Sudan University of Science and Technology

College of Graduate Studies

Association of Beta Fibrinogen 455 G-A Gene Polymorphism with Fibrinogen Level and Micro Vascular Complications in Diabetic Hypertensive Sudanese Patients

العلاقة بين الأشكال الجينية المتعددة 455 جي –اي لبيتا فبرينوجين ومستوى الفبرينوجين ومضاعفات الأوعية الصغرى لدى المرضى السودانيين المصابين بداء السكر وإرتفاع ضغط الدم.

A thesis Submitted in Fulfillment for the Requirement of PhD in Hematology and Immuno hematology

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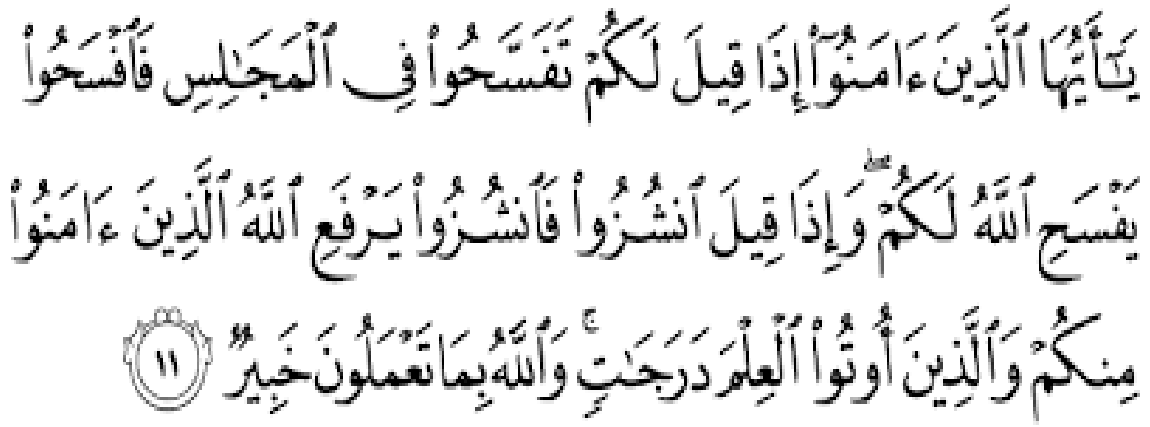
يَٰٓأَيُّهَا ٱلَّذِينَ ءَامَنُوٓاْ إِذَا قِيلَ لَكُمۡ تَفَسَّحُواْ فِي ٱلۡمَجَٰلِسِ فَٱفۡسَحُواْ
يَفۡسَحِ ٱللَّهُ لَكُمۡۖ وَإِذَا قِيلَ ٱنشُزُواْ فَٱنشُزُواْ يَرۡفَعِ ٱللَّهُ ٱلَّذِينَ ءَامَنُواْ
مِنكُمۡ وَٱلَّذِينَ أُوتُواْ ٱلۡعِلۡمَ دَرَجَٰتٖۚ وَٱللَّهُ بِمَا تَعۡمَلُونَ خَبِيرٞ ١١

سورة المجادلة (11)

Dedication

I dedicate this work to:

Souls of my mother and father

Brothers

Sisters

Persons who stand behind me at the time when I need them.

Acknowledgment

I would like to express my great fullness and sincere thanks to the supervisor Dr.Fathelrahman Mahdi Hassan and Dr. Amar Mohamed Ismail without their strict continues guidance this work could have not been a reality.

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Abstract

This was a case control study conducted on a total of 300 type 2 diabetic hypertensive Sudanese patients with micro vascular complications as case group and 100 non diabetic non hypertensive as control group at Khartoum teaching hospital in Khartoum state during the time period from January 2014 to April 2017**,** age range between 30 and 90 years، age mean 57.2years, male and female with or without complications. Blood sample was collected 5ml and divided into 3 ml in trisodium citrate 3.8% plasma was separated and stored at -20 ^{0}C for fibrinogen assay, 2 ml blood was drawn into EDTA, plasma was separated and stored at -20^{0}C for ELISA assay and leukocytes was used for genotyping, the study aimed to investigate the association between beta fibrinogen 455 G→A gene polymorphisms with fibrinogen, D-dimer, fibrinogen/fibrin degradation products (FDPs) levels with demographic and clinical data. Genotypes were determined by polymerase chain reaction by restriction fragment length polymorphism Hae III and fibrinogen was assessed by coagulometer, D-Dimer and FDPs measured by ELISA:

There were common increase of fibrinogen, D- dimer and FDPs levels in A/A allele's genotype in case study. There was risk factor when compared G/G with GA+A/A in dominant model (ood ratio=1.8) (*P*-value=0.010).There were more frequencies of female when compared to male in case and control groups, essential hypertension more common in case group than secondary. Micro vascular complications were present in fewer patients when compared to absent complications in patients group. There were no statistically significant association between alleles genotype A/A, G/G and G/A with absence or presence of neuropathy, retinopathy, nephropathy and male compared to female.

There were statistically significant increase in mean fibrinogen, D-dimer and FDPs when compared patients to control groups (*P*-value=0.00). There were no

statistically significant association in mean fibrinogen, D-dimer and FDPs when compared male to female in case group (*P*-value=0.131, 0.096 and 0.340) respectively. There were no statistically significant in mean fibrinogen, D-dimer and FDPs when compared essential and secondary hypertension stage. (*P*-value=0.687, 0.350 and 0.663) respectively. They were statistically significant increase in mean fibrinogen, D-dimer and FDPs in duration time of both diabetes mellitus and hypertension (*P*-value=0.006. 0.003 and 0.009) respectively. There were statistically significant increase in mean fibrinogen, D-dimer and FDPs when patients with neuropathy compared to those without neuropathy(*P*-value=0.012. 0.008 and 0.005) respectively .There were statistically significant increase in mean fibrinogen when patients with nephropathy compared to those without nephropathy (*P*-value=0.021), mean of D-dimer when patients with retinopathy compared to those without retinopathy(*P*-value=0.004) and mean fibrinogen, D-dimer and FDPs when compared between age group in study group (*P*-value=0.001, 0.003 and 0.047) respectively.

خلاصة الدراسة

هذه دراسة حالة وضبط أجريت في مامجموعه 300 للمرضى السودانيين المصابين بمرض السكرى النوع الثانى وضغط الدم ولديهم مضاعفات الاوعيه الصغرى ويمثلون مجموعه الحالة و 100شخص اصحاء ويمثلون المجموعة الضابطة بمستشفي الخرطوم التعليمي بولاية الخرطوم فى الفترة ما بين يناير 2014 وحتى ابريل 2017 ،اعمارهم تتراوح بين 30 الى 90 سنه ومتوسط الاعمار57.2، ذكور واناث لديهم او ليس لديهم مضاعفات، عينةالدم جمعت 5مل وقسمت 3مل في 3.8% ثلاثى الصوديوم السترات،البلازما حفظت في -20 لقياس الفبرينوجين و2مل صبت في الادتا والبلازما فصلت وحفظت في -20 لقياس الاليزا وكريات الدم البيضاء استعملت لنوع الجينات، تهدف الدراسة لتشخيص العلاقة بين المتغير الجينى بيتا فبرينوجين 455 جى ايه و ومستويات الفبرينوجين ودى دايمر والفبرينوجين/منتجات تكسر الفبرين مع البيانات السريرية والديموجرافي .نوع الجينات حدد بتفاعل السلسله المتبلمر مع الانزيم القاطع اتش ايه اى ثلاثه .الفبرينوجين قيس بجهاز التجلط المتعدد ودى دايمر والفيرينوجين /منتج تكسر الفبرين قيسا بالاليزا:توجد زيادة عموما لمستويات الفبرينوجين ودى دايمر ومنتجات تكسر الفبرين بالاليل ايه ايه اكثر من جي جي واقلها جي ايه لمجموعة الدراسة. وجدت زياده فى عامل الخطورة للمتغيرات الجينيه السائده الجي الجي عند مقارنتها مع الجي ايه وايه ايه حيث ان (تناسب الاوود=1.8) (بقيمة معنوية=0.010). وجدت زيادة فى التردد للاليل ايه ايه عند مقارنتها بالاليل جي جي والجي ايه لمجموعتى الحالة الضبط . وجدت زياده فى التردد للاناث عند مقارنتهم بالذكور للمجموعتين. ضغط الدم الاساسى اكثر عموميه عند مقارنته بالثانوى لمجموعه الحاله.مضاعفات الاوعيه الصغرى ظهرت بصورة اقل عند مقارنتها بمن ليس لديهم مضاعفات لمجموعة الدراسه. لا توجد فروق ذات دلاله احصائيه بين الاليل ايه ايه ،جى جى وجى ايه عند مقارنتهم مع غياب او ظهور مضاعفات الاعصاب ، مضاعفات العيون ، مضاعفات الكبيبات الكلويه ،الذكور مقارنه بالاناث.توجد زيادة ذات دلالة احصائية لمتوسط الفبرينوجين ،دى دايمر وفبرينوجين /منتج تكسر الفبرين لدى مجموعة الحالة مقارنة بالمجموعة الضابطة (بقيمه معنوية=0.00). لا توجد فروق ذات دلاله احصائيه لمتوسط الفبرينوجين ودى دايمر وفبرينوجين /منتج تكسر الفبرين عند مقارنه الذكور بالاناث بالمجموعة الضابطة (بقيمة معنوية =0.131,0.096 و 0.340) علي التوالي، و عند مقارنة ضغط الدم الاساسى بالثانوى (بقيمة معنوية= 0.687 ، 0.350 و 0.663) علي التوالي. توجد زيادة ذات دلالة احصائية لمتوسط الفبرينوجين ودى دايمر وفبرينوجين /منتج تكسر الفبرين للفترة الزمنية للاثنين السكرى والضغط (بقيمه معنويه=0.003,0.006 و0.009) علي التوالي. توجد زيادة ذات دلالة احصائية لمتوسط الفبرينوجين ودى دايمر وفبرينوجين /منتج تكسر الفبرين عند مقارنه المرضى المصابين بمضاعفات

الاعصاب مع اولئك الذين ليس لديهم مضاعفات الاعصاب بمجموعة الحالة (بقيمه معنوية=0.008، 0.012 و0.005) علي التوالي .توجد زيادة ذات دلالة احصائية لمتوسط الفبرينوجين عند مقارنه المرضى المصابين بمضاعفات الكبيبات الكلويه مع اولئك الذين ليس لديهم مضاعفات الكبيبات الكلويه (بقيمة معنوية=0.021). توجد زيادة ذات دلالة احصائية لمتوسط دى دايمر عند مقارنة المرضي المصابين بمضاعفات العيون مع اولئك الذين ليس لديهم مضاعفات العيون بقيمة معنوية=0.004). توجد زيادة ذات دلاله احصائية لمتوسط الفبرينوجين ودى دايمرومنتج تكسر الفبرين عند المقارنة بين مجموعة الاعمار بالمجموعة الضابطة (بقيمة معنوية =0.001,0.003 و0.047) على التوالى.

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AA	Adenine Adenine
Apo(a)	Apolipoprotien -alpha
Apo(B100)	Apolipoprotien (B100)
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CVD	Cardiovascular Disease
CNS	Central Nervous System.
DASH	Dietary Approaches to Stop Hypertension.
DKA	Diabetes Keto Acidosis
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
DVT	Deep Venous Thrombosis
DBP	Diastolic Blood Pressure
DIC	Disseminated Intravascular Coagulation
EDTA	Ethyl Diamine Tetra Acitic Acid
ECM	Extra Cellular Matrix
FDPs	Fibrin Degradation Products
FEU	Fibrinogen Equivalent Units
FFP	Fresh Frozen Plasma
FP-A- B	Fibrinopeptides A and B
G A	Guanine Adenine.
GAIT	Genetic Analysis of Idiopathic Thrombophila
GDM	Gestational Diabetes Mellitus
HHS	Hyperglycemic Hyperosmolar State

HCV	Hepatitis C Virus
IDDM	Insulin Dependent Diabetes Mellitus
IFG	Impaired Fasting Glucose
IFT	Impaired Glucose Tolerance
IL-6	Interleukin-6
IFN g	Interferon- g
IRS-1	Insulin Receptor Substrate-1
IRS	Insulin Receptor Substrate-2
MAP	Mitogen-Activated Protein kinase
MI	Myocardial Infarction (MI)
MCP-1	Monocyte chemoattractant protein-1
NEFA	None esterified fatty acid
NIDDM	Non Insulin Dependent Diabetes Mellitus
NO	Nitric Oxide
NCEP	National Cholesterol Education Program
NaCl	Natrium Chloride
PAD	Peripheral Arterial Disease
P E	Pulmonary Embolism
PCI	Per Cutaneous Coronary Intervention
PCR	Polymerase Chain Reaction
PI-13	Phosphatidylinositol 3-kinase
RBC	Red Blood Cell
ROS	Reactive Oxygen Species
RAGE	Receptors for Advanced Glycosylation End
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species

RAGE	Receptors for Advanced Glycosylation End
RNA	Ribonucleic acid
SBP	Systolic Blood Pressure
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
TAFI	Thrombin Activatable Fibrinolysis Inhibitor.
T-PA	Tissue Plasminogen Activator
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
VLDL	Very Low-Density Lipoprotein
WHO–ISH	World Health Organization–International Society of Hypertension
WBC	White Blood Cell

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Chapter One

Introduction and literature review

1.1 Introduction

Diabetes mellitus (DM) is a metabolic disorders characterized by chronic hyperglycemia due to disturbance of carbohydrates, fats and protein metabolism associated with absolute or relative deficiencies in insulin or insulin action or both (Charles, 2008). Over 170 million people worldwide and about 1.9-7.0% of African population were affected (Santagida *et al*., 2002).

There are three main types: Type 1 diabetes mellitus, which is called (Insulin Dependent Diabetes Mellitus).Type 2 diabetes mellitus, which is called (Non Insulin Dependent Diabetes Mellitus) and gestational diabetes which is classified as type two diabetes mellitus (Nathan *et al*., 2005). The long term affects and complications of diabetes include progressive development of retinopathy, nephropathy and neuropathy with micro vascular and macro vascular diseases. Macro vascular disorder such as atherosclerosis are recognized as major causes of mortality in the diabetic population ,and are implicated in circulatory disturbances that are seen in diabetes. The circulatory disturbance in platelets count and activity coagulopathy, fibrinolytic aberration, haemorrlogical factors and change in endothelial metabolism. Many studies have shown that DM cause hypercoagulable state (Alvin *et al*., 2001).

Hypertension is sustained high blood pressure (≥140/90mmHg)(Pickering, 2005).

When hypertension coexists with overt diabetes, which is commonly does, the risk for cardiovascular disease, including nephropathy, is raised two fold. Improved control of blood pressure in diabetic patients has been shown to be effective in reducing the risk of cardiovascular complications (Hansson *et al*., 1998). Fibrinogen is a glycoprotein circulates in plasma at a concentration of approximately 9 micrometer. Fibrinogen molecules are elongated , 45-nanometer

long structures with 2 outer D domains that are connected by a coiled-coil segment to a central E-domain. They consist of 2 symmetric half molecules, each containing a set of 3 different polypeptide chains termed A, B and G. The 3 chains are encoded by 3 separate genes, fibrinogen alpha, fibrinogen beta, and fibrinogen gamma, clustered in a region of approximately 50 kilo base on chromosome 4q31.3, fibrinogen beta contains 8 exon (Mosesson *et al*., 2001).

Fibrinogen production in the liver is regulated by cytokines, mainly by interleukin-6, and is greatly enhanced by the acute phase response to inflammatory processes hence; fibrinogen elevation might simply reflect the low-grade inflammation associated with vascular disease. On the other hand, increased fibrinogen levels (due to inflammation or other mechanisms) may still participate in the pathogenesis of vascular lesions, i.e. be a true modifier of the atherosclerotic disease and contribute to its progression. Moreover, fibrinogen and fibrin degradation products might in turn enhance the inflammatory aspect of vascular lesions by regulating cytokine production and leukocyte-endothelial interactions (Flick *et al.*, 2004).

It is well known that elevated plasma fibrinogen levels can be affected by environmental and genetic factors. It has been reported that some of ten or more genetic polymorphisms of the fibrinogen gene that have been investigated to date may be involved in elevation of the plasma fibrinogen level (Komitopoulou *et al*., 2006). Fibrinogen levels had a heritability of 34% indicating that genetic factors have an important effect on the quantitative variation in this phenotype. It has been reported that a proportion of this variation can be explained by polymorphisms in the fibrinogen genes, especially in the fibrinogen beta chain gene, which regulates the limiting step in fibrinogen synthesis. Between 5% and 9% of fibrinogen variability could be explained by the beta chain polymorphisms, whereas 4.2% was determined by the fibrinogen alpha chain gene (Souto *et al*., 2000).

Polymorphisms of the beta-fibrinogen gene including the beta-455 Guanine/Adenine polymorphism, which is especially involved in the rate-limiting steps of the formation of the beta -chain have been shown to be closely related to elevation of the plasma fibrinogen level. Several studies have suggested that the 455Guanine/Adenine polymorphism is associated with an elevated plasma fibrinogen concentration (Maumus *et al*., 2007). Beta-fibrinogen 455 G/A polymorphisms are a gene mutation that may lead to alterations in the activity of fibrinogen. Previous study revealed the increased fibrinogen activity in the presence of homozygote Adenine /Adenine allele (Folsom *et al*., 2001).

Homozygote of the A allele of the fibrinogen beta -455 -G>A gene polymorphism, which is caused by a Guanine -to-Adenine substitution at position -455 in the 5, promoter region of the fibrinogen beta gene, show higher plasma fibrinogen levels than subjects with the GG genotype. Elevated plasma fibrinogen concentrations have been associated with increased plasma viscosity and platelet aggregability and, thus, may contribute to vascular disease **(**Humphries *et al*., 1995).

It was determined that the increased A/A allele is associated with increased cardiovascular events and increased prevalence of lacunar infarct in brain (Martiskainen *et al*., 2003).

D-dimer is the primary degradation product of cross-linked fibrin and therefore serves as a direct marker of ongoing coagulation with fibrinolysis. D-dimer, a fragment cleaved from cross linked fibrin as part of fibrin clot degradation, reflects thrombin production and fibrinolysis. A meta-analysis has suggested an independent 1.7-fold increased risk of coronary heart disease (CHD) for the highest versus lowest tertile of D-dimer. (Danesh *et al*., 2001).

The specificity of the relation of these hemostatic factors with cardiovascular disease nevertheless may be questioned, because they often are related positively

to risk of other chronic conditions, such as cancer or total mortality or show a moderate degree of correlation with markers of inflammation (Smith *et al*., 2005). There are two basic steps that lead to fibrin degradation. The initial step is the activation of plasminogen to plasmin by several important protein/enzymes as tissue Plasminogen Activator (t-PA) and urokinase Plasminogen Activator (u-PA). In the second and final step, the active plasmin that is able to complex, with fibrin, specifically degrades the complexes fibrin into soluble fibrin degradation products (Hajjar, 2003).

1.2 Literature review

1.2.1 Diabetes Mellitus

Diabetes Mellitus (DM) is group of metabolic disease in which a person has high blood glucose, because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced. This high blood glucose produces the classical symptoms of polyuria (urination), polydispsia (increase thirst) and polyphagia (increase hunger) (Lawrence *et al*., 2008**).**

1.2.1.1 Classification of DM

1.2.1.1.1 Type1 DM

It is referred to as insulin dependent diabetes mellitus (IDDM) or juvenile diabetes. It is characterized by loss of insulin producing beta cells of islets of langerhans in the pancreas leading to insulin deficiency. This type of diabetes can be further classified as immune mediated or idiopathic. The majority of type 1 diabetes is of immune mediate nature, where beta cell loss is T-cell mediate autoimmune attack.

There is no known preventive measure against type 1diabetes, which causes approximately 10% of diabetes mellitus cases in North America and Europe. Many affected people were healthy and normal weight when onset occurs. Sensitivity and responsiveness to insulin are usually normal, especially in early stages. Type1 may affect children or adults but it was traditionally termed "Juvenile diabetes" because its represent in the majority of diabetes cases in children (Lawrence *et al.*, 2008).

1.2.1.1.2 Type2 DM

It is referred to as non insulin dependent diabetes mellitus (NIDDM), and adult onset diabetes. It's characterized by insulin resistance which may be combined with reduced insulin secretion. The defective responsiveness of body tissue to insulin is believed to involve the insulin receptor. However, specific defect is not known. Type2 are most common type (Lawrence *et al.,* 2008).

1.2.1.1.3 Gestational diabetes

Gestational diabetes mellitus (GDM) resemble type 2 diabetes in several respects, involving a combination of relative inadequate insulin secretion and responsiveness. It occurs in about 2% -5% in all pregnancies and may improve or disappear after delivery.GDM is fully treatable but require careful medical supervision throughout the pregnancy. About 20% -50% of affected women develop type 2 (IDDM) in later of life and need for medication or control (Lawrence *et al.,* 2008).

Untreated gestational diabetes can affect the health of fetus or mother. Risk to baby include macrosomia (high birth baby), congenital cardiac and nervous system abnormities. Other types are pre-diabetes indicates a condition that occurs when a person blood glucose level are higher than normal but not higher enough for diagnosis of type 2 diabetes (Handelman, 2009).

1.2.1.2 Epidemiology and global prevalence

The number of people with diabetes is increasing due to population growth, aging, urbanization, and increasing prevalence of obesity and physical inactivity. Quantifying the prevalence of diabetes and the number of people affected by diabetes, now and in the future, is important to allow rational planning and allocation of resources. Estimates of current and future diabetes prevalence have been published previously (Amos *et al*., 2010). The "top three" countries are the same as those identified were (India, China, and U.S.). Bangladesh, Brazil, Indonesia, Japan, and Pakistan also appear in the lists for both 2000 and 2030. The Russian Federation and Italy appear in the list for 2000 but are replaced by the Philippines and Egypt for 2030, reflecting anticipated changes in the population size and structure in these countries between the two time periods. It was estimated that there were 151 million people with diabetes in this subpopulation in 2000, despite methodological differences, this was similar to the present estimate for a

comparable population of 147 million. The IDF has subsequently released estimates of the numbers of people with diabetes for 2003 and forecasts for 2025 of 194 million and 334 million, respectively (International Diabetes Federation, 2003). Even if the prevalence of obesity remains stable until 2030, which seems unlikely, it is anticipated that the number of people with diabetes will more than double as a consequence of population aging and urbanization. In the light of the observed increase in prevalence of obesity in many countries of the world and the importance of obesity as a risk factor for diabetes, the number of cases of diabetes in 2030 may be considerably higher than stated here. Increasing evidence of effective interventions, including changes in diet and physical activity or pharmacological treatment to reduce prevalence of diabetes, provides an impetus for wider introduction of preventive approaches (Tuomilehto *et al*., 2001).

1.2.1.2.1 Prevalence according to W.H.O

World Health Organization reported in 1998 that between 1995 and 2025 the number of the adult population affected by diabetes mellitus in the developing countries is expected to increase by 170% from 84 to 228 million (Global burden of diabetes 2005). In a recent report, the total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild *et al*., 2004). In Saudi Arabia prevalence of DM is 23.7% with maleshaving higher prevalence than females. Diabetes mellitus is more prevalent among Saudis living in urban areas as compared to rural areas (Al-Nozha *et al.,* 2004). The prevalence of diabetes and glucose intolerance is also extremely high among adult Arab Americans (32.2 %) in Michigan and represents a major clinical and public health problem. Both excess body fat and physical inactivity predispose to type 2 (Jaber *et al*., 2003).

1.2.1.2.2 Epidemiology of DM in Sudan

The prevalence of DM in the Sudan, as in many other low-income countries, is increasing to epidemic proportions, leading to the emergence of a public health problems of major socio-economic impact. Before 1989 all knowledge about DM in the Sudanese population was based on a few hospital-based studies, but later a series of investigations explored epidemiology and characteristics of the disease in collaboration with Uppsala University, Sweden (Abdelgadir *et al*., 2004).

1.2.1.2.2.1 DM Type 1in Sudan

Prevalence of type 1 DM was estimated at 0.1 % among school children7-14 years of age. (Abdelgadir *et al*., 2004). The natural history of DM in the Sudanese population is strongly linked to obesity, approximately 75% of the diabetic patients have type 2 diabetes mellitus, and 40% of them were obese and have a family history of diabetes (Elmahadi ,1998).

DM in this population often had a poor glycaemic control, with a high prevalence of acute and chronic complications and a low quality of life. May due to limited resources, most of the patients (51%) had reduced or abandoned insulin therapy due to non-availability or non affordability of this drug. Most patients did not receive a satisfactory diabetes care and education, leading to lower rate of clinic attendance (55%), and dietary non-compliance (78.5%). In Sudan, a public health approach should be the cornerstone in providing an acceptable diabetes care. The formulation of a national diabetes management program is needed, with an objective also of working towards prevention of this rapidly increasing disease. Further, public health education is needed regarding the symptoms of the disease and risk factors for its development, so that those who have the disease can present early (Abdelgadir *et al*., 2004).

Community-based programmes for optimal diabetes management should be based on diabetes centers, which should be an integral part of the healthcare delivery

system from the primary care at the local governmental level, to the tertiary centers for the management of the most serious complications. Social support is essential to reduce social and psychological problems, often present in patients and their relatives, in order to cope with the burden of diabetes, its treatment and social consequences. A few studies have been carried out in Sudan focused on DM risk factors such as obesity and glycaemic control. However, the knowledge base is still weak, and financial resources are needed for research as well as for prevention and man (Abdelgadir *et al*., 2004).

1.2.1.2.2.2 DM Type 2 in Sudan

Recent information indicates that type 2 DM is common among the adult population of northern Sudan. The prevalence is estimated to be 3.4% over25 year of age. It was found to be 5.5% in the Northern State and 8.6% in Khartoum State and lowest in the western desert-like parts, 0.9%. The prevalence was higher in a certain communities in the Northern State, particularly the Danagla tribe where it reached 10.4%. However, no significant urban/rural difference was observed (Abdelgadir *et al*., 2004).

1.2.1.3 Causes of DM

Causes of DM is that body tissues receptors not respond to insulin (Even when insulin level are normal) this form is uncommon. Genetic mutation (autosomal or mitochondrial) can lead to defect to beta cell function. Abnormal insulin action may also have been genetically determined in some cases. Any disease that causes extensive damage to pancreases may lead to diabetes, also some drugs impaired insulin secretion by damage pancreases beta cell (Lawrence *et al*., 2008).

Table 1.1

Comparison between type1 and type2 diabetes (Lwerance *et al*., 2008).

Feature	Type 1diabetes	Type 2 diabetes
Onset	Sudden	Gradual
Age of onset	In young	In adult
Body habit	Thin or normal	Obese
Ketoacidosis	Common	Rare
Auto antibodies	Usually	Absence
Endoenous insulin	Low or absence	Normal or decrease
Prevalence	Less prevalence	More prevalence

1.2.1.4 Signs and symptoms

The classical symptoms of DM are polyuria, polydipasia and polyphagia .Prolonged high blood glucose causes glucose absorption, whish lead to change in the shape of lenses of eyes, resulting in vision changes: Blurred vision is a common complain leading to diabetes diagnosis type 1 case of rapid vision change, but in type 2 it is more graduate (Cooke and Dlotnic,. 2008).

1.2.1.5 Pathophysiology

Insulin is a principle control signal for conversion of glucose to glycogen for internal storage in liver and muscle cells. Lowered glucose level result in both reduction of insulin release from beta cells and reverse in conversion of glycogen to glucose when glucose level fall. This mainly controlled by glucagon hormone which acts in opposite manner to insulin. Glucose produced from internal liver cells stores (as glycogen) re –enter the blood stream, muscle cell lack the necessary export mechanism. Normally liver cells do this when the level of the insulin is low

.Insufficient insulin or cells respond poorly to insulin (insulin insensitivity or resistance) or defect in insulin, glucose will not be absorbed properly by those body cells that require it nor it is stored appropriately in the liver and muscles. The net effect is a persistent high level of blood glucose. Poor protein synthesis and other metabolic derangement, such as acidosis (Cooke and Dltonic, 2008).

Table 1.2.

Diabetes criteria (WHO, 2006):

Condition	**2hours glucose**	**Fasting glucose**
Normal	<140mg/dl	<110mg/dl
Impaired Fasting Glucose	<140mg/dl	<120mg/dl
Impaired Glucose Tolerance	>140mg/dl	<126mg/dl
Diabetes Mellitus	>200mg/dl	>126mgdl

1.2.1.6 Diagnosis of DM

DM is characterized by recurrent or persistent hyperglycemia and demonstrates by the following: Fasting blood glucose level >120mg/dl. 2hours after 75gram oral glucose load in tolerance test > 200mg/dl. Positive results are confirmed by repeated above method several days of absence of hyperglycemia (Handelman, 2009). It is preferable to measure a fasting blood glucose level because of ease of measurement and considerable time commitment of formal glucose tolerance testing, which take two hours to complete and offers prognostic advantage over the fasting test. According to current definition, two fasting glucose measurement above 125mg/dl is considered diagnostic for diabetes mellitus (Santaguda *et al.*, 2006).

Individual with fasting glucose level from 100 -125mg/dl are considered to have impaired fasting glucose. Patients with plasma above 140mg/dl, but not over 200mg/dl, 2hours after 75gram oral glucose load are considered to have impaired

glucose tolerance. Of these two pre-diabetic states, the later in particular is major risk factor for progression to full blown DM. (Nathan *et al.*, 2005).

1.2.1.7 Complications

1.2.1.7.1 Acute complications

Severely elevated blood glucose level due to lack of insulin or relative deficiency of it which leads to:

In type 1: occur either glucose urea whish result in loss of fluid and electrolytes in urine or causes to inability to store fat and protein along with breakdown of existing fats and proteins stored which result in ketosis and release of ketoses in the blood which turn into acidic which called DKA (Diabetes Keto Acidosis), have many symptom such as nausea, vomiting and abdominal pain, which can be developed to shock, coma and event death.

In type 2: stress and medications (e.g. corticosteroids), this elevation can lead to hyperosmolar state (Increase blood osmalarity), whish lead to hyperosmlar coma which occurs in elderly patients. Abnormally low blood glucose level due to too mush insulin or other glucose lowering medications can lead to central nervous system (CNS) symptoms, such as confusion, dizziness, weakness and termers in case of glucose is less than 65mg/dl occurred irreversible brain damage (Nathan *et al*. ,2005).

1.2.1.7.1.1 Diabetic ketoacidosis

Diabetic ketoacidosis (DKA), the most common endocrinal emergency remains a life-threatening condition despite improvements in diabetes care (Savage,*et al*,.2011). The mortality and morbidity rates remain high worldwide, especially in developing countries and among non-hospitalized patients, which highlight the importance of early diagnosis and implementation of effective preventive and management strategies. The adage "The child is not a miniature adult" is most appropriate when considering DKA. The fundamental pathophysiology of DKA is

the same in children as in adults; however, the child differs from the adult in a number of characteristics which raise some important considerations in management (American Diabetes Association, 2006).

The purpose of this chapter is to briefly review the pathophysiology of DKA and discuss recommended treatment protocols and current standards of care pertaining to children, adolescents and adults with type 1 or 2 diabetes presenting with DKA. The information provided is based on evidence from published studies and internationally accepted guidelines whenever possible and, when not, supported by expert opinion or consensus (Savage *et al*., 2011).

1.2.1.7.1.1.1 Definition of diabetic ketoacidosis

The biochemical criteria for DKA include the following triad (Dunger *et al*.,2004)

Hyperglycemia (blood glucose more than 11 mmol/L (200 mg/dL])

Venous pH less than 7.3 and/or bicarbonate less than 15 mmol/L

Ketonemia and ketonuria

1.2.1.7.1.1.2 Pathophysiology of diabetic ketoacidosis

Diabetic ketoacidosis (DKA) results from absolute or relative deficiency of circulating insulin and the combined effects of increased levels of the counter regulatory hormones: catecholamines, glucagon, cortisol and growth hormone (Kitabchi *et al*., 2006).

Absolute insulin deficiency occurs in the following conditions:

Undiagnosed type 1 diabetes mellitus (T1DM); DKA is reported be the first presentation in about 25% of cases especially in those less than 5 years old (American Diabetes Association, 2006).

Relative insulin deficiency, on the other hand, occurs when the concentrations of counter regulatory hormones increase in response to stress in conditions such as: **s**epsis, trauma or gastrointestinal illness with diarrhea and vomiting.

The combination of low serum insulin and high counterregulatory hormone concentrations results in an accelerated catabolic state with increased glucose production by the liver and kidney (via glycogenolysis and gluconeogenesis), impaired peripheral glucose utilization resulting in hyperglycemia and hyperosmolality, and increased lipolysis and ketogenesis, causing ketonemia and metabolic acidosis (Dunger *et al*., 2004). Hyperglycemia and hyperketonemia cause osmotic diuresis, dehydration, and electrolyte loss. This stimulates stress hormone production, which induces insulin resistance and leads to a vicious circle, worsening the hyperglycemia and hyperketonemia. Fatal dehydration and metabolic acidosis will ensue if management is not initiated. Poor tissue perfusion or sepsis may lead to lactic acidosis which can aggravate the ketoacidosis (Kitabchi *et al*., 2006).

1.2.1.7.1.1.3 Frequency of diabetic ketoacidosis

Type 2 diabetes mellitus (T2DM), associated with increased rates and severity of obesity, and may account for as much as one half of newly diagnosed diabetes in those aged 10 to 21 years, depending on the socioeconomic and ethnic composition of the population. Acute decompensation with DKA has been recognized to occur at the time of diagnosis in as many as 25% of children with type 2 diabetes mellitus (T2DM) (Hathout *et al*.,2001).

In children with established diabetes (recurrent DKA) (Dunger ,*et al*,.2004)

The risk of DKA in established T1DM is 1–10% per patient per year

Risk is increased in the following conditions (Rewers *t al*.,2002): Poor metabolic control or previous episodes of DKA, peripubertal and adolescent girls. Psychiatric disorders, including those with eating disorders, difficult or unstable family circumstances, omission of insulin, limited access to medical services and insulin pump therapy.

1.2.1.7.1.1.4 Diagnosis of diabetic ketoacidosis

Although DKA is defined by the biochemical triad of ketonemia, hyperglycemia and acidemia, several exceptions do exist which may provide a diagnostic dilemma for the physician in the emergency room. Examples of such are:

"Euglycemic ketoacidosis'': Partially treated children and children who have consumed little or no carbohydrate may present rarely with mildly increased blood glucose concentrations and absent or mild metabolic acidosis, ketonemia and ketonuria: This may occur in the Hyperglycemic Hyperosmolar State (HHS) or if the patient experiences severe vomiting which may lead to alkalosis which can mask the present acidosis. Hyperglycemic hyperosmolar state (HHS), also referred to as hyperosmolar nonketotic coma, may occur in young patients withT2DM, but rarely in T1DM subjects.

The criteria for HHS include;

Plasma glucose concentration >33.3 mmol/L (600 mg/dL), arterial pH >7.30, serum bicarbonate >15 mmol/L, small ketonuria, absent to mild ketonemia, effective serum osmolality >320 mOsm/kg and stupor or coma.

It is important to recognize that overlap between the characteristic features of HHS and DKA may occur. Some patients with HHS, especially when there is very severe dehydration, have mild or moderate acidosis. Conversely, some children with T1DM may have features of HHS (severe hyperglycemia) if high carbohydrate containing beverages have been used to quench thirst and replace urinary losses prior to diagnosis (Canarie *et al*.,2007).

1.2.1.7.2 Chronic complications

1.2.1.7.2.1 Micro vascular complications

1.2.1.7.2.1.1 Retinopathy

Diabetic retinopathy is the leading cause of blindness in working-age individuals. There is increasing evidence that established risk factors for diabetic retinopathy

including duration of diabetes, hyperglycemia, and hypertension, only explain a limited amount of the variance in the risk of diabetic retinopathy **(**Mohammed *et al*., 2007).

1.2.1.7.2.1.2 Nephropathy

Diabetes mellitus is the most frequent cause of chronic kidney failure in both, developed and non developing countries (Reutens *et al*., 2008).

There are several risk factors for the development of diabetic nephropathy. They can be divided into those that cannot be altered (genetic factors, age, and race) and those that can and must be changed (hyperglycemia, hypertension, dyslipidemia, and GFR (Tap *et al*., 2004).

Diabetic nephropathy, also known as Kimmelstiel-Wilson syndrome or nodular diabetic glomerulosclerosis or intercapillary glomerulonephritis,is a clinical syndrome characterized by albuminuria (>300 mg/day or >200 mcg/min) confirmed on at least two occasions 3-6 months apart, permanent and irreversible decrease in glomerular filtration rate (GFR) and arterial hypertension (Adler *et al*., 2003).

There is a high prevalence rate of hypertension in patients with type 1 DM (40%) and type 2 DM (70%), even before albuminuria can be found. Evidence from several large clinical studies indicates a causal relationship between the increased arterial pressure and diabetic nephropathy (Patel, 2007).

Moreover, at least three factors have been shown to contribute to the development of increased arterial pressure in this metabolic disorder including hyperinsulinemia, excessive extracellular fluid volume, and increased arterial rigidity. Hyperinsulinemia contributes to the development of increased arterial pressure via insulin resistance in type 2 DM or via administration of insulin per *se*. Randeree et al. study in 80 patients with type 2 DM wh started treatment with

exogenous insulin showed an increase in their blood pressure from 132/81 mm Hg to 149/89 mm Hg (Randeree *et al*., 1992).

Strict blood pressure control is important in the prevention of progress of diabetic nephropathy and other complications in patients with type 2 DM. The optimum lower range of systolic blood pressure is not clearly defined. According to the UKPDS study, a reduction in systolic blood pressure by 10 mm Hg decreases the risk of development of diabetic complications by 12%; the risk is the lowest where systolic blood pressure values are below 120 mm Hg (Fioretto *et al*., 2006).

The association between diabetic nephropathy and retinopathy;

Retinopathy has easily recognizable clinical manifestations and always precedes the clinically manifest signs of nephropathy in the same patient. The vice versa is not the case. A small number of patients with advanced retinopathy have glomerular histological changes and microalbuminuria, but most have no biopsy evidence of kidney disease (Chavers *et al*.,1994).

The association between diabetic nephropathy and retinopathy is weaker in patients with type 2 DM. In a study carried out by Parving in 35 patients with type 2 DM and proteinuria (> 300 mg/day), 27 of these patients had biopsy evidence of nephropathy (Parving *et al*.,1992). Diabetic retinopathy was present in 15 of these 27 patients and in none of the eight patients without diabetic nephropathy. Further analysis showed that approximately one-third of patients without retinopathy had no biopsy evidence of diabetic nephropathy (Christensen *et al*., 2000). Thus, patients with type 2 DM and significant proteinuria and retinopathy were most likely to develop diabetic nephropathy, whereas those with proteinuria but without retinopathy had a greater likelihood of having an underlying non-diabetic kidney disease (Huang et *al*., 2007).

1.2.1.7.2.1.3 Neuropathy

.The most common symptomatic complication is diabetic neuropathy, found in 50 percent of patients with diabetes over the age of 60 (Corriveau *et al*., 2008).

Polyneuropathy is a common problem for people with diabetes, leading to pain and impaired sensation and movement in the limbs, which in turn can cause leg ulcers. (Chalk *et al*., 2007). Diabetic neuropathy plays a significant role in falling among elderly patients (Maurer *et al*., 2005).

Postural sway in these patients is increased, especially with the eyes closed (Ahmmed and Mackenzie, 2003). Peripheral neuropathy caused by diabetes causes significantly impaired sensation in the feet, reducing patients' ability to control their balance properly during daily activities (Boucher *et al*., 1995). Poor balance can be due to proprioception impairment (Akbari *et al*., 2006). Balance problems are also caused by movement-strategy impairment, biomechanical structural disorders, and disorientation (De Oliveira,*et al*,.2008).

In the elderly, slips or falls are one of the most common causes of injury or death (Kim and Robinson, 2006). People with diabetic neuropathy have balance disorders even with open eyes, making them vulnerable to falls.(Speers *et al*., 2002). Proper postural control depends on the spatiotemporal coordination of information received from the vestibular, vision, and somatosensory systems. Damage to one or more of these systems increases the prevalence of falling in older people (Priplata *et al*., 2004). Patients with diabetic neuropathy had weaker stability measures in all balance indices compared with nondisabled subjects. They also demonstrated that using visual inputs can improve balance among these patients(Aly *et al*., 2008).

Vibration and passive tactile cues have been used to activate the sensory afferent system to improve balance in diabetic patients (Menz *et al*., 2006). Aerobic exercise is also effective in reducing the risk or severity of peripheral neuropathy

in patients(Balducci *et al*., 2006). Exercise therapy, including balance exercises, leads to increased oxygen pressure in the lower limbs, skin, and chests of diabetic patients, improving skin blood flow (Williams *et al*., 2007). Group exercise therapy is effective in improving balance in older people and reduces the risk of falling [Robitaille *et al*.,2005).

Any changes in shear stress and pressure on the soles of the feet during standing tasks can stimulate mechano-receptors to the higher nervous centers, which leads to increased balance ability in patients with diabetic neuropathy (Hijmans *et al*., 2008).

1.2.1.7.2.2 Macrovascular complications

1.2.1.7.2.2 .1Thrombosis and Hypercoagulability

Persons with type I or type II diabetes are at increased risk of arterial thrombosis, manifested as myocardial infarction and coronary artery disease (Tehrani *et al.*,2010). The causes are multi factorial, and may involve regulation of tissue factor expression and plasminogen activator inhibitor-1 (particularly in patients with type II diabetes) (Westrick and Eitzman, 2007), but part of this risk may be attributable to increased platelet activity. There is increasing evidence that platelets are hyper activated in patients with both type I and type II diabetes, and platelet hyperactivity may therefore play a contributory role in the increased risk of arterial thrombosis associated with diabetes (Tehrani *et al.*,2010). In addition, diabetic patients frequently show resistance to conventional antiplatelet drugs used as antithrombotic therapies, such as aspirin and clopidogrel . Therefore, finding novel means to reduce cardiovascular risk that will maintain efficacy in diabetic populations is an important area of research. However, the mechanisms by which hyperglycemia or other diabetic sequelae contribute to arterial thrombosis remain poorly understood (Anfossi *et al* .,2008).

Diabetes mellitus is considered to be a hypercoagulable state and is recognized as a risk factor for arterial thromboembolism in coronary, cerebral, mesenteric and peripheral vessels (Carr, 2001). However, venous thromboembolism has rarely been reported a complication of diabetic ketoacidosis or a hyperosmolar hyperglycemic state (Shujaat and Shapiro, 2004). Upper extremity venous thrombosis accounts for only 4% of cases of venous thrombosis; symptomatic cases are uncommon, accounting only for 0.15% of upper extremity venous thromboses, including catheter-related thrombosis (Mustafa *et al*., 2003).

Venous thromboembolism shares many risk factors with atherosclerotic cardiovascular disease, including obesity, hypertension, dyslipidemia, smoking, and diabetes (Piazza and Goldhaber, 2010). The majority of epidemiological studies demonstrate an increased risk of deep vein thrombosis and pulmonary embolism among diabetic patients (Ageno *et al*., 2008).

Independent of platelet dysfunction, diabetes induces a hypercoagulable state. Increased levels of PAI-1 decrease fibrinolytic activity and tissue factor, as well as factors VII and XIII are increased. There is also a relative decrease in antithrombin III and protein C. Many of these abnormalities also correlate with the presence of hyperglycemia and pro insulin split products. Von Willebrand’s factor and factor VIII are also both increased, possibly due to endothelial dysfunction (Beckman *et al*., 2002).

Cardiovascular disease (CVD) is the leading cause of death and disability in developed nations and is increasing rapidly in the developing world, it is estimated that CVD will surpass infectious disease as the World's leading cause of death and disability. Acute coronary syndromes occur when an unstable plaque ruptures and activates coagulation at the site, blocking blood flow and causing ischemic injury to the heart. Thrombogenesis is the final process whereby the exposed tissue factor

triggers the activation of coagulation and the freshly formed clot fills the coronary artery lumen (Shitrit *et al*., 2002).

The hemostatic system may play an important role in the initiation and progression of atherosclerosis. Various hemostatic proteins, such as fibrinogen and von Willebrand factor, have been found to be independently associated with future coronary events in healthy individuals as well as in patients with documented atherosclerosis (Koenig, 2001)**.**

1.2.1.7.2.2.2 Atherosclerosis

Patients with DM have an over tenfold risk for cardiovascular disease in their lifetime. Cardiovascular disease is one of the most morbid complications of DM with men and women being equally at risk, essentially eliminating the protection against cardiovascular disease characteristic of premenopausal women. DM predisposes to higher rates of coronary artery disease (CAD), cerebral vascular disease, and peripheral arterial disease (PAD). Aggressive blood sugar control has been shown to decrease some cardiovascular sequels in diabetics, particularly in type I DM; however it does not eliminate all risk and intensive glycemic control for type II diabetics has not proven to be beneficial and may even be detrimental (Nathan *et al*., 2005).

CAD is the most morbid cardiovascular complication of DM with a two- to fourfold increased risk (Beckman *et al*., 2002). Compared to cardiovascular disease in non diabetics, diabetic patients have a greater overall coronary plaque burden and a higher rate of multi vessel disease. The proportion of stenotic segments is directly proportional to the duration of disease (Gao *et al*., 2011) . In combination, these factors place diabetic patients at greater risk for myocardial infarction (MI). In fact, diabetics without a prior MI are at equal risk for MI as non diabetics with a prior MI. After MI, complications and death are higher in DM. The increased risk also extends to those undergoing cardiac procedures. After per cutaneous coronary

intervention (PCI), diabetic patients are at both higher risk for death and need for re intervention (Mathew, *et al*,. 2004). Diabetic patients who undergo coronary artery bypass grafting (CABG) are at higher risk for both complications and death, particularly in those with insulin-dependent type II DM, with no benefit seen in those who have had tight postoperative glycemic control (Kubal, *et al*,.2005). Similarly to CAD, DM also carries a two- to fourfold increased risk of PAD. The distribution of lower extremity lesions in DM shows a higher propensity of atherosclerotic disease in the deep femoral artery, as well as in all vessels below the knee. Not surprisingly, DM is the leading risk factor for on-traumatic lower extremity amputations and diabetic patients have a higher frequency of infra geniculate arterial interventions (Beckman *et al*.,2002). The development of diabetes-related atherosclerosis follows the same histological course as atherosclerosis in non diabetic patients. This includes endothelial injury, smooth muscle cell proliferation, foam cell development and in filtration, platelet activation, and increased inflammation. Sites of lesions are determined by altered hemodynamic forces and external sources of injury to the endothelial cells. Increased endothelial permeability leads to the retention of deleterious low-density lipoproteins (LDL) that interact with the underlying extracellular matrix (ECM). This interaction retains the LDL in the vessel wall where it can undergo oxidation by reactive oxygen species (ROS). This oxidized LDL can then stimulate the overlying endothelial cells to up regulate cellular adhesion molecules, chemotactic proteins, growth factors, and inhibit nitric oxide (NO) production. These activities recruit monocytes and macrophages, which interact with highly oxidized aggregated LDL to form foam cells. Pro-inflammatory cytokine production by activated macrophages stimulates proliferation of vascular smooth muscle cells. Intimal smooth muscle cells subsequently produce an ECM that gives rise to a fibrous cap. The resulting complex plaque is vulnerable to destabilization, rupture,

and superimposed thrombosis leading to an acute vascular occlusio (Lusis, 2000). Atherosclerotic plaques in the presence of diabetes generally have increased calcification, necrotic cores, receptors for advanced glycosylation end products (RAGE), and macrophage and T-cell infiltration. There is also a higher incidence of healed plaque ruptures and vascular remodeling (Virmani *et al*., 2006).

Hyperglycemia increases the production of reactive oxygen species as a consequence of mitochondrial dysfunction, which in turn promotes atherosclerotic lesion formation by up regulation of protein kinase C, activation of the hexosamine and polylol pathways, and accumulation of advanced glycation end products with up regulation of RAGE receptors. In many tissues, glucose uptake is mediated by insulin independent glucose transporters Therefore, a rise in intracellular glucose concentrations parallels serum levels (Giacco and Brownlee, 2010).

Obesity and a sedentary lifestyle are both predisposing factors for the development of insulin resistance and type II DM. In insulin resistance, there is an inadequate response by fat, muscle, and liver cells to insulin stimulation. Independent of hyperglycemia, insulin resistance is a risk factor for atherosclerosis. Insulin resistance in the type II diabetic patient is characterized by decreased insulin production, central stimulation for increased oral intake, and increased gluconeogenesis in the liver, decreased uptake by peripheral tissues, and increased lipolysis of adipocytes leading to increased non esterified fatty acid secretion (Kahn *et al*., 2006). NEFA can be deposited in and cause dysfunction of skeletal muscle, liver, and pancreatic b cells, all of which contributes to insulin resistance Adipose tissue contributes to insulin resistance by releasing NEFA and pro-in fl amatory cytokines, such as tumor necrosis factor- a (TNF- a), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1). Decreased skeletal muscle uptake of glucose is a side effect of insulin resistance and a significant contributor to hyperglycemia in type II DM. Increased intracellular NEFA in skeletal muscle is

deleterious by competing with glucose for substrate oxidation and by increasing the intracellular content of fatty acid metabolites such as DAG, fatty acylcoenzyme A (fatty acetyl-CoA), and ceramide. In turn, these can activate a serine/threonine kinase cascade leading to serine/threonine phosphorylation of insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2), reducing the ability of these receptors to undergo tyrosine phosphorylation and propagate the normal insulin signal. The downstream target of these receptors is phosphatidylinositol 3-kinase (PI3), which normally mediates insulin's physiologic anti inflammatory signal by decreasing NF- k b activation, ROS formation, expression of adhesion molecules, and increasing NOS production (Muntoni and Muntoni, 2011). PI3 blockade is also associated with decreased uptake of glucose and increased gluconeogenesis in the liver. DAG also has deleterious downstream affects through PKC production. The alternate pathway to PI3, mitogen-activated protein kinase (MAP-kinase), is hyper stimulated when the PI3 pathway is blocked by a compensatory increase in insulin production. This alters insulin's effect from anti-atherogenic to proatherogenic, as unopposed stimulation of the MAP-kinase pathway contributes to vascular hypertrophy, hypertension, increased PAI-1 production, and arrhythmias (Montagnani *et al*.,2002). The presence of increased adipose tissue is an important contributor to insulin resistance; however it is the distribution of this body fat that plays a key role in determining insulin sensitivity as intra abdominal visceral fat puts one at much higher risk. Even in lean individuals, body fat distribution can markedly affect the degree of insulin resistance if there is increased visceral intra abdominal fat. Mesenteric fat, more than peripheral fat, interferes with insulin's ability to suppress lipolysis leading to higher NEFA production (McLaughlin *et al*., 2011). These mesenteric adipocytes tend to be larger and contribute to a pro inflammatory environment in DM type II by increasing interferon- g (IFN g) expression, macrophage attraction, and up

regulation of MCP-1 and NF- k b (Muntoni and Muntoni, 2011). Differences in adipocytes, combined with the proximity of the liver to intra abdominal fat, result in greater exposure to NEFAs in liver than in peripheral tissues. In fact, the liver can be insulin resistant at a time when the peripheral tissues are not. Increased delivery of NEFA to the liver also increases gluconeogenesis, as well as production of very low-density lipoprotein (VLDL) (Ginsberg, 2000). Beta cells, which contain insulin, are also affected by insulin resistance as they are unable to fully compensate for impaired insulin uptake. b -cell dysfunction can exist in obese individuals with high central fat even in the presence of normoglycemia. The beta -cell is unable to produce insulin rapidly enough in response to high glucose levels. Insulin resistance further contributes to this effect by NEFA inhibiting insulin mRNA expression and insulin secretion. There are also half as many b cells in type II DM due to hyperglycemia-related toxicity. Impaired insulin secretion leads to decreased signaling in the hypothalamus, leading to increased food intake and weight gain, decreased inhibition of hepatic glucose production, reduced efficiency of glucose uptake in muscle, and increased lipolysis of mesenteric adipocytes with higher NEFA levels. An increase in body weight and NEFA production further contributes to insulin resistance (Kahn *et al*., 2006).

1.2.1.7.2.2.3 Foot gangrene

Venous thrombosis in diabetes has been described in the portal and cerebral vein (Schweigart *et al.*, 2008).Although our patient had peripheral arterial disease which is a major etiological factor for diabetic foot gangrene, deep venous thrombosis (DVT) was the identified precipitating cause for the foot gangrene. Left foot gangrene in association with left calf swelling occurred after admission into hospital. This is the first report of diabetic foot gangrene precipitated by DVT. Foot gangrene likely resulted from further compromise of an already precarious distal lower limb arterial circulation with the occurrence of the DVT. Deep venous

thrombosis will lead to a rise in venous pressure distal to the site of thrombosis while peripheral arterial disease which was already present results in reduced lower limb arterial pressures. Foot gangrene is the likely consequence of a further decline in capillary perfusion pressure from the raised left lower limb venous pressure in the face of existing lower limb arterial pressures. Peripheral arterial disease at presentation as a cause of limb pains and reduced distal lower limb pulsations was confounded by the presence of peripheral neuropathy and dehydration. Although limb coldness is consistent with arterial disease, the finding of calf swelling meant that co-morbidities such as venous thrombosis, cellulitis, necrotizing fasciitis, abscess and ruptured popliteal cyst needed to be excluded. Diabetic foot gangrene in association with limb swelling is typically associated with cellulitis and necrotizing fasciitis. These were however, unlikely in our patient as the left foot and calf were not warm as may be expected with an infection. Ultrasonography apart from confirming venous thrombosis also excluded cystic conditions like abscess and popliteal cyst. The other techniques for the diagnosis of DVT include impedance plethysmography and contrast venography(Alexander *et al.,* 2002). Advanced age, dehydration and immobilization are factors that may have contributed to the development of DVT in our patient.Although our patient was a male with long standing diabetes and hypertension, and had all risk factors for PAD (Shammas, 2007), the occurrence of gangrene and subsequent death may have been prevented if there had been regular monitoring for PAD. Peripheral arterial disease is objectively assessed by determining the ankle-brachial pressure-index (ABPI), with an ABPI < 0.4 indicating critical limb ischaemia.(Collins *et al*.,2007) Electively performed salvage revascularization procedures guided by arteriography may prevent the occurrence of gangrene in critically ischaemic limbs(Akbari *et al*.,2006) At the occurrence of gangrene, tissue viability can

further be determined by measuring transcutaneous oxygen tension.(Carter and Tate, 2006).

Deep vein thrombosis should be excluded in diabetic foot gangrene associated with calf swelling particularly where features of local sepsis are lacking. Foot gangrene associated with deep vein thrombosis in diabetes mellitus may be avoided by early detection and treatment of peripheral vascular disease, as well as appropriate thrombo prophylaxis during hospitalization or extended periods of immobility

1.2.1.7.2.2.4 Cardiovascular diseases

The conditions, i.e., impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and overt diabetes, appear to be associated with an increased risk of CVD to a variable degree. Recently, IFG and IGT have been officially termed "pre-diabetes." Both categories, IFG and IGT, are risk factors for future diabetes and CVD (American Diabetes Association 2006).

1.2.1.8 Management of DM

DM is a chronic disease which is difficult to cure. Management concentrates on keeping blood glucose level as close to normal (Euglycemia) as possible without presenting patients danger. This can usually be with close dietary management, exercise and using of appropriate medications (Insulin only type 1 DM).Oral medications used on type 2. Patient's education, understanding and participation are vital since the complications of diabetes are less common and less severs in people who have well managed blood glucose level. Wider health problem may accelerate the deleterious effect of diabetes. This includes smoking, elevated cholesterol level, obesity, high blood pressure and lack of regular exercise (Pignon *et al.,* 2010).

1.2.2 Hypertension

Blood pressure itself is the pressure exerted by the blood on the walls of the blood vessels. Each time the heart beats (about 60-70 times a minute at rest), it pumps

blood into the arteries. Blood pressure is at its highest when the heart beats, pumping the blood. This is called systolic blood pressure. When the heart is at rest, between beats, blood pressure falls. This is diastolic pressure. Blood pressure follows a circadian rhythm in a normal individual. Blood pressure falls during sleep and rises rapidly just before we wake, which is when the risk of cardiovascular events is the highest (Pickering, 2005).

Normal blood pressure: is defined as level: 120/80 mmHg, Pre hypertension: is systolic blood pressure of 120 – 139 and or diastolic blood pressure 80 – 89 mmHg. This group of patients is at increased risk for progression to hypertension and has significantly greater risk to develop future cardiovascular events than those with normal blood pressure. Therefore, they should be identified and managed separately. Clustering of cardiovascular risk factors (e.g., diabetes, dyslipideamia, obesity, and impaired glucose tolerance) is more prevalent in this group than in individuals with normal blood pressure. Isolated systolic pressure is defined as high systolic pressure with normal diastolic pressure (W.H.O, 2005)

1.2.2.1 Guidelines of hypertension

Hypertension is one of the conditions for which disease-specific guidelines have been generated by different organizations:

1.2.2.1.1 **World Health Organization Guidelines** W.H.O (2003).

Table1.3 World Health Organization Guidelines

W.H.O/I.S.H	**Systolic B.P**	**Diastolic B.P**
Optimal	<120	< 80
Normal	<130	<85
High Normal	130-139	85-89
Stage 1(mild hypertension) subgroup borderline	140-159 140-159	90-99 90-94
Stage2(moderate hypertension)	160-179	100-109
Stage 3(sever hypertension)	>180	110
Isolate systolic hypertension Subgroup boarder line	>140 140-149	<90 <90

1.2.2.1.2 Joint National Committee Guideline (JNC, 2003)

Table1.4 Joint National Committee Guideline

E.SH./E.S.C //W.H.O/I.S.H,2003	**Systolic B.P**	**Diastolic B.P**
Normal	<120	<80
High Normal	130-139	85-89
Stage 1	140-159	90-99
Stage2	160-179	100-109
Stage 3	>180	>110
JNC: Joint National Committee on Prevention, Detection ,Evaluation and Treatment of High Blood Pressure		

According to the guidelines issued by the World Health Organization–International Society of Hypertension (WHO–ISH) and the sixth report of the Joint National Committee on prevention, detection, evaluation, and treatment of high blood pressure (JNC), hypertension in adults is defined as a resting systolic blood pressure (SBP) of 140 mmHg or greater and/or a diastolic blood pressure (DBP) of 90 mmHg or greater in adults who are not taking antihypertensive medication. Patients taking antihypertensive medication may also be defined as hypertensive, even though their blood pressure may be below these cut-off points (J.N.C/W.H.O, 2003).

1.2.2.2 Epidemiology of hypertension

Hypertension is the third leading killer in the world. There are one billion hypertensive patients globally, and four million people die annually as a direct result of hypertension. In the Eastern Mediterranean Region, the prevalence of hypertension averages 26% and it affects approximately 125 million individuals (World Health Organization, 2005).

1.2.2.3 Pathophysiology

Hypertension, or high blood pressure, is a chronic and often asymptomatic medical condition in which systemic arterial blood pressure is elevated beyond normal. As such, the heart is forced to work harder to overcome the increased systemic pressure in order to deliver blood to tissues, which puts strain on the heart and arteries. Over time, the additional strain leads to cardiovascular dysfunction and is a primary contributing cause of potentially deadly sequelae such as congestive heart failure, myocardial infarction, pulmonary embolism, cerebral aneurysm and kidney failure (Pierdomenico, 2009).

1.2.2.4 Classification

Provided that the readings are taken as the mean of two or more properly measured blood pressure readings, on two or more visits.

Normal blood pressure: is defined as level _ 120/80 mmHg. Pre hypertension: is SBP of 120 – 139 and or DBP 80 – 89 mmHg. This group of patients is at increased risk for progression to hypertension and has significantly greater risk to develop future cardiovascular events than those with normal blood pressure. Therefore, they should be identified and managed separately. Clustering of cardiovascular risk factors (e.g., diabetes, dyslipideamia, obesity, and impaired glucose tolerance) is more prevalent in this group than in individuals with normal blood pressure.

Isolated systolic pressure is defined as high systolic pressure with normal diastolic pressure (World Health Organization, 2005).

1.2.2.5 Hypertension stages

Hypertension is two stages according to level of the blood pressure (JNC. 2003)

Table1.5 Hypertension Stages.

Classification	Systolic B.P	Diastolic B.P
Normal	<120	<80
Pre hypertension	130-139	80-89
Stage 1	140-159	90-99
Stage 2	>160	>100

Abnormally high blood pressure is generally divided into two main categories: essential hypertension and secondary hypertension.

1.2.2.5.1 Essential hypertension

Essential hypertension or primary hypertension is the most prevalent type, affecting between 90-95 percent of patients diagnosed with hypertension (Carretero, 2000). The cause of essential hypertension has not been directly identified, but its pathophysiology is assumed to be multifactorial. Many causal factors have been linked to essential hypertension including a sedentary lifestyle, tobacco smoking, excessive stress, visceral obesity, hypokalemia (potassium deficiency), high sodium intake and other poor dietary habits, sodium sensitivity, alcohol consumption, vitamin D deficiency, and obesity (Kyrou, 2006). In fact, more than 85 percent of patients diagnosed with essential hypertension have a body mass index (BMI) greater than (Haslam, 2005).Increased risk of essential hypertension has been associated with advancing age, inherited genetic mutations, family history of hypertension, low birth weight, insulin resistance, elevated levels of renin (a kidney hormone), and sympathetic nervous system over-activity. (Rahmouni, 2005). The sympathetic nervous system is responsible for the "flight or fight" response, which causes vasoconstriction of certain arteries and the release of adrenalin and other hormones and neurotransmitters. Chronic stress / anxiety,

overconsumption of caffeine, kidney disease, and cerebral and glandular tumors all contribute to sympathetic over stimulation. Essential hypertension can be further subcategorized as stage I or stage II (depending on progression and symptomatology), isolated systolic hypertension (where diastolic readings are considered normal), or as medication resistant hypertension if pharmaceutical intervention does not make a significant impact on blood pressure readings. Exercise-related hypertension is defined as excessively high blood pressure during intense exercise, although the normal range of systolic pressures during exercise is between 200 and 230 mmHg. Those people who exhibit exercise-related hypertension may be at risk of developing essential hypertension while at rest (Rost , 2007).

1.2.2.5.2 Secondary hypertension

The remaining 5-10 percent of hypertension case is classified as secondary hypertension. Secondary hypertension results from an identifiable cause, usually a disease that affects hormone synthesis and excretion. Secondary hypertension is approached differently than essential hypertension, in that the underlying cause of the elevated blood pressure is treated and managed. In contrast, essential hypertension is treated directly with medications that affect vasoconstriction of arteries and with lifestyle modifications. Secondary hypertension often results as a consequence of a compromise or imbalance of the hormone-dependent endocrine system, which is responsible for regulating blood volume and heart function. Common disease conditions that lead to secondary hypertension include atherosclerosis, diabetes, kidney disease, adrenal gland tumors, Cushing's syndrome, hyperthyroidism, hypothyroidism, obesity, metabolic disorders, preeclampsia during pregnancy, sleep apnea, congenital defects of the aorta and heart, alcoholism, and toxicity from prescribed and illicit drugs, especially cocaine and methamphetamines(Mayo, 2011) .

1.2.2.6 Etiology

In 90% of all cases the reason for hypertension remains unclear. This is called essential hypertension. Hypertension is generally a product of genetic predisposition with environmental and lifestyle factors (Beevers, 2001).

There is no significant difference in the development of hypertension between men and women although the prevalence of hypertension increases sharply with age, especially for women (Kearney, 2005).

1.2.2.7 Diagnosis

Blood pressure is measured in millimeters of mercury (mmHg), corresponding to the height of a column of mercury that could be supported in a mercury sphygmomanometer, a device which until recently was the standard method of measuring blood pressure. Blood pressure is now frequently measured using accurate electronic devices calibrated in mmHg as the standard unit for blood pressure measurement. The most accurate method for measuring blood pressure in the office is to listen for Korotkoff sounds in the brachial artery as cuff pressure is reduced (Pickering, 2005).

1.2.2.8 Signs and symptoms

It is important to note that mild-to-moderate essential hypertension is usually asymptomatic, which is why it is sometimes called the "silent killer." Accelerated and severe hypertension is associated with signs and symptoms that include headache, drowsiness, confusion, blurred vision, nausea and vomiting. These symptoms are collectively called hypertensive encephalopathy, which is caused by reversible small blood vessel congestion and brain swelling (Fauci, 2008). Some additional hormone-related signs that suggest hypertension is likely include abdominal obesity, fat accumulation on the upper thoracic spine ("buffalo hump") and wide purple scars on the abdomen known as striae. A hormone disorder known as Cushing's syndrome is also highly correlated with hypertension. Other causes of

secondary hypertension are accompanied by additional symptoms specific to these diseases. For example, hyperthyroidism causes hypertension, but also weight loss, tremors, heart rate abnormalities and increased sweating. Hypertension during pregnancy is one symptom of preeclampsia. Preeclampsia can progress to a life threatening condition called eclampsia, which is the development of protein in the urine, generalized swelling and severe seizures. Eclampsia occurs when substances from the placenta cause endothelial dysfunction in the maternal blood vessels of susceptible women. Kidney and liver damage can ensue, which causes the release of vasoconstrictive factors in the blood and increased blood pressure. However, a slight increase in blood pressure is often considered normal during the later stages of pregnancy because cardiac output and blood volume is increased to provide sufficient circulation in the utero-placental arterial bed (David and Jones, 2010). Severe, uncontrolled hypertension eventually leads to tissue and organ damage as noted above. Specific symptoms of organ damage includes ischemic stroke and paralysis, intracerebral hemorrhage and central nerves system insult, subarachnoid hemorrhage, vascular dementia, retinopathy, left ventricular dysfunction, myocardial infarction, angina pectoris, congestive heart failure, renal failure, albuminuria, and intermittent claudication (Fauci, 2008).

1.2.2.9 Complications of hypertension

1.2.2.9.1 Retinopathy

In the initial, vasoconstrictive stage, there is vasospasm and an increase in retinal arteriolar tone owing to local auto regulatory mechanisms. This stage is seen clinically as a generalized narrowing of the retinal arterioles. Persistently elevated blood pressure leads to intimal thickening, hyperplasia of the media wall, and hyaline degeneration in the subsequent, sclerotic, stage. This stage corresponds to more severe generalized and focal areas of arteriolar narrowing, changes in the arteriolar and venular junctions, and alterations in the arteriolar light reflex (i.e.,

widening and accentuation of the central light reflex, or "copper wiring") (Wong and Mitchell, 2004).

This is followed by an exudative stage, in which there is disruption of the blood–retina barrier, necrosis of the smooth muscles and endothelial cells, exudation of blood and lipids, and retinal ischemia. These changes are manifested in the retina as microaneurysms, hemorrhages, hard exudates, and cotton-wool spots. Swelling of the optic disk may occur at this time and usually indicates severely elevated blood pressure (i.e., malignant hypertension). Because better methods for the control of blood pressure are now available in the general population, malignant hypertension is rarely seen. In contrast, other retinal vascular complications of hypertension, such as macroaneurysms and branch-vein occlusions, are not uncommon in patients with chronically elevated blood pressure. These stages of hypertensive retinopathy however, may not be sequential (Pache *et al*.,2002).

1.2.2.9.2 Nephropathy

Renal risk appears to be more closely related to systolic than to diastolic blood pressure (Marin *et al*., 2005). Black men are at greater risk than white men for developing ESRD at every level of blood pressure (Swift and Macgregor, (2004).

The atherosclerotic, hypertension-related vascular lesions in the kidney primarily affect the preglomerular arterioles (Marín *et al*., 2005), resulting in ischemic changes in the glomeruli and postglomerular structures (Loscalzo *et al*., 2008). Glomerular injury may also be a consequence of direct damage to the glomerular capillaries due to glomerular hyperperfusion. Glomerular pathology progresses to glomerulosclerosis (Stoian *et al*., 2007). The renal tubules may also become ischemic and gradually atrophic. The renal lesion associated with malignant hypertension consists of fibrinoid necrosis of the afferent arterioles, sometimes extending into the glomerulus, and may result in focal necrosis of the glomerular tuft. Clinically, macroalbuminuria (a random urine albumin/creatinine ratio more

than 300 mg/g) or microalbuminuria (a random urine albumin/ creatinine ratio 30–300 mg/g) are early markers of renal injury. These are also risk factors for renal disease progression and for cardiovascular disease(Loscalzo *et al*., 2008).

1.2.2.9.3 Encephalopathy

Hypertension is an important risk factor for brain infarction and hemorrhage. Approximately 85% of strokes are due to infarction and the remainder are due to hemorrhage, either intracerebral hemorrhage or subarachnoid hemorrhage. The incidence of stroke rises progressively with increasing blood pressure levels, particularly systolic blood pressure in individuals more than 65 years. Treatment of hypertension convincingly decreases the incidence of both ischemic and hemorrhagic strokes (Loscalzo *et al*., 2008).

Infarct due to occlusion of a "strategic" larger vessels or multiple lacunar infarcts due to occlusive small vessel disease resulting in sub cortical white matter ischemia (Pantoni *et al*., 2009).

Cerebral blood flow remains unchanged over a wide range of arterial pressures (mean arterial pressure of 50–150 mmHg) through a process termed auto regulation of blood flow. Signs and symptoms of hypertensive encephalopathy may include severe headache, nausea and vomiting (often of a projectile nature), focal neurologic signs, and alterations in mental status. Untreated, hypertensive encephalopathy may progress to stupor, coma, seizures, and death within hours (Refain*et al*., 2008). It is important to distinguish hypertensive encephalopathy from other neurologic syndromes that may be associated with hypertension, e.g., cerebral ischemia, hemorrhagic or thrombotic stroke, seizure disorder, mass lesions, pseudo tumor cerebri, delirium tremens, meningitis, acute intermittent porphyria, traumatic or chemical injury to the brain, and uremic encephalopathy(Loscalzo *et al*,.2008).

1.2.2.9.4 Cardiomyopathy

Hypertensive heart disease is the result of structural and functional adaptations, leading to left ventricular hypertrophy, diastolic dysfunction and chronic heart failure. Abnormalities of blood flow due to atherosclerotic coronary artery disease (Steinmetz and Nickenig, 2009). Abnormalities of diastolic function, ranging from asymptomatic heart disease to overt heart failure, are common in hypertensive patients. Patients with diastolic heart failure have a preserved ejection fraction, which is a measure of systolic function. Diastolic dysfunction is an early consequence of hypertension-related heart disease and is exacerbated by left ventricular hypertrophy and ischemia (Hennersdorf and Strauer, 2007).

1.2.2.10 Complications associated to diabetes and hypertension

The chief reason why people with diabetes develop high blood pressure is hardening of the arteries. Diabetes tends to speed up the process of atherosclerosis. The other fact about diabetes is that it affects both large and small blood vessels in the body. Over time, blood vessels become clogged with fatty deposits, become non-compliant and lose their elasticity. The process of atherosclerosis is a lot faster in diabetic individuals whom do not have good control of their blood sugars. The high blood pressure eventually leads to heart failure, strokes, heart attacks, blindness, kidney failure, loss of lipids and poor circulation of blood in the legs. When the blood supply to the feet is compromised, the chance of infections and amputations also increases. All diabetics should know that even mild elevations in blood pressure can be detrimental to health. Studies have shown that diabetics with even a slight elevation in blood pressure have 2-3 times the risk of heart disease compared to individuals without diabetes (Diabetes associated to Hypertension, 2010).

Blood pressure readings do vary but experts recommend that blood pressure should not range above 140/80. Secondly, high blood pressure is a silent disease and thus

it is vital for all diabetics to regularly check their blood pressure or have it checked at a doctor's office on a regular basis. The American Diabetes Association recommends that all diabetics get their blood pressure measured by a health care professional at least 2-5 times a year (Medical *journal* of Australia, 2010).

1.2.2.11 Managements

Lifestyle modification, these include: weight control, regular exercise, and low-fat and low-sodium diet, refer to nutritionists, weight-loss programs, and exercise programs can be productive. Data regarding dietary changes in children with hypertension are limited. A no-salt-added diet with more fresh fruits and vegetables combined with low-fat dairy and protein like to the DASH (Dietary Approaches to Stop Hypertension).Exercise combined with diet in adolescents had a greater antihypertensive effect than diet alone. Pharmacologic Therapy: Introduce one medication at a time at the lowest dose, and then increase the dose until therapeutic effects are seen, side effects are seen, or the maximal dose is reached (Linda, 2004).

1.2.2.12 Epidemiology of diabetes mellitus and hypertension

The biggest increase in diabetes mellitus cases is expected in China and India. India currently, has around 40 million cases of diabetes mellitus and these numbers are projected to increase to 87 million by the year 2030 (Hasan and Khatoon, 2012). A patient who suffers from type 2 diabetes mellitus has a 2–4 times greater risk of death from cardiovascular causes than the patient without DM. The most common cause of death in the diabetic patient is heart disease. In addition, peripheral vascular disease, end-stage renal disease, blindness and amputations are common co-morbidities in diabetic patients (Ehud *et al*., 2008). .Genetic and environmental factors are also reported to play a key role in hypertension, 90% of which are better classified as idiopathic. High blood pressure in adults has a high

impact on the economy and on the quality of life of individuals with important implications for resource expenditures (Erhun *et al*., 2005).

Several studies conducted in different ethnic groups show a close association between hypertension and diabetes mellitus, where the prevalence of hypertension is significantly higher in the patients with non insulin-dependent diabetes mellitus (NIDDM or type II diabetes mellitus). Both systolic and diastolic hypertension has been reported, and conclusive evidence indicates that the link between diabetes mellitus and essential hypertension is hyperinsulinemia (Sowers *et al*., 2001). The prevalence of hypertension is 1.5–2.0 times more in those with diabetes mellitus than in those without diabetes mellitus, whereas almost one-third of the patients with hypertension develop diabetes mellitus later (Sahay, 2007). The presence of hypertension in diabetic patients substantially increases the risks of coronary heart disease, stroke, nephropathy and retinopathy. When hypertension coexists with DM, the risk of CVD is increased by 75%, which further contributes to the overall morbidity and mortality of already high risk population. Generally hypertension in type 2 diabetic persons clusters with other CVD risk factors such as micro-albuminuria, central obesity, insulin resistance, dyslipidaemia, hypercoagulation, increased inflammation and left ventricular hypertrophy (Sowers *et al*.,2001).

1.2.2.13 Relationship between hemostasis, diabetes and hypertension

Patients with diabetes mellitus have increased arterial stiffness, which develops due to the increased glycation of proteins and consequent development of arteriosclerosis. Decreased arterial elasticity in patients with glucose intolerance or diabetes mellitus contributes to the increased systolic pressure as an independent mortality risk factor (Cruickshank *et al*.,2002).

Derangements in haemostatic parameters are risk factors for the development of poly arterial disease: peripheral (PAD) and coronary artery disease (CAD). Haemostatic dysbalances have their origin in life habit (smoking), are associated

with some diseases (diabetes), or are otherwise identified as congenital coagulopathies (Clement *et al*., 2000).

Endothelial dysfunction is characterized by a change of the actions of the endothelium toward reduced vasodilatation and a pro inflammatory state. It is associated with most forms of cardiovascular disease, such as hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes and chronic renal failure. Mechanisms that participate in the reduced vasodilatation in endothelial dysfunction include reduced nitric oxide generation, oxidative excess and reduced production of hyperpolarizing factor. Up regulation of adhesion molecules, generation of chemokines such as macrophage chemoattractant peptide-1 participate in the inflammatory response state (Verma and Anderson, 2002).

1.2.3 Fibrinogen

1.2.3.1 Structure

The fibrinogen molecule is a glycoprotein containing two copies of each of three polypeptide chains (α, β, and γ) encoded by three distinct genes fibrinogen alpha, fibrinogen beta, and fibrinogen gamma located on the long arm of chromosome 4 at position q23–32.1.The fibrinogen gamma gene contains ten exons and is oriented in tandem with the fibrinogen alpha gene, which contains six exons. They are transcribed in the direction opposite the fibrinogen beta gene, which is located downstream of the fibrinogen alpha gene and contains eight exons (kant, *et al., 1*

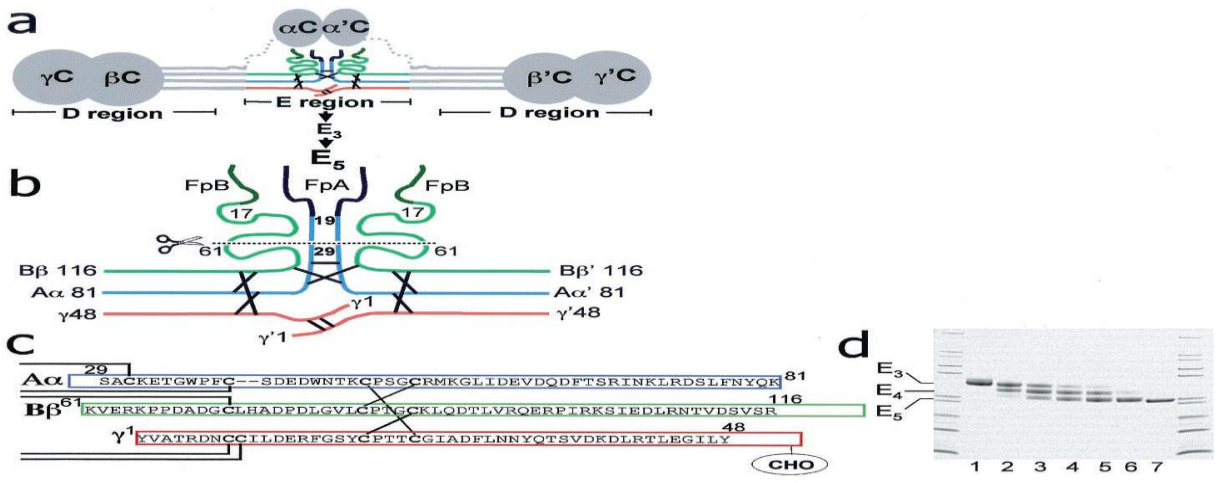

Figure 1.1 Fibrinogen structure

(*a*) Schematic diagram of the intact fibrinogen dimer highlighting the central location of the E region. Here, the N-terminal regions of the Aa, Bb and g chains from the two halves of the molecule are covalently linked by 11 disulfide bonds. The C-terminal regions of the chains form globular domains (depicted by circles). Coiled coils are depicted by parallel lines, and disordered segments are dotted.

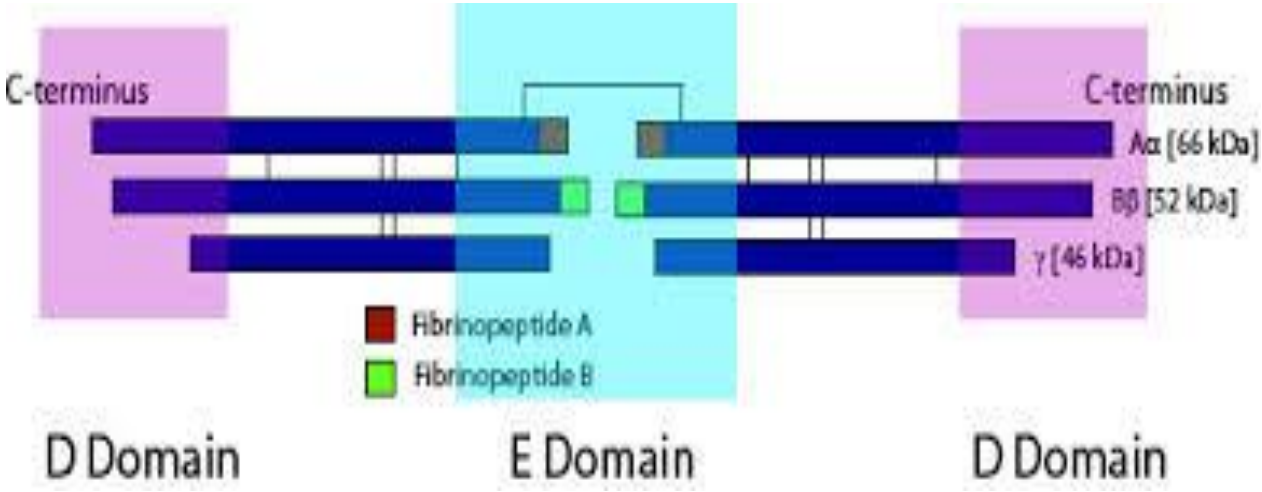

Figure 1.2 Fibrinogen domain structure

The most common haplotypes of each of the 3 fibrinogen genes have been reported by Seattle SNPs. These haplotypes are thought to represent all frequent gene and protein variants that exist in Americans of European descent. Alternative

splicing may occur in both the *FGA* and the *FGG* genes. The predominant alpha chain of circulating fibrinogen contains 610 amino acid residues, whereas the alternative alpha chain (1%-2% of A chains) 9 contains 846 amino acid residues. The beta chain consists of 461 amino acids. The most abundant form of the beta chain consists of 411 amino acid residues, whereas the variant beta chain (7%-15% of chains10 contains 427 amino acid residues (Mosesson *et al*., 2001).

1.2.3.2 Function

Fibrinogen is the precursor of fibrin, the end-product of blood coagulation and is an essential component of the hemostatic system. Its level influences platelet aggregation, blood viscosity, and endothelial cell injury, mechanisms that play a role in atherosclerosis and arterial and venous thrombosis. Among the components of the coagulation system, elevated fibrinogen has been associated most consistently with cardiovascular disorders such as myocardial infarction and stroke (Libby, 2001).

Fibrinogen is converted to fibrin through limited proteolysis by thrombin, which exposes polymerization sites on the fibrin monomers. These monomers spontaneously associate to form insoluble fibrin. Activated factor XIII (subunit A) forms covalent bonds between adjacent fibrin monomers (Mosesson *et al*., 2001).

During normal blood coagulation, a coagulation cascade activates the zymogen prothrombin by converting it into the serine protease thrombin. Thrombin then converts the soluble fibrinogen into insoluble fibrin strands. These strands are then cross-linked by factor XIII to form a blood 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 . Both the activation of Factor XIII by thrombin and plasminogen activator (t-PA) are catalyzed by fibrin (Muszbek,et al,.2008). Fibrin specifically binds the activated coagulation factors factor 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(Kaiser, 2003). Recent research has shown that fibrin plays a key role in the inflammatory response and development of rheumatoid arthritis (Gilliam, *et al*,.2011) .

1.2.3.3 Physiology

In its natural form, fibrinogen can form bridges between platelets, by binding to their GpIIb/IIIa surface membrane proteins; however, its major function is as the precursor to fibrin. Fibrinogen, the principal protein of vertebrate blood clotting, is a hexamer, containing two sets of three different chains (α, β, and γ), linked to each other by disulfide bonds. The N-terminal sections of these three chains contain the cysteines that participate in the cross-linking of the chains. The C-terminal parts of the α, β and γ chains contain a domain of about 225 amino-acid residues, which can function as a molecular recognition unit. In fibrinogen as well as in angiopoietin, this domain is implicated in protein-protein interactions. In lectins, such as mammalianficolins and invertebrate tachylectin 5A, the fibrinogen C-terminal domain binds carbohydrates. On the fibrinogen α and β chains, there is a small peptide sequence (called a fibrinopeptide). These small peptides are what prevent fibrinogen from spontaneously forming polymers with itself(Spraggon *et al*.,1997).

1.2.3.3 Structure–function Relationship

Fibrinogen is secreted into the bloodstream as a disulphide-linked hexamer composed of two identical heterotrimers, each consisting of one Aa, one Bb and one c chain with molecular masses of 67 (610 residues), 57 (461 residues) and 47 kDa (411 residues), respectively (Asselta and Duga, 2006). The hexamer is characterized by a symmetrical structure with a central E domain, connected to two peripheral D. The three chains are encoded by paralogous genes (FGA, FGB and

FGG coding for Aa, Bb and c chains, respectively), clustered in a 50-kb region on chromosome 4 (4q31.3–4q32.1) [10].Conversion of fibrinogen to fibrin occurs after removal of fibrinopeptides A (FPA) and B (FPB) by thrombin from the N-termini of the Aa and Bb chains at the Arg16–Gly17 and the Arg14–Gly15 bonds, respectively. FPA release takes place faster and earlier than FPB release and is sufficient to induce clot formation. Abnormalities at the thrombin cleavage site can cause impaired release of FPA, inhibiting conversion of fibrinogen to fibrin leading to bleeding. FPB (Bb 1–14) cleavage occurs more slowly and contributes to lateral fibril and fibre association. Absent or slow FPB release with delayed polymerization of the fibrin monomers can cause a bleeding phenotype while impaired FPB release results in abnormalities of polymerization that are associated with thrombotic events. Finally, the soluble fibrin clot is stabilized by the amidolytic action of activated factor XIII (FXIII) to form gamma– gamma dimers and alpha polymers. Plasmin cleavage sites include regions between D and E domains in all the three chains producing fragments Y, D and E. Abnormal fibrinogens that exhibit defective cross-linking by activated FXIII have been associated with abnormal wound healing while abnormalities that interfere with plasminogen binding or activation on the fibrin clot result in clinical thrombosis (Asselta and Duga, 2006).

1.2.3.4 Molecular basis of fibrinogen abnormality

1.2.3.4.1 Congenital Deficiency

1.2.3.4.1.1 Afibrinogenemia and hypofibrinogenemia mutations

Mutations causing afibrinogenemia have been detected in all three genes; the majorities found to date are in FGA which are mainly deletions, frame shift, nonsense or splicing mutations. These can lead to deficiency of fibrinogen by several mechanisms: these can act at the DNA level, RNA level by affecting mRNA splicing or stability or at the protein level by affecting protein synthesis,

assembly or secretion. In a database compiled by Hans and Biot these mutations have been associated with clinical bleeding and thromboses there being no genotype–phenotype correlation which could possibly be related to modifier genes or common variant thrombophilic genes (Hans and Biot, 2001). There seems to be considerable overlap between the causative mutations accounting for afibrinogenemia and hypofibrinogenemia. In many cases asymptomatic patients are in fact heterozygous for null mutations which in homozygosity or heterozygosity would cause afibrinogenemia. Mutations of the FGA gene which include null mutations, large deletions (11 kb, 1238 bp and 15 kb in the FGA gene), frameshift (IVS4+A>G and)1138C>T in the FGA gene), splice-site and early truncating nonsense mutations are the most frequently identified mutations irrespective of geographic 1154 S.S (Neerman-Arbaz, 2006). In contrast, the spectrum of mutations in FGG and FGB includes an excess of missense mutations in the C-terminal globular domains (Vu *et al*., 2005). It is important to identify these mutations for designing efficient strategies for mutation detection in new cases. In the majority of patients with afibrinogenemia or hypofibrinogenemia for whom the causative mutation has been identified there is no intracellular accumulation of the mutant fibrinogen chain. However, missense mutations identified in the FGG can cause fibrinogen deficiency in the heterozygous state owing to intracellular retention of the mutant gamma chain and progressive liver disease with hepatocellular intracytoplasmic inclusions ((Neerman-Arbaz, 2006).

Congenital fibrinogen deficiency (afibrinogenemia) or disturbed function of fibrinogen has been described in a few cases (Acharya and Dimichele, 2008).It can lead to either bleeding or thromboembolic complications, or is clinically without pathological findings. More common are acquired deficiency stages that can be detected by laboratory tests in blood plasma or in whole blood by means of thrombelastometry (Langn *et al*., 2009).

1.2.3.4.1.2 Dysfibrinogenemia mutations

Consistent with dominant transmission of dysfibrinogenemia, the majority of patients with dysfibrinogenemia are heterozygous for missense mutations in one of the three fibrinogen genes leading to delayed or absent FPA release or defective fibrin polymerization. Mutations at these sites are estimated to account for approximately 45% of dysfibrinogenemia mutations (Hans and Biot, 2001). The majority of these mutations are in FGA. Thus, dysfibrinogenemia can cause either a bleeding disorder or thrombophilia; some mutations can cause both. Not all are symptomatic; many are in fact discovered following routine laboratory tests before surgery, by prolonged Thrombin Time. Individuals (65%) with FGG mutations are asymptomatic with 5% having bleeding symptoms and 30% thrombosis. A defective binding of thrombin to abnormal fibrin which leads to increased thrombin levels has been implicated in thrombosis and a defective stimulatory function of abnormal fibrin in the tissue plasminogen activator- mediated fibrinolysis has also been implicated in thrombotic events (Roberts *et al*., 2001). These thrombophilic mutations have been found predominantly in the C-terminal domain of the A a chain and the thrombin cleavage site of the Bb chain (McDonagh J *et al*., 2001). Interestingly, dysfibrinogens Marburg and Bern V both a chain mutation form clots which are fragile with failure to form normal fibrin aggregates leading to bleeding symptoms. However, there is also impaired fibrinolysis leading to thrombotic complications (Roberts *et al*., 2001).

Dysfibrinogenemias are infrequent congenital or acquired qualitative abnormalities of fibrinogen that are most commonly correlate diagnosed in adults. Congenital dysfibrinogenemia is caused by heterozygosity for a mutation within any of the 3 fibrinogen chain genes (4q28.1, 4q28.2, and 4q28.3 for *FGG*, *FGA*, and *FGB*, respectively), most commonly in the first 2 genes. Congenital dysfibrinogenemia is associated predominantly with defective fibrinopeptide release and/or with

retarded fibrin polymerization, and is in most cases detected incidentall. Clinically overt manifestations of dysfibrinogenemia involve bleeding, usually related to trauma, surgery, or childbirth (in 20% of cases) and/or thrombosis (in 25% of cases). Less common manifestations of congenital dysfibrinogenemia involve an increased risk of miscarriages; umbilical cord bleeds, and prolonged wound healing. In a vast majority of congenital dysfibrinogenemia, there is a discrepancy between the levels of clottable fibrinogen (determined most frequently using the Clauss method) and immunologically measured fibrinogen. Functional fibrinogen levels are typically lower than its antigen concentration, with fibrinogen activity antigen ratio being in most cases approximately 1:2. Thrombin time is commonly prolonged in congenital dysfibrinogenemia. Acquired dysfibrinogenemia is a rare abnormality that can be observed in a subset of patients diagnosed with liver disease (hepatoma, chronic active hepatitis, cirrhosis, and isolated obstructive jaundice), multiple myeloma, autoimmune disorders, and in some cases of cancer; dysfibrinogenemia may also be induced by medications (e.g., isotretinoin, glucocorticoid, antileukemic agents. This type of dysfibrinogenemia is typically associated with normal fibrinogen activity and antigen levels as well as results of routine coagulation tests, including thrombin time. Acquired dysfibrinogenemia is usually associated with a prothrombotic state and largely results from interactions of fibrinogen molecules with other plasma proteins, e.g., paraproteins, and posttranslational modifications of fibrinogen molecules, e.g., oxidation, which have not been well described yet (Undas *et al*., 2011)

1.2.3.4.2 Acquired fibrinogen deficiency

Acquired deficiency is found after hemodilution, blood losses and/or consumption such as in trauma patients, during some phases of disseminated intravascular coagulation (DIC), and also in sepsis. In patients with fibrinogen deficiency, the correction of bleeding is possible by infusion of fresh frozen plasma (FFP),

cryoprecipitate (a fibrinogen-rich plasma fraction) or by fibrinogen concentrates. There is increasing evidence that correction of fibrinogen deficiency or fibrinogen polymerization disorders is very important in patients with bleeding (Fries *et al*., 2009) .

1.2.3.4.3 Clinical outcome and genetic basis of hyperfibrinogenemia

Abnormalities of fibrinogen have been reported to affect the risk for deep venous thrombosis. In a large case-control study, elevated levels of plasma fibrinogen were found to increase the risk for thrombosis, primarily in the elderly (Mosesson, *et al*., 2001).

The precise mechanism of this effect is unknown, though multiple mechanisms have been proposed (VanHylckama, 2003). In addition, genetic variants of fibrinogen (dysfibrinogenemias) have been found in patients with thrombosis and prolonged thrombin time. Most of these patients have a mutation in the *FGA* or the *FGG* gene, though the precise relation between carriership of these mutations and venous thrombosis is poorly documented (Mosesson *et al*., 2001).

It is well known that plasma fibrinogen levels are regarded as an independent risk factor for overall ischemic stroke, as well as for all main etiological subtypes. Moreover, it is proved that fibrinogen levels are significantly higher in large vessel and cardio embolic strokes as compared to small vessel or cryptogenic strokes. There are several factors that could affect plasma fibrinogen concentrations. Among them, the A allele of the β-fibrinogen –455G/A gene single nucleotide polymorphism (SNP) is mentioned .It was shown that the A allele of this SNP is associated with high plasma fibrinogen levels (Jood *et al*., 2008).

The clinically most important and prevalent conditions associated with both elevated fibrinogen level and cardiovascular disease are type 2 diabetes mellitus, and the closely related insulin resistance syndrome (IRS) which affect 10-25 % of the general population in developed countries (Vantyghem, 2004).

However, the mechanisms leading to hyperfibrinogenemia in insulin-resistant and type 2 diabetic subjects have not been elucidated so far, even though a potential role of low-grade inflammation, hyperinsulinemia and albuminuria has been discussed (Zanettim *et al.,* 2001).

Furthermore, the phenomenon of clustering of features within IRS and the high heritability of its components, have led some researchers to propose the existence of "insulin resistance genes" with a pleiotropic effect on metabolism. These genes are supposed to induce changes in multiple metabolic traits with the subsequent development of IRS features including hyper fibrinogenemia (DeLange *et al.,* 2003).

Recently, as part of the Genetic Analysis of Idiopathic Thrombophila (GAIT) Project, quantified the genetic contribution to susceptibility of thrombosis and related phenotypes in the Spanish population. Of the quantitative risk factors studied, fibrinogen levels had a heritability of 34%, indicating that genetic factors have an important effect on the quantitative variation in this phenotype (Souto, 2000).

It has been reported that a proportion of this variation can be explained by polymorphisms in the fibrinogen genes, especially in the fibrinogen beta chain gene, which regulates the limiting step in fibrinogen synthesis. In these studies, reported that between 5% and 9% of fibrinogen variability could be explained by the beta chain polymorphisms, whereas 4.2% was determined by the fibrinogen alpha chain gene. Therefore, it appears that polymorphisms in the genes encoding fibrinogen chains do not explain the total variance of circulating levels of fibrinogen. Thus, other genetic factors are likely involved in the quantitative variation of this phenotype (Humphries *et al*., 1995).

However, the relationship between fibrinogen gene polymorphisms and disease is not clear. Positive findings were reported between the *G* allele of the -455 G/A

polymorphism in the fibrinogen beta chain gene and coronary artery disease. Also, the -455 G/A polymorphism has been related to the progression of atheroma, but it was the *A* allele that was associated with deleterious effects (Lane and Grant, 2000).

1.2.3.5 Diagnostic use

Fibrinogen levels can be measured in venous blood. Normal levels are about 1.5-3 g/L, depending on the method used. In typical circumstances, fibrinogen is measured in citrated plasma samples in the laboratory; however the analysis of whole-blood samples by use of Thrombelastometry (platelet function is inhibited with cytochalasin D) is also possible (Lane and Grant, 2000).

1.2.3.5.1 Fibrinogen high levels

Higher levels are, amongst others, associated with cardiovascular disease (>3.43 g/L). It may be elevated in any form of inflammation, as it is an acute-phase protein; for example, it is especially apparent in human gingival tissue during the initial phase of periodontal disease (Pag and Schroeder,1976). Fibrinogen levels increase in pregnancy to an average of 4.5 g/l, compared to an average of 3 g/l in non-pregnant people (Salvia and Vinita, 2003).

1.2.3.5.2 Fibrinogen low levels

Low levels of fibrinogen can indicate a systemic activation of the clotting system, with consumption of clotting factors faster than synthesis. This excessive clotting factor consumption condition is known as disseminated intravascular coagulation. DIC can be difficult to diagnose, but a strong clue is low fibrinogen levels in the setting of prolonged clotting times (Prothrombin Time or Activation Partial Thromboplastin Time), in the context of acute critical illness such as sepsis or trauma. Besides low fibrinogen level, fibrin polymerization disorders that can be induced by several factors, including plasma expanders, can also lead to severe

bleeding problems. Fibrin polymerization disorders can be detected by viscoelastic methods such as thrombelastometry (Lane and Grant, 2000).

1.2.4 Fibrin Degradation Products

1.2.4.1 Structure

A balance between blood clot development and blood clot lysis is important for maintaining the integrity of the cardiovascular system and the continuous blood flow. Fibrinolysis serves as the completion step of homeostasis whereby unnecessary fibrin is removed from the vascular system. (Hajjar, 2003).

Fibrin is generated in a process mediated by thrombin from fibrinogen, Fibrin clot formation, being the final step in blood coagulation, results from a series of rapid events initiated by thrombin cleavage of the A, α- and B, β chains of fibrinogen. Thrombin mediated release of FPA and, much slower, FPB from the amino termini of the A α- and B β chains of fibrinogen, respectively, results in the formation of Fn monomer with the structure (α, β, γ)2. When protofibrils grow sufficiently long, they undergo lateral aggregation to make a fibrin fiber (Hoffman, 2008).

Impaired lateral aggregation usually yields clots made up of thin fibers with many branch points. Fibrin clot resistance to enzymatic degradation is largely determined by covalent cross linking catalyzed by activated FXIII of transglutaminase activity (Muszbek *et al.*,2008).

The proper balance between fibrin formations, its cross linking, and degradation is necessary to protect the vascular system from excess blood loss and also from obstructed blood flow (Cesarman *et al*., 2005).

Dissolution of a fibrin clot results is mediated by the interaction of tissue plasminogen activator (tPA) and plasminogen on a fibrin surface. The presence of fibrin greatly accelerates by at least 2 orders of magnitude plasmin generation catalyzed by tPA, which is limited proteolysis of plasminogen. Plasmin bound to fibrin is protected from the action of α 2- antiplasmin. The thrombin activatable

fibrinolysis inhibitor down regulates plasminogen activation and fibrinolysis. Fibrin structure directly affects the rate of fibrinolysis. In vivo fibrinolysis appears to be a heterogenous process, locally extremely rapid while other sites remain unlyzed, but determinants of this process are not fully elucidated. Plasmin mediated fibrin degradation leads to the release of specific cross-linked fibrin degradation products, termed D-dimers, which are a sensitive marker of in vivo fibrin formation and lysis (Wiesel, 2007)

A blood clot is caused by an injury to an artery or vein that activates the coagulation cascade. Fibrin threads are produced during the coagulation process. These fibrin threads form a mesh to clump platelets because they are cross-linked to form blood clots. When the injury heals, the clot is broken down by plasmin for removal. These broken fibrin fragments are called fibrin degradation products (FDPs). Clot is degraded by plasmin. Plasmin converts fibrinogen initially into X component, which is cleaved into component Y and component D. Component Y is cleaved into component D and component E. Component D is also called D-dimers. Normally undetectable, D-dimer levels are elevated when a clot is broken down ((Daniels, 2009).

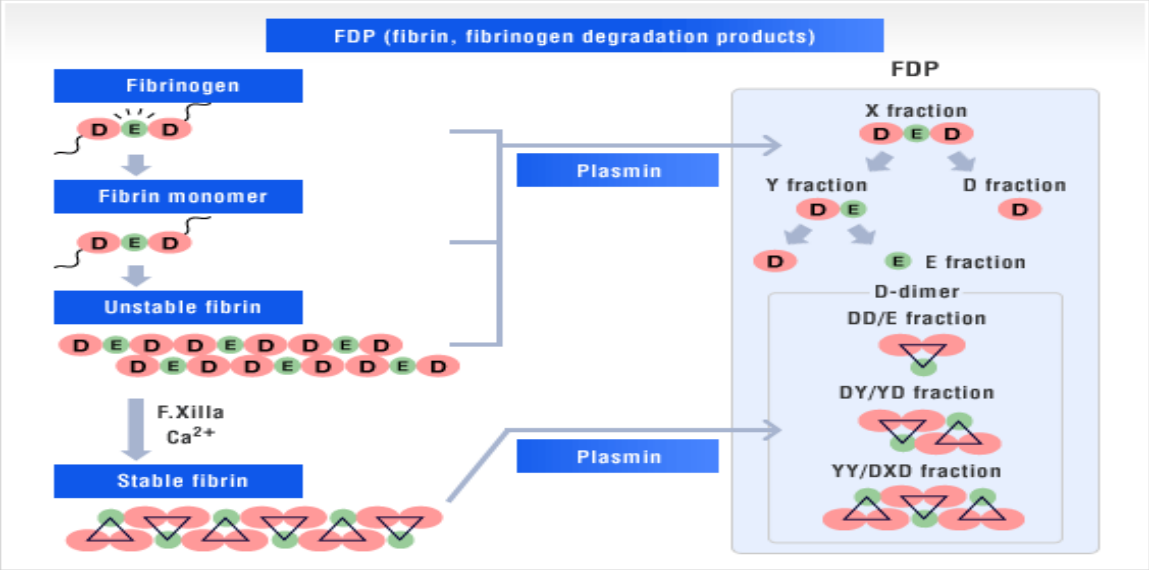

Figure1.3Fibrin degradation products structure.

1.2.4.2 Abnormality

The hemostatic system may play an important role in the initiation and progression of atherosclerosis. Various hemostatic proteins, such as fibrinogen and von Willebrand factor, have been found to be independently associated with future coronary events in healthy individuals as well as in patients with documented atherosclerosis (Koenig, 2001).

1.2.4.3 Indications/Applications

It is used to diagnose disseminated intravascular coagulation. Fibrin split products are used to rule out venous thromboembolism (including deep vein thrombosis and pulmonary embolism) (Adam *et al*., 2009).

1.2.4.4 Considerations

False-positives results on FDPs testing may be caused by rheumatoid factor (Wallach *et al*., 2006).

1.2.4.4.1 High levels

Chronic conditions (eg, renal failure, liver failure) in elderly persons may cause an increase in FDPs levels. An FDPs level of more than 40 mg/mL is considered critical (Daniels, 2009).

1.2.4.4.2 Low levels

Decreased FDP levels do not have clinical significance (Wallach *et al*., 2006).

1.2.4.4.3 Reference range

The reference range of FDPs levels is less than 10 mcg/mL (conventional units) or less than 10 mg/L (SI units) (Duh and Cook, 2012).

1.2.5 D-Dimer

1.2.5.1 Structure

The circulating enzyme plasmin, the main enzyme of fibrinolysis, cleaves the fibrin gel in a number of places. The resultant fragments, "high molecular weight polymers", are digested several times more by plasmin to lead to intermediate and then to small polymers (FDPs). The cross-link between two D fragments remains intact, however, and these are exposed on the surface when the fibrin fragments are sufficiently digested. The typical D-dimer containing fragment contains two D domains and one E domain of the original fibrinogen molecule. D-dimers are not normally present in human blood plasma, except when the coagulation system has been activated, for instance because of the presence of thrombosis or disseminated intravascular coagulation. D-dimer was originally described in the 1970s, and found its diagnostic application in the 1990s (Adam *et al.*, 2009).

1.2.4.2 Molecular weights

The plasmin enzyme induces the degradation metabolism of the stabilized fibrin. Resulting products are numerous due to the different possible cleaving places on the fibrin. Degradation products, called D or Y fragments are therefore heterogeneous and have different molecular weights varying from 350 kilo Dalton

to 2000 kDa. The final degradation product is the D-dimer (184.6 kD), which is often linked by hydrophobic interactions to the E fragment. Recently, the crystal structure of the D-D–E complex (Spraggon *et al*., 1997).

1.2.5.3 Clinical significant of DD measurement

Plasmin is the fibrinolytic enzyme derived from its inactive precursor, plasminogen, by the action of thrombin and plasminogen activators. The main plasminogen activators are tissue plasminogen activator (tPA) and pro-urokinase, which is activated into urokinase by, among others, the contact system of coagulation. Plasmin is neutralised by a 2 antiplasmin thereby restricting its fibrinogenolytic activity and localizing the fibrinolysis on the fibrin clot(Durieux P *et al*.,2001). Fibrin is the main component of a thrombus. It is formed by the activation of the coagulation system. Its production is followed by activation of the fibrinolytic system, resulting in plasmin generation and subsequent fibrin lysis. Under normal physiological conditions there is a balance of the two opposing processes. Dissolution of crosslinked fibrin (XL-Fg) leads to formation of specific degradation products, including DD, which can be measured in both whole blood and plasma using monoclonal antibodies directed against epitopes located in the DD fragment. The activity of DD is considered to reflect the overall activity of clot formation and lysis. Because DD is not artificially generated in vitro during blood collection, its measurement more consistently reflects in vivo haemostatic activity than do other assays for coagulation or fibrinolytic activities that may be activated in vitro. Its absence excludes the presence of intravascular thrombus (Lowe *et al*., 2001).

1.2.5.4 Indication

D-dimer testing is of clinical use when there is a suspicion of deep venous thrombosis (DVT), pulmonary embolism (PE) or disseminated intravascular

coagulation (DIC). It is under investigation in the diagnosis of aortic dissection (Suzuki *et al.*,2010).

For DVT and PE, there are possible various scoring systems that are used to determine the *a priori* clinical probability of these diseases (Wells *et al.*, 2003).

For a very high score, or pretest probability, a D-dimer will make little difference and anticoagulant therapy will be initiated regardless of test results, and additional testing for DVT or pulmonary embolism may be performed.For a moderate or low score, or pretest probability: A negative D-dimer test will virtually rule out thromboembolism: the degree to which the D-dimer reduces the probability of thrombotic disease is dependent on the test properties of the specific test used in the clinical setting: most available D-dimer tests with a negative result will reduce the probability of thromboembolic disease to less than 1% if the pretest probability is less than 15-20%.If the D-dimer reads high, then further testing (ultrasound of the leg veins or lung scintigraphy or CT scanning) is required to confirm the presence of thrombus. Anticoagulant therapy may be started at this point or withheld until further tests confirm the diagnosis, depending on the clinical situation. In some hospitals, they are measured by laboratories after a form is completed showing the probability score and only if the probability score is low or intermediate. This reduces the need for unnecessary tests in those who are high-probability. (Rathbun *et al.*, 2004).

Performing the D-dimer test first can avoid a significant proportion of imaging tests and is less invasive. Since the D-dimer can exclude the need for imaging, specialty professional organizations recommend that physicians use D-dimer testing as an initial diagnostic (Fesmire *et al*., 2011).

1.2.4.5 D-Dimer assays

There are many different D-Dimer assays currently on the market. They may be grouped by laboratory method with advantages and disadvantages of each type.

Extensive discussion of the merits of individual assays is beyond the scope of this article. The classic enzyme-linked immunosorbent assay (ELISA) is highly sensitive but is also time consuming, labor intensive, and impractical for use as an emergency test (Brill-Edwards and Lee 1999). Slide latex agglutination assays have also been evaluated. Results are qualitative (or semiquantitative if serial dilutions are used). Although rapid, their sensitivities are not high enough to reliably exclude venous thrombosis. They should not be used in any diagnostic algorithm for DVT or PE (Carter *et al*., 1999).

1.2.5.6: Evaluation of different D-dimer assays

Whole blood RBC agglutination tests are based on a hybrid monoclonal antibody that binds the D-Dimer antigen and RBCs, leading to agglutination. The Simplired™ D-Dimer assay (Agen Biomedical Limited M, Brisbane, Australia) is rapid and can be performed at the bedside with only a few drops of capillary blood. The result is qualitative. Sensitivities ranging from 61% to 100% have been reported. Interobserver variability with this assay has been noted because the test result must be interpreted visually. Experienced laboratory personnel performed best. Immunofiltration assays are based on the binding of gold labeled monoclonal antibodies to D-Dimer antigen fixed to a filtration membrane. A qualitative result is interpreted visually or semi-quantitatively using a reflectometer. These assays are rapid and have the added advantage of availability for bedside point of care testing. Sensitivities range from 81% to 100% (Van der, *et al*., 2000). Automated rapid ELISA assays have recently been introduced with turnaround times of 1 hour. These assays have all the benefits of the classical ELISA assay but rapid availability of results facilitates their use as an emergency test. The Vidas DDimer ® (Biomerieux M, Marcy L'etoile and France) is the only rapid ELISA assay available with reported sensitivities ranging from 94% to 100%. Latex enhanced photometric immunoassays are quantitative, turbidimetric or colorimetric assays

that use latex particles coated with human monoclonal antibody to the D-Dimer antigen. These assays can be performed either in the laboratory or at the bedside. Turnaround times are rapid, facilitating their use as an emergency test. In limited studies the Tinaquant D-Dimer BM™, STA LIAtest D-Di™ (both of Roche Diagnostics M, Indianapolis, IN), Turbiquant D-Dimer™ (Merck M, Whitehouse, NJ), and IL-D-Dimer™ (Beckman Coulter M, Fullerton, CA) performed best with sensitivities approaching that of the rapid ELISA tests, 92% to 100% (Van der, *et al*., 2000).

1.2.4.1 Characteristics of the ideal D-dimer assay are listed in table below

Table1.6 Characteristics of the ideal D-dimer assay

High sensitivity
Rapid lab turnaround
Availability for 24 hours a day/7 days a week
Point of care
Low inter observer variability
Low coefficient of variation

The current assays use different monoclonal antibodies and there is an overall lack of standardization. Identical antibodies may react differently in different assay formats. The calibrators used in one assay may give a different result when used in a different assay. The units also vary with some reported in ng/ml of D-Dimer units and others in fibrinogen equivalent units (FEU). Results from one assay cannot necessarily be extrapolated to another (Dempfle, 2000).

1.2.5.7 Test properties

Various kits have 93-95% sensitivity and about 50% specificity in the diagnosis of thrombotic disease (Schrecengost *et al*., 2003).

1.2.5.7.1 False positive readings

Can be due to various causes: liver disease, high rheumatoid factor, inflammation, malignancy, trauma, pregnancy, recent surgery as well as advanced age (Van *et al*., 2012).

1.2.5.7.2 False negative readings

Can be due to if the sample is taken either too early after thrombus formation or if testing is delayed for several days. Additionally, the presence of anti-coagulation can render the test negative because it prevents thrombus extension. False values may be obtained if the specimen collection tube is not sufficiently filled (false low value if under filled and false high value if over filled). This is due to the dilution effect of the anticoagulant (the blood must be collected in a 9:1 blood to anticoagulant ratio). Likelihood ratios are derived from sensitivity and specificity to adjust pretest probability. In interpretation of the d-dimer, for patients over age 50 a value of ageX10 may be abnormal (Van *et al*., 2012).

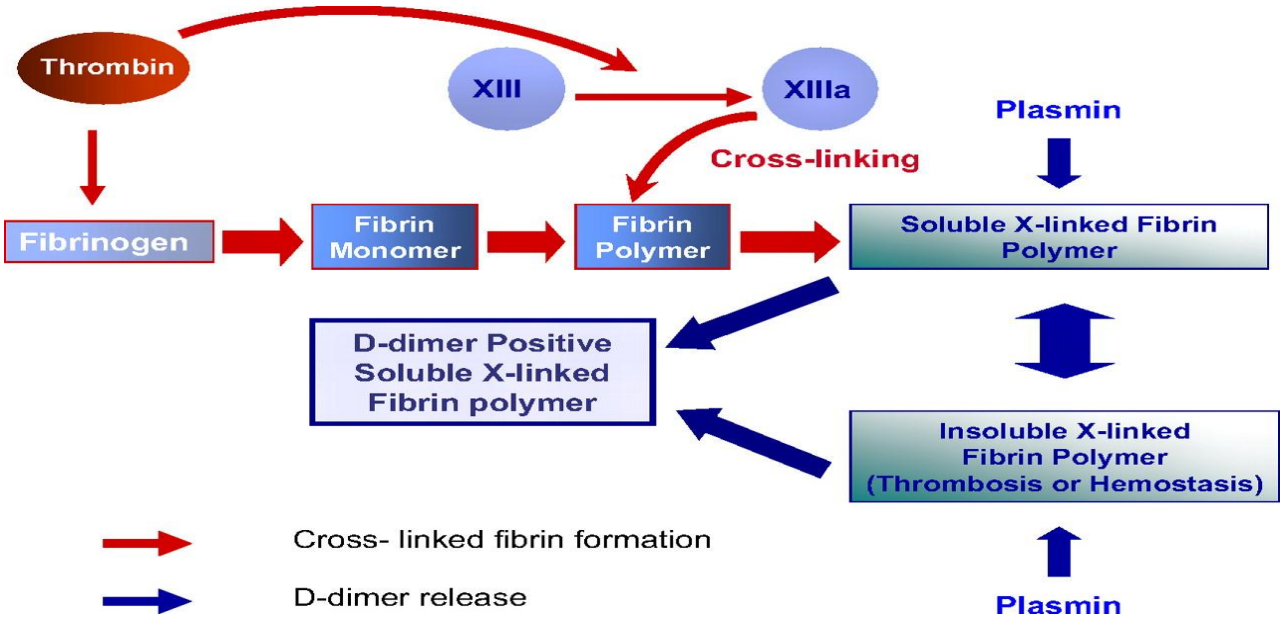

Figure 1.4 D dimer Structure Digram

1.2.5.8 Function

Fibrin D-dimer can be considered as a global marker of activation of the hemostatic system and therefore of fibrin turnover (ongoing fibrin formation and degradation). Fibrin D-dimer is the degradation product of cross-linked fibrin and has been gaining increasing interest as an indicator of coronary artery disease. Fibrinolytic variable such as D-dimer are indicative of atherogenesis and thrombogenesis (Koenig, 2001).

1.2.5.9 Abnormalities

Importantly, elevated D-dimer levels predict the risk of future coronary events independently of conventional risk factors in initially healthy middle-aged male and female subjects. Increased serum D-dimer levels have been associated with increased risk of myocardial infarction, stroke, and peripheral vascular disease. The specificity of the relation of these hemostatic factors with cardio vascular disease nevertheless may be questioned, because they often are related positively to risk of other chronic conditions, such as cancer or total mortality or show a moderate degree of correlation with markers of inflammation (Danesh, 2001).

D-dimer levels rise earlier than cardiac injury markers (including myoglobin) in acute ischemic events and can detect ongoing thrombus formation, dissolution in patients that are undetectable by conventional methods. He even suggested that elevated D-dimer could identify patients most suitable for lytic or antiplatelet therapy, whereas patients without D-dimer elevation were more suitable for interventional therapies. Higher levels of D-dimer were associated with more frequent cardiovascular events in patients with artificial heart valves, and higher risk of recurrence in patients with venous thrombosis. Measurements of D-dimer provided reliable pathways for monitoring anticoagulant therapy (Vene *et al*., 2003). We have recently reported that plasma fibrin D-dimer, a marker of the formation and lysis of cross-linked fibrin , showed a strong and independent

association with incident ischaemic heart disease in the Caerphilly Study, we have suggested that increased plasma D-dimer levels in persons developing ischaemic heart disease might reflect increased activation of blood coagulation, because similar elevations of coagulation activation markers and D-dimer have been observed in patients with chronic arterial disease, and because increased plasma D-dimer levels can be normalized by anticoagulation with warfarin (Lowe,1998).

1.2.6 Rationale

Studies have shown that the risks of complications in people with diabetes are linked to their blood pressure level, with greater risks among those with higher blood pressure levels. While studies have shown that lowering blood pressure in people with diabetes and “hypertension” can avoid some of these complications.

Diabetes is linked to micro vascular disturbances causing neuropathy, retinopathy and nephropathy, as well as macro vascular complications such as stroke, coronary heart disease, and non healing foot ulcers. Observations over-the-years that one-third of the diabetic patients develop diabetic nephropathy which on long term leads to chronic renal problems. It is well appreciated both that coexisting hypertension exacerbates diabetic nephropathy and that diabetic nephropathy somehow results in a markedly increased risk of hypertension. Diabetic retinopathy is a common micro vascular complication of diabetes. It is the leading cause of blindness among working-aged adults around the world. It has been reported that a proportion of this variation can be explained by polymorphisms in the fibrinogen genes, especially in the fibrinogen beta chain gene, which regulates the limiting step in fibrinogen synthesis. Hemostatic factors especially fibrinogen, has been implicated as a cause of atherosclerosis and its complications. Elevated levels of plasma fibrinogen were found to increase the risk for complications, primarily in the elderly. Elevated D-dimer and FDPs levels were associated with a hypercoagulable state and marker of inflammation. Molecular detection of genes polymorphism, immune assays of coagulation factors and evaluation of coagulation profile is highly recommended in diabetic hypertensive patients to know the early complications.

1.2.7 Objectives

1.2.7.1 General objective

To associate between beta fibrinogen 455 G→A gene polymorphisms with fibrinogen, levels and micro vascular complications in diabetic hypertensive Sudanese patients.

1.2.7.2 Specific objectives

1- To detect beta fibrinogen 455 G→A gene polymorphisms in type2 diabetic hypertensive Sudanese patients
2- To measure fibrinogen, D-Dimer and fibrinogen/fibrin degradation products level in case and control groups.
3- To associate beta fibrinogen 455G→A gene polymorphisms with fibrinogen, D-dimer, FDPs levels in case.
4- To correlate the duration times of both diabetes and hypertension have same onset and complications with patient's demographic data (age, sex) and microvascular complications.

Chapter Tow

2. Materials and Methods

2.1 Study design

This was a case- control study.

2.2 Study area

This study was conduct at Khartoum teaching hospital in Khartoum state.

2.3 Study population

Type2 diabetic hypertensive Sudanese patients. The study was included (300) of Sudanese type 2 diabetic hypertensive patients and (100) non diabetic non hypertensive individuals as control.

2.4 Data collection

Demographic and clinical data was collected through questionnaires, (non self).

Inclusion criteria:-

Type2 diabetic hypertensive Sudanese patients with or without micro vascular complications (males and females in all age groups) were included.

Exclusion criteria:-

Diabetic patients who received any anticoagulant and the presence of any disease known that affect fibrinogen level such as liver disease. The studied patients were not on any lipid lowering treatment affecting fibrinogen levels such as fibrates.

2. 5 Ethical considerations

The objectives of the study were explained at the beginning to all individual under study. The study was approved from Sudan University of Science and Technology. Written consent was obtained from each participant in the study.

An interview by questionnaire with subjects was conducted to obtain the clinical data, questionnaire including informative data (age, gender, duration time of both DM and HTN with or without microvascular complications).

2.6 Methods

2.6.1 Sample collection

Blood was collected five ml and divided into three ml in trisodium citrate 3.8% plasma was separated and stored at -20 ^{0}C for fibrinogen assay, tow ml of blood was drawn into EDTA, plasma was separated and stored at -20^{0}C for ELISA assay and leukocytes was used for genotyping.

2.6.2 Fibrinogen assay

The plasma fibrinogen level was measured by von Clauss method it is afunctional assay based upon the time of fibrin clot formation with addition of bovine thrombin using semiautomated coagulation analyzer (Kselemed Coagulometer K-3002 Auto) (Clauss, 1957).

2.6.2.1 CLauss method

Fibrinogen in plasma was determined by using semi automated procedure.

2.6.2.2 Test principle

The determination of fibrinogen with thrombin clotting time was based on the method originally described by fibrinogen was transformed into fibrin and clot formation time was inversely proportional to the concentration of fibrinogen.

2.6.2.3 Fibrinogen samples

Samples were collected, into the siliconzed blastic tube, 9 parts of freshly venous blood was drawn into 1 part of trisodium citrate 3.8%. The plasma was separated after centrifugation for 15 minute ‘at 1500 rpm. .

2.6.2.4 Procedure

The assay procedure was consisted of placed 200µl of diluted plasma (diluted 1:10 by the combination of 100µl of plasma +900 µl of buffer) in a test tube preheated to 37 ^{0}C was incubated for an additional 2 min at 37 ^{0}C , then was added 100µl of the fibrinogen reagent. A stopwatch was started, and the clotting time was

measured. The time (seconds) was assessed until clot formation was readied and last was converted into mg/dl by calibration graph.

2.7 D-dimer assay

Blood was collected in tubes containing EDTA at baseline and was centrifuged to collected plasma and was stored at -20^{0}C. The binding of the antibody was measured quantitatively by enzyme-linked immunosorbent assays (model Automated ELISA liquid handing wit). D-dimer reference range (20-400 µg/l.).

2.7.1 Principle of the test

The simple step ELISA was employed an affinity tag was labeled captured antibody and a reporter was conjugated detector antibody which immune was captured the sample analyte in solution. Capture antibody/analyte/detector antibody were in turned immobilized via immune affinity of an anti-tag antibody was coated the well. Assay was performed by samples or standards were added to the wells and were followed by the antibody mixed. After incubation, the wells were washed to remove unbound material. TMB substrate was added and during incubation was catalyzed by HRP, generating blue coloration. This reaction was stopped by additional of stop solution completing any color change from blue to yellow. Signal was generated proportionally to the amount of bound analyte and the intensity was measured at 450 nm. Optionally, instead of the endpoint was readied, development of TMB was recorded kinetically at 600 nm.

2.7.2 Procedure

Appropriate number of antibodies were coated well strips were removed, all reagents were equilibrated to room temperature and prepared, samples and standards as instructed. Standard and samples were added to appropriate wells. Antibody cocktail was added to all wells then was incubated at room temperature, aspirated and each well was washed. TMB Substrate was added to each well and

incubated. Stop solution was added at a defined endpoint. Alternatively, was recorded color development kinetically after TMB substrate was added.

2.7.3 Technical hints

Samples were generated values higher than the highest standard were further diluted in the appropriate sample dilution buffers. Foaming or bubbles were avoided when was mixed or reconstituted components. Cross contamination of samples or reagents were avoided by changed tips between samples, standard and reagent additions. Plates were ensured properly covered during incubation steps. Completed removal of all solutions and buffers during washed steps were necessary to minimized background. As a guide, typical ranges of sample concentration for commonly were used sample types are shown below in sample preparation. All samples were mixed thoroughly and gently. Multiple freezed /thawed of samples were avoided. ELISA plates were incubated on a plate shaker during all incubation steps. When was generated positive control samples, it was advisable to change pipette tips after each step. The provided antibody diluents and sample diluents contain protease inhibitor aprotinin. Additional protease inhibitors were added if required. Samples or standards were added to the well before the additional of the antibody cocktail.

1X wash buffer PTP

X Wash Buffer PT was prepared by diluting 10X wash buffer PT with deionized water. 50 mL 1X Wash Buffer PT was made by combined 5 mL 10X wash buffer PT with 45 mL deionized water, mixed thoroughly and gently.

Antibody cocktail

Antibody cocktail was prepared by diluting the capture and detector antibodies in antibody diluent 4BI. 3 mL of the antibody cocktail were made by combined 300 µL 10X capture antibody and 300 µL 10X detector antibody with 2.4 mL antibody diluent 4BI and was mixed thoroughly and gently.

Standard preparation

Serially diluted standard was prepared immediately prior to use. Afresh set of positive controls were prepared for every used. The D-dimer lyophilized purified protein was reconstituted by 100 µL water by pipette, mixed thoroughly and gently when was added. Holded at room temperature for 3 minutes and mixed gently. The 150 ng/mL stock standard solution was prepared. Eight tubes were labeled, standards 1– 8. 320 µL sample diluent NS was added into tube number 1 and 150 µL of sample diluent NS into numbers 2-8. Stock standard was used to prepare the following dilution series. Standard 8 contains no protein and was used as the blank control.

Plasma

Plasma was collected by using EDTA. Samples were centrifuged at 2,000 rounds per time for 10 minutes. Samples were diluted into ample diluent NS and assay. Un-diluted plasma samples were stored at -20ºC.

Plate preparation

The 96 well plate strips were included and supplied ready to use. Unused plate strips were immediately returned to the foil pouch was contained the desiccant pack, resealed and stored at 4°C. For each assay was performed, a minimum of two wells were used as the zero control. For statistical reasons, was recommended each sample was assayed with a minimum of two replicates (duplicates). Differences in well absorbance or "edge effects" were not observed with this assay.

2.7.4Assay procedure

All materials and prepared reagents were equilibrated to room temperature. Excess micro plate strips were removed from the plate frame, returned them to the foil pouch was contained the desiccant pack, resealed and returned to 4ºC storage.

50 µL of all sample or standard was added to appropriate wells. 50 µL of the antibody cocktail was added to each well. The plate was sealed and incubated for 1

hour at room temperature on a plate shaker set to 400 rpm. Each well was washed with 3 x350 µL 1X wash buffer PT by aspirating from wells then dispensing 350 µL. Wash Buffer PT into each well. Complete removal of liquid at each step was essential for good performance. After the last washed was inverted the plate and blot it against clean paper to wells to remove excess liquid. 100 µL of TMB substrate was added to each well and incubated for 10 minutes in the dark on a plate shaker set to 400 rpm. 100 µL of stop solution was added to each well. Plate was shacked on a plate shaker for 1 minute to mix. The OD at 450 nm was recorded. This is an endpoint reading. Alternative to 13.7 – 13.8: instead of the endpoint reading at 450 nm, recorded the development of TMB Substrate kinetically. Immediately after additional of TMB development solution was began recorded the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode:	**Kinetic**
Wavelength:	**600 nm**
Time:	**up to 15 min**
Interval:	**20 sec - 1 min**
Shaking:	**Shake between readings**

2.7.5 Calculations

Subtract average zero standard from all readings. Average the duplicate readings of the positive control dilutions and plot against their concentrations. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations were examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from

the standard curve plotted. Samples producing signals greater than that of the highest standard further were diluted and reanalyzed, and then multiplying the concentration was found by the appropriate dilution factor.

2.8 Fibrin degradation product assays

Plasma levels of FDPs were measured by immunoassays based on specific monoclonal antibodies. The binding of the antibody was measured quantitatively by ELISA (model automated ELISA liquid handing wit. FDP reference range I0-40 mg/l.

Note: kits were recognized D-Dimer and other Fibrin Degradation Products.

2.8.1 Application details

2.8.1.1 ELISA plate

The just opened ELISA Plate was appeared water-like substances, which was normal and not have any impact on the experiment results.

2.8.1.2 Sampling

The interval of sample was added between the first well and the last well was not too long, otherwise was caused different pre-incubation time, which significantly was affected the experiment's accuracy and repeatability. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate not was exceeded 10 minutes. Parallel measurement was recommended.

2.8.1.3 Incubation

Evaporation and ensure accurate results were avoided by proper adhesion of plate sealers during incubation steps was necessary. Wells not were allowed to sit uncovered for extended periods between incubation steps. Strict was compliance with the given incubation time and temperature.

2.8.1.4 Washing

The wash procedure was critical. Insufficient washing was result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells was

pat dry against absorbent paper in the washing process. But don't put absorbent paper into reaction wells directly. The residual liquid was cleared and fingerprint in the bottom before measurement, so was not affected the micro-titer plate reader.

2.8.1.5 Reagent preparation

As the volume of detection Ab and HRP conjugated was very small, liquid was adhered to the tube wall or tube cap when was binged transported, centrifugal it for 1 minute at 1000rpm. Pipette the solution for 4-5 times before was pippeted. Standards carefully were reconstituted; working solutions of detection Ab and HRP conjugate according to the instructions. To minimize imprecision that was caused by pipetting, pipettes were calibrated. It was recommended to suck more than 10μL for once pipetting. Standard solution was not reused, working solution of detection Ab and HRP conjugate, which was diluted. Standard repeatedly was used and divided the standard into small pack according to the amount of each assay, at -20°C to -80°C was saved and repeated freezing and thawing was avoided.

2.8.1.6 Reaction time control

Control reaction time strictly was followed the product description.

2.8.1.7 Substrate

Substrate solution was easily contaminated. Stop solution: As it was an acid solution, pay attention to the protection of your eyes, hands, face and clothes when were used this solution.

2.8.1.8 Mixing

Micro-oscillator was used better at the lowest frequency, as sufficient and gentle was mixed particularly important to reaction result. If there was no micro-oscillator available, the ELISA plate frame was knocked gently with finger before reaction.

2.9 Genotype determination

2.9.1 DNA xtraction

DNA was extracted by the Vivantis DNA blood kits (*Malasia*) according to manufacture instructions.

2.9.1.1 Method

All steps were carried out at room temperature unless stated otherwise. Wash Buffer 1 and wash buffer 2 (concentrate) was diluted with absolute ethanol before use. Solutions were reconstitution. Precipitation forms in buffer BB were incubated at temperature 65°C with occasional mixed until precipitate was completely dissolved. Water path was pre-seted to 65°C. Elution buffer was pre-heated at 65°C. 200µl of buffer BB was added into a 200µl blood sample in a microcentrifuge tube. Thoroughly was mixed by pulsed-vortexing. 20µl of Proteinase K was added and mixed immediately. At 65°C for 10 min was incubated. 200µl of absolute ethanol was added. Immediately and thoroughly was mixed to obtain a homogeneous solution. To column was loaded. The column was washed with 500µl wash buffer 1 and was centrifuged at 5,000 rpt for 1 min. Flow through was discarded. The column was washed with 500µl wash buffer 2 and centrifuged at 5,000 rpt for 1 min. Flow through was discarded. Column was washed again with 500µl wash buffer 2 and centrifuged at maximum speed for 3 minutes. The column was placed into a clean microcentrifuge tube. 100µl of preheated elution buffer was added; TE buffer or sterile water was directly onto column membrane and stood for 2 min. Centrifuged at 5,000 rpt for 1 min to elute DNA. DNA was Stored at 4°C or -20°C.

2.9.1.2 Genotyping

Beta fibrinogen 455 G/A gene polymorphisms were detected by PCR-RFLP method. As a negative control, PCR mixture without DNA sample was used to ensure contamination free PCR product. Primers for DNA -fragments in the

promoter region of the fibrinogen gene -455 G/A polymorphism were; forward 5-AGGGTCTTTCTGATGTGT-3,

reverse 5-AAGTTAGGGCACTCCTCA-3.

PCR amplification was performed with a 50μL reaction volume containing 1 μL of DNA template, 1 μL of each of the sense and antisense primers,with ready master mix was consisted of deoxynucleotide triphosphate, MgCl2, of AmpliTaq GoldTM polymerase. The amplification conditions were as follows: an initial denaturing step at temperature 96^0C for 7 min, was followed by 35 amplification cycles of denaturation at temperature 94 ^{0}C for 30 sec, annealing at temperature 55^0C for 30 sec, and extension at temperature 72^0C for 30 sec, and a final extension step at temperature 72^0C for 10 min. PCR products (336-bp) were electrophoresed on a 1.2% agarose gel. 5 μL of amplified PCR products was mixed with 5 μL of (restrictive enzyme Hae III and buffer), were allowed to react at temperature 37^0C for one hour. The final product was electrophoresed on a 2% gel containing ethedium bromide, and its genotypes were analyzed by UV transilluminator

2.9.1.3 Interpretation

The PCR product of -455A allele was not cleaved by HaeIII generating a 336-bp band, whereas the PCR product of -455G allele was cleaved by the enzyme generating 215 and 121-bp fragments. The G/A heterozygotes were generated three bands: 336, 215, and 121-bp bands.

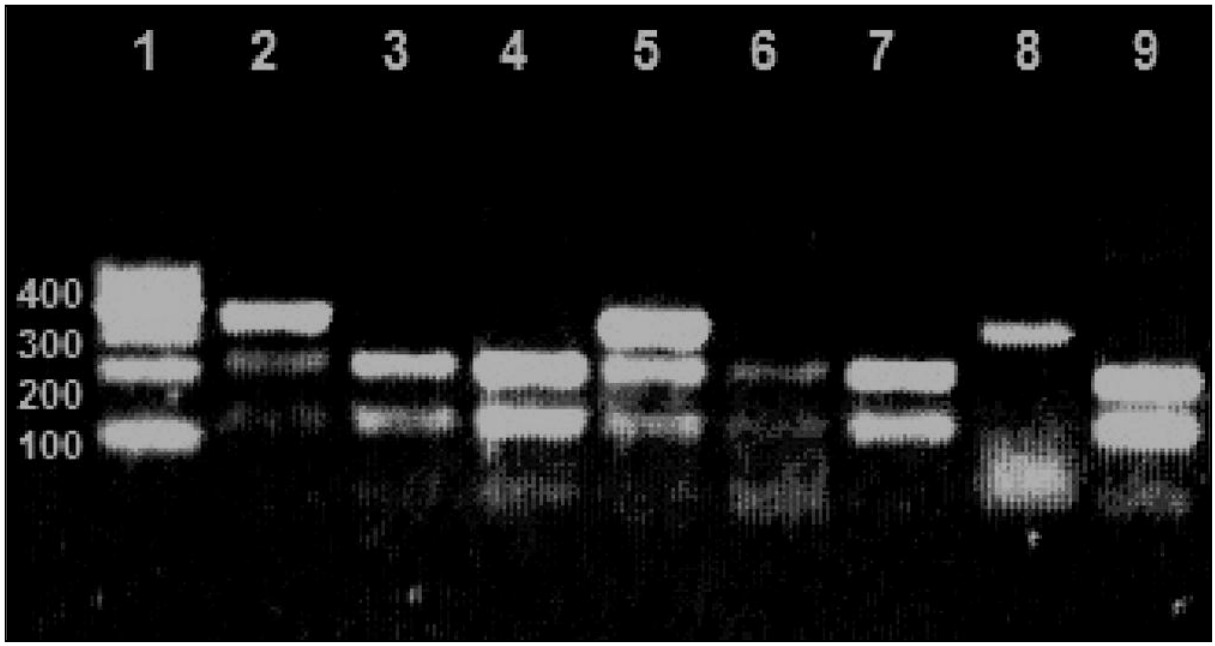

Figure 2.1PCR-RFLP, Lane 1: marker ladder 100, 200, 300, 400, 500 bp; lane 3, 4, 6, 7 and 9: GG genotype (215, 121 bp); lane 8: AA genotype (336 bp); lane 2, 5, and 8: G/A genotype (336, 215, 121 bp).

2.10 Data analysis

The data of this study was analyzed by SPSS computer program version 16.0 software. Independent sample T- Test was analyzed means, standard deviation and probability value (*P*-value) of parameters of fibrinogen, D-dimer and FDPs for case and control and all data that were classified in two groups Cross tabulation and Chi square were used to place frequencies of each variable to each group. Probability value < 0.05 was considered statistically significant.

Chapter Three
Results

Table3.1

Frequency of demographic and clinical data in case group

There were more females than males. Essential hypertension and A/A genotype were more common. Micro vascular complications were present in less in patients group.

(Table.3.1)

Variable		Frequency (N= 300)	Percent %
Sex	**male**	**94**	**31.3**
	Female	**206**	**68.7**
Hypertension Stage	**Essential**	**296**	**98.7**
	Secondary	**4**	**1.30**
Retinopathy	**Present**	**111**	**37.0**
	Absent	**189**	**63.0**
Nephropathy	**Present**	**9**	**3.00**
	Absent	**291**	**97.0**
Neuropathy	**Present**	**37**	**12.3**
	Absent	**263**	**87.7**
Duration of diseases	**Less than 5**	**160**	**53.3**
	More than 5	**140**	**46.7**
Age group/years	**Less than 40**	**129**	**43**
	More than 40	**171**	**57**

Table 3.2`

Comparison between fibrinogen, D dimer and FDPs levels in patients and control group.

There was statistically significant increase in mean fibrinogen, D-dimer and FDPs in patients when compared to control group.

Table 3.2

Parameters	**Case Mean ±SD**	**Control Mean ±SD**	***P*-value**
Fibrinogen/ (mg/dl)	**545±155**	**323±155**	**0.000**
Ddimer/ (ng/ml)	**672±400**	**330±71.1**	**0.000**
FDPs/ (ng/ml)	**293±110**	**159±28.4**	**0.000**

Table 3.3

Comparison between fibrinogen, D dimer and FDPs levels according to gender in Patient group.

There were no statistically significant differences in mean fibrinogen, D-dimer and FDPs according to gender.

Table 3.3

Parameters	Male Mean ±SD	Female Mean ±SD	*P*-value
Fibrinogen/ (mg/dl)	565±165	236±150	0.131
Ddimer/ (ng/ml)	729±463	646±367	0.096
FDPs/ (ng/ml)	302±121	289±105	0.340

Table 3.4

Comparison between fibrinogen, D dimer and FDPs levels according to type of hypertension.

There were no statistically significant differences in mean fibrinogen, D-dimer and FDPs when compared in patients with essential and secondary hypertension.

Table .3.4

Parameters	Essential HTN Mean ±SD	Secondary HTN Mean ±SD	*P*-value
Fibrinogen/ (mg/dl)	545±158	514±120	0.687
D-dimer/ (ng/ml)	675±402	486±171	0.350
FDPs/ (ng/ml)	293±110	269±102	0.663

Table 3.5

Correlations between duration time of both diabetes mellitus and hypertension with fibrinogen, D-dimer and FDPs in study group.

There was statistically significant positive correlation between fibrinogen, D-dimer and FDPs with duration time of both diabetes mellitus and hypertension.

		Duration	Fibrinogen	Ddimer	FDP
Duration	**Pearson Correlation**	**1**	**.480****	**.481****	**.318****
	Sig. (1-tailed)		**.000**	**.000**	**.000**
Fibrinogen	**Pearson Correlation**	**.480****	**1**	**.748****	**.632****
	Sig. (1-tailed)	**.000**		**.000**	**.000**
Ddimer	**Pearson Correlation**	**.481****	**.748****	**1**	**.611****
	Sig. (1-tailed)	**.000**	**.000**		**.000**
FDP	**Pearson Correlation**	**.318****	**.632****	**.611****	**1**
	Sig. (1-tailed)	**.000**	**.000**	**.000**	

****. Correlation is significant at the 0.01 level (1-tailed).**

Table 3.6

Comparison between fibrinogen, D dimer and FDPs levels according to neuropathy.

There were statistically significant increase in mean fibrinogen, D-dimer and FDPs in patients with neuropathy compared to those without neuropathy.

Table 3.6

Parameters	With Neuropathy Mean ±SD	Without Neuropathy Mean ±SD	*P*-value
Fibrinogen/ (mg/dl)	**615±178**	**535±149**	**0.012**
Ddimer/ (ng/ml)	**884±506**	**642±376**	**0.008**
FDPs/ (ng/ml)	**356±145**	**283±101**	**0.005**

Table 3.7

Comparison between fibrinogen, D dimer and FDPs levels according to nephropathy.

They were statistically significant increase in mean Fibrinogen in patients with nephropathy compared to those without nephropathy, although D dimer and FDPs were increase in patients with nephropathy but the difference was not statistically significant.

(Table .3.7)

Parameters	With Nephropathy Mean ±SD	Without Nephropathy Mean ±SD	*P*-value
Fibrinogen/ (mg/dl)	**662±165**	**441±154**	**0.021**
D-dimer/ (ng/m)l	**1020±659**	**661±386**	**0.138**
FDPs/ (ng/ml)	**346±111**	**291±110**	**0.144**

Table .3.8

Comparison between fibrinogen, D dimer and FDPs levels according to retinopathy.

There was statistically significant increase in mean, D-dimer in patients with retinopathy compared to those without retinopathy, although fibrinogen and FDPs were increase in patients with retinopathy but the difference was not statistically significant.

Table .3.8

Parameters	With Retinopathy Mean ±SD	Without Retinopathy Mean ±SD	*P*-value
Fibrinogen/ (mg/dl)	**563±172**	**531±140**	**0.094**
D-dimer/ (ng/ml)	**765±478**	**613±335**	**0.004**
FDPs/ (ng/ml)	**304±121**	**285±101**	**0.151**

Table 3.9

Comparison between fibrinogen, D dimer and FDPs levels according to age groups in case study.

There was statistically significant increase in mean fibrinogen, D-dimer when compared between less than40 years and more than 40 years, but decrease in mean of FDPs.

Table 3.9

Parameters	Age group/years	Mean ±SD	*P*-value
Fibrinogen (mg/dl)	**Less than 40** **More than 40**	**513±139** **569±162**	**0.001**
D-dimer/ (ng/ml)	**Less than 40** **More than 40**	**594±289** **731±460**	**0.003**
FDPs/ (ng/ml)	**Less than 40** **More than 40**	**278±100** **303±116**	**0.047**

Table .3.10

Association between fibrinogen, D dimer and FDPs levels according to beta fibrinogen 455G→A polymorphisms within patients group.

There was statistically significant increase in mean fibrinogen, D-dimer and decrease in FDPs when compared mutant and wild gene.

Table 3.10

Parameters	Mutant Mean ±SD GA+AA	Wild Mean ±SD GG	*P*-value
Fibrinogen/ (mg/dl)	408±113	367±150	0.000
D-dimer/ (ng/ml)	431±230	411±409	0.000
FDPs/ (ng/ml)	195±94.2	309±104	0.000

Table 3.11

Genotyping distribution under dominant and recessive inheritance models and alleles frequency for polymorphism in patient and control group.

The difference for the dominant between patients and control group was statistically significant with OR at 95% CI was 1.8 (1.66−3.78) and recessive was no statistically significant with OR at 95% CI was 0.77 (0.522−1.162) .The frequency of A allele was higher in patients (69.3%) than control group (63.5%) although the different was not significant at *P*-value= 0.130 .

Table 3.11

455G/A		**Patients**	**Control**	
Dominant Model	**G/G**	**71(23.7%)**	**37(37.0%)**	***P*-value=0.010** **OR(95%CI=1.8(1.66−3.78)**
	G/A+A/A	**229(76.3)**	**63(63.0%)**	
Recessive Model	**G/G+G/A**	**113(37.7%)**	**37(37.0%)**	***P*-value =0.90** **OR(95%CI=0.9(0.60−1.5)**
	A/A	**187(30.3%)**	**63(63.0%)**	
Allele Frequency	**G**	**184(30.7%)**	**73(36.5%)**	***P*-value =0.130** **OR(95%CI=1.29(0.926-1.81)**
	A	**415(69.3%)**	**127(63.5%)**	

For all analyses, the homozygote carriers of the major allele were used as the reference group G/G. CI, confidence interval (95%); OR, ood ratio; Case, diabetic type2hypertesiv patients with microvascular complications; Control, non diabetic non hypertensive individual. *P*-value Probability value (0.05).

Table3.12

Association between genotype and retinopathy in case groups.

There was no statically significant association between genotype A/A, G/G and G/A with present or absent of retinopathy in patients group,

Table3.12

Genotype	Retinopathy		Total	*P*-value
	Present *N*(%)	Absent *N*(%)		
A/A	67 (35.8%)	120 (64.2%)	187(100.0%)	0.69
G/G	26 (36.6%)	45(63.4%)	71(100.0%)	
G/A	18(42.9%)	24(57.1%)	42(100.0%)	
Total	111(37.0%)	189(63.0%)	300(100.0%)	

Table.3.13

Association between genotype and nephropathy in case group.

There was no statistically significant association between genotype A/A, G/G and G/A present or absent of nephropathy of patients group

Table.3.13

Genotype	Nephropathy		Total	*P*-value
	Present N(%)	Absent N(%)		
A/A	7(3.7%)	180(96.3%)	187(100%)	0.43
G/G	2(2.8%)	69(97.2%)	71(100%)	
G/A	0(0%)	42(100%)	42(100%)	
Total	9(3.0%)	291(97.0%)	300(100%)	

Table.3.14

Association between genotype and neuropathy in case groups.

There was no statistically significant association between genotype A/A, G/G and G/A with present or absent of neuropathy of patients group.

Table.3.14

Genotype	Neuropathy		Total	*P*- value
	Present N(%)	Absent N(%)		
A/A	25(13.4%)	162(86.6%)	187(100.0%)	0.75
G/G	8(11.3%)	63(88.7%)	71(100.0%)	
G/A	4(9.5%)	38(90.5%)	42(100.0%)	
Total	37(12.3%)	263(87.7%)	300(100.0%)	

Chapter Four

4.1Discussion

This was a case control study conducted on a total of 300 type 2 diabetic hypertensive Sudanese patients with micro vascular complications as case group and 100 non diabetic non hypertensive as a control group at Khartoum teaching hospital in Khartoum state during the time period from January 2014 to April 2017**,** age range between 38 and 90 years age mean 57.2, patients with or without complications to investigate the association between beta fibrinogen 455 G→A gene polymorphisms with fibrinogen, D-dimer, FDPs with demographic and clinical data. Genotypes were determined by polymerase chain reaction a restriction fragment length polymorphism with restrictive enzyme Hae III and Fibrinogen was assessed by coagulometer, D-Dimer and Fibrinogen/Fibrin Degradation Products was measured by ELISA.

Females were more frequent than males in study group. Essential hypertension more frequent than secondary hypertension, A/A genotype more dominant than G/G and G/A. There was statistically significant increase in mean fibrinogen, when compared patients to control groups (*P-v*alue=0.000), this was agreement with the study by Khan *et al.*, 2005, (Niranjan and Vijay. 2012). Who were found statistically significant increase of fibrinogen between patients and control individuals, thus may due to Mechanism contributing to systemic vascular disease in diabetes with hypertension are platelet adhesion and aggregation defects, coagulation and lipoprotein abnormalities, alterations in vascular endothelium, vascular smooth muscle abnormalities and disagree `with study by Kamath and Lip who were found plasma fibrinogen is an important component of the coagulation cascade. Increasing evidence from epidemiological studies suggests that elevated plasma fibrinogen levels are associated with an increased risk of cardiovascular disorders, including ischemic heart disease, stroke and other thrombo-embolism

(Kamath and Lip, 2003). The fibrinogen was traditionally considered to be a haemostatic factor, but a recent analysis from the Cardiovascular Health Study showed elegantly that fibrinogen clusters with inflammatory and not with haemostatic factors. **(**Sakkinen *et al*., 2000). There was statistically significant increase in mean D-dimer and when compared patients to control groups (*P*-value=0.00), this was agreement with related study done by (Palanisamy *et al*., 2011) and disagreement with study done by Leonardo, who were found that significant of D-dimer between study population and control individuals, have reported a strong and independent association between fibrinogen and the presence and severity of diabetic hypertension and D-dimer concentrations were significant related to the severity of target organ damage in ischemic heart disease hypertensive patients and that hypertension per se may confer a hypercoagulable state, suggest that elevation of D-dimer ,the principle breakdown fragment of fibrin and a reliable indicator of the overall state of activation of the coagulation pathways, is associated with increased risk of future myocardial infarction(Leonardo *etal*.,2000). Also, D-dimer is one of the more widely studied haemostatic variables for association with CHD. Increased D-dimer levels indicate increased fibrin turnover (Danesh *et al*., 2001). There was statistically significant increase in FDPs when compared patients to control groups (*P*-value=0.00).May due to an elevation of circulating FDPs that reflect, at least partly, a more rapid degradation of fibrinogen given that it is a protein with short half life. FDPs including D-dimer are produced by clot degeneration (Amin *et al.*, 2014). In normal subjects, plasma FDPs levels are below the detectable levels. In response to the fibrous deposition in the inflammatory area, the body produces more fibrinogen and FDPs, which counteract the thrombin (Amin *et al*., 2014). There was no statistically significant difference in mean fibrinogen, D-dimer and FDPs when females compared to males in patient group (*P*-value=0.131, 0.096 and 0.340),

respectively. This was in agreement with the study by Erem who did not found statistically significant association with gender variable. There was no statistically significant between fibrinogen, D-dimer and FDPs levels when compared in patients with essential and secondary hypertension (*P-v*alue=0.687, 0.350 and 0.663) respectively. My suggest that in about 90% of cases of hypertension has unknown cause (primary or essential hypertension), the remainder were mostly secondary to renal disease or (less often) to renal artery stenosis (renovascular hypertension), endocrine abnormalities, vascular malformations, or neurogenic disorders (Mitchell *et al.,* 2006). Thrombosis often appears to complicate the course of patients with hypertension (Osman and Muddathir, 2013)**.** They were statistically significant significant positive correlation between fibrinogen, D-dimer and FDPs with duration time of both diabetes mellitus and hypertension (r=0.480**, 0.481** and 0.318**)respectively(*P*-value=0.000). This was in agreement with the study by Abdulrahaman and Dallatu., who were found that significant fibrinogen, D-dimer and FDPs level in duration time of both diabetes mellitus and hypertension has the same onset time (Abdulrahamanand Dallatu,2012). This may indicate that both diabetes mellitus and hypertension complications may start irrespective to diabetic hypertension in onset, and this was related to glucose control and diabetes mellitus management rather than duration of onset of the diseases. (Asakawa *et al*., 2000). In addition, it was disagree with other studies reported positive relationship between the mean platelets volume and duration of diabetes which gives evidence that the risk of micro vascular complications increases with duration of diabetes (Akinsegun *et al*., 2014). There were statistically significant increase in mean fibrinogen, D-dimer and FDPs in patients with neuropathy compared to those without neuropathy (*P*- value=0.012. 0.008 and 0.005) respectively. This was agreement with related study done by Bolman*etal*; who were found significant in neuropathy in fibrinogen and D-dimer of diabetic

hypertensive patients).May were suggested, the effects of micro vascular disease are most profound in the retina, kidneys and peripheral nerves. This was disagreement with study by Fong, who was explained that several biochemical pathways were proposed to link hyperglycaemia and micro vascular complications. These include formation of advanced glycation end products (AGEs), polyol accumulation, oxidative stress and activation of protein kinase C (PKC). These processes are thought to modulate the disease process through effects on cellular metabolism, signaling and growth factors (Fong *et al*., 2004). There was statistically significant increase in mean fibrinogen in patients with nephropathy compared to those without nephropathy (*P*.value=0.021). This was agreement with related study done by Bolman*et al*; who were found statistically significant increase in nephropathy of fibrinogen in study group, may due to fibrinogen and albumin are two liver synthesized proteins, with different functions. Patients with increased protein loss such like nephritic syndrome and renal failure, production of albumin and fibrinogen increased (Zanetti *et al*., 20012), suggesting coordinate changes in hepatic protein production in response to albuminuria. Chronic complications of uncontrolled diabetes begins with loss of a small amount of albumin (Microalbuminuria), this may lead to increase production of fibrinogen (Sacks *et al*., 2011). Microalbuminuria, a marker of microangiopathy, is an early renal manifestation of diabetes. Persistent hyperglycemia causes hyper filtration, advanced glycation products and activation of cytokines. All this causes glomerular damage and endothelial dysfunction leading to increased systemic vascular permeability (Roberto *et al*., 2001). It modulate the metabolism of cardiovascular risk factors like fibrinogen, Lp(a) and left ventricular mass (Rutter *et al*., 2000). Although DD and FDPs were increase in patients with nephropathy but the different was not statically significant*(P*-value=0.138 and 0.144), this was agreement with results by Van *et al*, suggest an increased prevalence of

microalbuminuria associated with poorer glycaemic control and augmented reactive fibrinolysis (probably after preceding coagulation activation). Recently, we described an increase of coagulation activation with a decreased efficiency of glyco-metabolic control, (Van *et al*., 1990). There were no statistically significant in mean fibrinogen and FDPs in patient with retinopathy compared to those without retinopathy (*P*-value=0.094 and 0.151) respectively. This was agreement with the related study by Fujisawa *et al*, who did found that no statistically significant different in Japanese diabetic type 2 hypertensive populations. Retinopathy refers to progressive pathologic alterations in the retinal microvasculature leading to areas of retinal non perfusion, increased vascular permeability and the pathologic proliferation of retinal vessels (Robert, 2004). Thus may due to Patients with diabetic retinopathy show some alterations in their hemorreological determinants. Plasma fibrinogen concentration, which is a major determinant of plasma viscosity, is increased in diabetic retinopathy patients. The packed cell volume is another important determinant of whole-blood viscosity, which has been reported to be increased in diabetic retinopathy patients (Vekasi *et al*., 2001). Structural changes of the hemoglobin molecule due to glycosylation have been reported to contribute to increased viscosity of blood (Symeonidis, *et al*., 2001) and also affect the oxygen release in diabetic patients. The increased packed cell volume observed in the Caucasian diabetic patients could be a compensatory mechanism due to the impaired oxygen release in the diabetic patients. (James *et al*., 2004) There was statistically significant increase of D-dimer in patients with retinopathy compared to those without retinopathy(*P*-value=0.004). No data published was found. Fibrinogen, D-dimer and FDPs were statistically significant increase between age groups *(p*-value=0.001, 0.003 and 0.047) respectively. This was agreement with the study done by Leonardo *et al*, who did found that statistically significant between age groups in case study. In

general, fibrinogen levels increased with age, smoking, body size, diabetes, fasting serum insulin, LDL cholesterol, lipoprotein(a), leukocyte count, and menopause, and decreased with ethanol intake, physical activity, HDL cholesterol, and female hormone use (Folsom *et al*.,2001).

I was chose to examine the 455G/A polymorphism because of recent in vitro evidence suggesting that this polymorphism is associated with altered transcriptional activity in fibrinogen promoter/luciferase reporter constructs and influences the binding of a specific, but as yet unidentified, nuclear protein that regulates transcription (Anderson *et al*.,1993). Our results were in contrast agreement with those of previous studies (Humphries *et al*., 1995) that have demonstrated an association between this polymorphism and fibrinogen levels. The explanation for the difference in the results of these studies may involve heterogeneity in the genetic factors regulating fibrinogen levels in patients and healthy subjects. Moreover, multiple other variables in diabetes could influence the interaction between genotype and circulating fibrinogen levels. Along these same lines, fibrinogen gene polymorphisms have been shown to influence the effect of smoking on fibrinogen levels in healthy subjects (Thomas *et al.,* 1994). No evidence was found for linkage between the fibrinogen locus and them regulation of plasma fibrinogen levels (Soria *et al*., 2005). There were common graduate increase of means in fibrinogen, D- dimer and FDPs levels in A/A allele's genotype than G/G and less frequent were G/A alleles in case study.

There was statistically significant and risk factor when the homozygote minor allele was compared with the homozygote major allele and increase risk factor when was compared heterozygote allele with homozygote minor allele. This agreement with study done by Abbas Dehghan, who did found that the most significant SNP in our study was rs1800789 which is located on the FGB gene. The FGB gene encodes the fibrinogen ß chain. A well-characterized SNP at this

locus is rs1800787 (–148C/T) which resides 965 base pairs away from our top SNP (rs1800789) and was in high known that rs1800787 directly affects gene transcription in basal and IL6-stimulated conditions in luciferase expression studies . (Verschuur M*et al*., 2005). There were increased frequencies of A/A alleles in patients and control groups. This is in agreement with the study by (Lam KS*et al.*, 1999), who did found difference between control individuals and patients, A alleles genotype frequency were found in diabetic patients with hypertesion . However, in our study, A/A alleles genotype and allele A frequency were more common among the case and control groups. This was agreement with the related study by Kessler C, he was found homozygosity for the A allele was more common in patients with cardiovascular diseases resulting from large-vessel disease. These data demonstrate that the A/A alleles genotype of the beta fibrinogen gene polymorphism 455G→A substitution occur significantly more frequently in patients with hypertension (Kessler *et al.*, 1995).

There was no statistically significant association between alleles genotype A/A,G/G and G/A with absent or present neuropathy (*P*-value= 0.75). This was agreement with the study by Ajjan and others researchers, who were found others factors like inflammation and diabetes may contribute to this increase (Ajjan *et al.*,2009). There was no statistically significant association between alleles genotype A/A, G/G and G/A with absent or present nephropathy (*P*- value=0.61). Allelic association in Caucasians, as well as in Hong Kong Chinese (O.C.K. Ma *et al*, unpublished observations) with a C-148T change located close to the consensus interleukin-6 response element and may therefore be linked to increased fibrinogen gene transcription in response to interleukin-6 released from lung macrophages following tissue damage induced by smoking. A genotype-environment interaction was also apparent in the diabetic patients in whom the genotype effect was much smaller, probably because of the predominant effect of nephropathy, the most

important factor contributing to their increased fibrinogen concentrations, (Thomas *et al*., 1994). There was no statistically significant association between alleles genotype A/A,G/G and G/A with absent or present retinopathy in patients group (*P*-value= 0.69), no similar others studies were result found . There was no statistically significant association between alleles genotype A/A,G/G and G/A when compared male to female in patient group(*P*- value= 0.61). Related to study done by (Hamphreis *et al.*, 1995 and Lam *et al* 1998).Who explained that, it is well known that genetic and environmental factors interact in determining plasma concentrations of haemostatic factors. In this study, the effect of the A/A genotype was strongest among male control subjects, in keeping with a gender-genotype interaction (Hamphreis *et al.*, 1995). The higher fibrinogen concentrations in women have been attributed to environmental influences such as the menopause or oral contraceptives (Lam *et al.*, 1998).

4.2 Conclusion

These results of the study was concluded that;

- Fibrinogen, D-dimer and FDPs were statistically significant increase in mean patients compared to control group, in patient with and without neuropathy, according to duration time of diabetic hypertension and between age group.
- They were statistically significant increase in mean fibrinogen in patients with nephropathy compared to those without nephropathy.
- There was statistically significant increase in mean of D-dimer in patients with retinopathy compared to those without retinopathy.
- There were more frequent of A/A in patients compared to control groups.

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4.3 Recommendations

From the results of this study, I recommended that:

1. Patients with type II diabetes mellitus and hypertension should check their, fibrinogen, D-dimer and fibrinogen/fibrin degradation products for early detection of complications
2. We recommended for further studies to evaluate others related genes to diabetic hypertension disorders in different area of Sudan and its association with complications.

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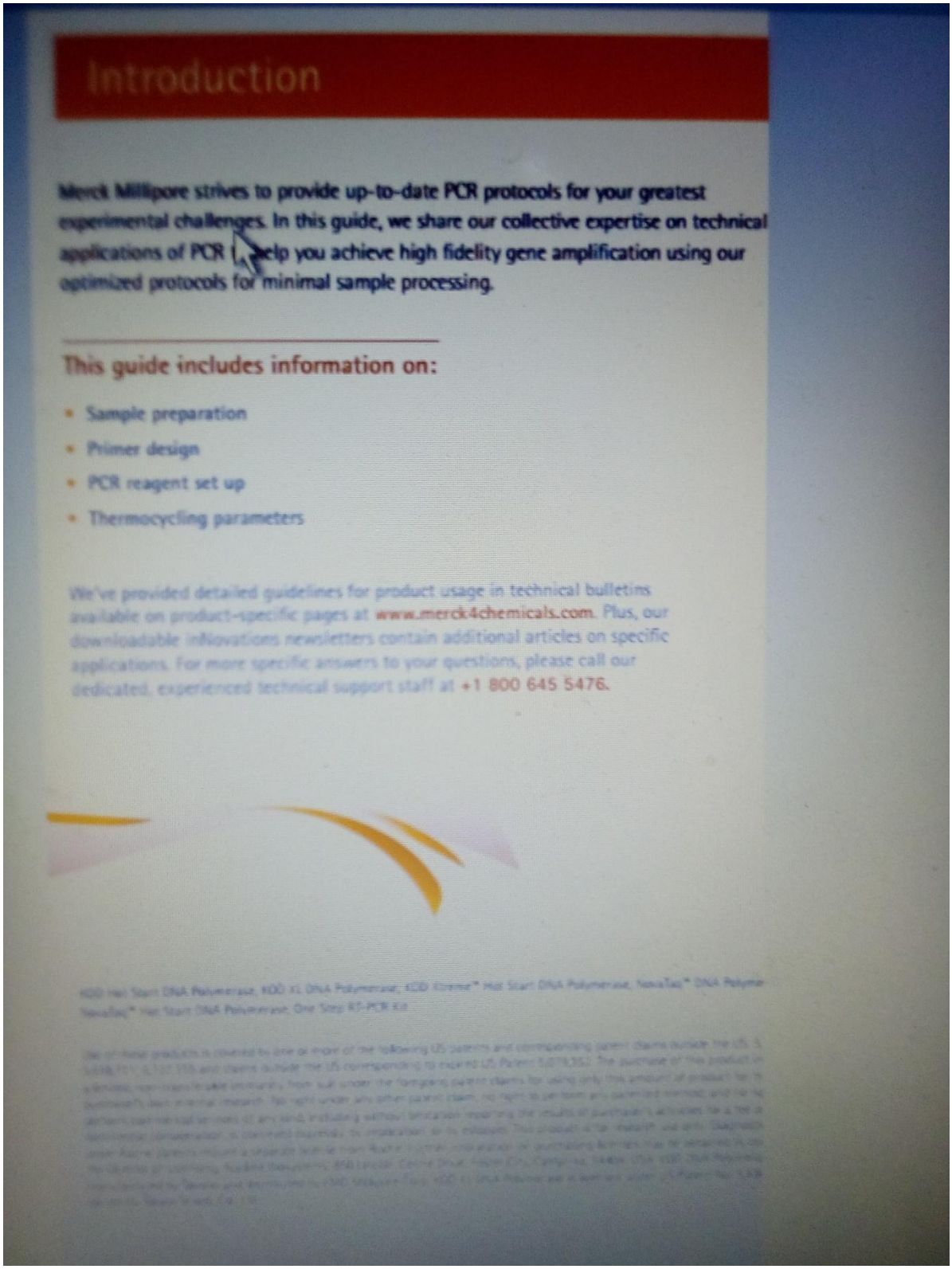


Appendix (I) PCR Protocol.

Sandwich ELISA

abcam
discover more

The Sandwich ELISA measures the amount of antigen between two layers of antibodies (i.e. capture and detection antibody). The antigen to be measured must contain at least two antigenic sites capable of binding to antibody, since at least two antibodies act in the sandwich.

Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in Sandwich ELISA systems. Monoclonal antibodies recognise a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible.

The advantage of Sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive (up to 2 to 5 times more sensitive than direct or indirect).

General note:

Sandwich ELISA procedures can be difficult to optimise and tested match pair antibodies should be used. This ensures the antibodies are detecting different epitopes on the target protein so they do not interfere with the other antibody binding. Therefore, we are unable to guarantee our antibodies in sandwich ELISA unless they have been specifically tested for sandwich ELISA. Please review antibody datasheets for information on tested applications.

General Procedure:

Coating with Capture antibody

1. Coat the wells of a PVC microtiter plate with the capture antibody at a concentration of 1-10 µg/ml in carbonate/bicarbonate buffer (pH9.6).

If an unpurified antibody is used (eg ascites fluid or antiserum), you may need to compensate for the lower amount of specific antibody by increasing the concentration of the sample protein (by 10ug/ml).

2. Cover the plate with an adhesive plastic and incubate overnight at 4°C.

3. Remove the coating solution and wash the plate twice by filling the wells with 200 µl PBS. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.

Blocking and Adding Samples

4. Block the remaining protein-binding sites in the coated wells by adding 200 µl blocking buffer, 5% non fat dry milk/PBS, per well.

5. Cover the plate with an adhesive plastic and incubate for at least 1-2 h at room temperature or, if more convenient, overnight at 4°C.

6. Add 100 µl of appropriately diluted samples to each well. For accurate quantitative results, always compare signal of unknown samples against those of a standard curve. Standards (duplicates or triplicates) and blank must be run with each plate to ensure accuracy. Incubate for 90 min at 37°C.

For quantification, the concentration of the standard used should span the most dynamic detection range of antibody binding. You may need to optimize the concentration range to ensure you obtain a suitable standard curve. For accurate quantitation, always run samples and standard in duplicate or triplicate.

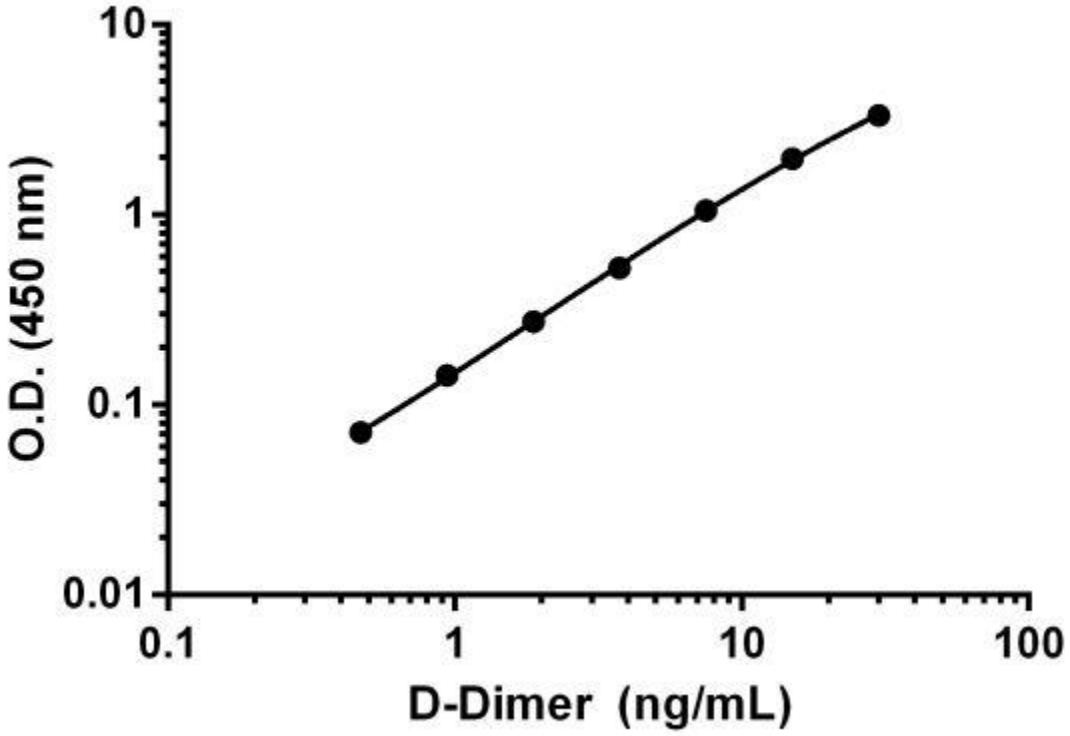
7. Remove the samples and wash the plate twice by filling the wells with 200 µl PBS.

Incubation with Detection antibody and then Secondary antibody

8. Add 100 µl of diluted detection antibody to each well.

Appendix (II) ELISA Protocol

Typical Data:-



Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed. Appendix (III) typical standard curve

Fibrinogen reagents

Bovine thrombin: Buffered lyophilized bovine thrombin, preservatives Plasma Calibration "Cal-Fib": Lyophilized human plasma, stabilizers and preservatives.

Control Plasma "Pat-Fib":Lyophilized human plasma, stabilizers and preservatives. (Appendix IV)

Fibrinogen material required

Test tubes for analysis, coagulometer cuvette ,sterirre , auomicropipettes 0.1, 1 ml and gloves, tipsand automated coagulation analyzer. .(Appendix V)

D-dimer and FDPs reagents preparation

-Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells.

-Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations. .(Appendix VI)

Materials required

- Micro plate reader capable of measuring absorbance at 450 or 600 nm
- Method for determining protein concentration (BCA assay recommended)
- Deionizer water
- PBS (1.4 mM KH2PO4, 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4)
- Multi- and single-channel pipettes
- Tubes for standard dilution
- Plate shaker for all incubation steps
- Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors)

.(Appendix VII)

DNA extraction kits

The GF-1 Blood DNA Extraction Kit was designed for rapid and efficient purification of genomic DNA from up to 400µl whole blood. This kit was used a specially treated glass filter membrane fixed into a column to efficiently bind DNA in the presence of high salt. kit was applied the principle of a mini-column spin technology and the use of optimized buffers to ensure that only DNA was isolated while cellular proteins, metabolites, salts and other low molecular weight impurities were removed during the subsequent washed steps. High-purity genomic DNA was then eluted in water or low salt buffers and has an A260/280 ratio between 1.7 and 1.9 was made it ready to used in many routine molecular biology applications such as restriction enzyme digestion, PCR, Southern blotting, DNA fingerprinting, and other manipulations

Components

GF-1 columns 100, Collection tubes 100, Blood Lysis Buffer, (Buffer BB) 24ml, Wash Buffer 1(concentrate)*30ml, Wash Buffer 2(concentrate)*34ml, Elution Buffer 1.5ml 10ml 20ml Proteinase K*. (Appendix VIII).

Standard Curve Measurements			
Conc. O.D. (ng/mL)	**O.D. 450 nm**		**Mean O.D**
	1	**2**	
0	**0.051**	**0.049**	**0.050**
0.475	**0.121**	**0.123**	**0.122**
0.95	**0.193**	**0.193**	**0.193**
1.9	**0.325**	**0.321**	**0.323**
3.8	**0.561**	**0.587**	**0.574**
7.5	**1.112**	**1.083**	**1.100**
15.0	**2.043**	**2.001**	**2.022**
30	**3.388**	**3.358**	**3.373**

Example of D-Dimer standard curve. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

Appendix (IX) Standard Curve Measurements.

Sudan University of Science and Technology

College of Postgraduate Studies

Hematology Department

Investigations the association between beta fibrinogen 455 G→A gene polymorphisms with fibrinogen, D-dimer, fibrinogen/fibrin degradation products levels and micro vascular complications in type2 diabetic hypertensive Sudanese patients.

No…….

Age…….....

Gender…….

Duration of both DM and HTN……

Hypertension stage……..

Retinopathy …….

Nephropathy…….

Neuropathy……

<u>Investigations</u>

Fibrinogen……mg/dl.

D-dimer……ng/dl

FDPs…… ……../ng/dl.

455G/A genotype…….

Appendix X

Informed Consent
اعلام موافقة

هذه دعوة مني الباحث :عادل عبد الرحمن عيسى احمد طالب دكتوراة –مختبرات طبية-امراض الدم بجامعة السودان للعلوم والتنكلوجيا لمشاركتكم في برنامج بحث هدفه تشخيص الجينات المرتبطة بمستويات الفبرينوجين ودى دايمر ومنتجات تكسر الفبرين لدى السودانيين المصابين بمرض السكر النوع الثاتى وارتفاع ضغط الدم.

اذا رغبتم في انجاح هذا البرنامج فاني وفريق البحث سنقوم :

باخذ عينة من الدم لقياس مستويات الفبرينوجين ودى دايمر ومنتجات تكسر الفبرين ولتشخيص الجينات المرتبطة بها بملء استمارة بمعلومات تخصكم لها علاقة بموضوع البحث.

اى معلومة تخصكم بالاستمارة سوف تكون سرية.

مشاركتكم في البرنامج تسعدنا وتساهم بانجاح هدف البحث.

لكم كامل الحرية فى اختيار عدم المشاركة او الانسحاب من برنامج البحث في اى وقت تشاءون.

يمكنكم الحصول على اجابة لاى سؤال عن برنامج البحث.

التاريخ.....

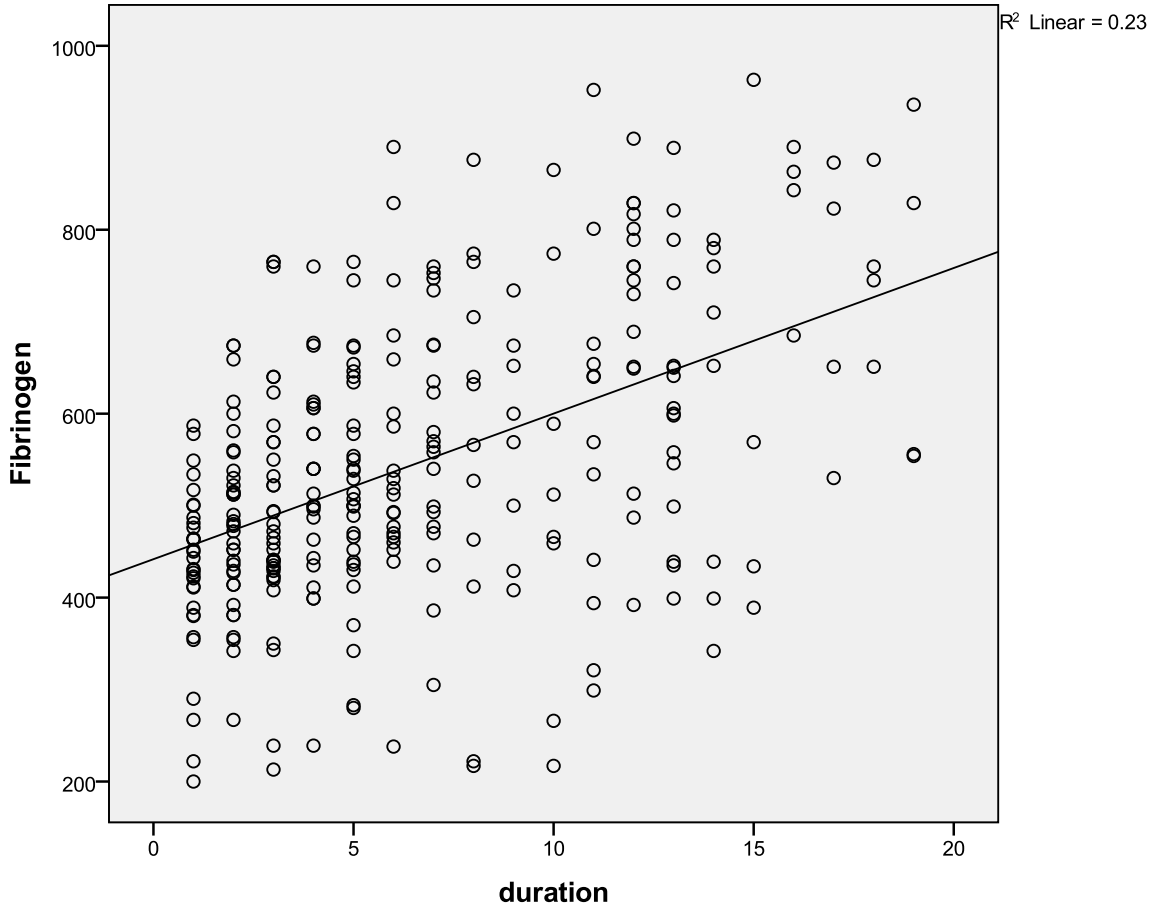
توقيع المتبرع .....

توقيع الباحث.....

Appendix XI

Correlations between duration time of both diabetes and hypertension and fibrinogen in study group.

There was statistically significant positive correlation between fibrinogen with duration time of both diabetes mellitus and hypertension.

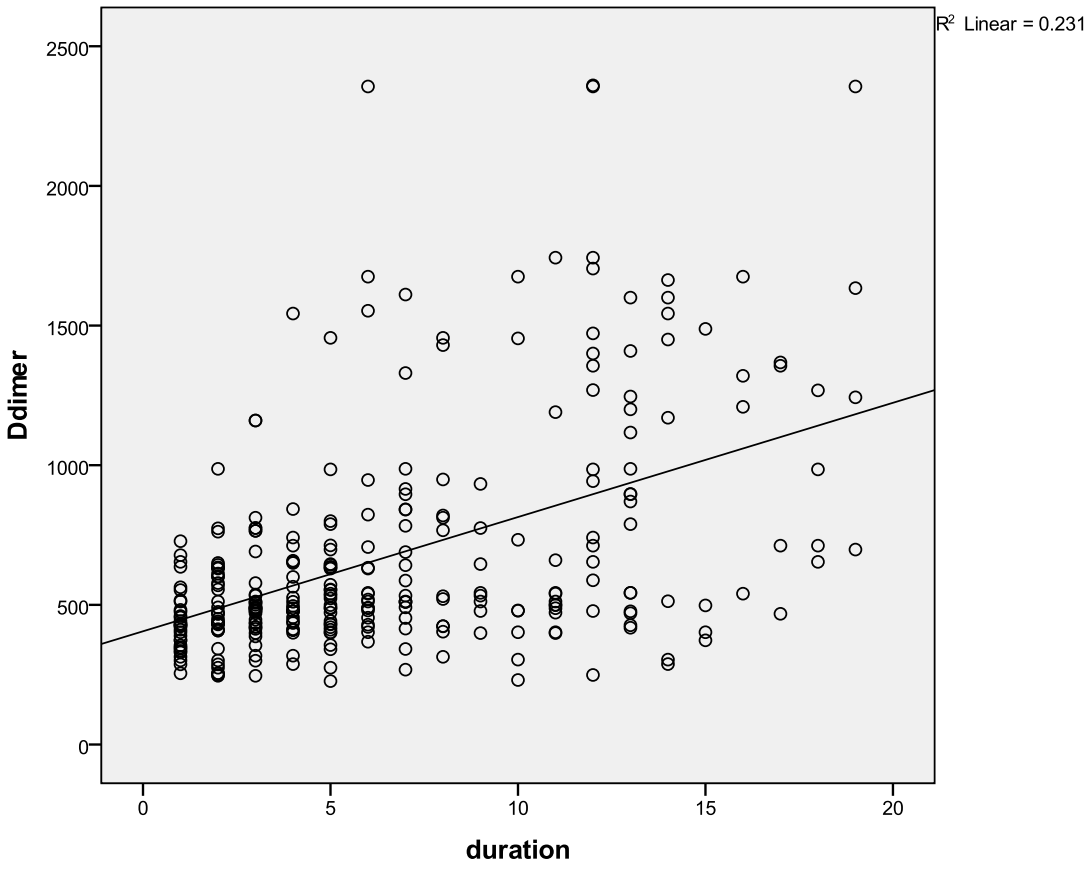


Scatter plot shows the relationship between fibrinogen (mg/dl) with duration time of both diabetes mellitus and hypertension(r=0.480, *P*-value=0.000).

Appendix XII

Correlations between duration time of both diabetes mellitus and hypertension with D-dimer in study group.

There was statistically significant positive correlation between D-dimer with duration time of both diabetes mellitus and hypertension.

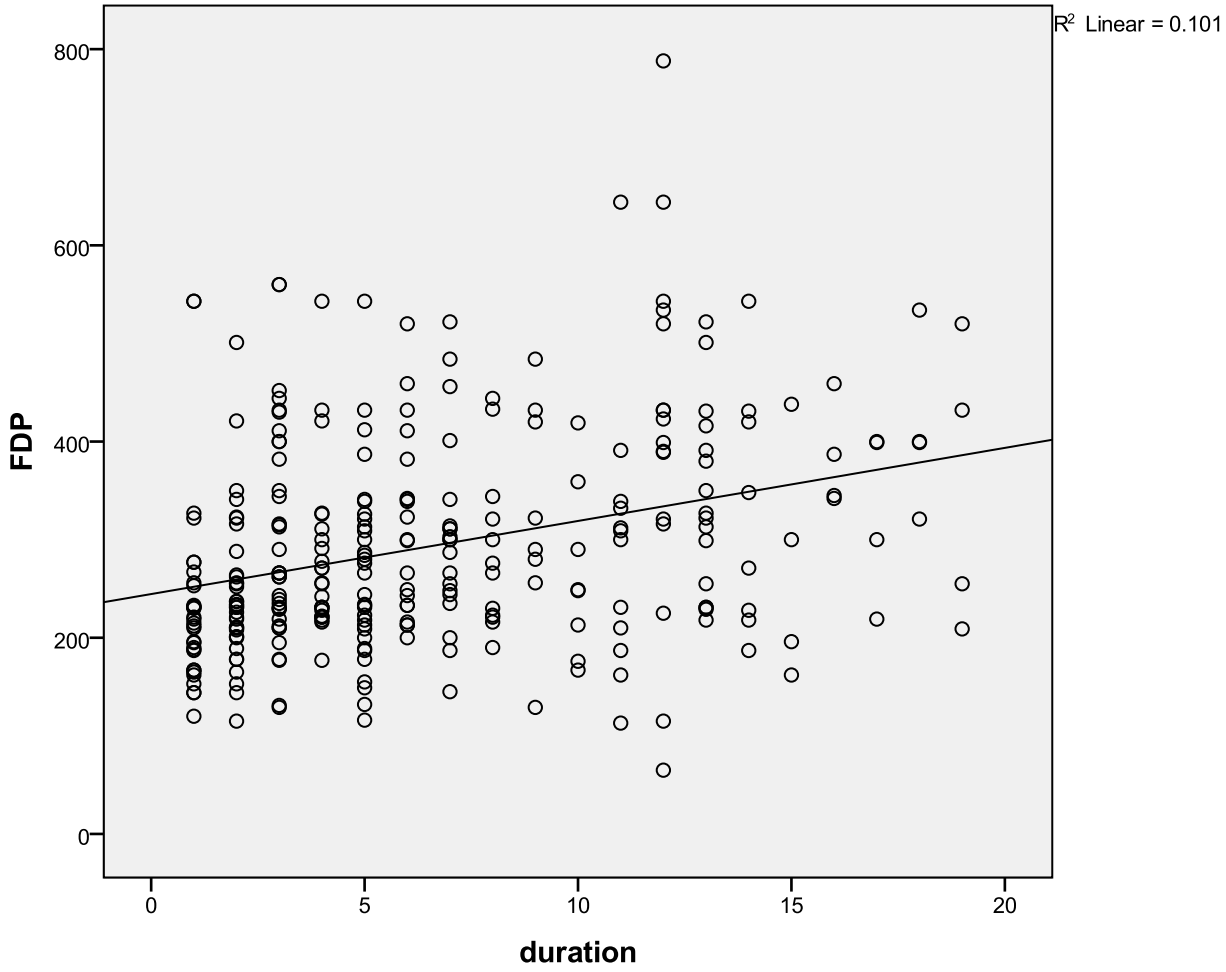


Scatter plot shows the relationship between D-dimer(ng/dl) with duration time of both diabetes mellitus and hypertension(r=0.481, *P*-value=0.000).

Appendix XIII

Correlations between duration time of both diabetes mellitus and hypertension with FDPs in study group.

There was statistically significant positive correlation between FDPs with duration time of both diabetes mellitus and hypertension.



Scatter plot shows the relationship between FDPs (ng/dl) with duration time of both diabetes mellitus and hypertension(r=0.318, *P*-value=0.000)

Appendix XIV