Sudan University for Sciences and Technology

College of Graduate Studies

Association between Angiotensin Converting Enzyme Gene Insertion/Deletion Polymorphism and Cardiovascular Diseases among Diabetic Patients

العلاقة بين تعدد الشكل الجيني إضافة / حذف للإنزيم المحول للأنجيوتينسين وأمراض القلب وسط مرضي السكري

A dissertation is submit for partial fulfillment for the requirement of MSc degree in Medical Laboratory Sciences (Hematology and Immunohematology)

By:

Mona Mohammed Suleiman salama

Bachelors of the Sciences, Medical Laboratory Sciences, Kordofan University(2013)

Supervisor:

Dr. Elshazali Widaa Ali

June 2017

الآية

قال تعالي :-

(قَالُوا سُبْحَانَكَ لاعَلَمْ لَنَا إِلاَّ مَا عَلَّمْتنا إِنكَ أَنْتَ الْعَلِيمُ الْحَكِيم)

صدق الله العظيم

سورة البقرة الآية (32)

Dedications

To the soul of my father who gave his life for our future.

To my precious mother.

To wonder sisters and my brother.

To my cousin mohammed alqali

Acknowledgements

Abstract

Cardiovascular diseases(CVDs) are the most prevalent cause of mortality and morbidity among people with diabetes mellitus. On the other hand, diabetes mellitus deserves to be designated a major risk factor for cardiovascular diseases.

The insertion/deletion (I/D) polymorphism of the angiotensin-converting enzyme (ACE) gene is considered to contribute to an interpersonal variability in serum ACE levels and thereby providing a plausible basis for increased susceptibility to thrombotic events.

This study aimed to investigate the association between ACE gene I/D polymorphism and risk of cardiovascular diseases in diabetic patients.

A total of 100 subjects were recruited for this case control study, 50 diabetic patients with CVDs and 50 healthy volunteers as a control group. Blood samples were collected from all participants in EDTA anticoagulant container, genomic DNA was extracted by salting out method, and the ACE I/D polymorphism was analysed using polymerase chain reaction (PCR). Amplified fragments separated on 2% agarose gel stained with ethidium promide and demonstrated by gel documentation system.

Patients' data was collected by structured interview questionnaire and analysed by statistical package for social sciences (SPSS), version 19.

The DD genotype of ACE I/D polymorphism was more frequent in both diabetic patients with CVDs and control group(72%,74%) respectively than ID genotype(28%,26%) respectively, while the II genotype was absent in both groups.

ACE I/D polymorphism was found to have has no effect on age of incidence of CVDs in diabetic patients($Pvalue$ 0.886).

There was no interaction observed between ACE genotypes and other known CVDs risk factors in diabetic patients

The results showed no statistically significant difference in mean duration of DM when compared in the two ACE genotypes, Mean± SD (12.28±8.410, 12.21±8.894 for DD,ID) respectively, ($pvalue$ 0. 981).

There was no statistically significant association between ACE I/D polymorphism and risk of cardiovascular diseases in Sudanese diabetic patients.

المستخلص

تعتبر أمراض القلب اكثر سبب لحدوث الوفيات في مرضي السكري، من جهة أخري يشكل مرض السكري عامل خطير لحدوث أمراض القلب.

يعتبر تعدد الشكل الجيني للإنزيم المحول للأنجيوتينسين يعتبر مساهم في تغيير مستوي الإنزيم في الدم وبالتالي يوفر قاعدة مناسبة لزيادة التعرض للجلطات.

هدفت هذه الدراسة لفحص تعدد الشكل الجيني إضافة/حذف وأمراض القلب وسط مرضي السكري.

شملت الدراسة 100 شخص، 50 مريض مصاب بالسكري والقلب و 50 أصحاء.أخذت عينة دم، أستخلص الحمض النووي(DNA) ، تم تحليل تعدد الشكل الجيني للإنزيم المحول للأنجيوتينسين بالتفاعل البلمرة التسلسلي وتم فصل الجزء المستهدف من ال (DNA) بواسطة 2% من الجل وصبغ بالإثيديوم برومايد وأظهرت بنظام توثيق الجل.

معلومات المريض جمعت بواسطة استبيان من خلال معاينة وحللت بواسطة الحزمة الإحصائية للعلوم الإجتماعية.

حذف/حذف نمط جيني هوالأكثر تكرارا بين المرضي والأصحاء(72%،74%) متتالية، يتبعه إضافة/حذف نمط جيني(28%،26%) متتالية، ولا يوجد إضافة/إضافة نمط جيني.

الإنزيم المحول للأنجيوتينسبن لا يؤثر علي عمر حدوث أمراض القلب في مرضي السكري (القيمة 0.886).

لا يوجد تفاعل بين الإنزيم المحول للأنجيوتينسبن نمط جيني والعوامل المؤثرة في أمراض القلب عند مرضي السكري.

لا يوجد إختلاف في متوسط فترة مرض السكري عندما قورنت مع النوعين من الإنزيم المحول للأنجيوتينسبن للأنماط الجينية المتوسط± الإنحراف المعياري (12.28±8.410 ، 12.21±8.894) لل حذف/حذف ، إضافة/حذف متتاليا، (القيمة 0.981).

لاتوجد علاقة بين تعدد الشكل الجيني للإنزيم المحول للأنجيوتينسين وأمراض القلب في مرضي السكري السودانيين.

List of contents

Chapter One

Introduction and Literature Review

Chapter Two

Materials and Methods

Chapter Three

Results

Chapter Four

Discussion, Conclusions and Recommendations

List of tables

List of figures

List of Abbreviations

ADA	American diabetes association
ACE	Angiotensin converting enzyme
AF	Atrial fibrilation
AS	Atherosclerosis
AVR	Aortic valve regurgitation
Bp	Base pair
CVD	Cardiovascular sisease
CAD	Coronary artery disease
CHD	Coronary heart disease
CM	Cardiomyopathy
DM	Diabetes mellitus
HbA1c	Hemoglobin A1c
HDL	High density lipoprotien
IDDM	Insulin dependent diabetes mellitus
I/D	Insertion/deletion
ICAM-1	Intercellular adhesion molecule-1
ICA	Islet cell antibody

LDL	low density lipoprotein
MODY	Maturity onset diabetes of the young
MCP-1	Monocyte chemoattractant protein 1
MI	Myocardial infarction
MVR	Mitral valve regurgitation
MYO	Myocardial hyperatrophy
NO	Nitric oxide
NIDDM	Non-insulin dependent diabetes mellitus
PDGF	Platelet-derived growth factor
PAI-1	Plasminogen activator inhibitor 1
PCR	Polymerase chain reaction
rxn	Reaction
RAS	Renin-angiotensin system
SPSS	Statistical package for social sciences
TE	Tris EDTA
T2	Type 2
VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cell
VEGF	Vascular endothelial growth factor

Chapter one

Introduction and Literature Review

1.1.1 Diabetes mellitus

Diabetes mellitus(DM) is a chronic disease which has been described as a state of raised blood glucose associated with premature mortality. It arises when the pancreas fails to produce enough insulin (type 1 diabetes), or when the body cannot effectively use of the insulin produced (type 2 diabetes) (International Diabetes Federation., 2001).

1.1.1.1 Epidemiology of diabetes mellitus

Diabetes is the most common endocrine disorder (Bastaki., 2005). Its incidence and prevalence has significantly increased in recent decades, mainly because of an increase in type 2 diabetes, which represents almost 90% of all cases of diabetes. The World Health Organization (WHO) estimates that, by 2025, there will be 300 million diabetic patients (5.4% of the world population). Older patients are most affected by diabetes, as the disease prevalence increases with age, at least up until 75 years. The progressive aging of the global population could explain about half of the predicted increase of diabetic patients in the near future (Monteiro *et al*., 2005).

1.1.1.1.1 Diabetes in Sudan

The prevalence and incidence rates of DM in Sudan, as in many other low-income countries, are increasing to epidemic proportions, leading to emergence of a public health problem of major socio-economic impact. In the northern states the crude prevalence in 1992 reached 3.4% in those ≥25 years of age. It was found to be

5.5% in the northern state and 8% in Khartoum state. The prevalence was particularly high (10.8%) in a certain community in northern state. Type 1 DM is not rare in Sudan, the prevalence being approximated to 0.1% among children7-14 years of age (Elrayah-Eliadarous., 2007).

1.1.1.2 Classification of diabetes mellitus

The diagnostic criteria and the classification of diabetes was first put forward by the WHO in 1965, then by the National Diabetes Data Group in 1979, and this was followed by simplified recommendations by the WHO in 1980. These WHO recommendations were modified slightly in 1985. The latest recommendations have been published by the American Diabetes Association (ADA) in 1997 and by the WHO in 1999. Both groups agree on the recommendations and criteria .

Diabetes mellitus may be categorized into several types but the two major types are type 1 and type 2. On the basis of aetiology, the term type 1 and type 2 were widely used to describe Insulin Dependent Diabetes Mellitus (IDDM) and Non-Insulin Dependent Diabetes Mellitus (NIDDM), respectively. The term juvenile -onset diabetes has sometimes been used for IDDM and maturity-onset for NIDDM (Bastaki., 2005).

1.1.1.2.1 Type 1 diabetes mellitus

Is present in patients who have little or no endogenous insulin secretory capacity and who therefore require insulin therapy for survival. The two main forms of clinical type 1 diabetes are type 1a (about 90% of type 1 cases in Europe) which is thought to be due to immunological destruction of pancreatic ß cells resulting in insulin deficiency; and type 1b (idiopathic, about 10% of type 1 diabetes), in which there is no evidence of autoimmunity. Type 1a is characterized by the presence of

islet cell antibody (ICA), anti-glutamic acid decarboxylate, Islet Antigen 2 Antibody or insulin antibodies that identify the autoimmune process with ß-cell destruction. Autoimmune diseases such as Grave's disease, Hashimoto's thyroiditis and Addison's disease may be associated with type 1 diabetes mellitus. There is no known etiological basis for type 1b diabetes mellitus. Some of these patients have permanent insulinopaenia and are prone to ketoacidosis, but have no evidence of autoimmunity. This form is more prevalent among individuals of African and Asian Origin (Bastaki., 2005).

1.1.1.2.2 Type 2 diabetes mellitus

Type 2 is the most common form of diabetes and is characterized by disorders of insulin action and insulin secretion, either of which may be the predominant feature. Both are usually present at the time that this form of diabetes is clinically manifest. By definition, the specific reasons for the development of these abnormalities are not yet known (Consultation,W. H. O., 1999).

1.1.1.2.3 Gestational Diabetes mellitus

Gestational diabetes is carbohydrate intolerance resulting in hyperglycaemia of variable severity with onset or first recognition during pregnancy. It does not exclude the possibility that the glucose intolerance may antedate pregnancy but has been previously unrecognized. The definition applies irrespective of whether or not insulin is used for treatment or the condition persists after pregnancy. Women who become pregnant and who are known to have diabetes mellitus which antedates pregnancy do not have gestational diabetes but have "diabetes mellitus and pregnancy" and should be treated accordingly before, during, and after the pregnancy (Consultation,W. H. O., 1999).

1.1.1.2.4 Other types

Include genetic defects of the pancreatic ß cell or in insulin action pathways (insulin receptor mutations or post-receptor defects) as well as disease of the exocrine pancreas (e.g., Pancreatitis, pancreatic reaction, or cystic fibrosis) are less common causes of DM. Endocrinopathies producing insulin counterregulatory hormones excess (e.g., Cushing's syndrome, acromegaly) may result in DM. Among several monogenic forms of DM which have been identified, maturity onset diabetes of the young (MODY) is a familial form of NIDDM with autosomal-dominant inheritance, which usually develops in childhood, adolescence or young adulthood, and presents primarily insulin-secretion defects. MODY is not a single entity, but involves genetic, metabolic, and clinical heterogeneity. Mutations in six genes cause most cases of MODY (MODY 1 - MODY 6). The prevalence of MODY is unknown but about 2-5% of patients with type 2 diabetes may in fact have MODY (Bastaki., 2005).

1.1.1.3 Risk factors of diabetes mellitus

The exact causes of type 1 diabetes are unknown. It is generally agreed that type 1 diabetes is the result of a complex interaction between genes and environmental factors, though no specific environmental risk factors have been shown to cause a significant number of cases (World Health Organization., 2016)

The risk of type 2 diabetes is determined by an interplay of genetic and metabolic factors. Ethnicity, family history of diabetes and previous gestational diabetes combine with older age, overweight and obesity, unhealthy diet, physical inactivity and smoking to increase risk (World Health Organization., 2016).

Excess body fat, is the strongest risk factor for type 2 diabetes, both in terms of clearest evidence base and largest relative risk. Overweight and obesity, together with physical inactivity, are estimated to cause a large proportion of the global diabetes burden. Higher waist circumference and higher body mass index are associated with increased risk of type 2 diabetes. Several dietary practices are linked to unhealthy body weight and/or type 2 diabetes risk, including high intake of saturated fatty acids, high total fat intake and inadequate consumption of dietary fibre. High intake of sugar-sweetened beverages, which contain considerable amounts of free sugars, increases the likelihood of being overweight or obese, particularly among children. Recent evidence further suggests an association between high consumption of sugar-sweetened beverages and increased risk of type 2 diabetes (World Health Organization., 2016).

1.1.1.4 Pathophysiology of Diabetes Mellitus

1.1.1.4.1 Type 1 diabetes mellitus

Cellular-mediated autoimmune destruction of the β cells of the pancreas, causing an absolute deficiency of insulin secretion. This disease is usually initiated by an environmental factor or infection (usually a virus) in individual with a genetic predisposition and causes the immune destruction of the β cells of the pancreas and, therefore, a decreased production of insulin .

One or more of the following markers are found:

1)Islet cell autoantibodies.

2)Insulin autoantibodies.

3)Glutamic acid decarboxylase autoantibodies.

4)Tyrosine phosphate autoantibodies (Bishop *et al.*, 2005).

1.1.1.4.2 Type 2 diabetes mellitus

Under normal physiological conditions, plasma glucose concentrations are maintained within a narrow range, despite wide fluctuations in supply and demand, through a tightly regulated and dynamic interaction between tissue sensitivity to insulin (especially in liver) and insulin secretion In type 2 diabetes these mechanisms break down, with the consequence that the two main pathological defects in type 2 diabetes are impaired insulin secretion through a dysfunction of the pancreatic β-cell, and impaired insulin action through insulin resistance .Type 2 diabetes mellitus has a greater genetic association than type 1 DM, the pathogenesis of type 2 diabetes mellitus is characterized by impaired insulin secretion and insulin resistance (Ozougwu *et al*., 2016).

Individuals with NIDDM have detectable levels of circulating insulin, unlike patients with IDDM. On the basis of oral glucose tolerance testing the essential elements of NIDDM can be divided into four distinct groups:

1) Those with normal glucose tolerance.

2) Chemical diabetes (called impaired glucose tolerance).

3) Diabetes with minimal fasting hyperglycemia (fasting plasma glucose less than 140 mg/dl).

4) Diabetes mellitus in association with overt fasting hyperglycemia (fasting plasma glucose greater than 140 mg/dl).

The individuals with impaired glucose tolerance have hyperglycemia inspite of having highest levels of plasma insulin, indicating that they are resistant to the action of insulin. In the progression from impaired glucose tolerance to diabetes

mellitus, the level of insulin declines indicating that patients with NIDDM have decreased insulin secretion. Insulin resistance and insulin deficiency are common in the average NIDDM patients. Insulin resistance is the primary cause of NIDDM, however some researcher contend that insulin deficiency is the primary cause because a moderate degree of insulin resistance is not sufficient to cause NIDDM. Most patients with the common form of NIDDM have both defects. Recent evidence has demonstrated a role for a member of the nuclear hormone receptor super family of proteins in the etiology of type 2 diabetes (Ozougwu *et al*., 2016).

1.1.1.5 Signs and Symptoms of diabetes mellitus

include polydipsia (excessive thirst), polyphagia (increased food intake), polyuria (excessive urine production), rapid weight loss, hyperventilation, mental confusion, and possible loss of consciousness (due to increased glucose to brain) (Bishop *et al*., 2005).

1.1.1.6 Diagnosis of diabetes mellitus

The identification of patients with diabetes or pre-diabetes by screening allows for earlier intervention, with potential reductions in future complication rates, although randomized trials are lacking to definitively show benefit. The patient described in the vignette has risk factors (obesity, hypertension, and a family history of diabetes) and should be screened. About 25% of patients with type 2 DM already have microvascular complications at the time of diagnosis suggesting that they have had the disease for more than 5 years at the time of diagnosis. As a result there are different approaches to diagnose diabetes among individuals.

In 1997 ADA recommendations for diagnosis of DM focus on fasting plasma glucose while WHO focuses on the oral glucose tolerance test (Baynest., 2015).

1.1.1.6.1 Diagnosis of both types of diabetes

1.1.1.6.1.1 Random plasma test

The simplest test and doesn't require fasting before taking the test.

If 200 or more than 200 mg/dl of blood glucose it probably indicates diabetes but has to be reconfirmed.

1.1.1.6.1.2 Fasting plasma glucose test

There should be eight hours fasting before taking this test. Blood glucose more than 126 mg/dl on two or more tests conducted on different days confirms a diabetes diagnosis (Baynest., 2015).

1.1.1.6.1.3 Oral glucose tolerance test

When random plasma glucose test is 160-200 mg/dl and the fasting plasma test is 110-125 mg/dl, then this test is conducted.

Blood test evaluates body's response to glucose, and requires fasting at least eight but not more than 16 hrs.

This test considered normal if glucose level at two hours is less than 140 mg/dl. A fasting level of 126 mg/dl or greater and two hour glucose level of 200 mg/dl or higher confirms a diabetes diagnosis (Baynest., 2015).

1.1.1.6.1.4 Glycated proteins

Proteins react spontaneously in blood with glucose to form glycated derivatives. The extent of glycation of proteins is controlled by the concentration of glucose in blood and by the number of reactive amino groups present in the protein that are accessible to glucose for reaction. All proteins with reactive sites can be glycated and the concentration of the glycated proteins that can be measured in blood is a marker for the fluctuation of blood glucose concentrations during a certain period. From a clinical diagnostic point glycated proteins with a longer life time in blood are of interest, since they reflect the exposure of these proteins to glucose for longer periods (Baynest., 2015).

1.1.1.6.1.5 Glycated hemoglobin

The life span of hemoglobin in vivo is 90 to120 days. During this time glycated hemoglobin A forms, being the ketoamine compound formed by combination of hemoglobin A and glucose. Several subfractions of glycated hemoglobin have been isolated. Of these, glycated hemoglobin A fraction hemoglobin A1c (HbA1c) is of most interest serving as a retrospective indicator of the average glucose Concentration. HbA1c is recommended as an essential indicator for the monitoring of blood glucose control. The blood HbA1c$\geq 6.5\%$ is considered as diabetes. In the prediabetic range, the patient should be counseled or treated for pre-diabetes. If the result of the repeat test is entirely normal (which is unlikely), rescreening in 6 months should be considered (Baynest., 2015).

1.1.1.7 Compliacations of Diabetes mellitus

The long-term vascular complications of diabetes include retinopathy, nephropathy, neuropathy and macrovascular disease. The outcomes are:

• visual impairment and blindness due to diabetic retinopathy.

• renal failure and hypertension due to diabetic nephropathy.

• pain, paraesthesiae, muscle weakness and autonomic dysfunction due to diabetic neuropathy.

• cardiac disease, peripheral vascular disease and stroke due to macrovascular disease (Donaghue *et al*., 2009).

Clinically evident diabetes-related vascular complications should be rare in childhood and adolescence. However, early functional and structural abnormalities may be present a few years after the onset of the disease. Childhood and adolescence is a period during which intensive education and treatment may prevent or delay the onset and progression of complications (Donaghue *et al*., 2009).

1.1.1.7.1 Macrovascular Complications

Macrovascular complications include fatal and non - fatal coronary heart disease (CHD) events, stroke and peripheral arterial disease. Cardiovascular disease (CVD) accounts for most (> 75%) of the premature mortality and shortened life expectancy among patients with diabetes (Bilous & Donnelly., 2010).

1.1.1.7.1.1Pathophysiologic features of macrovascular complications

Unlike microvascular disease, which occurs only in patients with diabetes mellitus, macrovascular disease resembles that in subjects without diabetes. However, subjects with diabetes have more rapidly progressive and extensive CVD, with a greater incidence of multivessel disease and a greater number of diseased vessel segments than nondiabetic persons. Although dyslipidemia and hypertension occur with great frequency in type 2 diabetic populations, there is still excess risk in diabetic subjects after adjusting for these other risk factors. Diabetes itself may confer 75% to 90% of the excess risk of coronary disease in these diabetic subjects, and it enhances the deleterious effects of the other major cardiovascular risk factors. In subjects with or without diabetes, atherosclerosis begins with endothelial dysfunction or injury. These endothelial changes or injury induce the secretion of chemokines such as monocyte chemoattractant protein 1 (MCP-1), increase the expression of endothelial adhesion molecules for leucocytes and platelets, and enhance permeability to lipoproteins and other plasma constituents. This leads to recruitment of monocyte-macrophages to the subendothelial space and to the infiltration of plasma low-density lipoprotein (LDL), which binds to arterial proteoglycan. The retained LDL then undergoes oxidation and is taken up by macrophages (Larsen *et al.*,2003).

Activated macrophages and other leukocytes, as well as adherent aggregated platelets, stimulate smooth muscle cell proliferation and elaboration of extracellular matrix, culminating in the formation of a complex lesion filled with prothrombotic material contained by a fibrin cap. Rupture of this fibrin cap by matrix metalloproteinases causes thrombus formation and arterial occlusion. Because macrovascular disease also occurs in nondiabetic subjects, diabetes is

thought to accelerate the process by increasing endothelial cell dysfunction and by exacerbating dyslipidemia (Larsen *et al.,* 2003).

The pathogenesis of endothelial cell dysfunction in diabetic arteries appears to involve both insulin resistance and hyperglycemia. In vitro studies suggest that insulin has both antiatherogenic and proatherogenic effects One major antiatherogenic effect is the stimulation of endothelial nitric oxide (NO) production. NO released from endothelial cells is a potent inhibitor of platelet aggregation and adhesion to the vascular wall. Endothelial NO also controls the expression of genes involved in atherogenesis. It decreases expression of the chemoattractant protein MCP-1, and of surface adhesion molecules such as CD11/CD18, P-selection, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). Endothelial cell NO also reduces vascular permeability and decreases the rate of oxidation of LDL to its proatherogenic form. Finally, endothelial cell NO inhibits proliferation of vascular smooth muscle cells. Two major proatherogenic effects of insulin are the potentiation of platelet-derived growth factor (PDGF)-induced vascular smooth muscle cell (VSMC) proliferation and the stimulation of VSMC plasminogen activator inhibitor 1 (PAI-1) production (Larsen *et al.,* 2003).

Hyperglycemia also inhibits arterial endothelial NO production, both in vivo and in vitro. Similarly, hyperglycemia potentiates PDGF-induced VSMC proliferation and stimulates endothelial cell PAI-1 production. In addition, hyperglycemia has a variety of other proatherogenic effects on endothelial cells, platelets, and monocyte/macrophages. These include increased expression of MCP-1, up-regulation of adhesion molecules such as ICAM-1 and VCAM-1, potentiation of

collagen-induced platelet activation, and increased secretion of collagen type IV and fibronectin (Larsen *et al.,* 2003).

Atherosclerosis: is the major threat to the macrovasculature for patients with and without diabetes. The gen¬eral pathogenesis of atherosclerosis has been reviewed elsewhere, but several factors specific to diabetes are worth mentioning here. Clinically, dyslipidemia is highly correlated with atherosclerosis, and up to 97% of patients with diabetes are dyslipi-demic. In addition to the characteristic pattern of increased triglycerides and decreased high density lipoprotein (HDL) cholesterol found in the plasma of patients with diabetes, abnormalities are seen in the structure of the lipoprotein particles. In diabetes, the predominant form of LDL choles¬terol is the small, dense form. Small LDL particles are more atherogenic than large LDL particles because they can more easily penetrate and form stronger attachments to the arterial wall, and they are more susceptible to oxidation. Because less cholesterol is carried in the core of small LDL par¬ticles than in the core of large particles, subjects with predominantly small LDL particles have higher numbers of particles at comparable LDL choles¬terol levels (Dokken *et al*., 2008).

diabetic cardiomyopathy: there are two main types of cardiomyopathy: (1) primary cardiomyopathy, where the cardiac function is aggravated by a defect in the heart itself, and (2) secondary cardiomyopathy, where cardiac performance is affected because of a systemic syndrome. Cardiomyopathy leads to heart failure, which can be either diastolic heart failure, with preserved ejection fraction, or systolic heart failure, with reduced ejection fraction. Diabetes can lead to heart failure, not only by augmenting the impact of classical cardiovascular risk factors (e.g. accelerating the appearance and progression of coronary artery disease through

macroangiopathy), but also via a direct deleterious effect on the myocardium. This condition is known as diabetic cardiomyopathy, defined as the presence of myocardial involvement in patients with diabetes, characterized by dilatation and hypertrophy of the left ventricle, with the concomitant appearance of diastolic and/or systolic dysfunction, and its presence is independent of the coexistence of ischemic or hypertensive or valvular heart disease. Myocardial fibrosis and myocyte hypertrophy are the most frequently proposed mechanisms to explain cardiac changes in diabetic cardiomyopathy. Several studies have shown that diabetes causes defects in cellular calcium transport, defects in myocardial contractile proteins and an increase in collagen formation which result in anatomic and physiological changes in the myocardium (Trachans., 2014).

Aortic regurgitation (AR): is characterized by diastolic reflux of blood from the aorta into the left ventricle due to malcoaptation of the aortic cusps. Its clinical presentation is variable and depends on a complex interplay of a number of factors, including acuity of onset, aortic and left ventricle compliance, hemodynamic conditions, and severity of the lesion. Although chronic AR is generally well tolerated for many years, acute AR may lead to rapid cardiac decompensation and, if untreated, to early death (Bekeredjian &Grayburn., 2005).

AR results from malcoaptation of the aortic leaflets due to abnormalities of the aortic leaflets, their supporting structures (aortic root and annulus), or both. Diseases that primarily affect the leaflets include bicuspid aortic valve and other congenital abnormalities, atherosclerotic degeneration, infective endocarditis, rheumatic heart disease, connective tissue or inflammatory diseases, antiphospholipid syndrome, and use of anorectic drugs. The leaflets can also be

affected by trauma, due either to chest wall or deceleration injury, or a jet lesion, due to dynamic or fixed subaortic stenosis (Bekeredjian &Grayburn., 2005).

Acute myocardial infarction: is an event of myocardial necrosis caused by an unstable ischemic syndrome. The disorder is diagnosed and assessed on the basis of clinical evaluation, the electrocardiogram ,biochemical testing, invasive and noninvasive imaging, and pathological evaluation(Jeffrey *et al*., 2017).

mechanism for acute myocardial infarction is rupture or erosion of a vulnerable, lipid-laden, atherosclerotic coronary plaque, resulting in exposure of circulating blood to highly thrombogenic core and matrix materials in the plaque (Jeffrey *et al*., 2017).

Angina :Complete, sudden blockage of an artery is not the only problem. Even a reduced blood supply will reduce the oxygen supply to heart muscle, and an oxygen-starved heart muscle responds with a characteristic feeling of pain or discomfort called angina. When its arteries are narrowed by atherosclerosis, a heart may still get enough oxygen to pump blood at rest. On the other hand, exercise increases the work of the heart, and narrowed arteries cannot always deliver the excess oxygen required by an exercising heart. Under these circumstances, a person with narrowed coronary arteries will develop angina when exercising (Michael & Sheryl., 2015).

Arrhythmias: another significant result of sudden ischemia is a change in the heart's rhythm. Such changes can be serious. The arrhythmias (notably, ventricular fibrillation) that sometimes result from heart ischemia are the cause of most sudden deaths after an acute myocardial infarction (Michael & Sheryl., 2015).

1.1.1.7.2 Microvascular Complications

1.1.1.7.2.1 Diabetic retinopathy

Diabetic retinopathy may be the most common microvascular complication of diabetes. It is responsible for ~ 10,000 new cases of blindness every year in the United States alone (Fowler., 2008).

Approximately 25% of Type 1 patients have some retinopathy after five years. These numbers increase to almost 60% after 10 years and greater than 80% after 15 years. In Type 2 patients older than age 30 with a known duration of diabetes of less than five years, 40% of patients taking insulin and 24% of those not taking insulin are found to have retinopathy. After 10 years, the numbers increase to 53% and 84%, respectively. Proliferative diabetic retinopathy is found in approximately 2% of type 2 patients who have diabetes for less than five years, and 25% who have had diabetes for 25 years or more (Ferrucci *et al*., 2016).

Aldose reductase may participate in the development of diabetes complications. It is the initial enzyme in the intracellular polyol pathway. This pathway involves the conversion of glucose into glucose alcohol (sorbitol). High glucose levels increase the flux of sugar molecules through the polyol pathway, which causes sorbitol accumulation in cells. Osmotic stress from sorbitol accumulation has been postulated as an underlying mechanism in the development of diabetic microvascular complications, including diabetic retinopathy. Oxidative stress may also play an important role in cellular injury from hyperglycemia. High glucose levels can stimulate free radical production and reactive oxygen species formation. Growth factors, including vascular endothelial growth factor (VEGF), growth hormone, and transforming growth factor β, have also been postulated to play

important roles in the development of diabetic retinopathy. VEGF production is increased in diabetic retinopathy, possibly in response to hypoxia. In animal models, suppressing VEGF production is associated with less progression of retinopathy (Fowler., 2008).

1.1.1.7.2.2 Diabetic nephropathy

Diabetic nephropathy is the leading cause of end stage renal disease worldwide and is associated with increased cardiovascular risk. The earliest clinical manifestation is of microalbuminuria. Tight blood glucose and blood pressure control reduce the risk of microalbuminuria. Once microalbuminuria is present, the rate of progression to end stage renal disease and of cardiovascular disease can be delayed by aggressive management of blood pressure, glucose, and lipids. Inhibition of the renin-angiotensin system is important to reduce intraglomerular pressure but other classes of antihypertensive agent may also be needed to gain adequate control of systemic blood pressure. Such measures can at least half the rate of progression of nephropathy and cardiovascular disease (Marshall., 2004).

The classical definition of diabetic nephropathy is of a progressive rise in urine albumin excretion, coupled with increasing blood pressure, leading to declining glomerular filtration and eventually end stage renal failure. Patients generally have diabetic retinopathy. Recently, greater appreciation of the close links between nephropathy and cardiovascular disease have lead to the inclusion of premature cardiovascular disease, cardiovascular risk increasing in parallel with albuminuria. Diabetes causes unique changes in kidney structure. Classic glomerulosclerosis is characterized by increased glomerular basement membrane width, diffuse mesangial sclerosis, hyalinosis, microaneurysm, and hyaline arteriosclerosis Tubular and interstitial changes are also present. Areas of extreme mesangial

expansion called Kimmelstiel-Wilson nodules or nodular mesangial expansion are observed in 40– 50% of patients developing proteinuria . Micro- and macroalbuminuric patients with type 2 diabetes have more structural heterogeneity than patients with type 1 diabetes (Gross *et al*., 2005).

1.1.1.7.2.3 Diabetic neuropathy

The diabetic neuropathies are heterogeneous, affecting different parts of the nervous system that present with diverse clinical manifestations. They may be focal or diffuse. Most common among the neuropathies are chronic sensorimotor distal symmetric polyneuropathy and the autonomic neuropathies. distal symmetric polyneuropathy is a diagnosis of exclusion (Boulton., 2005).

Causative factors include persistent hyperglycemia, microvascular insufficiency, oxidative and nitrosative stress, defective neurotropism, and autoimmune-mediated nerve destruction (Vinik., 2013).

The precise nature of injury to the peripheral nerves from hyperglycemia is not known but likely is related to mechanisms such as polyol accumulation, injury from advanced glycosylated end products, and oxidative stress. Peripheral neuropathy in diabetes may manifest in several different forms, including sensory, focal/multifocal, and autonomic neuropathies. More than 80% of amputations occur after foot ulceration or injury, which can result from diabetic neuropathy (Fowler., 2008).

1.1.2 Angiotensin converting enzyme

Renin angiotensin system (RAS) is a complex regulator of blood pressure, water homeostasis, cardiovascular remodeling and vascular tone. This system is composed of several key proteins including angiotensinogen, ACE and angiotensin II and its receptors affect hemostasis through different mechanisms. The fibrinolytic system constitutes the endogenous defense mechanisms against intravascular thrombus formation and is activated by the presence of a fibrin clot within the vasculature. Fibrinolysis starts when plasminogen, mediated by activators, is converted to plasmin, a proteolytic enzyme. Two important plasminogen activators in the vascular system are tissue-type plasminogen activator and urokinase. Fibrinolysis is regulated by the balance between the activity of plasminogen activators and their inhibitors. Plasminogen activator inhibitor type 1 is the most important physiologic inhibitor of tissue-type plasminogen activator and urokinase in plasma. Clinical and experimental studies have defined the relationship between RAS and the fibrinolytic system. Angiotensin I is converted to Angiotensin II by ACE which binds to endothelial cells and stimulates the production of plasminogen activator inhibitor type I Plasminogen activator inhibitor type 1 thus down-regulating fibrinolysis. In addition, ACE degrades bradykinin, an important mediator of the tissue type plasminogen activator, which also contributes to decrease fibrinolysis, hence increasing the thrombotic risk (Munhoz *et al.*, 2005).

1.1.2.1 Polymorphism of ACE

The ACE gene is 21kb long, consisting of 26 exons and 25 introns and located in chromosome 17p23. It is characterized by an insertion/deletion polymorphism based on the presence (Insertion I) or absence (Deletion D) of a 287-bp Alu repeat

sequence in intron 16, resulting in three genotypes DD homozygote, II homzygote and ID heterozygote. The DD genotype is associated with a two fold increase in plasma ACE activity over that of II genotype, with intermediate level of heterozygote ID (mekki & Ali., 2015).

1.1.2.2 ACE inhibitors

Angiotensin converting enzyme (ACE) inhibitors plays an important role in hypertension and cardiovascular protection management, both alone and in various combinations with other pharmacological agents from different classes. This position is supported by the significant hemodynamic, cardiac effect, renal effect, antithrombotic and neurohormonal effects of these pharmacological agents, effects demonstrated in clinical trials with a significant number of patients analysed. ACE inhibitors have proven their efficacy and safety in blood pressure control, as well as a substantial influence in reducing cardiovascular risk, at the same time having target organ protection. The main result of ACE inhibitors therapy is to improve quality of life and reduce cardiovascular morbidity and mortality (ŢÂNŢU *et al*., 2014).

The inhibitors of angiotensin converting enzyme I represent a group of first-line drugs used in the treatment of hypertension, in the treatment of heart failure, and of the patients with myocardial infarction, both in the early phase of the disease and in the long-term treatment of ischemic heart disease. It also became increasingly clear the cardiovascular preventive role, their administration in high risk patients resulted in decreased mortality risks, decreased risks of vascular accidents and myocardial infarction .ACE inhibitors have the basic structure of 2-methyl-propyl-Lproline; they are classified into three types, chemically different depending on the

ligand group of the zinc ion: sulfhydryl, carboxyle and phosphoryl. These structures affect directly tissue distribution and elimination pathways (ȚÂNȚU *et al.*, 2014).

1.2 Previous studies

Many previous studies were reported an association between the ACE I/D-polymorphism and coronary heart disease in patients with diabetes mellitus (Klemm *et al*., 2003).

Fujisaw *et al* (1995), examined 267 NIDDM patients, 61 of them with myocardial infarction(MI), and 136 patients without MI, the result was found an association between ACE polymorphism and MI in the patients with NIDDM, Homozygotes for the DD polymorphism were found more frequently in diabetic patients with MI (Fujisaw *et al*.,1995).

Narne *et al* (2012) screened 283 T2DM patients, inclusive of 160 patients with angiographically defined CAD, 73 patients with MI, 89 patients without MI and 121 T2DM individuals with no evidence of CAD, the result did not reveal any statistically significant association between ACE gene I/D polymorphism and the attendant risk for CAD in T2DM. However, a significant association of this polymorphism with MI in T2DM+CAD patients was observed (Narne *et al*., 2012).

Klemm *et al* (2003) investigated 691 patients with diabetes mellitus prospectively characterized for the presence/ absence of coronary heart disease. The result showed no association between ACE I/D polymorphism and the occurrence of CHD in T2DM patients (Klemm *et al*., 2003).

Zintzaras *et al* (2008) conducted a meta-analysis involving 118 studies; when made a subgroup analysis for DM, showed a significant association between ACE I/D polymorphism and CAD (Zintzaras *et al*., 2008).

A study by Ganesan *et al* (2011) included 520 individuals, of them 160 had CVD + T2DM, 90 were CVD patients without T2DM, 150 had T2DM with no cardiovascular complications, and 120 were age and sex matched health controls, the result with respect to ACE I/D polymorphism showed a higher percentage of D/D genotype in CVD + T2DM patients but the association was not statistically significant (Ganesan *et al*., 2011).

Chuang *et al* (1997) recruited 197 normal controls, 70 patients with NIDDM and CHD to study the association of ACE I/D polymorphism with CHD, they reported that, ACE I/D polymorphism was not associated with the occurrence of CHD in T2DM patients (Chuang *et al*., 1997).

Rationale

Cardiovascular diseases are the most prevalent cause of mortality and morbidity among people with diabetes mellitus. The diabetes mellitus deserves to be designated a major risk factor for cardiovascular diseases.

The renin-angiotensin system (RAS) is a complex regulator of blood pressure, cardiovascular remodeling and vascular tone. This system is composed of several key proteins including angiotensinogen, ACE and angiotensin II and its receptors affect hemostasis through different mechanisms. Angiotensin I is converted to angiotensin II by ACE which binds to endothelial cells and stimulates the production of plasminogen activator inhibitor type I (PAI-1) thus down-regulating fibrinolysis.

 A 287 bp insertion/deletion (I/D) polymorphism in intron 16 of the ACE gene is considered to contribute to an interpersonal variability in serum ACE levels and thereby providing a plausible basis for increased susceptibility tothrombotic events. Concentration of ACE are elevated in CAD and other CVDs. As a result, there is an increase angiotensin 11 levels. This has generally harmful, vasoconstrictive effects that increase oxisative stress, promote inflammation and thrombosis, damage the endothelium and lead to the development of atherosclerosis.

ACE inhibitor interfere with the formation of hormone angiotensin 11 that can narrow blood vessels. ACE lower blood pressure and lower the chances of heart attack.

To our knowledge, there is no study in sudan addressing ACE I/D polymorphism as risk factor for CVD in diabetic patients.

Objectives

General objective

To study the association between ACE I/D gene polymorphism and cardiovascular diseases in diabetic patients.

Specific objectives

1- To determine the frequency of ACE I/D genotypic variants in diabetic patients with cardiovascular disease using polymerase chain reaction (PCR).

2- To correlate ACE I/D polymorphism with disease duration, age and gender.

3- To compare the genotype distribution with risk factors of CVDs in DM.

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Study design

This was case control and a hospital based study.

2.1.2 Study area and duration

Study conducted in Sudan Heart Centre, Khartoum, Sudan from December 2016-June 2017.

2.1.3 Study population

Diabetic patients.

2.1.4 Inclusion criteria

Diabetic patients diagnosed with CVDs .

2.1.5 Exclusion criteria

Diabetic patients without CVDs.

2.1.6 Ethical consideration

Informed consent was obtained from each participant before sample collection.

2.1.7 Data collection

Patients data(age, sex, duration of DM, types of CVDs and other chronic disease) were   collected using structured interview questionnaire.

2.1.8 Sample collection

Blood samples(3ml) were collected from patients upon their consent in EDTA container.

2.2 Methods

2.2.1 Laboratory methods

DNA extraction, PCR and agarose gel electrophoresis.

2.2.1.1 DNA extraction

900 µl of red cell lysis buffer to 300 µl of whole blood in 1.5 ml eppendorf. Cells were centrifuged at 8000 rpm for 3 minutes and the supernatant was discarded.

This step was repeated 2-3 times till RBCs lysis was complete and a white pellet of WBCs was obtained (Sugana *et al.*, 2014).

To the cell pellet, 300 µl of white cell lysis buffer and 40 µl of 10% Sodium dodecyl sulphate were added Mixed thoroughly and incubated at 37˚c for 5 minutes. At the end of incubation, 100 µl of 6M NaCl was added and vortexed to precipitate the proteins. Cells were centrifuged at 8000 rpm for 5 minutes.

Precipitation of DNA The supernatant was transferred into a new eppendorf tube containing 300 µl of isopropanol. DNA was precipitated by inverting the eppendorf slowly. Further, the eppendorfs were centrifuged at 8000 rpm for 10 minutes to pellet down the DNA. Supernatant was discarded, 70% ethanol was added and mixed slowly to remove any excess salts. Finally the tubes were centrifuged at 8000 rpm for 5 minutes to pellet down the DNA. Supernatant was discarded and DNA air-dried. After thorough drying, 50 µl of TE buffer was added to dissolve the DNA.

1% agarose gel electrophoresis use to detect genomic DNA isolated from human blood samples (Sugana *et al.*, 2014).

2.2.1.2 Polymerase chain reaction

PCR was used for molecular detection of ACE insertion /deletion polymorphism. The sequences of the forward, reverse and internal primers (Table 2.1).

PCR was carried out by added the template DNA (DNA extracted)from whole blood, 100pmol/µL of ACE primer and Distilled water(water for injection) to Maxime PCR Pre Mix Kit (i-Taq) for 20µL rxn (Table 2.2).

The amplification process consisted of initial denaturation of 94˚C for 3 min; 35 cycles each consist of 94˚C for 1 min ,58˚C for 30 sec, and 72˚C for 30 sec; then final extension at 72˚C for 5 min. Amplification was done using forward, reverse and internal primer.

2.2.1.3 Agarose gel electrophoresis

PCR products were electrophoresed on 2% agarose gel containing ethidium bromide and analyzed under UV light. 5µL of the PCR product were loaded on the gel along with 4µL of 100bp deoxyribonucleic acid (DNA) ladder was applied with each batch of patients samples.

The gel was run at 1X TBE(Tris borate EDTA) buffer (10X TBE: Tris base , boric acid and Na EDTA).

The sizes of the different fragments obtained were 490 bp (II), 190 bp (DD), and 490 and 190 bp (ID) figure (2.1).

2.2.2 Statistical analysis

Data was analyzed by statistical package for social sciences (SPSS), version 19. Qualitative data was represented as frequency and Percentage. Quantitative data was presented as mean ± SD. Association between qualitative variables was tested using Pearson's Chi square (χ2) and Fisher's exact tests. Binary logistic regression

analysis was used to investigate the interaction between qualitative risk factors. The alleles frequencies were tested using the conventional formula (Allele frequency= homozygous+1/2X heterozygous). Hardy-Weinberg equilibrium was calculated by goodness of fit test.

Chapter Four

Discussion, Conclusion, and Recommendations

4.1 Discussion

This study aimed to investigate the association between ACE I/D polymorphism with CVDs in diabetic patients.

The result of the present study showed an increased frequency of DD genotype in both DM patients with CVDs and healthy control group than ID genotype, while the II genotype was totally absent in both groups; There was no statistically significant association between ACE I/D polymorphism and CVDs in DM patients. This finding agrees with that of Ganesan *et al* (2011).

This finding agrees with studies by Klemm *et al* (2003) who reported that, ACE I/D polymorphism was not associated with the occurrence of CHD in T2DM patients (Klemm *et al*., 2003). In contrast, study by Narne *et al* (2012) reported there was no any statistically significant association between ACE gene I/D polymorphism and the attendant risk for CAD in T2DM (Narne *et al*., 2012). The present result was also inconsistent with a meta-analysis involving 118 studies by Zintzaras *et al*(2008) which showed a significant association between ACE I/D polymorphism with CAD when a subgroup analysis for diabetes mellitus was performed (Zintzaras *et al*.,2008.). Variations in these studies and our studies may be due to differences in patients selection criteria, as some of these studies concerning with CHD and in the others only the NIDDM patients were included.

In the present study, the ACE gene I/D polymorphism was not associated with patients' age and gender. This disagrees with study by Klemm *et al* (2003) who introduced the age and gender as main risk factors for CHD (Klemm *et al*., 2003.).

The mean duration of Diabetes mellitus was not significantly different in patients with DD genotype compared to those with ID genotype. Narne *et al (2012)* reported similar result (Narne *et al*.,2012).

The analysis of the present study revealed no interaction between ACE I/D polymorphism and other known risk factors of CVDs in diabetic patients.

4.2 Conclusions

• The DD genotype of ACE I/D polymorphism was more frequent in both diabetic patients with CVDs and control group than ID genotype, while the II genotype was absent in both groups.

• There was no statistically significant association between ACE I/D polymorphic genotypes and CVDs in DM patients, age and mean duration.

• There was no interaction observed between ACE genotypes and other known CVDs risk factors in diabetic patients

4.3 Recommendations

Further studies should be conducted to identify the causes of CVDs in diapetic patients.

ACE level should be measured in the future studies.

Further studies should be conducted with large sample size.

References

Bastaki, A.(2005) Diabetes mellitus and its treatment. *International journal of Diabetes and Metabolism. 13*(3). p.111.

Baynest, H. W.(2015). Classification, Pathophysiology, Diagnosis and Management of Diabetes.

Bekeredjian, R., &Grayburn, P. A. (2005) Valvular Heart Disease Circulation. 112(1), 125-134.
Bilous, R., &Donnelly, R. (2010). Introduction to diabetes. Handbook of Diabetes, 4th edition, 1-4.

Bishop, M. L., Fody, E. P., & Schoeff, L. E. (2005). Clinical chemistry, principles, procedures, correlation. Quality assurance, 3,4.

Boulton, A. J., Vinik, , A. I., Arezzo, J. C., Bril, V., Feldman, E. L., Freeman, R.,… & Ziegler, D. (2005). Diabetic neuropathies. Diabetes care, 28(4), 956-962.

Consultation,W. H. O. (1999). Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications.

Dokken, B. B. (2008). The Pathophysiology of Cardiovascular Disease and Diabetes: Beyond Blood Pressure and Lipids. Diabetes spectrum, 21(3), 160-165.

Donaghue, K. C., Chiarelli, F., Trotta, D., Allgrove, J., Dahl-Jorgensen, K. (2009). Microvascular and macrovascular complications associated with diabetes in children and adolescents. Pediatric Diabetes 10 10(s 12), 195–203.

Elrayah-Eliadarous, H. (2007*).* Economic burden of diabetes on patients and their families in Sudan. Institution för folkhäsovetenskap/Department of Public Health Sciences.

Ferrucci, S., & Yeh, B.(2016). Diabetic Retinopathy by the numbers: a guide to following and educating patients who face this sight-threatening diagnosis. Review of Optometry, 153(6), 36-43.

Fowler, M.J. (2008) Microvascular and macrovascular complications of diabetes. Clinical diabetes. 26(2). p.77-82.

Fujisaw, T., Ikegami, H., Shen, G. Q., Yamato, E., Takekawa, K., Nakagawa, Y., … & Ohishi, M. (1995). Angiotensin 1- converting enzyme gene polymorphism is associated with myocardial infarction, but not with retinopathy or nephropathy, in NIDDM. Diabetes Care, 18(7), 983-985.

Ganesan, M., Bhaskar, S., Mani, R., Idris, M. M., Khaja, N., Gulla, S., …& Hasan, Q.(2011). The relationship of ACE andCETP gene polymorphisms with cardiovascular disease in a cohortof Asian Indian patients with and those without type2 diabetes. *Journal of diabetes and its complications, 25*(5), 303-308.

GROSS, J. L., . De Azevedo, M. L., Silveiro, S. P., Canani, L. H., Caramori, M. L., &Zelmanovitz, T.(2005). Diabetic nephropathy: diagnosis, prevention, and treatment. Diabetes care, 28*(1), 164-176.*

International Diabetes Federation .(2001). Diabetes and Cardiovascular Disease: Time to Act. International Diabetes Federation.

Jeffrey L. Anderson, M.D., and David A. Morrow, M.D.(2017) Acute Myocardial Infarction. *the new England journal of medicine*.

Klemm, T., Mittrach-Schorin, S., Neumann, S., Gerike, T., Krankenberg, H., Schuler, G., & Paschke, R. (2003). No association between angiotensin converting-enzyme gene insertion/deletion Polymorphism and the occurance of macroangiopathy in patients with diabetes mellitus type 2. Hormone and metabolic research, 35(01), 43-47.

Larsen, R. P., Kronenberg, H. M., & Melmed, S. Williams Textbook of Endocrinology 10th edition,2003. Philadelphia United States of America.

Marshall, S. M. (2004). Recent advances in diabetic nephropathy. *Postgraduate Medical Journal 2004*,80. 949: 624–633.

Mekki, L. B., & Ali, E. W. (2015). Genotype distribution of angiotensin converting enzyme insertion/deletion polymorphism in sudanese patients with ischemic stroke. *Int. J. Adv. Pharm., Biol. Chem, 4*, 223-227.

Mellitus. *J Diabetes Metab, 6*(541), 2.

Michael, J., Sheryl, M.(2015) Coronary Artery Disease (CAD)WILD IRIS MEDICAL EDUCATION INC.

Monteiro, P., Gonçalves, L., &Providência, L. A. (2005) Diabetes and cardiovascular disease: the road to cardioprotection. Heart. 91(12). P.1621-1625.

Munhoz, T. P., Scheibe, R. M., & Schmitt, V. M. (2005). Angiotensin converting enzyme (ACE) DD genotype: relationship with venous thrombosis. Revista Brasileira de hematologia e hemoterapia, 27(2), 87-90.

Narne, P., Ponnaluri, K.C., Singh, S., Siraj, M. and Ishaq, M.(2012) Relationship between angiotensin-converting enzyme gene insertion/deletion polymorphism, angiographically defined coronary artery disease and myocardial infarction in patients with type 2 diabetes mellitus. *Journal of the Renin-Angiotensin-Aldosterone System. 13*(4). p.478-486

Ozougwu, J C., Obimba, K. C., Belonwu, C. D., & Unakalamba, C. B. (2013). The pathogenesis and pathophysiology of type 1 and type 2 diabetes mellitus. *Journal of Physiology and Pathophysiology, 4*(4), 46-57.

Ruiz,J., Blanche, H., Cohen, N., Velho, G., Cambien, F.,Cohen, D., …Froguel, P. (1994). Insertion/deletion polymorphism of the angiotensin-converting enzyme gene is strongly associated with coronary heart disease in non-insulin-dependent diabetes mellitus. Proceeding of the National Academyof Sciences,91(9), 3662-3665.

Sugana, S. A. J. J. A., Nandal, D., Kamble, S., Bharatha, A., & Kunkulol, R. (2014). Genomic DNA isolation from human whole blood samples by non enzymatic salting out method. *Int J pharm pharm sci, 6, 198-199.*

ŢÂNŢU, M., Belu, E., Bobescu, E., Armean, S. M., Armean, P., CONSTANTIN, M., & Domnariu, C. D. (2014). Role of angiotensin converting enzyme (ACE) inhibitors in hypertension and caediovascular protection management.

Trachans, K., Sideris, S.,Aggeli, C., Poulidakis, E., Gatzoulis, K., Tousoulis, D., & Kallikazaros, I. O. N. N. I. S.(2014) Diabetic Cardiomyopathy: From Pathophysiology to Treatment. *Hellenic J Cardiol* 2014; 55: 411-421.

Vinik, A. I., Nevoret, M. L., Casellini, C.,& Parson, H. (2013). Diabetic Neuropathy. Endocrinology and metabolism clinics of North America, 42(4), 747-787.

World Health Organization. (2016). Global report on diabetes. World Health Organization.

Zintzaras, E., Raman, G., Kitsios, G., & Lau, J. (2008). Angiotensin-converting enyme insertion/deletion gene polymorphic variant as a marker of coronary artery disease: a meta-analysis. Archives of Internal Medicine, 168(10), 1077-1089.

Appendix

Table 2.1 primer sequences and length

Name of the gene	Primer sequences		Possible bands
ACE	Forward-5'-CTG GAG ACC ACT CCC ATC CTT TCT - 3'		490 bp
	Reverse-5'-GAT GTG GCC ATC ACA TTC GTC GTC AGA T- 3'		190 bp
	Internal-5'-TGG GAT TAC AGG CGT GAT ACA G - 3'		

Table 2.2 PCR reaction mixture

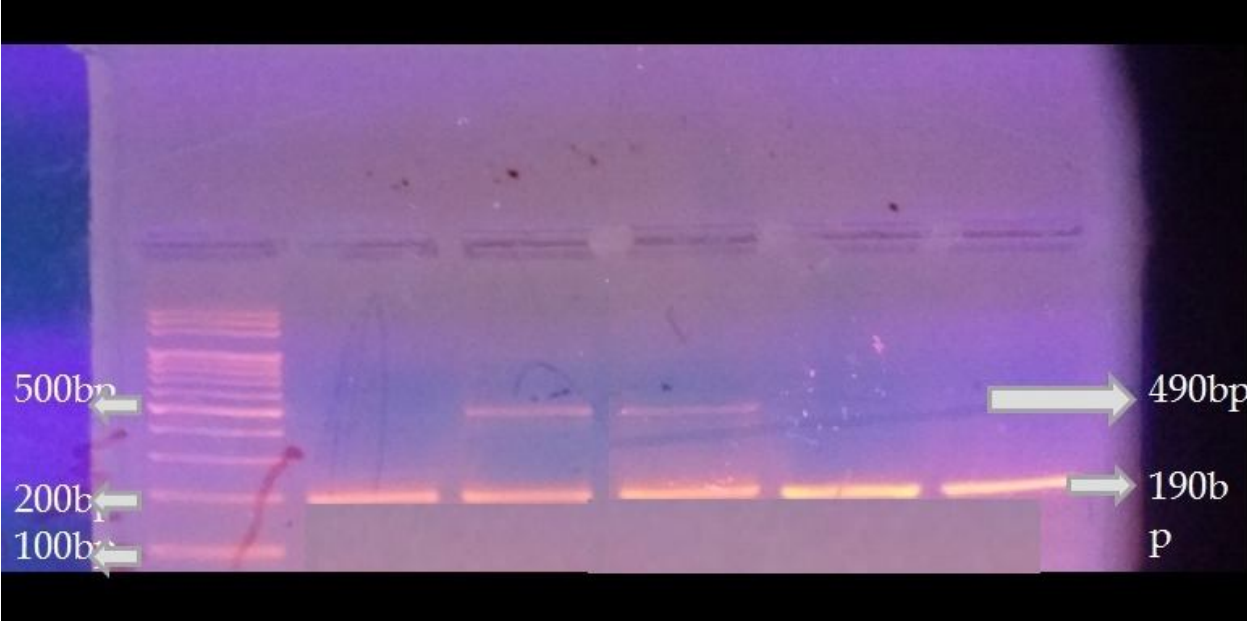| PCR reaction mixture	volume
Template DNA	2µ/L
Primer (F: 100Pmol/µL)	1µ/L
Primer (R: 100Pmol/µL)	1µ/L
Primer (I: 100Pmol/µL)	1µ/L
Distilled water	15µ/L
Total reaction volume	20µ/L

Figure 2.1 Amplified fragments of ACE

Sudan University of Science and Technology

College of Graduate Studies

Questionnaire of Association between Angiotensin Converting Enzyme Gene Insertion/Deletion Polymorphism and Cardiovascular Diseases among Diabetic Patiens

Patient informations

Name ……………..

Gender   M□   F□

Age …………………

Duration of diabetes mellitus …………..

Type of cardiovascular diseases ……….

Other diseases ………………………………