for HbA1C estimation. Bio-Quant Glycated Haemoglobin (HbA1C) Enzymatic assay kit produced by BioSupply of United kingdom was used to determine HbA1C in whole blood. This was measured primarily to identify the average plasma glucose concentration over the past 3-4 months and hence
indirect indicator of glycaemic control in T2DM patients. The detailed procedure and settings used for Glycated Haemoglobin estimation is shown in appendix 15. The method was adapted for use on the Bechman synchron CX-7 fully automated chemistry analyser at Konkola Mine Hospital in Chililabombwe, Zambia.

3.11 Determination of haemostatic profiles of T2DM patients.

The haemostatic profiles that were analysed in both T2DM patients and control participants were vWF, Fibrinogen levels, Prothrombin Time and Activated Patial Thromboplastin Time. The mean result of each variable was calculated and compared in T2DM patients and control participants and the independent T-test was used to evaluate if the differences were statistically significant.

3.12 Prevalence of hypercoagulability in T2DM patients

vWF and Fibrinogen plasma concentrations were measured in both T2DM patients and control participants and categorized as normal (vWF ≤ 2.6g/l and Fibrinogen ≤ 3.2g/l) or hypercoagulable vWF > 2.6 IU/mL and Fibrinogen > 3.2g/l). The proportion of participants who were hypercoagulable was compared between T2DM and control participants.

3.13 Risk factors and patient attributes associated with a hypercoagulable state in T2DM patients.

Hypercoagulation in T2DM patients was determined by estimating vWF and fibrinogen and the results obtained were compared with the potential risk factors for hypercoagulation such as age, sex, glycaemic control, duration of T2DM and awareness of T2DM risk factors and complications.

3.13.1 Association of age and hypercoagulation in T2DM patients

Age of T2DM patients was categorized and each category’s frequency of hypercoagulability (vWF > 2.6 IU/mL and Fibrinogen > 3.2g/l) was determined and compared among different categories to determine if the differences were statistically significant.
3.13.2 Glycaemic control and hypercoagulation in T2DM patients

Glycated haemoglobin (HbA1C) was used as an indirect measure of glucose control in T2DM patients. Glycated haemoglobin of less than or equal to 7% was regarded as good glycaemic control, while HbA1C of greater than 7% was regarded as poor glycaemic control. Using the chi-square test the frequency of hypercoagulability was compared between those with good glycaemic control (HbA1c ≤ 7%) and poor glycaemic control (HbA1c >7.0%) and analyzed for any significance difference in hypercoagulation. Logistic regression was used to obtain the odds ratios and determine the strength of association between glycaemic control and hypercoagulation.

3.13.3 Levels of knowledge about T2DM risk factors, complications and management in relation to hypercoagulation.

Investigating the level of awareness about diabetes mellitus risk factors, complications and management among T2DM patients was determined by administering a general questionnaire. Participants were asked to mention the risk factors, complications and management of T2DM. Participants who mentioned 2 or less risk factors/complications and less than 2 ways of managing type diabetes mellitus, were deemed to have inadequate knowledge and those who mentioned 3 or more risk factors/complications and more than 2 ways of managing T2DM were deemed to have adequate knowledge about T2DM risk factors/complications and management of T2DM. The proportion of hypercoagulability between those with adequate knowledge and inadequate knowledge was analysed statistically using the chi-square and determined any significance difference between these two groups. Logistic regression was used to determine the strength of association between hypercoagulability and level of knowledge about T2DM risk factors and complications.
3.13.4 Duration of T2DM and risk of hypercoagulation

Data for duration of T2DM for each patient was obtained from the questionnaire. The duration was categorized into ≤5 years, 5 to 10 years and > 10 years. Using the chi-square test, the proportion of hypercoagulability was compared among the three categories and determined whether the differences in the proportions were statistically significant. Logistic regression was used to determine the strength of association between duration of T2DM and hypercoagulation.

3.14 Suitability of PT-APTT as markers for hypercoagulability

The relationship between PT, APTT, vWF and Fibrinogen was assessed by using the spearman method to determine the correlation coefficient.

Sensitivity, Specificity, Positive and negative Predictive values for PT and APTT as markers for hypercoagulability were determined. Sensitivity ("positivity in disease") refers to the proportion of participants who have the target condition (reference standard positive) and give positive test results. Specificity ("negativity in health") is the proportion of participants without the target condition and give negative test results. Positive predictive value is the proportion of positive results that are true positive (i.e. have the target condition) whereas negative predictive value is the proportion of negative results that are true negatives (i.e. do not have the target condition). vWF/fibrinogen concentration was used as a biomarker for hypercoagulability and this was compared to PT and APTT results for each patient. The cross tabulation tables obtained from SPSS between vWF/fibrinogen results in comparison to PT/APTT were transferred to CEBM (Center for evidence Based Medicine) Statistics Calculator for calculation of sensitivity, specificity, positive and negative predictive values.
3.15 Data analysis

The Statistical Package for Social Science (SPSS version 16) and CEBM Statistics Calculator was used to analyse the results statistically. Analysis of distribution was made using the Kolmogoroff-Smirnoff test.

All the parameters were normally distributed and hence reported as the mean +/- standard deviation. The significance of the differences between patients and controls for normally distributed parameters were determined using the independent samples T-test for continuous variables and Chi-square test for categorical variables. Risk factors and patient attributes associated with hypercoagulability in T2DM were determined by Binary Logistic regression analysis. Odds ratios and their 95% confidence intervals are reported. A level of correlation between PT-APTT and vWF/Fibrinogen was determined by spearman method. Sensitivity, Specificity and Positive predictive value for PT-APTT as markers for hypercoagulability were calculated from a 2x2 table computed in CEBM Statistics Calculator. P-values of less than 5% were taken as significant.
4.0 RESULTS

4.1 Sample population distribution by demographic and biochemical factors

A total of 385 subjects aged 21-83 years participated in the study out of which 167(43.4%) were males and 218(56.6%) were females. 213 participants were T2DM patients and 172 were control subjects. The age of T2DM participants ranged from 21 years to 79 years with the majority of participants [80 (37.6%)] being in the range of 51-60. The mean age was 45 years old (SD 4.31). While that of control subjects also ranged from 21 years to 83 years with the majority 65(37.8%) being in the range of 41-50 years. The mean age for control subjects was 44.3 (SD 4.21). Very few T2DM patients and control participants had attained tertiary education 22(10.3 %) and 7(4.1%) respectively. The unemployed comprised the majority of T2DM participants than control subjects [122(57.3%)] and [105(61.1%)] respectively as shown in Figure 4.1.

Figure 4.1 further shows that the proportion of T2DM patients who had good glycaemic control was lower [100(46.9%)] than those who had poor glycaemic control [113(53.1%)]. This study further reveals that T2DM patients who had higher glucose levels was lower as compared to those who had normal levels [61(28.6%)] and [152(71.4%)] respectively.

Figure 4.1 also shows that a higher proportion of T2DM patients had adequate knowledge about the risk factors of T2DM [108(50.7%)] as compared to control participants [48(27.4%)]. A higher proportion of T2DM patients [55(25.8%)] had higher levels of cholesterol as compared to control subjects [6(3.5 %)] and the frequency of hypertension in control participants was lower as compared to T2DM patients, [13(7.6 %)] and [126(59.2 %)] respectively. A higher proportion of T2DM patients were found to be obese as compared to control participants, [62(29.2 %)] and [8(4.6 %)] respectively.
Figure 4.1: Demographic variables of study participants

NIDDM—Non Insulin Dependent Diabetes Mellitus

Figure 4.2 BMI, Biochemical characteristics and related knowledge on T2DM risk factors, complications
4.2 Haemostatic profile of T2DM

Table 4.1 Haemostatic profiles in T2DM patients and control Subjects. Independent t-test parameters.

<table>
<thead>
<tr>
<th>Status</th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>t-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF (IU/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>172</td>
<td>2.6</td>
<td>2.2</td>
<td>6.00</td>
<td>0.004</td>
</tr>
<tr>
<td>Diabetic</td>
<td>213</td>
<td>7.4</td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>172</td>
<td>2.3</td>
<td>1.6</td>
<td>8.36</td>
<td>0.003</td>
</tr>
<tr>
<td>Diabetic</td>
<td>213</td>
<td>4.3</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT (Seconds)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>172</td>
<td>12.5</td>
<td>2.9</td>
<td>5.41</td>
<td>0.168</td>
</tr>
<tr>
<td>Diabetic</td>
<td>213</td>
<td>12.4</td>
<td>3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APTT (Seconds)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>172</td>
<td>32.2</td>
<td>4.2</td>
<td>7.58</td>
<td>0.000</td>
</tr>
<tr>
<td>Diabetic</td>
<td>213</td>
<td>24.7</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

An independent-sample t-test was conducted to compare the haemostatic profiles in T2DM and control participants. Table 4.1, reveals that mean fibrinogen concentration for T2DM patients (4.3 ±2.5 g/l) was significantly higher than control participants (2.3±1.6 g/l) t-value = 8.36; P-value = 0.003.

The mean Vonwillebrands factor concentration for T2DM patients (7.4 ±4.1 IU/ml) was significantly higher than control participants (2.6±2.2 IU/ml). t-value= 6.00; P-value = 0.0004.

The mean Prothrombin Time for T2DM patients (12.4±3.3 seconds) was lower than control participants (12.5 ±2.9 seconds) but the difference was not significant t-value=5.41; P-value = 0.168.
The mean Activated Partial Thromboplastin Time for T2DM patients (24.7 ±3.8 seconds) was significantly lower than control participants (32.2±4.2 seconds). t-value= 7.58; P value = 0.000.

Table 4.2 Haemostatic profiles of T2DM patients in males and females. Independent t-test

<table>
<thead>
<tr>
<th>Status</th>
<th>Status</th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>t-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF (IU/mL)</td>
<td>Male</td>
<td>93</td>
<td>2.6</td>
<td>1.2</td>
<td>6.71</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>120</td>
<td>4.6</td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>Male</td>
<td>93</td>
<td>3.4</td>
<td>2.0</td>
<td>2.31</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>120</td>
<td>4.3</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT (Seconds)</td>
<td>Male</td>
<td>93</td>
<td>11.9</td>
<td>3.3</td>
<td>3.18</td>
<td>0.172</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>120</td>
<td>11.2</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APTT (Seconds)</td>
<td>Male</td>
<td>93</td>
<td>32.0</td>
<td>3.0</td>
<td>6.72</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>120</td>
<td>28.3</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2 reveals results of an independent-sample t-test that was conducted to compare the haemostatic profiles in T2DM male and female patients. The table reveals that the mean fibrinogen concentration for T2DM female patients (4.3±2.4 g/l) was significantly higher than male participants (3.4 ±2.0 g/l) t-value= 2.31; P-value= 0.017.

The mean Vonwillebrands factor concentration for T2DM female patients (4.6 ±2.3 IU/ml) was significantly higher than male participants (2.6 ±1.2 IU/ml) t-value=6.71; P-value = 0.001.
The mean Prothrombin Time for T2DM female patients (11.2± 2.8 seconds) was lower than male participants (11.9±3.3 seconds). The difference in the two means was not significant t-value=3.18; P = 0.172

Table 4.2 further shows that the mean APTT for T2DM female patients (28.3±3.5 seconds) was lower than male participants (32.0±3.0 seconds). The difference between the two means was significant t-value 6.72; P = 0.001.

4.2.1 Prevalence of hypercoagulability in T2DM patients and control participants.

Table 4.3: Comparison of hypercoagulability prevalence between T2DM patients and control participants and between male and female T2DM patients.

<table>
<thead>
<tr>
<th>Status</th>
<th>Total No.</th>
<th>N</th>
<th>%</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Participants</td>
<td>172</td>
<td>22</td>
<td>12.7</td>
<td>0.001</td>
</tr>
<tr>
<td>T2DM patients</td>
<td>213</td>
<td>126</td>
<td>59.2</td>
<td></td>
</tr>
</tbody>
</table>

VWF and fibrinogen plasma concentrations, which were continuous variable in SPSS, were recoded so as to categorise the results into two categories; those who had vWF and fibrinogen plasma concentration of greater than 2.6 IU/mL and 3.2 g/l respectively were categorized as hypercoagulable and those whose vWF and fibrinogen results were ≤ 2.6 IU/ml and 3.2 g/l respectively were regarded as normal. Thereafter a chi-square test of independence was done to determine the proportion of hypercoagulability in T2DM patients and control subjects. Table 4.3 reveals that T2DM patients had higher prevalence of hypercoagulability [126(59.2%)] than control participants [22(12.7%)]. The difference was significance P=0.001
4.3 Risk factors and patient attributes associated with a hypercoagulable state in T2DM patients

A chi-square test of independence was conducted to determine the differences in the proportion of T2DM patients who were hypercoagulable in relation to different demographic variables and other selected factors.

Table 4.4 Proportion of T2DM patients with hypercoagulability according to patient attributes

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total</th>
<th>N</th>
<th>%</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21-30</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>31-40</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>41-50</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>51-60</td>
<td>64</td>
<td>0</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>&gt;61</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>93</td>
<td>38</td>
<td>40.9</td>
<td>0.000</td>
</tr>
<tr>
<td>Female</td>
<td>120</td>
<td>88</td>
<td>73.3</td>
<td></td>
</tr>
<tr>
<td><strong>Glycaemic control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>100</td>
<td>34</td>
<td>34.0</td>
<td>0.003</td>
</tr>
<tr>
<td>Poor</td>
<td>113</td>
<td>92</td>
<td>81.4</td>
<td></td>
</tr>
<tr>
<td><strong>Duration of T2DM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 5 years</td>
<td>67</td>
<td>14</td>
<td>20.9</td>
<td>0.000</td>
</tr>
<tr>
<td>5 to 10 years</td>
<td>49</td>
<td>22</td>
<td>44.9</td>
<td></td>
</tr>
<tr>
<td>Greater than 10 years</td>
<td>97</td>
<td>90</td>
<td>92.8</td>
<td></td>
</tr>
<tr>
<td><strong>Body Mass Index</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>84</td>
<td>28</td>
<td>33.3</td>
<td>0.000</td>
</tr>
<tr>
<td>Overweight</td>
<td>67</td>
<td>42</td>
<td>62.7</td>
<td></td>
</tr>
<tr>
<td>Obese</td>
<td>62</td>
<td>56</td>
<td>90.3</td>
<td></td>
</tr>
<tr>
<td><strong>Knowledge on T2DM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adequate Knowledge</td>
<td>108</td>
<td>48</td>
<td>44.4</td>
<td>0.001</td>
</tr>
<tr>
<td>Inadequate knowledge</td>
<td>105</td>
<td>78</td>
<td>74.3</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.4 reveals that at the 5% level the proportions of subjects that were hypercoagulable differed significantly among the 21-30, 31-40, 41-50, 51-60 and > 60 age groups in T2DM Patients. $\chi^2 = 72.6$, $P=0.001$. The proportion of T2DM Patients who were hypercoagulable was higher in the age range of 51-60 years and above 60 years [60(93.8%)] and [18(94.7%)] respectively. This shows that there was a significant correlation between age and hypercoagulation.

Table 4.4 further reveals that at the 5% level the proportions of male T2DM patients who were in hypercoagulable state [38(40.9%)] was lower than in female T2DM patients [88(73.3%)]. This difference was significant. $\chi^2 = 20.5$, $P=0.000$.

The proportion of T2DM patients who were hypercoagulable was higher in those with poor glycaemic control [92(81.4%)] as compared to those with good glycaemic control [34(34.0%)]. The difference was significant $\chi^2 = 39.3$, $P=0.003$. Therefore glycaemic control was correlated with the prevalence of hypercoagulability in T2DM patients.

The results indicate that hypercoagulability in T2DM patients was dependent on the duration of the disease. Those who had the disease for over 10 years had a higher proportion of hypercoagulability [90(92.8%)] than those who had it in less than 5 years [22(44.9%)]. $\chi^2 = 90.4$, $P=0.000$. Hence there was a significant correlation between duration of T2DM and hypercoagulability (Table 4.4).

The proportion of T2DM patients who were hypercoagulable was lower in those who had adequate knowledge about the risk factors of T2DM [48(44.4%)] than in patients who had inadequate knowledge [78(74.3%)]. This difference was statistically significant. $\chi^2 = 19.6$, $P=0.000$. 

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The results obtained as shown in table 4.4 further indicates that hypercoagulability in T2DM patients was dependent on body mass index. The proportion of obese patients who were hypercoagulable was significantly higher \([56(90.3\%)]\) than those who had a normal body mass index \([28(33.3\%)]\). \(\chi^2 = 48.4, P=0.000\). Hence there was a significant correlation between body mass index of T2DM and hypercoagulability.
4.4 Determinants of hypercoagulability in T2DM patients using binary logistic regression.

Logistic regression was used to determine the risk factors associated with hypercoagulability in T2DM.

Table 4.5 Univariate and multivariate regression results: likelihood of hypercoagulability among T2DM patients based on patient attributes

<table>
<thead>
<tr>
<th>Factor</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% C.I</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male®</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td><strong>3.98</strong></td>
<td><strong>2.23-7.10</strong></td>
</tr>
<tr>
<td><strong>Age (Years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21-30®</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>31-40</td>
<td>1.00</td>
<td>0.15-2.62</td>
</tr>
<tr>
<td>41-50</td>
<td><strong>2.12</strong></td>
<td>0.58-4.23</td>
</tr>
<tr>
<td>51-60</td>
<td><strong>2.61</strong></td>
<td>1.20-4.80</td>
</tr>
<tr>
<td>≥61</td>
<td><strong>2.66</strong></td>
<td>1.05-4.87</td>
</tr>
<tr>
<td><strong>Glycaemic Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good®</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td><strong>7.50</strong></td>
<td>4.53-10.95</td>
</tr>
<tr>
<td><strong>Duration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5 years®</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>5-10 years</td>
<td><strong>3.08</strong></td>
<td>1.37-6.97</td>
</tr>
<tr>
<td>&gt;10 years</td>
<td><strong>8.67</strong></td>
<td>5.48-11.85</td>
</tr>
<tr>
<td><strong>Body Mass Index</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal®</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Overweight</td>
<td><strong>1.52</strong></td>
<td>1.06-4.32</td>
</tr>
<tr>
<td>Obese</td>
<td><strong>5.33</strong></td>
<td>2.26-11.48</td>
</tr>
<tr>
<td><strong>Knowledge about T2DM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adequate knowledge®</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>Inadequate Knowledge</td>
<td><strong>3.48</strong></td>
<td>1.96-6.18</td>
</tr>
</tbody>
</table>

OR: odds ratio; AOR: adjusted odds ratio; ®: Reference group
Table 4.5 reports the results of multivariate regression analysis revealing patient attributes that were also independent risk factors for hypercoagulability in T2DM patients. Sex was significantly associated with hypercoagulability. T2DM female patients were 3.98 (95% CI [2.23-7.10]) times more likely to be hypercoagulable than the male patients. Even after adjusting for confounders such as obesity, hypercholesterolemia and hypertension, the odds of female T2DM patients being hypercoagulable was still significant AOR being 4.42 (95% CI [2.77-10.63]).

In an unadjusted model, Age was significantly associated with hypercoagulability in T2DM patients. Participants aged 51-60 years and those aged 61 years and above were 2.61 (95% CI [1.20-4.80]) and 2.66 (95% CI [1.05-4.87]) more likely to be hypercoagulable than those in the age range of 21-30 years. Even after adjusting for cofounders participants whose age was between 51-60 years and above 61 years were more at risk of hypercoagulability than those in the age range of 21-30 years giving an AOR of 2.53 (95% CI [1.21-4.25]) and 1.45 (95% CI [1.19-3.16]) respectively.

In an unadjusted model, T2DM patients who had poor glycaemic control 53.1% (OR=7.50; 95% CI [4.53-10.95]) were more likely to be hypercoagulable than those who had good glycaemic control. The odds of being hypercoagulable were still high even after adjusting for age, hypercholesterolemia and obesity, giving an AOR of 6.12 (95% CI 2.27-8.36).

Duration of T2DM was significantly associated with hypercoagulability. Participants who had the disease between the duration of 5 – 10 years and those who have had the disease more than 10 years were OR=3.08 (95% CI 1.37-6.97) and OR= 8.67 (95% CI 5.48-11.85) times more likely to be hypercoagulable than those who had the disease for the duration of less than 5 years. The odds of being hypercoagulable was still high even after adjusting for
age, hypercholesterolemia and obesity, giving an AOR of 2.20(95% CI 1.07-4.44) and 5.28(CI 95% 3.01-8.21).

Body Mass Index was significantly associated with hypercoagulability in T2DM patients. Participants who were overweight were 1.52(95% CI 1.06-4.32) more likely to be hypercoagulable than those whose BMI was normal. The odds of being hypercoagulable in participants who were obese were 5.33(95% CI 2.26-11.48). After adjusting for Age and cholesterol levels, the odds of hypercoagulable in the obese group was 4.54(95% CI 2.88-10.59). In the overweight group the odds of hypercoagulability became 1.05(95% CI 0.75-2.86). The null value is 1, and because this confidence interval does include 1 in adjusted model, the result indicates a statistically insignificant difference in the odds of overweight and normal individuals in terms of hypercoagulability.

In univariate analysis T2DM patients who had inadequate knowledge on the risk factors, complications and management of T2DM were 3.48 more likely to be hypercoagulable than those with adequate knowledge OR 3.48(CI 95% 1.96-6.18). However after adjusting for glycaemic control, age, hypercholesterolemia and obesity, the odds of hypercoagulability was the same in those with adequate knowledge about the risk factors and complications and those who had inadequate knowledge, AOR being 1.00(CI 95% 0.80-2.20).
4.5 Suitability of PT-APTT as markers for hypercoagulability in T2DM patients

Figure 4.2 Correlation between vWF and Fibrinogen plasma concentration

Figure 4.2 shows that there was a statistically significant positive correlation between vWF plasma concentration and Fibrinogen concentration. This correlation was very strong.

[R=0.895; R²=0.8019, P=0.000].

Figure 4.3 Correlation between vWF plasma concentration and APTT
Figure 4.3 indicates that vWF was negatively correlated with APTT and the correlation was strong and statistically significant. \[ R = -0.783; R^2 = 0.6123; P = 0.001 \].

Figure 4.4 Correlation between vWF plasma concentration and Prothrombin time

![Figure 4.4](image)

\[ R^2 = 0.12, P = 0.002 \]

Figure 4.4 shows that there was a very weak negative correlation between vWF plasma concentration and prothrombin time. \[ R = -0.346; R^2 = 0.12; P = 0.002 \]

Table 4.6 Comparison of vWF/Fibrinogen results and PT test results in T2DM patients

<table>
<thead>
<tr>
<th>PT test</th>
<th>vWF/ Fibrinogen</th>
<th>Normal</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypercoagulable (vWF &gt; 2.6 IU/mL)</td>
<td>Normal (vWF ≤ 2.6 IU/mL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fibrinogen &gt; 3.2 g/l</td>
<td>Fibrinogen ≤ 3.2 g/l</td>
<td></td>
</tr>
<tr>
<td>Abnormal tests</td>
<td>70</td>
<td>8</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>TP</td>
<td>FP</td>
<td></td>
</tr>
<tr>
<td>Normal tests</td>
<td>56</td>
<td>79</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>FN</td>
<td>TN</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>126</td>
<td>87</td>
<td>213</td>
</tr>
</tbody>
</table>

TP: True Positive  TN: True Negative  FP: False Positive  FN: False Negative
Table 4.7 Comparison of vWF results and APTT test results in T2DM patients

<table>
<thead>
<tr>
<th>APTT test</th>
<th>vWF/ Fibrinogen</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypercoagulable</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Abnormal tests</td>
<td>(vWF &gt;2.6 IU/mL)</td>
<td>vWF ≤2.6 IU/mL</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen&gt;3.2g/l</td>
<td>118</td>
<td>4</td>
<td>122</td>
</tr>
<tr>
<td>Normal tests</td>
<td>8</td>
<td>83</td>
<td>91</td>
</tr>
<tr>
<td>Total</td>
<td>126</td>
<td>87</td>
<td>213</td>
</tr>
</tbody>
</table>

TP: True Positive  
TN: True Negative  
FP: False Positive  
FN: False Negative

Table 4.8 Quality evaluation of PT and APTT parameters

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT</td>
<td>55.6</td>
<td>90.8</td>
<td>89.7</td>
<td>58.5</td>
</tr>
<tr>
<td>APTT</td>
<td>93.7</td>
<td>95.4</td>
<td>96.7</td>
<td>91.2</td>
</tr>
</tbody>
</table>

Table 4.6 and 4.7 illustrates the crosstabulations of vWF and fibrinogen results in comparison with the PT and APTT test results. Table 4.6 reveals that 126(59.1%) of the participants were hypercoagulable. Out of these results, 70(32.9%) participants results were true positives, implying that both vWF/fibrinogen test and prothrombin time were abnormal. 8(3.7%) of the Prothrombin test results were false positives as the patients were not hypercoagulable because the vWF and fibrinogen results were below 2.6 IU/mL and 3.2g/l respectively. 56(26.3%) of the participants prothrombin results were false negatives, implying that the prothrombin test results were normal but the participants were in actual fact hypercoagulable. 79(37.1%) normal prothrombin test results were true negatives because both vWF and fibrinogen patients results were normal, implying that the patients were not hypercoagulable.
Table 4.7 reveals that 118(54.3%), 4(1.9%), 8(3.7%) and 83(39.0%) APTT test results were True Positives, False Positives, False negatives and True Negatives respectively.

Table 4.8 reveals that APTT tests were more sensitive 93.7% (95% CI [88.0-96.7]) than PT test which had a sensitivity of 55.6% (95% CI [46.8-63.9]). Both APTT and PT tests had better specificity; however APTT was higher 95.4% (95% CI [88.8-98.2]) than PT test 90.8% (95% CI [82.9-94.3]).

APTT test had a higher PPV 96.7% (95% CI [91.9-98.7]) than PT test 89.7% (95% CI 81.0 94.7]). PT test gave a much lower NPV 58.5% (95% CI [50.0-66.5]) as compared to APTT tests which had a higher NPV 91.2% (95% CI [83.6-94.5]).

This entails that APTT is a better predictor of hypercoagulable state than PT in T2DM patients. Furthermore, APTT is a better discriminator for non-hypercoagulable states in T2DM patients.
CHAPTER 5

5.0 DISCUSSION

5.1 Haemostatic profile of T2DM patients

Activated Partial thromboplastin time (APTT) in the diabetic subjects was significantly shorter than that of control participants while Prothrombin time (PT) of diabetic subjects was insignificantly shorter than that of non-diabetic controls. The results for Prothrombin Time accords that of Zhao et al., (2011) who found insignificant shortened PT in T2DM patients as compared to control subjects. The results are also consistent with Lippi et al., (2009), who found shortened PT and APTT in T2DM patients than in non-diabetes control subjects, but only the APTT results were significantly shorter. Boekel et al., (2002) reported that PT and APTT tests are standard screening tests for function of the coagulation system and their utility in monitoring therapeutic anticoagulation is widely accepted. Prolonged APTT values have clinical relevance as an indicator of factor deficiency or the presence of coagulation inhibitors. Shortened APTTs are generally considered to be laboratory artifacts arising from problematic venepunctures. However, there is mounting evidence that shortened APTT values in some cases may reflect a hypercoagulable state, which is potentially associated with increased thrombotic risk and adverse cardiovascular events (Ng et al., 2009 and Lippi et al., 2009). Shortened APTTs may result from an accumulation of circulating activated coagulation factors in plasma caused by enhanced coagulation activation in vivo (Ng et al., 2009 and Lippi et al., 2009).

Acang et al., (2005) observed that there were significantly shortened PT and APTT values, in diabetic patients, especially in patients with long-term diabetes with chronic complications, which are consistent with the results of this study. The insignificant PT results supports the hypothesis that there is less involvement of the extrinsic pathway in hypercoagulability state.
in diabetic conditions due to the fact that injury occurring to the vascular system in diabetic patients does not involve tissue factor from outside the vascular system (Soltani et al., 2011). Korte et al. (2008) reported that patients presenting with shortened APTT values had increased thrombin generation, were in a complex hypercoagulant state and were twice at increased risk for thromboembolism than those who had normal APTT values. Tripodi et al. (2004) found that hypercoagulability detected by shortened APTT values was independently associated with venous thromboembolism (VTE) and hypothesized that shortened APTT could be considered as a risk marker for VTE. Shortened APTT in T2DM as reported in this study could be a risk factor for hypercoagulability in T2DM patients in accordance with various studies that have demonstrated shortened APTT to be associated with an increased risk of thrombosis and hence hypercoagulability (Abdullah et al., 2010, Legnani et al., 2006 and Landi et al., 2003).

The mean fibrinogen concentration of persons with diabetes in this study was significantly higher than the concentration in the controls (P < 0.05). This higher fibrinogen concentration found in the diabetic group agrees with Mark’s report (2001) of elevated fibrinogen concentration as one of the risk factors for atherosclerosis among diabetics. The findings also accords that of Zhao et al., (2011) who reported increased Fibrinogen concentrations among Chinese T2DM patients. Fibrinogen may induce thrombus formation by affecting platelets and erythrocytes to aggregation and by promoting increased blood viscosity (Merril et al., 2001). Kannel et al., (2005) reported that fibrinogen is often elevated in T2DM patients and this elevation is associated with poor glycaemic control. The increase in fibrinogen levels in T2DM patients may be due to chronic fibrinogen hyper secretion. Increased fibrinogen levels in diabetes may be related to the associated low-grade inflammation. Interleukin (IL)-6 levels are elevated in diabetes and this cytokine is able to stimulate hepatocytes to produce fibrinogen, representing an important link between inflammation and hypercoagulation. In an
hyperglycemic environment, fibrinogen can become hyperglycosylated (Zhao et al., 2011). When this abnormal fibrinogen clots, the resulting fibrin structure is composed of small diameter fibers that are markedly resistant to degradation by plasmin hence increase in hypercoagulability (Zhao et al., 2011). A definite association exists between fibrinogen and atherothrombogenesis. However, the nature of the link is unclear. Although epidemiological and clinical studies suggest that the link is causal, no definite evidence exists. Kannel et al., (2005) and Meade TW, (1986) reported that elevated plasma fibrinogen levels are associated with an increased risk of cardiovascular disorders, including ischaemic heart disease (IHD), stroke and other thromboembolism. Machlus et al (2011) demonstrated that high fibrinogen leads to increased formation of thrombi that are more resistant to proteolytic degradation and hence causes sustained thrombosis. Therefore the increase in fibrinogen as reported in this study supports the hypothesis that T2DM patients are at risk of thrombosis.

In the current study, the mean von willebrands factor concentration was higher in T2DM patients than the control participants. This accords the results obtained by Mohamed et al., (2005), Alzahrani et al., (2010) and Soltani et al., (2011) who found increased vWF levels in T2DM patients than control participants. vWF is also known as a marker for endothelium damage and in turn is increased in T2DM patients (Frankel et al., 2008; Bonnetti et al., 2003 Meigs et al., 2006). vWF is produced by endothelial cells and megakaryocytes throughout the body (Blann et al., 2009). Levels of circulating vWF are increased following endothelial cell damage. Several mechanisms contribute to the endothelial dysfunctional phenotype in T2DM patients including altered glucose metabolism, impaired insulin signaling, low-grade inflammatory state, and increased reactive oxidant species (ROS) generation (Fig. 2.1). Enhanced oxidative stress in the hyperglycaemic milieu accelerates the glycoxidation of proteins and lipids to generate advanced glycation end products (AGEs) (Santili et al., 2009). AGEs accumulate in the vessel wall, where they may directly disturb cell structure and
function and hence exposure of the vWF which is always found in the sub endothelium layer. Wahlberg et al., (2000) and Nilsson et al., (2009) reported that raised vWF is associated with increased thromboembolic events, for example, in patients with deep venous thrombosis. Nossent and his colleagues (2006) demonstrated also vWF secretion to be a biomarker of thrombosis risk. They reported a dose–response relationship between vWF and thrombosis risk, with vWF being significantly higher in thrombotic subjects than in controls. Therefore increased vWF as reported in this study further demonstrates that T2DM patients are at risk of thrombosis.

The mean Fibrinogen and Vonwillebrands factor were were significantly increased in female T2DM patients than in male patients. The mean APTT in female T2DM patients was significantly lower than in male patients. Though the mean Prothrombin Time was higher in male than female T2DM patients the difference was not significant indicating that the difference in hypercoagulability between male and female T2DM patients may be arising from the intrinsic pathway of coagulation as opposed to the extrinsic pathway. The haemostatic profiles obtained for PT and APTT in females correlates very well with the results obtained by Ukaejiofo et al., (2007) among the Nigerian T2DM patients, in which both PT and APTT were prolonged in male patients as compared to female T2DM patients. In the current study, female T2DM patients had significantly higher fibrinogen and vWF levels than the male patients indicating that female T2DM patients are more hypercoagulable than the male patients. Infact in bivariate logic regression it was found that women were four times at risk of hypercoagulability than the male patients (Fig 4.7). Similar results were reported by Soedama et al., (2008), who found significantly higher fibrinogen levels in female T2DM patients than male patients. Bruno et al., (2000) also reported higher fibrinogen levels in female T2DM patients than male patients. This finding supports the hypothesis
which states that females are more hypercoagulable than males. In a study conducted to determine the course of coagulation after injury and to determine whether there is a gender difference, Screiber et al., (2005) reported that Women are more hypercoagulable than men early after injury. The results accords that of Michael W et al., (2002), who found significantly higher FVII: C, Vonwillebrands and PAI-1 levels in women than in men with T2DM. The finding of higher PAI-1 and vWF levels in diabetic women therefore may indicate an important sex-specific interference in the haemostatic system by diabetes that could increase vascular risk (Michael W et al., 2002). A mechanism independent of insulin resistance by which female sex could be associated with higher vWF, FVIII: C and PAI-1 levels in T2DM is not clear. Differences caused by sex hormone levels do not explain the findings. In women higher estrogen levels are associated with lower PAI-1 levels while in healthy postmenopausal women not receiving estrogen replacement, PAI-1 levels are no higher than in men of the same age (Gebara et al., 2008). The study results are in conflict with the known epidemiology that men have more cardiovascular events than premenopausal women due to the estrogen, which regulates menstruation, protects women against cardiovascular disease by increasing the high-density lipoprotein (HDL), which prevents blockages in the arteries by carrying cholesterol away from the arteries and out of the body. Therefore Premenopausal women have much higher levels of HDL cholesterol than men of the same age because of an increase in estrogen levels (Pilote L et al., 2007). However research findings have found T2DM women to have less HDL levels than diabetic men hence the reason why T2DM female patients are more hypercoagulable than male patients (Pilote L et al., 2007). The reason for this is unclear and it requires further research to be done.

In the present study no assessment of sex hormone levels was done. Investigation of the relationship among female hormonal status, haemostatic variables, and the features of insulin resistance in premenopausal and postmenopausal T2DM women may help in the evaluation
of the importance of estrogens and vWF and PAI-1 in thrombotic development in women with T2DM.

5.2 Prevalence of hypercoagulability among T2DM patients.

In the current study participants who had vWF and fibrinogen results greater than 2.6 IU/mL and 3.2 g/l respectively were categorized as hypercoagulable. Those who had vWF and fibrinogen concentration of less than or equal to 2.6 IU/mL and 3.2 g/l respectively were regarded as not hypercoagulable. The overall prevalence of hypercoagulability among T2DM patients was 59.2% while that of control subjects was 12.7%. This finding accords that of Pandolfi et al., (2007) who found a prevalence of 30%. This shows that the prevalence of hypercoagulability among T2DM patients in Zambia is very high as compared to the general population. As illustrated in table 4.4 most diabetic patients had inadequate knowledge about the risk factors, complications and management of T2DM. This may lead to poor glycaemic control among these patients and consequently damage to the endothelium may occur thereby activating the coagulation system leading to hypercoagulable state.

5.3 Risk factors and patient attributes associated with a hypercoagulable state in T2DM patients.

5.3.1 Age and hypercoagulability

The research revealed that patients aged 51 years and above were at risk of hypercoagulability than those who were below 51 years old. These results are consistent with the findings of past studies. Bruno et al., (2000) and Soltani et al., (2011) reported a significant correlation between age and hypercoagulability in T2DM patients. The risk of hypercoagulability with increasing age could be attributed to the changes that occur to the vascular system as a result of aging thus tilting the scale to hypercoagulability in older patients.
5.3.2 Glycaemic control and hypercoagulability

This study reveals that type 2 diabetic patients who had poor glycaemic control were at risk of hypercoagulability than those with good glycaemic control. The results are consistent with many past studies. Chantal et al., (2010) found significant association between glycaemic control and hypercoagulability. The results in this study also accords that of Osende et al., (2008), who reported a correlation between improved glycaemic control and blood thrombogenicity. Poor glycaemic control may lead to hypercoagulability because of the effects of glucose on the endothelium. Long term high glucose levels damage the endothelium by accelerating glycosylation of proteins and lipids to generate advanced glycation end products (AGEs) (Santilli et al., 2009). AGEs accumulate in the vessel wall, where they may directly disturb cell structure and function. Furthermore, the receptor for AGEs (RAGE) activation on endothelial cells inhibits nitric oxide (NO) biosynthesis by endothelial NO synthase (eNOS) down regulation, with increased generation of ROS. ROS as a negative effect on NO by forming the highly oxidant peroxynitrite ion, which in turn uncouples eNOS to produce superoxide anion and asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS. NO is important in haemostasis because it inhibits platelet aggregation therefore a reduction of NO may lead to unregulated platelet aggregation (Devangelio et al., 2007).

5.3.3 Duration of T2DM and hypercoagulability

In this study a good statistically significant correlation was found between the prevalence of hypercoagulability and the duration of diabetes that was consistent with findings of other studies. Participants who had the disease for a period of 10 years and above were more likely to be hypercoagulative than those who had it for duration of less than 5 years. Infact the odds of being hypercoagulable in T2DM patients increased with the increase in the duration of diabetes. Chantal et al., (2010) and Barbic et al., (2010) reported a significant correlation
between hypercoagulability and the duration of diabetes. The reason for this correlation may be due to the effects of hyperglyceamia on the endothelium over a prolonged period of time. Damage to the endothelium tend to increase with the increase in the duration of T2DM. Prolonged hyperglycaemia accelerates the glyoxidation of proteins and lipids to generate advanced glycation end products (AGEs) (Santilli et al., 2009). AGEs accumulate in the vessel wall, where they may directly disturb cell structure and function by downregulating the production of Nitric oxide (NO), which plays a role in haemostasis by inhibiting platelet aggregation (Devangelio et al., 2007).

5.3.4 Knowledge about T2DM risk factors, complications and management as a function of hypercoagulability.

In chi-square test of independence the proportion of individuals who were hypercoagulable was significantly higher in individuals who had inadequate knowledge about the risk factors and complications of T2DM. In the univariate analysis the odds of hypercoagulability in participants who had inadequate knowledge about T2DM risk factors and complications was higher than in those who had adequate knowledge. In the multivariate analysis after adjusting for glycaemic control the adjusted odds ratio was 1.00 indicating that the risk of hypercoagulability in those who had adequate knowledge and inadequate knowledge about the risk factors and complications of T2DM was the same. This is inconsistent with the results obtained by Blankenfeld et al., (2006), who reported that the existence of diabetes related complications was a significant predictor of poor knowledge in T2DM patients. However in this study Blankenfeld and colleagues did not adjust for glycaemic control. This may be the reason why there was such a discrepancy. Blankenfeld et al., (2006) further reported that patients who have inadequate knowledge about the risk factors and complications of the disease are more likely not to adhere to treatment or may be inconsistent in going to the Hospital for review so as to have the glucose levels checked. The results are
also in contrast with Ulvi et al., (2009) who reported a positive correlation between hypercoagulability and levels of knowledge about the risk factors and complications of T2DM patients among the rural community in Pakistan. T2DM patients with inadequate knowledge about the risk factors, complications and management of diabetes are less likely to understand why glucose levels should be monitored and hence usually tend to have poor glycaemic control and consequently increased hypercoagulability. Patients with adequate knowledge on the other hand may be very careful with their lifestyles because they know the complications that may occur if they don’t adhere to proper treatment and risk free life style and hence able to have better glycaemic control.

5.3.5 Body Mass Index and hypercoagulability

Obesity was also significantly associated with hypercoagulability. T2DM patients who were obese were likely to be hypercoagulable. These results are consistent with most of the past studies. Muthu et al., (2009), Soltani et al., (2011) and Kozek et al., (2004) found increased hypercoagulability with increased levels of cholesterol. Obesity was first proposed to be a risk factor for the development of atherosclerosis and T2DM over 40 years ago (Kozek et al., 2004). Metabolic alterations accompanying the visceral distribution of fat lead to arterial hypertension, dyslipidemia, insulin resistance and subsequently to T2DM. This phenomenon is associated not only with classical atherosclerotic risk factors but also with coagulation and fibrinolysis abnormalities (Sakkinen et al., 2009). Hypercoagulation in obesity is thought to be caused primarily by the synthesis of factors activating coagulation and inhibiting fibrinolysis (for example factor VII activator and the fibrinolytic inhibitor PAI-1) in adipose tissue (Kozek et al., 2004). Hemostatic abnormalities may also result from the synthesis in adipose tissue of cytokines that are mediators of inflammation and insulin resistance, such as interleukin 6 and TNF-alpha. In addition to this direct effect, the metabolic and lipid alterations that accompany obesity and T2DM are likely to indirectly influence coagulation.
properties in these patients. Hypercholesterolemia, obesity and hypertension are interrelated. Both obesity and hypercholesterolemia have been implicated in the development of T2DM and both are also associated with hypercoagulation. Studies have shown that raised plasma insulin levels with insulin resistance appears to be an atherogenic factor. Insulin stimulates cholesterol synthesis in smooth muscle cells and macrophages of the arterial walls and also stimulates the proliferation and migration of smooth muscle cells. Prospective data is needed to clarify whether these factors preceded T2DM or they are a consequence of the disease (Fein Et al., 2002). Diabetes and high blood pressure are closely related diseases.

5.4 Suitability of Prothrombin (PT) and Activated Partial Thromboplastin Time (APTT) as markers for hypercoagulability state in T2DM patients.

The results in this study show that APTT test was more sensitive than PT test in detecting hypercoagulability in T2DM as the test had less false negative results than PT test and therefore APTT tests have higher probability of detecting hypercoagulable states in T2DM than PT test. Both APTT and PT tests had acceptable specificity. However APTT had a higher specificity than PT test. This implies that APTT had less false positive results than PT test results and hence able to discern properly between T2DM patient who were at risk of hypercoagulability and those not at risk.

APTT test had a higher PPV than PT test. This can be interpreted to mean that the proportion of participants who had increased vWF and Fibrinogen and thus hypercoagulable and had positive test results was higher in APTT than PT test.

APTT test results had a high NPV as compared to PT which had a very low NPV. This means that APTT had a higher proportion of T2DM patients whose test results were negative and had normal levels of vWF and fibrinogen and hence not hypercoagulable than PT test results. From the literature searched so far, no diagnostic study has been done to specifically evaluate suitability of PT and APTT in detection of hypercoagulable states among T2DM patients.
Loog T.W (2002) reported that the acceptable sensitivity, PPV and NPV should be above 90%. From the results obtained in this study APTT was found to be a suitable screening test for hypercoagulability in T2DM patients because all the parameters were above 90%. Though PT had acceptable specificity, the test is not suitable for use as a screening test for hypercoagulability in T2DM patients because it had very low sensitivity and negative predictive values. This entails that APTT is a better predictor of hypercoagulable state than PT in T2DM patients. Furthermore, APTT is a better discriminator for non-hypercoagulable states in T2DM patients.

Positive and negative predictive values vary according to the prevalence of the condition under study Loong (2002). Therefore it would be wrong for predictive values determined for one population to be applied to another population with a different prevalence. In this case APTT tests could only be used among T2DM patients and not the general population because hypercoagulability may be very low in the population and hence low predictive values even if the test is highly sensitive and specific. The better sensitivity, specificity NPV and PPV obtained for APTT test results in determining hypercoagulability in T2DM patients can further be supported in this study by a very strong correlation obtained between vWF results and APTT as compared to PT, which gave a very low negative correlation with vWF plasma concentration.
5.5 Study limitations

This study was a cross-sectional analytical study and was therefore unable to determine whether hypercoagulability preceded the development of T2DM or it’s a consequence of diabetes. Therefore a prospective study such as cohort study need to be conducted so as to determine the time sequence between exposure and development of hypercoagulability in T2DM patients. As hypercoagulability is often present at the time of diagnosis of T2DM, such a study might have to begin in younger, nondiabetic subjects identified as having a high subsequent risk of developing T2DM. Further more in Zambia people do not go for routine check-up and many are only diagnosed when they present with complications therefore for many of the participants the actual duration of diabetes may not be known.

The other limitation is that the study was not carried out countrywide and therefore the results cannot be generalized for the whole country.
CHAPTER 6

6.0 CONCLUSION

The main aim of this study was to determine the haemostatic profiles of T2DM patients. It was observed that T2DM patients had increased levels of vonwillebrands factor and fibrinogen concentration than healthy non-diabetic control participants. T2DM patients had shorter APTT-PT and higher fibrinogen and vWF than the healthy non-diabetic control participants contributing to increased prevalence of hypercoagulability in diabetic patients than control participants. Therefore we conclude that T2DM patients are more hypercoagulable than non-diabetic healthy individuals. It was also found that hypercoagulability was more prevalence in female T2DM than male patients.

Age, sex, duration of T2DM, BMI, and glycaemic control were found to be risk factors for hypercoagulability in T2DM patients. There was good correlation between vWF and APTT and APTT had acceptable sensitivity, specificity, PPV and NPV. Hence we conclude that APTT can be used as a marker for hypercoagulability in T2DM.
CHAPTER 7

7.0 Recommendations

1) A cohort study need to be conducted on T2DM patients who participated in this study so as to follow them up and determine those who will develop thrombosis later on in life. This is only when APTT may be introduced as a probable marker for hypercoagulability.

2) Diabetes mellitus patients need to be educated about the risk factors, complications and management of T2DM so as to improve on glycaemic control and prevent complications.

3) The use of aspirin as preventative measure of thrombosis in T2DM should be intensified and vWF antagonists may be added as potential medication so as to prevent cardiovascular complications in these patients.
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Central Statistical Office (CSO), Ministry of Health (MOH), Tropical Diseases Research Centre (TDRC) and University of Zambia. (2007) Zambia Demographic and Health Survey.


APPENDIX 1

INFORMATION SHEET (ENGLISH)

HAEMOSTATIC PROFILE OF T2DM PATIENTS AT NDOLA CENTRAL HOSPITAL-NDOLA, ZAMBIA.

INTRODUCTION

I, Mwambungu Alick, a student of Master of Science degree in Haematology at the University of Zambia is requesting for your participation in the research study mentioned above. Before you decide whether or not to participate in the study, I would like to explain to you the purpose of the study, any risk or benefits and what is expected of you. Your participation in this study is entirely voluntary. You are under no obligation to participate. If you decide that you do not participate, no privileges will be taken away from you. If you agree that you participate, you will be asked to sign this consent in the presence of the study staff.

PURPOSE OF THE STUDY

The purpose of the study is to assess the risk of developing clots in T2DM patients and to evaluate whether this risk is the same for both male and female patients. The study will also evaluate whether Prothrombin and Activated partial Thromboplastin Time could be used as markers for hypercoagulable state. The study will further try to establish risk factors and patient attributes associated with increased risk of thrombosis in T2DM patients.

The information obtained in this study will help the health workers to determine T2DM patients at risk of developing clots so as to institute early treatment and also to reduce the risk factors associated with thrombosis development in patients with T2DM. The study results will also be used as a basis for introducing PT and APTT as cost effective laboratory tests in the management of T2DM patients.
PROCEDURES

Your height and weight will be measured. Blood samples will also be collected from you by a qualified health worker. A total of four (4) blood containers will be used for all the laboratory tests that will be done. Three (3) ml of venous blood will directly be delivered into each of the containers. Therefore in total twelve (12) ml of blood will be collected from you. A member of the research team will ask you a few questions and your responses will be recorded on the questionnaire and the interview is expected to last about 10 minutes.

RISKS AND DISCOMFORTS

No risks are anticipated in this particular study though you will experience slight pain when blood is collected from you. Should there be any injuries arising from the study, the researcher will take full responsibility and will cover all the expenses pertaining to treatment.

BENEFITS

There are no immediate benefits to you for participating in this study. However, the information which will be obtained will help the policy makers in the Ministry of Health take measures in reducing macro and micro vascular complications in patients with T2DM. No gift or money will be given in exchange for the information obtained.

CONFIDENTIALITY

Privacy and confidentiality will be maintained. Your name will not appear anywhere on the forms but will instead be identified by numbers. You will thus be assured that the information you will provide will not be used against you in any way. Personal information will not be released without your permission except when required by law.

The MOH and the UNZA Research Ethics Committee will review your records again but this will be done with confidentiality.
APPENDIX 2

INFORMATION SHEET (BEMBA TRANSLATION)

HEAMOSTATIC PROFILE OF T2DM PATIENTS AT NDOLA CENTRAL HOSPITAL.

IFYANTANSHI

Ine Mwambungu Alick, umusambi wa Science mu Haematology pe sukulu likalamba ilya UNIVERSITY OF ZAMBIA. Ndelomba ukuti mwingaibimbamo mukufwaikisha ukulosha ku mutwe. Ubuchindami bwaubu ubusambilisho kumona abalwele ba bulwele bwa shuga pa cipatala ca Ndola Central Hospital. Ilyo ta mula pingulapo ukwankulako muli ukukusambilisha kwa uyu ndefwaya ukumilondolweleko imifwaile muli uku ukusambilila, ubufwayo ubuli bonse nelyo usumuma ne cilekabilwa muli imwe.

Uku ibimbamo muli uku ukusambilila kuipelesha kwenu tamule patikishiwa ukuibimbamo. Lelo nga mwapingulapo uku kana ibimbamo cili fye bwino. Nga mwapingulapo ukuibimbamo mukepushiwa ukusaina ukuti namusumina ukuibimbamo pantashi ya muntu,uli nga kamboni.

UBUFWAYO BWA KU FWAILIKISHA

ubu busambililo bu kalenga ukwishisha abalwele ababili na Diabetes Mellitus type 2. Ubulondoloshi bu ka sendwa bukafwa aba bomfi mu cipatala ukumona abalwele ba T2DM abali pa bwafya bwakukwata ubulwele bwa ku kosa umulopa, pakuti ukundwapwa kwingatendenka mu kwangufyanya.

INSHILA YA KUBOMBELAMO

Ukubutuka kwa mulopa, ukulepa nelyo ukufina fikapimwa. Umulopa ukasendwa muli imwe kumubonfi wa mu cipatala uwa fikapo muli uyu umulimo. Umubomfi wafya kufwailikisha
akemupusha amepusho aya nono lelo amasuko yenu yakalembwa muli Questionnaire nefi fili no kuposa insa amkumi yatatu.

IFYAKWENEKELA
Tapali ubusanso ubuli bonse ubuli no kufumamo muli uku ukufwailikisha nangu cakuti mu konfwako ubukali panono ilintu bale mifunya umulopa.

UBUSUMA BULIMO
Tekuti mononkelemofye lilyaline fye mwaibimbamo lelo ifyebo fikapokwa fikafwa aba panga amafunde kuciputulwa ca bumi, ukupanga ifipimo mukucefya ifintu ifinono ne fikulu ifipanga ubwafya mumi shipa sha balwele ba Diabetes Mellitus type 2. Takwakabe ubupe nelyo indalama isha ka pelwa pa kukabushana kwa fyebo.

IFYANKAMA
Ukwishibika kwa fyebo ne nabekeshiba ta fyakeshibikwe. Ishina lyenu talyakalembwe apali ponse. Muci fulo caico mukeshibikwa kunambala.

Na mulifyo mukacetekela ukuti ifyebo mukapela tafyakeshibikwe ukwabula ukusiminisha kwenu kano fye abama funde bakabila ico. Iciputulwa ca bumi, isukulu likalamba ilya mu Zambia (UNZA) iciputulwa ici fwailikisha nangula isukulu ilya bumi kutibapita mufyebo fyenu nakabili lelo ici cikacitwa mu nkama.
APPENDIX 3

CONSENT FORM

The purpose of this study has been explained to me and I understand the purpose, benefits, risks and discomforts, and confidentiality of the study.

I further understand that: if I agree to take part in this study, I have the right to withdraw at any time without having to give an explanation and that taking part in this study is purely voluntary.

I__________________________________________ (names)

agree to take part in this study.

Signed___________________     Date___________________ (participant)

Patient’s signature or thumb print.

Signed: _________________________ Date____________________________ (Witness)

Signed: _________________________ Date ______________________________ (Researcher)
PERSONS TO CONTACT FOR PROBLEMS OR QUERIES

1. Mwambungu Alick
   UNZA, School of Medicine
   P.O Box 50110
   Lusaka
   Cell: 0966967326

2. The Chairperson
   UNZA Biomedical Research Ethics Committee
   P.O Box 50110
   Lusaka
   Phone: 260-211-250753
APPENDIX 4

IPEPALA LYA KUSUMINA (BEMBA TRANSLATION)

Imifwaile ya kusambilila nailondololwa kuli ine kabili ningufwikisha imifwaile, ubusuma, ifibimbilwemo ne nkama shilimo.

Kabili ninjishiba ukuti: ngacakuti na sumina ukusendako ulubali ninshi ni kwata isambu shakuleka panshita iyili yonse ukwabula ukupela ubulondoloshi nakabili ukusendako ulubali muli uku ukusambilila ukwakuitemenwa.

Ine __________________________________________________________ (ishina)

Nasumina ukusendako ululubali muli uku kusambilila

Ukusaina______________ ubushiku _________________________ (uibimbilemo)

Ukusaina kwa mulwele nagula icikumo

Ukusaina______________ ubushiku _________________________ (kamboni)

Ukusaina______________ ubushiku _________________________ (kafwailikisha)
### APPENDIX 5: HAEMOSTATIC PROFILE OF T2DM PATIENTS AT NDOLA CENTRAL HOSPITAL QUESTIONNAIRE

#### PART 1. DEMOGRAPHIC INFORMATION

<table>
<thead>
<tr>
<th>Participant’s code……….</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

1. Place of residence       High density (1)  
    Low density (2)  

2. Age of Participant        [ ]

3. Gender: 1. Male 2. Female [ ]

4. Level of education        None (0)  
    Primary school (1)  
    Secondary School (2) [ ]  
    College/University (3)  
    Post Graduate (4)

5. Employment status         Employed (1) [ ]  
                               Unemployed (2)

**Interviewer’s Identification**

Data collected by: (State full names) ……………………………………….  
Date ………………………  
Day Month Year
PART: 2 PHYSICAL MEASUREMENTS

6. Height (Meters) [   ]

7. Weight (Kilograms) [   ]

8. Blood Pressure (Systolic) [   ]
   (Diastolic) [   ]

9. Are you on any medication for High blood Pressure?  No (0)
   Yes (1)

Interviewer’s Identification

Data collected by: (State full names) ..................................................
Date .........................
   Day    Month    Year
PART 3. PARTICIPANTS AWARENESS LEVEL ABOUT T2DM COMPLICATIONS, RISK FACTORS AND MANAGEMENT.

10. What are the risk factors, complications and management of T2DM?

i) Risk factors

a. Weight
b. Inactivity
c. Family history
d. Race
e. Age
f. Hypertensive

ii) Management

a. Exercise
b. Diet
c. Drugs—Metformin
iii) Complications

a. Infections
b. Poor wound healing $\leq 2$ Correct answers in (i) and (iii) and $< 2$ in (ii) (0)
c. Gangrene

d. Skin ulceration

e. Eye disease $\geq 3$ Correct answers in (i), (iii) and $> 2$ in (ii) (1)
f. Erectile dysfunction
g. Nerve damage
h. Heart disease
i. Thrombosis

Interviewer's Identification

Data collected by: (State full names) ......................................................
Date ..........................

Day  Month  Year
PART 4: MISCELLANEOUS

11. For how long have you been known to be diabetic?
   < 5 years (1)
   5-10 years (2) [ ]
   > 10 years (3)

12. Any comments or questions you will like to say or ask me about my interview with you today?
   ……………………………………………………………………………………………………………………………
   ……………………………………………………………………………………………………………………………
   (Interviewer please thank respondent for the interviews and time).

   Interviewer’s Identification

Data collected by: (State full names) …………………………………
Date ……………………..
   Day    Month    Year
# APPENDIX 6: LABORATORY DATA COLLECTION FORMS

## Clinical Chemistry Laboratory Form

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 3.3 mmol/l</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.9–7.8 mmol/l</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 11.0 mmol/l</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Random Blood Glucose (mmol/l):

2. Random Cholesterol (mmol/l):

Participant’s code……

Name of Technologist/Scientist………………………………………

Signature……………………………………………………………

Counter checked by…………………………………………………

Designation…………………………………………………………

Date………………………………………………………………

91
1. Fibrinogen [ ] g/l
2. Prothrombin Time (PT) [ ] Seconds
3. Activated Partial Thromboplastin Time (APTT) [ ] Seconds
4. Von Willebrands level [ ] IU/ml
5. Glycated haemoglobin [ ] %

Name of Technologist/ Scientist……………………………………
Signature…………………………………………………………
Counter checked by………………………………………………
Designation……………………………………………………
Date……………………………………………………………...
APPENDIX 7: AUTHORIZATION LETTERS

THE UNIVERSITY OF ZAMBIA
SCHOOL OF MEDICINE

Telephone: 230611
Telegram: UNZA, Lusaka
Telex: UNZALL 22 4439
Email: solestinnzala@yahoo.com

-------------------------------------------------------------------------------------------------------------------------------------

05th September, 2012

Mr. Alick Mwambungu
Department of Pathology and Microbiology
School of Medicine
LUSAKA

Dear Mr Mwambungu,

RE: GRADUATES PROPOSAL PRESENTATION FORUM (GPPF)

Having assessed your dissertation entitled "Hemostatic Profile of Type 2 Diabetes Mellitus Patients at Ndola Central Hospital". We are satisfied that all the corrections to your research proposal have been done. The proposal meets the standard as laid down by the Board of Graduate Studies.

You can proceed and present to the Research Ethics.

Yours faithfully,

Dr. S. H. Nzala
ASSISTANT DEAN, POSTGRADUATE

CC: HOD – Pathology and Microbiology
16th November, 2012.

Your Ref: 006-09-12.

Mr. Alick Mwambungu,
School of Medicine,
Department of Microbiology & Pathology,
PO Box 50110,
Lusaka.

Dear Mr. Mwambungu,

RE: RE-SUBMITTED RESEARCH PROPOSAL: "HAEMOSTATIC PROFILE OF TYPE II DIABETES MELLITUS PATIENTS AT NDOLA CENTRAL HOSPITAL - NDOLA, ZAMBIA"

The above mentioned research proposal was re-submitted to the Biomedical Research Ethics Committee with recommended changes on 13th November, 2012. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- Ensure that a final copy of the result is submitted to this Committee.

Yours sincerely,

Dr. J.C. Muntali
CHAIRPERSON

Date of approval: 16 November, 2012  Date of expiry: 15 November, 2013
19th December, 2012

Mr Alick Mwambungu,
School of Medicine,
Department of Microbiology and Pathology,
University of Zambia,
P.O.Box 50110, Ridgeway
Lusaka.

Dear Mr Mwambungu,

Re: Request for Authority to Conduct Research

The Ministry of Health is in receipt of your request for authority to conduct Research on “Haemostatic Profile of Type II Diabetes Mellitus Patients at Ndola Central Hospital – Ndola, Zambia”. I wish to inform you that following submission of your research proposal to my Ministry, our review of the same and in view of the ethical clearance, my Ministry has granted you authority to carry out the study on condition that:

1. The relevant Provincial and District Directors of Health where the study is being conducted are fully appraised;
2. Progress updates are provided to MoH quarterly from the date of commencement of the study;
3. The final study report is cleared by the MoH before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the MoH, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Dr. P. Mwadi
Permanent Secretary
MINISTRY OF HEALTH

Cc: District Medical Officer
3rd January 2013

Mr. Alick Mwambungu
Biomedical College
Postal Agency
NDOLA

Dear Mr. Mwambungu,

RE: RESEARCH ON HAEMOSTATIC PROFILE OF TYPE 2 DIABETES MELLITUS PATIENTS.

Reference is made to your letter regarding the above mentioned subject matter.

I am pleased to inform you that Management has no objection for you to come and do your research on “Haemostatic Profile of Type Two Diabetes Mellitus Patients” at our institution.

While at this institution, you will not be entitled to any salary or accommodation. You will also be expected to abide by Ndola Central Hospital Disciplinary Code and Regulations.

Kindly report to the Human Resources Department as soon as you arrive at the hospital.

Yours faithfully

NDOLA CENTRAL HOSPITAL

CAROLINE BUTALE
A/SENIOR HUMAN RESOURCES MANAGEMENT OFFICER
For/ SENIOR MEDICAL SUPERINTENDENT

CC: Senior Medical Superintendent
    Human Resources – Training File
29th October 2012

University of Zambia- School of Medicine
Department of Microbiology and Pathology
P.O. BOX 50110
LUSAKA

Dear Mr Mwambungu,

RE: REQUEST TO USE HAEMATOLOGY AND CLINICAL CHEMISTRY LABS FOR ANALYSIS OF RESEARCH SAMPLES-MR. ALICK MWAMBUNGU

This serves to inform you that your request to use Haematology and Clinical Chemistry laboratories to analyse samples for your research project “Haemostatic Profile of Type 2 Diabetes Mellitus Patients at Ndola Central Hospital” has been granted.

Please be reminded that this permission is only for use of College equipment and not reagents. Therefore reagents for various tests will have to be sourced by you.

Yours faithfully,

NDOLA COLLEGE OF BIOMEDICAL SCIENCES

M.M. NGULUTA
HEAD-BIOMEDICAL COLLEGE
APPENDIX 8: SUPERVISOR’S CURRICULUM VITAE

Curriculum vitae

PERSONAL DETAILS

Name: Lidiya Korolova
Date of Birth: 9th December, 1953
Place of Birth: Kyiv, Ukraine
Nationality: Russian
Marital Status: Married
Date of Appointment in University: 4th November, 2000
Present Position: Lecturer I

ACADEMIC QUALIFICATIONS

<table>
<thead>
<tr>
<th>Year</th>
<th>Institution</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1971-1977</td>
<td>Kyiv State University</td>
<td>Master of Science Chemistry, Biology and Genetics</td>
</tr>
<tr>
<td>1985 - 1989</td>
<td>Scientific Research Institute of Haematology and Blood Transfusion, Kyiv.</td>
<td>PhD in Biology and Haematology</td>
</tr>
</tbody>
</table>

WORK EXPERIENCE

2000 to date - Lecturer I in Department of Pathology and Microbiology
1985 – 1989 - Award Doctorate in Biology and Haematology
1980 – 1993 - Worked as consultant on determining blood related diseases. In conjunction with other research workers.
carried out a number of haematological and immunological research on patients exposed to radiation during the Chernobyl Atomic Power Station disaster.

1980 – 1986  
Research Engineer in the Clinical and Experimental Laboratory of Haematology and Blood Transfusion. Lecturer II at Kyiv Medical School.

1977 – 1980  
Research Engineer in the Clinical and Experimental Laboratory of the Scientific Research Institute of Paediatrics, Obstetrics and Gynaecology.

ACTIVITIES AND ASSOCIATIONS

1972 – 1977  
Secretary of Council of young scientists and Researchers.

1971 – 1977  
Editor in University Newspapers

1977 – 1980  
Member of Ukrainian Research Association of Paediatricians, Obstetricians and Gynaecologists.

1980 – 1990  
Member of Ukrainian Association of Haematologists and Transfusionists.

1986 – 1992  
Ukrainian Committee of the effects of the Chernobyl Atomic Energy Power Station Disaster and the Effects Elimination

MEMBERSHIP – OTHER ORGANISATIONS

1977 – 1992  
Member of the Ukrainian Medical Trade Union

1977 – 1992  
Honorary Secretary of the Ukrainian Association of Scientists, Inventors and Innovators

1980 – 1992  
Advisor of the Ukrainian Red Cross and Red Crescent Societies.

1980 – 1992  
Honorary Member of Blood donors
1989 -1992  Member of the Scientific Council of the Ukrainian Institute of Haematology and Blood Transfusion

PUBLICATIONS


L. Korolova, Immunological Studies of the Spleen Slides in Patients Suffering from Idiopathic Thrombocytopenia, Symposium of the Scientific Research and Clinical Practice in Kharov, 1986.


Korolova L., Invention of the Prognosis of the Trend of Post-Surgical period of the Patients Suffering from some Blood diseases of the Splenectomy Moscow, 1992.
APPENDIX 9: GLUCOSE ESTIMATION

GLUCOSE liquicolor

GOD-PAP Method
Enzymatic Colorimetric Test for Glucose Method without Deproteinisation

Package sizes

<table>
<thead>
<tr>
<th>Type</th>
<th>Contents</th>
<th>10120</th>
<th>10121</th>
<th>10123</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kit</td>
<td>12 x 3 ml</td>
<td>1 x 100 ml</td>
<td>1 x 100 ml</td>
<td>9 x 3 ml</td>
</tr>
<tr>
<td>STD</td>
<td>1 x 3 ml</td>
<td>1 x 3 ml</td>
<td>1 x 3 ml</td>
<td>9 x 3 ml</td>
</tr>
</tbody>
</table>

Enzyme reagent

Enzyme reagent + phosphate buffer (pH 7.4)
4-aminoazobenzene + phenol
Glucose oxidase
Azo dye
Stabilizers

Calculation of the Glucose Concentration

\[ c = \frac{110 \times \Delta A_{550} \text{ [mg/dL]}}{\Delta A_{550} \text{ [mmol/L]}} \]

Performance Characteristics

- Linearity: The test is linear up to a glucose concentration of 400 mg/dL or 222 mmol/L. Dilute the sample 1:2 with distilled water, if the glucose concentration of the sample is over this limit and repeat the determination. Multiply the result by 2.
- Typical performance data can be found in the Verifilox report, accessible via www.verification.com or www.human.de/instruments/glucomat.pdf

Normal Values

- Serum, plasma (fasting): 75-135 mg/dL or 4.2-7.5 mmol/L
- Urine: 120-1500 mg/dL

Quality Control

- All control sera with glucose values determined by this method can be used.
- We recommend to use our quality control sera HUMANCOL based on serum or plasma and our SECKOL based on human serum.

Automation

- Proposals to apply the reagents on analysers are available on request.
- Each laboratory has to validate the application in its own responsibility.

Notes

1. The test contains sodium azide. Do not swallow. Avoid contact with skin and mucous membranes.
2. Sera which interfere with the test and should not be used as a sample: Triglycerides up to 2500 mg/dL, homoglobin up to 350 mg/dL and ascorbic acid up to 25 mg/dL do not interfere with the test.
3. A slight brownish sediment may develop in the kit; this does not influence the functionality of the kit. Do not mix this sediment during pipetting.

References

1. Barkam D. Trinder P. Analyt 97 (1972)

Pipetting Scheme

<table>
<thead>
<tr>
<th>Microlitre</th>
<th>Macro</th>
<th>Semi micro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipettor</td>
<td>1000 µL</td>
<td>1000 µL</td>
</tr>
<tr>
<td>or sample</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

Mix, incubate for 20 min. at 20-25°C or 5 min at 37°C. Measure the absorbance of the reagent blank and the sample against the reagent blank within 60 min (550 nm).
APPENDIX 10: CHOLESTEROL ESTIMATION

CHOLESTEROL liquicolor
C/10D-PAP-Method: Enzymatic Oxiunometric Test for Cholesterol with Lipid Clearing Factor (LCF)

Package Sizes
- Ref. 1 1017 4 x 50 ml Complete test kit
- Ref. 1019 3 x 250 ml Complete test kit
- Ref. 1038 4 x 100 ml Complete test kit
- Ref. 1015 9 x 3 ml Standard

Method
The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinonemine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

Reaction Principle
Cholesterol + H2O → cholesterol + lactic acid
Cholesterol + O2 + 4-aminoantipyrine + phenol

Contents
- Ref. 1 4 x 10 ml 3 x 250 ml or 4 x 10 ml Enzyme reagent
- Phosphate buffer (pH 5.5) 100 mM
- 4-Aminoantipyrine 0.3 mM
- Phenol 5 mM
- Peroxidase > 5 U/Ml
- Cholesterol oxidase > 160 U/Ml
- Sodium arsenite 0.01 M

STD
- 1 ml Standard
- Cholesterol 200 mg/dl or 5.17 mmol/1

Reagent Preparation
The Ref. 1 and the Ref. 1015 are ready for use.

Reagent Stability
The reagents are stable up to their open expiry date, even after opening when stored at 2-8 °C. The opened reagent is stable for 2 weeks at 15-25 °C. Contamination must be avoided.

Specimen
Serum, heparinized or EDTA-plasma.

Note: Lipemic specimens usually generate turbidity of the sample, which acts as falsely elevated results. The C/10D-PAP-Method gives better results than before Lipo Clearing Factor (LCF). The LCF clears up turbidity caused by lipemic specimens.

Asar
Wavelength: 550 nm; λg 545 nm
Optical path: 1 cm
Temperature: 25 ± 3 °C
Measurement: Against reagent blank; only one reagent blank per series is required.

Pipetting Scheme

<table>
<thead>
<tr>
<th>Pipette into cuvettes</th>
<th>Reagent blank</th>
<th>Sample or control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 100 µL</td>
<td>19 µL</td>
<td>1000 µL</td>
</tr>
<tr>
<td>200 µL</td>
<td>1000 µL</td>
<td></td>
</tr>
</tbody>
</table>

Mix, incubate 10 min at 30, 35°C or 5 min at 37°C. Measure the absorbance of the sample against the reagent blank (A0) within 30 min.

Calculation of the Cholesterol Concentration

1. With Factor

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>C [mg/dl]</th>
<th>C [mmol/1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>546 nm</td>
<td>840 ± 1 µA</td>
<td>21 ± 1 µA</td>
</tr>
<tr>
<td>510 nm</td>
<td>513 ± 1 µA</td>
<td>14.2 ± 1 µA</td>
</tr>
</tbody>
</table>

2. With Standard

Only the standard recommended by HUMAN (enclosed in kit or separately available, Ref. 1015) should be used.

\[
C = \frac{A_{492} \times 200}{A_{492\text{std}}} \quad [\text{mg/dl}]
\]

or

\[
C = \frac{517 \times A_{492}}{A_{492\text{std}}} \quad [\text{mmol/1}]
\]

Performance Characteristics

Linearity
The test is linear up to a cholesterol concentration of 150 mg/dl (19.3 mmol/1). Store samples with a higher cholesterol concentration 1 + 2 with physiological saline (0.9%) and repeat the determination. Multiply the results by 3.

Typical performance data can be found in the Verification Report, accessible via www.duat.de/index.php?h=3300110218.pdf)

Clinical Interpretation

- Suspect values: 200 mg/dl or 5.7 mmol/1
- Elevated values: 250 mg/dl or 6.7 mmol/1

The European Atherosclerosis Society recommends to decrease the cholesterol level to approximately 180 mg/dl for adults up to 35 years and to approximately 200 mg/dl for adults over 35 years.

Quality Control
All control sera with values determined by this method may be employed. We recommend to use our Oxford serum based HUMANrol or our human serum based SORODOS quality control sera.

Anotation

Provisions to apply the reagents on analysers are available on request. Each laboratory has to validate the application in its own responsibility.

Notes

1. The test is not influenced by hemoglobin values up to 200 mg/dl or by bilirubin values up to 5 mg/dl.
2. The reagents contain ascorbic acid as preservative (9.5%). Do not swallow. Avoid contact with skin and mucous membranes.

References

2. Richmond, W., Clin. Chem. 19, 1350 (1973)
5. ISO 16223 Medical devices... Symbols to be used with medical device labels, labelling and information to be supplied.
APPENDIX 11: PT ESTIMATION

Quantitative determination of Prothrombin time (PT)

**Method**

Sample: Plasma or serum (EDTA) [Without anticoagulants, Avoid freezing the specimen.]

PROCEDURE

1. Place the sample into the PT Test instrument (Spinreact), and stabilize at 37°C.
2. Press the start button (1) and observe the absorption of the sample.
3. Record the reading at 2 minutes (2).
4. Calculate PT.
5. Obtain the test result on the PT test strip.

**Calculations**

It is possible to report the results in seconds, but it is recommended to report PT (INR) or International Normalized Ratio (INR).

**PT ratio** (INR) = PT of patient/PT of normal internal control (in sec)

**International Sensitivity Index (ISI)**

The prothrombin ratio can be converted into internationally comparable values by means of the International Sensitivity Index (ISI). The result obtained is in International Normalized Ratio (INR).

**Quality Control**

Ensure that the test procedure is performed correctly and that the results are in the range of the control.

**Reference Values**

It is recommended to perform PT tests on the same day as the specimen is received.

**Performance Characteristics**

Precision: The results are determined as follows: the coefficient of variation (CV) of all normal controls is less than 10%.

**Interferences**

Do not use anticoagulants, EDTA or heparin as anticoagulants.

**Remarks**

Test performance, calibration, and control are important factors in the PT test. Always follow the manufacturer's instructions, the results must be validated by the laboratory.

**Packaging**

Ref. 1107322
- 5 x 1 ml
- 10 x 2 ml

Ref. 1107324
- 16 x 4 ml
- 9 x 4 ml

**Manufacturer**

Spinreact S.A., Ctra. Santa Coloma, s/n, 07616 SANT LLORENÇ DES Cardassar (Balears), 07616, Spain
Tel. +34 971 41 02 54 Fax: +34 971 41 02 55, email: info@spinreact.com
APPENDIX 12: APTT ESTIMATION

Quantitative determination of Activated Partial Thromboplastin Test (APTT)

Store at 2-8°C

PRINCIPLE OF THE METHOD
When phospholipid complex and calcium chloride (CaCl₂) are added to citrated plasma, the factors of intrinsic coagulation system are activated; the time for formation of a fibrin clot is then measured. 1-3

CLINICAL SIGNIFICANCE
The time measurement of APTT is the most common coagulation procedure performed in routine laboratories, apart from the PT. The APTT is particularly sensitive to defects of the intrinsic coagulation pathway (Factors VIII, IX, XI, XII). It is commonly used for monitoring heparin or anticoagulant therapy. 4-7

Clinical diagnosis should not be based on a single test result; it should integrate clinical and other laboratory data.

REAGENTS

R 1
Activator
Elastic acid

R 2
Starter
Calcium chloride (CaCl₂) 0.02M

Optional
CONTROL NORMAL  FEF: 1709104
CONTROL PATHOLOGIC  FEF: 1709105

PREPARATION
All the reagents are ready to use.
R 1: Stable for 1 month at 2-8°C after opening.

STORAGE AND STABILITY
All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8°C, protected from light and contaminations prevented during use.

A yellow sediment may form after prolonged storage. Do not use reagents over the expiration date. Do not freeze.

Signs of reagent deterioration:
- Presence of particles and turbidity,
- Quality control values outside established ranges,
- Precipitate color variations.

ADDITIONAL EQUIPMENT
- Coagulometer or stopwatch and bath at 37°C ± 0.5°C,
- General laboratory equipment

SAMPLES
Plasma from venous puncture diluted 1:10 in Trisodium citrate solution 3.8%

Mixing immediately the blood with anticoagulant. Avoid foaming the specimen.

Centrifuge the sample at 2500 x g for 15 min and transfer the plasma into a siliconized glass or plastic containers.

Turbid, icteric, hemolytic or hemolysed samples may generate erroneous results.

The sample is stable for 2 hours at room temperature (15-25°C) or 24 hours immediately frozen at below -20°C.

PROCEDURE
The reagent can be used by manual method, mechanical, photometric or other means of clot detection.

MANUAL METHOD
1. Pipette 100 µl of the reagents and the sample.
2. Mix thoroughly.
3. Pipette into a clean and dry tube:

Citrated plasma (µL) 100
R 1 (µL) 100

4. Mix and incubate exactly for 6 min. at 37°C (activation time).

5. Pipette:

R 2 (µL) 100

6. Mix thoroughly.
7. On addition of R 2 start stopwatch or timer on the coagulation analyzer and determine the coagulation time.

CALCULATIONS

It is possible to report the results as seconds or as APTT ratio, dividing the results of the sample (sec) by the results of plasma Control (sec).

APTT ratio = APTT of the patient in seconds / APTT of normal plasma (and ½ in seconds)

QUALITY CONTROL
Control sera are recommended to monitor the performance of assay procedures.

CONTROL NORMAL:
RED: 1709104
CONTROL PATHOLOGIC:
RED: 1709105

If control values are found outside the defined ranges, check the instrument, reagents and technique for problems.

Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

REFERENCE VALUES
An exhaustive study has been run with 265 samples of healthy people, and as a result it has been established the following reference values:

APTT (in seconds): 24 - 38 sec.

These values are for orientation purpose; each laboratory should establish its own reference range.

PERFORMANCE CHARACTERISTICS

Reagent Sensitivity:

Heparin conc. (units/mL) 0.05 APTT (sec)
0.0 29.0
0.1 38.5
0.2 60.1
0.3 63.1
0.4 69.9
0.5 98.8

Factor Sensitivity:

Activated factor must demonstrate > 30-40% factor activity.

<table>
<thead>
<tr>
<th>Factor</th>
<th>% activated APTT (sec)</th>
<th>% activated VIII APTT (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F VIII</td>
<td>42.0</td>
<td>100.0</td>
</tr>
<tr>
<td>F IX</td>
<td>44.0</td>
<td>100.0</td>
</tr>
<tr>
<td>F XI</td>
<td>46.0</td>
<td>100.0</td>
</tr>
<tr>
<td>F XII</td>
<td>48.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

These values should only be used as guidelines. Each laboratory should establish its own sensitivity to individual factors.

INTERFERENCES
Do not use sodium heparin, EDTA or heparin as anticoagulant.

Oral contraceptives, estrogens or pregnancy hormones in the test.

A list of drugs and other interfering substances with the determination has been recited.8-10

NOTES
1. All diluents must be clean and the use of trace amounts of detergents.
2. Always follow instrument manufacturer's instructions; the results must be validated by the test laboratory.

BIBLIOGRAPHY

PACKAGING
Ref: 1709201
R 1 x 4 mL
R 2 x 4 mL

CO65046  04 92

SPINREACT S.A.U.
Ctra Santa Coloma, T-11715 SANT POL DE MAR (Catalunya) SPAIN
Tel: +34 932 090 060 Fax: +34 932 090 061 email: atc@spinreacts.com

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APPENDIX 13: FIBRINOGEN ESTIMATION

Quantitative determination of Fibrinogen

1/1000

Store at 2-8°C

PRINCIPLE OF THE METHOD

Fibrinogen is precipitated by the addition of anticoagulant, which forms fibrin. The time for clot formation in dilute plasma is inversely proportional to the fibrinogen concentration in the sample.

CLINICAL SIGNIFICANCE

Fibrinogen (Factor I), probably synthesized by the liver, is the substance used in the blood to form a clot. Its determination is used to evaluate abnormal coagulation.

Preparation of reagents:

1. Dilute 2 mL of plasma with 2 mL of distilled water, cap well and mix gently to ensure complete mixing. Stability: 7 days at 24°C or 1 month at 4°C. Immediately freeze and store in the original container. Do not refreeze.

STORAGE AND STABILITY

All components of the kit are stable until the expiration date on the label when stored tightly covered at 2-8°C and protected from light. Do not store reagents below the expiration date.

Signs of reagent deterioration:

- Presence of particles and turbidity
- Quality control values outside established ranges
- Color variations

ADDITIONAL INFORMATION

- General laboratory equipment

SAMPLES

Put 2 mL of plasma into a tube containing 1:100 thrombin clot solution. Mix immediately and store at 4°C. Avoid freezing the specimen.

Cautions:

- Centrifuge the sample at 5000 rpm for 10 min and transfer the plasma to a clean, ice-cooled glass or plastic container.
- Fibrinogen, thrombin, or fibrinolytic samples may generate erroneous results.

NOTES

1. All laboratories must be clean and free of breast adhesions.
2. Always follow instrument manufacturer's instructions; the results must be validated by the test laboratory.

PROCEDURE

- The reagent can be used by the following procedures: mechanical, photo-optical, or other means of end clot detection.
- Dilute the plasma and control 1/10 with imidazole buffer. Prepare 50 µL plasma + 450 µL imidazole buffer. The diluted sample must be processed in 1 hour.

2. Prepare the following dilutions of the Coagulor in imidazole buffer.

<table>
<thead>
<tr>
<th>Diluents</th>
<th>Buffer</th>
<th>Calibrator Dilution</th>
<th>Concentration (mg/dL)</th>
<th>Correction (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/10</td>
<td>2/10</td>
<td>1/20</td>
<td>1/100</td>
<td>1/10</td>
</tr>
<tr>
<td>Imidazole</td>
<td>Buffer</td>
<td>3.9</td>
<td>2.9</td>
<td>1.8</td>
</tr>
<tr>
<td>COAGULATION CAL (mL)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Factor</td>
<td>10x40</td>
<td>10x100</td>
<td>10x100</td>
<td>10x100</td>
</tr>
<tr>
<td>Concentration (µL/mL)</td>
<td>0.25x + 0.35x + 0.5x + 1x + 2x + 3x</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Add 20 µL of R3 to 0.2 mL of each dilution, and allow it to reach 37°C for 4 hours.
4. Add 1 mL of R1 and time the clot formation. Do not allow thrombin R1.

Calculation:

1. Calculate the mean of duplicate clotting times immediately after reaction. Use all five of the control points to construct a log-log curve that plots fibrinogen concentration (mg/dL) vs. clotting time (s). The final curve must consist of at least three consecutive points. Constructing the curve with only the last linear points will produce the best recovery on control and patient samples.

2. The following curve is only orientative. It will change with lot and concentration of the calibrator, as well as with the instrument used.

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Concentration (mg/dL)</th>
<th>Concentration (µL/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.1</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>14.3</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>15.6</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>12.0</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>15.2</td>
<td>0.08</td>
<td>0.08</td>
</tr>
</tbody>
</table>

4. Find the clotting time of quality control and patient samples on the curve and read the corresponding fibrinogen value.

If dilution times for the 1/10 dilution fail to fall on the linear curve, prepare 1/20 or 1/100 dilutions as needed. If the sample is diluted 1/20, divide the result from the standard curve by 2; if the sample was diluted 1/100, multiply the curve result by 2 to get the final result.

QUALITY CONTROL

Control sera are recommended to monitor the performance of assay procedures.

1. Store at 2-8°C. If sera become cloudy, centrifuge to remove clots.
2. Control sera are recommended to monitor the performance of assay procedures.

REFERENCE VALUES

20-400 mg/dL.

PERFORMANCE CHARACTERISTICS

- Sensitivity: 1.0 mg/dL
- Specificity: 0.0 mg/dL

Accuracy:

- Correctness: 1.0 mg/dL
- Variability: 0.0 mg/dL

INTERFERENCES AND LIMITATIONS

Blood samples should be taken after 10 minutes at room temperature or 2 days refrigerated at 4°C.

- In case of interference, re-run the sample after centrifugation.
- In case of interference, re-run the sample after centrifugation.

BIBLIOGRAPHY


PRESENTATION

- Ref: 1709211
- CE 
- FIBRINOGEN

<table>
<thead>
<tr>
<th>Controls</th>
<th>8 x 2 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td></td>
</tr>
<tr>
<td>R3</td>
<td>3 x 3.5 mL</td>
</tr>
</tbody>
</table>
APPENDIX 14: vWF ESTIMATION

vWF (Human) ELISA Kit

Catalogue Number KA0512
96 assays
Version: 17
January 2012

Intended for research use only
Introduction

Background

Von Willebrand factor (vWF) is a multimeric glycoprotein that circulates in blood forming a noncovalent complex with procoagulant factor VIII. During normal homeostasis, the larger multimers of vWF are responsible for facilitating platelet plug formation by forming a bridge between platelet glycoprotein IB and exposed collagen in the subendothelium. The congenital dysfunctional state of vWF causes a moderate to severe bleeding diathesis-von Willebrand disease (VWD).

Principle of the Assay

The vWF ELISA kit is designed for detection of human vWF in plasma, serum, and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures vWF in less than 5 hours. An antibody specific for vWF has been pre-coated onto a microplate. Human vWF in standards and samples is sandwiched by the immobilized antibody and biotinylated-antibody specific for vWF, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.
General Information

Materials Supplied

List of component

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF Microplate: A polystyrene microplate coated with a monodonal antibody against vWF.</td>
<td>96(8x12) wells</td>
</tr>
<tr>
<td>Sealing Tapes: pressure-sensitive sealing tapes, which can be cut to fit the format of the individual assay.</td>
<td>3</td>
</tr>
<tr>
<td>Biotinylated vWF Antibody (50x): A 50-fold concentrated biotinylated polydonal antibody Against vWF</td>
<td>140 1-11</td>
</tr>
<tr>
<td>MIX Diluent Concentrate (10x): A 10-fold concentrated buffered protein base</td>
<td>30 ml</td>
</tr>
<tr>
<td>Wash Buffer Concentrate (20x): A 20-fold concentrated buffered surfactant</td>
<td>30 ml x 2</td>
</tr>
<tr>
<td>Streptavidin-Peroxidase Conjugate (SP Conjugate): A 100-fold concentrate</td>
<td>801-11</td>
</tr>
<tr>
<td>Chromogen Substrate: A ready-to-use stabilized peroxidise chromogen substrate Tetramethylbenzidine</td>
<td>8ml</td>
</tr>
<tr>
<td>Stop Solution: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction</td>
<td>12 ml</td>
</tr>
</tbody>
</table>

Storage Instruction

- Store components of the kit at 2-8°C or -20°C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20°C.
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8°C.
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8°C.
- Store Standard at 2-8°C before reconstituting with Diluent and at -20°C after reconstituting with Diluent.

Materials Required but Not Supplied

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 1-11, 20-200 1-11, 200-1000 1-11 and multiple channel).
- Deionized or distilled reagent grade water.
Precautions for Use

✓ Prepare all reagents (working diluents buffer, wash buffer, standards, biotinylated-antibody, and SP conjugate) as instructed prior to running the assay.

✓ Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.

✓ Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.

✓ This kit is for research only

✓ The stop solution is an acid solution.
Assay Protocol

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- MIX Diluent Concentrate (10x): If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8°C.
- vWF Standard: Reconstitute the 80 mU of human vWF Standard with 1 ml of MIX Diluent to generate a solution of 80 mU/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the Standard solution (80 mU/ml) 1:2 with equal volume of MIX Diluent to produce 40,20,10, 5 and 2.5 mU/ml. MIX Diluent serves as the zero standard (0 mU/ml). Any remaining solution should be frozen at -20°C and used within 30 days.

<table>
<thead>
<tr>
<th>Standard Point</th>
<th>Dilution</th>
<th>[vWF] (mU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1 part Standard (80 mU/ml)</td>
<td>80.00</td>
</tr>
<tr>
<td>P2</td>
<td>1 part P1 + 1 part MIX Diluent</td>
<td>40.00</td>
</tr>
<tr>
<td>P3</td>
<td>1 part P2 + 1 part MIX Diluent</td>
<td>20.00</td>
</tr>
<tr>
<td>P4</td>
<td>1 part P3 + 1 part MIX Diluent</td>
<td>10.00</td>
</tr>
<tr>
<td>P5</td>
<td>1 part P4 + 1 part MIX Diluent</td>
<td>5.00</td>
</tr>
<tr>
<td>P6</td>
<td>1 part P5 + 1 part MIX Diluent</td>
<td>2.50</td>
</tr>
<tr>
<td>P7</td>
<td>MIX Diluent</td>
<td>0.00</td>
</tr>
</tbody>
</table>

- Biotinylated vWF Antibody (50x): Spin down the antibody briefly and dilute the desired amount of the antibody 1:50 with MIX Diluent. Any remaining solution should be frozen at -20°C.
- Wash Buffer Concentrate (20x): If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute Wash Buffer Conc. 1:20 with reagent grade water.
- SP Conjugate (100x): Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20°C.

Sample Preparation

- Plasma: Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x g for 10 minutes and assay. Dilute samples 1:100 with MIX Diluent. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA or Heparin can also be used as anticoagulant).
Serum: Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x g for 10 minutes. Remove serum and assay. Dilute samples 1:100 with MIX Diluent. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

Cell Culture Supernatants: Centrifuge cell culture media at 3000 x g for 10 minutes to remove debris. Collect supernatants and assay. The samples can be stored at -20°C or below. Avoid repeated freeze-thaw cycles.

Assay Procedure

1. Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30°C).
2. Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
3. Add 50 µl of standard or sample per well, and cover wells and incubate for two hours. Start the timer after the last sample addition.
4. Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
5. Add 50 µl of Biotinylated vWF Antibody to each well and incubate for two hours.
6. Wash the microplate as described above.
7. Add 50 µl of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
8. Wash the microplate as described above.
9. Add 50 µl of Chromogen Substrate per well and incubate for about 20 minutes or until the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
10. Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
11. Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.
Data Analysis

**Calculation of Results**

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.

- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.

- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

![VWF Standard Curve](image)

Typical Standard Curve for VWF (Human) ELISA Kit.

**Performance Characteristics**

- The minimum detectable level of vWF was typically 2.5 mU/ml.

- Intra-assay and inter-assay coefficients of variation were 5.0% and 7.1% respectively.

- Standard has been calibrated against WHO reference standard.
• **Linearity**

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>Plasma</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 50</td>
<td>95%</td>
<td>96%</td>
</tr>
<tr>
<td>1 : 100</td>
<td>100%</td>
<td>101%</td>
</tr>
<tr>
<td>1 : 200</td>
<td>105%</td>
<td>101%</td>
</tr>
</tbody>
</table>

• **Recovery**

<table>
<thead>
<tr>
<th>Standard Added Value</th>
<th>3-30 mU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery %</td>
<td>88-118 %</td>
</tr>
<tr>
<td>Average Recovery %</td>
<td>99%</td>
</tr>
</tbody>
</table>

• **Cross-Reactivity**

<table>
<thead>
<tr>
<th>Species</th>
<th>% Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine</td>
<td>None</td>
</tr>
<tr>
<td>Bovine</td>
<td>None</td>
</tr>
<tr>
<td>Monkey</td>
<td>&lt; 40% (suggest dilution 1:40 for plasma/serum)</td>
</tr>
<tr>
<td>Mouse</td>
<td>None</td>
</tr>
<tr>
<td>Rat</td>
<td>&lt; 20% (Suggest dilution 1:10 for plasma/serum)</td>
</tr>
<tr>
<td>Swine</td>
<td>None</td>
</tr>
<tr>
<td>Rabbit</td>
<td>None</td>
</tr>
</tbody>
</table>

• **Reference Values**

Normal human plasma vWF concentration has been reported ranging approximately from 0.3 to 1.57 IU/ml. Normal citrated human plasma vWF values are 0.6 - 2.6 IU/mL for all patients.

• *Note: The conversion of IU and Iig is 1 International Unit (1 IU/ml) = 9.8 ug/ml*