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ASSOCIATION OF *ANGIOTENSIN CONVERTING ENZYME* GENE POLYMORPHISMS WITH RISK OF DIABETIC 2 AND ITS COMPLICATION AMONG PATIENTS VISITING BAHIRDAR FELEGEHIWOT REFERRAL HOSPITAL NORTH WEST, ETHIOPIA.

MSc. Thesis

By
Tadele Tamiru

Advisor: Dr. Nega Berhane

CO-ADVISOR: Mr. Wagaw Sendeku

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Tadele Tamiru

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LIST OF ABBREVIATIONS

ACE	*Angiotensin-Converting Enzyme*
AGT	*Angiotensinogen*
BMI	Body Mass Index
CKD	Chronic Kidney Disease
DM	Diabetes Mellitus
DN	Diabetic Nephropathy
DNA	Deoxyribose Nuclic Acid
DR	Diabetic Retinopathy
DNR	Diabetic Non-Retinopathy
eGFR	Estimated Glomerular Filtration rate
ESRD	End Stage Renal Disease
GFR	Glomerular Filtration Rate
HDL	High-Density Lipoprotein
IDF	International Diabetic Federation
I/D	Insertion/ Deletion
IDDM	Insulin Dependent Diabetes Mellitus
LDL	Low-Density Lipoprotein
NIDDM	Non -Insulin Dependent Diabetes Mellitus
RAS	Renin Angiotensin System
RAAS	Renin Angiotensin Aldosterone System
RBS	Random Blood Sugar
RPM	Revolution Per Minute
PCR	Polymerase Chain Reaction
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TAE	Trice Acetate Ethylene Diamine Tetra Acetic Acid
TC	Total Cholesterol
TG	Triglycerol

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ABSTRACT

Type 2 diabetes affect large population and able to develop micro and macrovascular complicati ons. There are many non- genetic and genetic factors or both are associated for the occurrence of type 2 diabetes mellitus (T2DM) and diabetic complications. Genes of the renin angiotensin syst em angiotensin converting enzyme insertion/deletion gene polymorphism has been associated wi th the risk of type 2 diabetes and its complications. The aim of the present study was to investigate the association of angiotensin converting enzyme gene polymorphism, risk of type 2 diabetes and its complications. A total of 222 subjects (111 T2DM and 111 healthy controls) were collected from Bahir Dar Felegehiwot referral hospital and Bahir Dar town, respectively by using non-probability purposive sampling techniques. Patients with T2DM were selected using non probability purposive sampling technique. Minidray fully automated analyzer was used for biochemical tests such as glucose, total cholesterol, triglycerol, urea and creatinine to assess the associated risk factors. The *ACE I/D* genotypes were identified by Polymerase chain reaction (PCR) using appropriate primers and PCR reaction conditions. The present study revealed that the frequency of *DD* genotype and *D* allele were higher in type 2 diabetes mellitus compared to healthy controls (*DD*, 64.2% Vs 35.6% $P < 0.001$) and (*D*, 79.3% Vs 59.9% $P < 0.001$). *DD* genotype showed three fold increase risk towards T2DM as compared to II Genotype (OR: 2.984, CI: 1.332 - 6.689, $P < 0.02$). The *D* allele carriers had five times high risk of getting diabetic as compared to *I* allele. (OR, *D* 2.178; CI: 1.168 – 3.232 $P < 0.001$ Vs *I* OR, 0.459; CI: 0.309 – 0.681 $P < 0.001$). To determine whether *ACE* gene polymorphism was associated with diabetic complication, patients with type 2 diabetes were divided into diabetic hypertension, diabetic retinopathy and diabetic nephropathy. The frequency of *ACE DD* genotype and *D* allele in patients with diabetic hypertension were significantly higher than T2DM patients (41.6% and 60.4% Vs 19.5% and 37.8% $P < 0.01$). The frequency of *ACE DD* genotype and *D* allele in patients with diabetic retinopathy were also higher than T2DM patients (28.6% and 44.1% Vs 19.5% and 37.8%). The frequency of *ACE* genotype in patients with diabetic nephropathy did not show significant association. This might be due to small sample size that lack statistical power. This study indicated that the frequency of *ACE DD* genotype and *D* allele were markedly higher in patients with type 2 diabetes. This genotype and allele were significantly associated with diabetic hypertension and diabetic retinopathy patients.

Keywords: *ACE* gene polymorphism; diabetes complications; diabetic hypertension; diabetic retinopathy; diabetic nephropathy

1. INTRODUCTION

Diabetes is a rising global health burden, which is a multifactorial, heterogeneous group of metabolic disorder with the common feature characterized by a deficiency or failure in maintaining normal glucose homeostasis; this is due to defective insulin secretion, resistance insulin action or due to a combination of both (Karuna *et al.,* 2013).

Diabetes mellitus is a serious condition with potentially stressful complications that affects all age groups worldwide (Guariguata *et al.,* 2013). The American Diabetes Association (ADA) divides diabetes mellitus into four categories (Kleinberger & Pollin, 2015). Type 2 diabetes mellitus (T2DM) is the second category and most diabetes is sorted in this category, a heterogeneous group of disorders caused by some combination of insulin resistance and impairment of insulin secretion (Kleinberger & Pollin, 2015). Insulin resistance is a condition in which the body's muscle, fat and liver cells does not use insulin effectively leads to high concentration of glucose in the circulation and occurred when the body can no longer produce enough insulin to compensate for the impaired ability to use insulin (Kaku, 2010; Skarfors *et al*., 1991 and Colditz *et al*., 1990).

According to reports by International diabetic federation (IDF), in 2013 approximately 382 million people had diabetes globally and the IDF has estimated this number will rise to 592 million by 2035 (Guariguata *et al.,* 2013). Among those type 2 diabetes, accounts about 350 million people worldwide and estimated 10 percent of the world's adult population (nearly 600 million people) will suffer from the disease by 2035 (Colagiuri *et al*., 2015). However, the largest increase of population with diabetes occur in sub-Saharan Africa, with a projected growth of 19.8 million in 2013 to 41.5 million by 2035 (Guariguata *et al.,* 2013). Among this, over 90% are type 2 diabetes in Sub-Saharan Africa (Hall *et al.,* 2011). This is due to a rapid uncontrolled urbanization, lifestyle changes towards western diets, reduced quality of food, late diagnosis, inadequate screening and diagnostic resources, poor control of blood sugar level, inadequate treatment at an early stage and smoking in sub-Saharan Africa (Kengne *et al.,* 2013; Mbanya *et al.,* 2010 and Vivian *et al.,* 2010). Since 1985 Ethiopia has been a member of IDF and the IDF estimated the number of diabetic among adults (20-79 years) in 2010 was 2.5% and the number

is expected to rise in to 3.5% by the year 2030 (Shaw *et al.,* 2010). Currently diabetes is a serious challenge in Ethiopia (Worku, 2010; Abera, 2000 and kasper and Lester, 1988).

Individuals who are unaware of diabetics disorder (Chronic hyperglycemia of diabetes) are at very high risk of chronic complications (Mbanya *et al.,* 2010). The complication may be macrovascular or microvascular origin and risk for diabetic hypertension. Macrovascular such as cardiovascular disease (CVD), coronary heart disease, peripheral vascular disease and cerebrovascular disease and microvascular complications such as, affecting the eyes cause of blindness (diabetic retinopathy), end stage renal disease (ESRD or diabetic nephropathy), diabetic foot and nerves (diabetic neuropathy (Tesfaye & Gill, 2011; Al-Khawlani *et al*., 2010 and Michael, 2008). The macro and microvascular complication of diabetes mellitus is also common in Ethiopia (Gebrekirstos *et al.,* 2015 and Tesfaye *et al*., 2015).

It is typically a multifactorial disease*,* there are many factors associated in the occurrence T2DM and the progress of its complication. Risk factors such as obesity (Body mass index (BMI)), over eating, stress as well as aging, dyslipidemia, hypertension*,* smoking, physical inactivity, dietary patterns, family history, and specific genes are the most frequently known risk factors for T2DM (Lyssenko *et al.,* 2008; Valdes *et al.,* 2007 and Holt, 2004). However, the genetic factors (genetic origins) of T2DM and its complication are not obviously understood because the genetics of T2DM is polygenic and multifactorial origins as a result of the interaction between the environment and multiple genes (Vivian *et al.*, 2010 and Strojek *et al.,* 1997).

At present many candidate genes have been associated with T2DM *(*Lyssenko *et al., 2007).* However, *g*enes that are encoding for the renin-angiotensin system (RAS) are the main genetic risk factors for T2DM and the progress of its complications (Giacchetti *et al.,2005 and* Jacobsen *et al.*, 2003). Among genes involved in the RAS, *Angiotensin converting enzyme (ACE)* gene encoding is a key enzyme. This gene is located at chromosome 17q23 consisting of 26 exons, 25 introns and it spans 21 kb (Erdos, 1990). The polymorphism of *ACE* gene due to the *insertion (I) or deletion (D)* of a 287 bp *Alu* repeat sequence near the 3′ end of intron 16 leads to three genotypes *DD, II* and *ID* (Skipworth *et al.,* 2011; Rivera *et al.,* 2004 and Rigat *et al.,* 1990). The main function of this gene is the conversion of *Angiotensin I* to vasoactive, natriuretic octapeptide *angiotensin II* in liver (Erdos, 1990) and inactivates a vasodilator peptide bradykinin (Kuoppala *et al.,* 2000). Alleles of *ACE* gene polymorphism are present in both healthy

individuals and T2DM patients with different frequencies and *DD* genotype individuals are at risk of T2DM and its complications (Jeffers *et al.,* 1997). This implies that, imbalances in RAS functions are related to insulin resistance and susceptible to T2DM. The present state of knowledge about *ACE* gene polymorphism in T2DM and its complication have a doubt because there were some studies in the literature that described a null association between the etiologies of T2DM and its complication with *ACE* gene polymorphism (Skipworth *et al.,* 2011and *Jacobsen et al., 2003*). However, most of the reported literature has indicated that *ACE* gene polymorphism is associated with T2DM and its complication in different populations (Hussein *et al.,* 2015; Nikzamir *et al.,* 2006; Yang *et al*., 2006 and Yoshida *et al*., 1996).

1.1 Statement of the Problem

Diabetes is a rising global health problem both in developed and developing continents, especially in Africa, sub-Saharan Africa due to late diagnosis, inadequate screening and lack of diagnostic resources, poor control of blood sugar, and inadequate treatment at an early stage of the disease (Guariguata *et al.,* 2013, Kengne *et al.,* 2013 and Sleire 2011). In Ethiopia, undiagnosed cases (late diagnosis) is highest proportion (82.6%) among the rural population and 63% among the urban population and family history of diabetes is highly associated with diabetes mellitus (Abebe *et al.,* 2014). This indicates that proper awareness of the public about the disease early diagnosis is not a practice as a result new cases already have diabetes with hypertension and other complication at the time of diagnosis (Seifu *et al.,* 2015 and Abebe *et al.,* 2014). Genetic polymorphism could be indicators for risk of T2DM and can serve as a marker so that may help for protecting the disease with better treatment out come and early diagnosis even before the onset of the disease. The role of *ACE* gene polymorphism in the pathogenesis of type 2 diabetes and its impact on the progress of diabetic complication has been investigated by different studies. However, there were no reported data of *ACE* gene polymorphism on the risk of T2DM and diabetic complications among the Ethiopian population. Therefore, the aim of this study was to investigate the association of risk factors, *ACE* gene polymorphism in patients with type 2 diabetes and its complications in the Ethiopian population, Bahir Dar Felegehiwot Referral Hospital.

1.2 Significance of the Study

Type 2 diabetes is a complex disorder resulting from an interaction between multiple genes and environmental factors. As a result, this study was conducted on both socio- demographic risk factors and one of the associate genes (*ACE*). It is important to look for the gene association in the Ethiopian population and compare it with previous studies on the same issue other than Ethiopian population. This will help scientists and physicians to rationalize research priorities regarding T2DM and its complication serve as a marker for early diagnosis of the disease and to order better therapeutic drugs that help to reduce the occurrences of diabetic complications. In addition, the result of this study would be used as a source of information to the community and health care professionals. Policymakers also get ideas about gene polymorphisms to develop policies and strategies for evidence-based approaches to a better treatment individual medicine for type 2 diabetes and its complications.

2. OBJECTIVES

2.1 General Objective

The general objective of this study was to assess the association of *angiotensin converting enzyme* gene polymorphism wit risk of diabetes 2 and its complication among patients visiting Bahir Dar Felegehiwot referral hospital.

2.2 Specific Objectives

The specific objectives of this study were

- ✓ To compare the frequency of *ACE* gene polymorphism in type 2 diabetes mellitus patients and healthy controls
- ✓ To evaluate the frequency of *ACE* gene polymorphism in patients with type 2 diabetes without complications and diabetic complications
- ✓ To determine the correlation of *ACE* gene polymorphism to risk of diabetic type 2 and degree of complications

3. LITERATURE REVIEW

3.1 Epidemiology of Type II Diabetes

Type 2 diabetes is mostly characterized due to insulin resistance and beta cell dysfunction (Kahn, 2003). Insulin resistance is a condition in which cells do not respond to the normal action of insulin secreted from the pancreatic β-cells. Consequently, cellular uptake of glucose does not occur and blood glucose level become elevated (Kahn, 2003).

Type 2 diabetes is also called non-insulin-dependent diabetes (NIDDM), the most common type of diabetes mellitus worldwide and it is a challenge both in the developed and developing countries (Colagiuri *et al*., 2015; Mbanya *et al*., 2010 and Dahiru *et al*., 2008). The other report by King *et al.,* (1998) indicated that the number of T2DM in 2000 was estimated approximately 150 million individuals with the disease and this number is expected to double by 2025 (King *et al.,* 1998). Similar report also indicated that T2DM represents 90% of the overall burden of diabetes worldwide with approximately 150 million cases (Diamond, 2003). Diabetic forum on 2015 indicate that, T2DM affects about 350 million people worldwide and if the tendency continues, an expected 10 percent of the world's adult population (nearly 600 million people) will suffer from the disease by 2035 (Colagiuri *et al*., 2015).

The prevalence of T2DM in Africa was mostly lower than 1%, except in South Africa (0·6–3·6%) and Coted'Ivoire (5·7%) between the 1960s and early 1980s (McLarty *et al*., 1990). However, this disorder is currently the most common sub-Saharan Africa, which is reported by Colagiuri *et al* (2015) and Hall *et al (*2011), over 90% of T2DM are in Sub-Saharan Africa having population prevalence ranged from 1% in rural Uganda to 12% in urban Kenya (Colagiuri *et al*., 2015 and Hall *et al.,* 2011).

Type 2 diabetes is also common in Ethiopia (Worku, 2010 and Abera, 2000). According to the 2011 report of the International Diabetes Federation (IDF), the figure of adults living with diabetes in Ethiopia was 3.5% (Whiting *et al*., 2011). Among those, Mekelle Ayder referral hospital, reported that, most of the patients, 216/263 (82%), had T2DM while 47/263 (18%) of them suffered from type I diabetes (T1DM), which indicates T2DM is common in Ethiopia (Ambachew *et al.,* 2015). The new report from 2011-2014 at Dilla referral hospital also reveal

that T2DM were highly predominated diseases across the year than T1DM (Alemu, 2015). The other study by Worku *et al* (2010) in the southwest Ethiopia of Jimma university specialized hospital indicate that, the larger proportion of 189 (62.0%) patients had T2DM and most of them are acute complications (Worku *et al*., 2010). However, the study by Brown *et al* (1998) in the northern Ethiopia, university of Gondar teaching referral hospital reveal that , T1DM are more common than T2DM which accounts 40% of the town and 75% of rural patients (Brown *et al.,* 1998). On the other hand, the study in another time in the northern Ethiopia, university of Gondar teaching referral hospital reveal that T2DM account 71% urban areas and 23% in the rural areas (Alemu & Watkins, 2004). T2DM is the fourth or fifth leading cause of death in most developed countries and the growing evidence indicate that, it has reached epidemic proportions in many developing counties like Ethiopia (Amos *et al*., 1997).

3.2 Insulin and its Resistance in Type 2 Diabetes

The pancreas islets of langerhans consist of insulin-releasing beta cells, glucagon-releasing alpha-cells, somatostatin-producing delta-cells, and pancreatic polypeptide-producing cells (Elayat *et al*., 1995). Of these, insulin attempts to maintain normoglycaemia through a constant supply of glucose between meals and conversion of the excess glucose to glycogen (glycogenesis) in the liver and muscles (Taylor and Agius 1988). Glucagon has the reveres action of insulin it converts liver glycogen to glucose (glycogenolysis) and muscle tissue glycogen into pyruvic acid or lactic acid in anaerobic conditions, which later changed to glucose in the liver (Taylor and Agius, 1988).

The insulin receptor (IR) is a heterotetramer consisting of two α and two β subunits those are linked by disulphide bonds (White *and* Kahn, 1994 and White *et al.,* 1988). β subunit has a hydrophobic transmembrane region, and an intracellular domain which has several tyrosine residues, a tyrosine kinase and an ATP-binding site (White *et al.,* 1988). Insulin binding to the α subunits activates β subunit tyrosine protein kinase and brings phosphorylation of tyrosine residues and activation of the β subunit kinase leads to transmission of the insulin signal, perhaps by initiating a phosphorylation/dephosphorylation cascade (White *et al.,* 1988 and Denton *et al.,* 1981). The alteration in the insulin-receptor tyrosine kinase activity induced by cyclic AMP-dependent protein kinase phosphorylation of serine residue of the receptor sites (β

subunit), and this could faithfully underlie catecholamine-induced insulin resistance (Tanti *et al.,* 1987). During the situation of extreme insulin resistance and T2DM, the process of signal transmission from the insulin receptor, a subunit insulin-binding site to activate the kinase appears to be defective at one or more sites (Caro *et al*., 1986). This leads to amino acid substitution in the ATP-binding region or the tyrosine kinase region and diminishes insulin action (Ellis *et al*., 1986). This impaired insulin secretion through a dysfunction of pancreatic β-cell and impair insulin action through insulin resistance are the main pathologic defect of type 2 diabetes (Holt, 2004).

3.3 Complications of Type 2 Diabetes and Diabetic Hypertension

The complications of T2DM are macro and microvascular origin. Macrovascular such as cardiovascular disease (CVD), coronary heart disease, peripheral vascular disease and cerebrovascular disease and microvascular complications such as, affecting the eyes cause of blindness (diabetic retinopathy), end stage renal disease (ESRD or diabetic nephropathy), diabetic foot and nerves (diabetic neuropathy (Tesfaye & Gill, 2011; Al-Khawlani *et al*., 2010 and Michael, 2008). Among those complications, microvascular complication is frequently occurred in T2DM. As a result, this study has paid great attention for diabetic nephropathy, diabetic retinopathy and diabetes with hypertension.

3.3.1 Diabetic Nephropathy

Diabetic nephropathy (DN) is a common and severe complication that leading the cause of chronic kidney disease in patients with T2DM and in a lesser proportion of subjects with T1DM (Wang *et al.,* 2008). However, the survival rate of T2DM patients with renal failure is much worse than that of patients with renal failure resulting from T1DM or other causes (Mogensen, 2003). In addition, T2DM occurs more frequently at a younger age and these individuals live longer with diabetes which expected to develop diabetic renal disease and ESRD due to a late complication (after 15 to 25 yr) (Mogensen, 2003).

Diabetic nephropathy is characterized by macro albuminuria >300 mg in a 24-hour urine or macro albuminuria and abnormal renal function as represented by an abnormality in serum

creatinine and serum urea and evidenced a decline in glomerular filtration rate (GFR) (Fontela *et al.,* 2014 and Shlomo *et al*., 2011). The progression of diabetes mellitus to DN monitored by serum urea and creatinine level, those are simple biomarkers available for nephropathy individuals and are higher in the diabetics especially in the case of DN (Bamanikar *et al.,* 2016*).* Males had elevated urea and creatinine levels compared to females, also age and period on treatment are associated with impairment of renal function in both sexes (Mafuratidze *et al*., 2014). The other study also indicates that age and plasma creatinine had a positive correlation (Amartey *et al*., 2015).

T2DM is characterized by hyperfiltration in the early stages but GFR values has decreasing when duration of diabetes increased with a mean GFR 103.508 ± 33.369 ml/min which indicates the progress of diabetic nephropathy (Agarwal *et al*., 2005). Similar study also indicated that longer duration of diabetes and lower diastolic blood pressure are associated with reduced estimated glomerular filtration rate (eGFR) among 2368 subjects with T2DM had 12% of eGFR <60ml/min/1.73m2 (Wall *et al*., 2010). Glomerular filtration rates below 60 mL/min/1.73m² represents a decrease of approximately 50% in normal renal function and leads to complications of chronic kidney disease (CKD) (Fontela *et al.,* 2014). The other report can classify renal failure in to different stages based on eGFR as Normal or high >90, mildly decreased 60-89, mildly to moderately reduced 45-59, moderately to severely declined 30-44, severely decreased 15-29 and Kidney failure <15 (KDIGO, 2013).

3.3.2 Diabetic Retinopathy

Diabetic retinopathy (DR) is a leading cause of visual impairment among diabetic patients. Some new diagnosis patients with T2DM have DR, which indicates there is a need for early screening of all type 2 DM patients (Jammal *et al*., 2013). Among 10 individuals, one of them has at risk for DR (Hu *et al*., 2015). Age at diagnosis, high total cholesterol, high low-density lipoprotein, triglycerides, microalbuminuria, elevated blood pressure, the use of anti-hypertensive drugs, cigarette smoking and duration of hypertension are associated risk factor for the development of DR (Hussain *et al., 2013;* Shammari *et al*., 2005 and Ohn *et al*., 1993). However, the study in China indicates that among the above risk factors only diabetic duration is the risk for the occurrence of DR (Hu *et al*., 2015).

Diabetes is one of the risk factor for retinopathy and other serious diseases in the Bangladesh population (Islam *et al.,* 2015). The study in the northern part of Ethiopia, diabetic clinic at Mekelle Hospital indicate that among 105 diabetic patients 21% of them had diabetic retinopathy (Gill *et al*., 2008). The other review reports in Gondar University referral hospital indicated that the prevalence of DR among Ethiopian diabetics has been estimated to be approximately 25% (Nigatu, 2012).

3.3.3 Diabetic with Hypertensive

Hypertension is not classified as micro or macrovascular complication rather it is a risk factor for diabetes mellitus patients (Reaven, 1988). Cellular insulin resistance is responsible for the development of hypertension through alteration substrate supply or energy needs of the cell and the resultant changes in substrate/energy requirements sensitize, either directly or by altering ion fluxes into the cell, the vascular smooth muscle response to amines such as nor epinephrine and *Angiotensin II* (DeFronzo & Ferrannini, 1991). The other study also recommended that, microvascular dysfunction may affect both peripheral vascular resistance that leads to hypertension (Serne *et al.,* 1999) and insulin-mediated glucose disposal that leads to insulin resistance (Clark *et al.,* 2003; Serné *et al.,* 2001and Serne *et al.,* 1999).

Individual who had a systolic blood pressure of 140mmHg and/or diastolic blood pressure of 90 mmHg considered to have hypertension. The studies reveal that systolic and diastolic blood pressure were risk factors for type 2 diabetes and diabetic complications (Unnikrishnan *et al.,* 2007).

3.4 Risk Factors for type 2 diabetes and its complications

There are many risk factors for T2DM and it's progression towards diabetic complications. Those risk factors can be non-genetic or genetic etiology or either of both (Kasper *et al.,* 2004). Among the non-genetic or either of both risk factors most of the studies showed that, the association of type 2 diabetic patients and its complication with advancing age and duration of diabetes, obesity, poor glycemic control, smoking, gender, lipids, hypertension, proteinuria, serum creatinine, eGFR, urea, higher BMI (Viswanathan *et al.*, 2012; Haque *et al.,* 2010 and Iranparvar *et al.,* 2006).

Many of the studies show that, there are many genes associated for the occurrence of T2DM and the progress of diabetic complications. Among the genetic risk factors, researcher addresses by family studies, and other genes such as genes in the rennin Angiotensin system, genes associated to dyslipidemia, hypertension and oxidative stress are confirmed as a genetic risk factors contribute for the occurrence of T2DM and the development diabetic complications both in T2DM and T1DM (Al-rubeaan *et al.,* 2014; Giacchetti *et al*., 2005 and White *et al*., 1998).

3.4.1 Non-genetic Risk Factors

3.4.1.1 Smoking

Numerous prospective studies has been reported that current smokers are having a risk factor for developing of T2DM (Hur *et al*., 2007; Sairenchi *et al.,* 2004 and Hu *et al*., 2001). The other studies reveal that, the association between smoking and T2DM has stronger among heavy smokers (more than 20 cigarettes/day) compared with light smokers (Willi *et al*., 2007 and Patja *et al.,* 2005). In addition, the follow-up study found that an increased risk of type 2 diabetes is occur in the first 2-3 years after smoking termination (Yeh *et al.,*2010 and Sairenchi *et al.,*2004).

In diabetes mellitus, numerous prospective studies found that cigarette smoking is able to increases the risk of microvascular and macrovascular complications such as in diabetic retinopathy smoking raises the risk of changes in the blood vessels inside the eye that can cause blindness in those patients over the age of 65 (Thornton *et al*., 2005). Cigarette smoking has its own effects on lipid profile such as triglyceride, cholesterol and low-density lipoprotein (LDL) levels are high in smoker individuals but high-density lipoprotein is lower in smokers than in non-smokers (Craig *et al*., 1989).

3.4.1.2 Age and Sex

Age and sex are globally identified risk factors for diabetes mellitus (Ebenezer *et al*., 2003). Individual who have an older age (>45) are having a chance to develop hyperglycemia and it is more critical in women than men (Veghari *et al*., 2010). The other similar report in Libya also indicate that, subjects having ≥50 years of age had nearly double risk of complications than those with <50 years of age (Roaeid and Kadiki. 2011). The report in Nigeria indicates that age was

associated with T2DM in both sexes (Ekpenyong *et al*., 2012). However, the recent study in Batticaloa district, Sri Lanka indicate that age above 50 years have more chance to develop diabetes than other age groups but not correlated with gender (Prasanth *et al*., 2015). The other study reported that, male genders are more prevalent in the high-risk group for T2DM (Marinho *et al.,* 2013). The study in Bangladesh indicate that sex is not associated with T2DM, however individuals in the middle (31-50 years) and older age group (>50 years) are exposed to the occurrence of T2DM (Al Sharmin and Munima, 2016).

Diabetes tends to increase with increasing age (high in 40-59 years) has triple risks of developing T2DM than younger ages (Whiting *et al*., 2011). The other report also supporting these idea, diabetes tends to increase with increasing age (high in 41-60 years) and tend to decrease in >60 years for both men and women (Ruhembe *et al*., 2014). Increasing in age is associated with higher risk of T2DM without difference regarding to gender (Bhalerao *et al*., 2014). Most of subjects with diabetes are age between 40 and 59 years and the mean age did not differ according to gender (Sagna *et al*., 2014).

Many studies have a conflict in the association or non-association between gender and diabetic complications. To solve this conflict some of the studies reveal that, gender has no any impact on progression of renal disease or survival (Hall *et al.,* 2011 and Mogensen, 2003). However, other studies showed that males having an independent risk factor or have strong association with diabetic nephropathy and the risk of developing diabetic nephropathy is 20 times higher compared to female (Ahmad *et al.,* 2014; Al-rubeaan *et al.,* 2014 and Abdulhakeem *et al.,* 2012). This implies that male is having an increased risk for diabetic and renal disease than females. The other study in diabetic retinopathy also shows that male genders are high risk for the development of diabetic retinopathy (Hussain *et al.,* 2013). However, the other report reveals that female genders are at high risk for the development of diabetic retinopathy (Ohn *et al*., 1993).

3.4.1.3 Duration of Diabetics

Diabetes duration mean that the survival of patient long time with diabetics. Such cases were studied by different researchers and some of the studies reveal that, patients with duration of diabetes of 7-14 and ≥14 years had nearly twofold and threefold increase in complications than

patients with duration of <7 years (Roaeid and Kadiki, 2011). Patients that have been diagnosed for the disease for more than 10 years having complications are higher than diagnosis of the disease for less than 10 years (Cortez *et al*., 2015). These imply that longer diabetes duration is the cause for the development of micro or macrovascular diabetic complication.

In microvascular complication, diabetic nephropathy (diabetes duration of 15 years) (Al-rubeaan *et al.,* 2014 and Kasper *et al.,* 2004). The other study also reveal that diabetic nephropathy is associated with a longer diabetic duration (Mafuratidze *et al.,* 2014 and Unnikrishnan *et al.,* 2007). Diabetic retinopathy increased with the duration of diabetes mellitus (5-10 yr: 5.2- fold; > 10 yr: 10-fold) (Kim *et al*., 2011). Diabetic duration is also associated with retinopathy (EL-Shazly *et al*., 2011 and Correa *et al*., 2003). With the mean duration of diabetes mellitus in all patients (13.35 ± 8.17 years) diabetic retinopathy is highest in women (11.91 ± 7.92 years) than in men (14.42 ± 8.20 years) (El-Babb *et al*., 2012).

3.4.1.4 Body Mass Index

Obesity and T2DM are worldwide public health problem in the 21st century that increase the prevalence of diabetes parallels that of obesity. Obesity is excess storage of fat in the body that leads to the development of cardiovascular morbidity, insulin resistance by muscle cells and resistance to the cellular actions of insulin which results the occurrence of T2DM and (Hribal *et al.,* 2002). This an impaired ability of insulin inhibits glucose output from the liver and promote glucose uptake in fat and muscle cell is due to adipose tissue releases increased amounts of glycerol, hormones, non-esterified fatty acids, pro-inflammatory cytokines and other factors associated to the development of insulin resistance (Hribal *et al.,* 2002 and Saltiel and Kahn, 2001).

Many studies have reported that increased BMI is a strong risk factor for T2DM (Almdal *et al*., 2008 and Knowler *et al.,* 1991). This occurs both in men (Almdal *et al.,* 2008 and Knowler *et al.,*1991) and women (Colditz *et al.,* 1990). Genes responsible for obesity and insulin resistance interact with environmental factors such as increased fat/ calorie intake and decreased physical activity resulting in the development of obesity and insulin resistance followed ultimately by the development of T2DM (Kahn *et al.,*2006 and O'Rahilly *et al.,*2006). Those obese individuals having elevated plasma levels of free fatty acids are known to cause muscle insulin resistance

(Yaturu, 2011). People with severe obesity are at greater risk of T2DM than obese people with a lower BMI calculated as the weight in kilograms divided by the height in meters squared (Gatineau *et al.,* 2014 and Iranparvar *et al.,* 2006). BMI is long-term predictors of renal function in men than in women anthropometric and metabolic parameters are less predictive eGFR (Nagel *et al.,* 2013).

3.4.1.5 Dyslipidemia

Resistance to insulin has a direct link to the changes in lipid profiles in NIDDM, by developing a number of alterations in lipid metabolism and lipoprotein composition that make more pathogenic in patients with T2DM and usually it is associated with higher concentrations of Triglycerol (TG) and lower concentrations of high- density lipoprotein (HDL) (Schaefer *et al.,* 2009). The mechanisms of circulating free fatty acids (FFA) for insulin-mediated effects on microvascular function are not completely understood. The elevated FFA induces an increase in reactive oxygen species production (Lu *et al.,* 1998). This leads to cause vascular endothelial dysfunction indirectly via increased release of the vasoconstrictor substance (Piatti *et al.,* 1996).

Type 2 diabetes is one of the free radical diseases increases diabetic complications with increased lipid peroxides (Kumawat *et al.,* 2012). Increased oxygen free radicals result in the lipid peroxidation of cellular lipids leads to macrovascular and microvascular complications in T2DM (Barathmanikanth *et al.,* 2010). The main features of type 2 diabetic patients have plasma lipid alterations of Hypertriglyceridemia with decreased HDL (Miller *et al.,* 2011). The presence of High plasma triglycerides and low plasma HDL cholesterol levels are an indicator of prediabetic state for individuals who had insulin resistance syndrome (Fabbrini *et al.,* 2010 and Mitsuyoshi *et al.,* 2009).

Hypercholesterolemia is higher in T2DM and it is also a common risk factor for the complication of progressive DN, DR , contributes to high cardiovascular morbidity and mortality of chronic kidney disease (CKD) patients and diabetic hypertension (Bakris, 2011 and Moorehead *et al.,* 2005). Hypercholesterolemia characterized by high TG > 150 mg/dL and HDL > 40 mg/dL in men and cholesterol levels > 50 mg/dL and a predominance of small dense low-density lipoprotein (LDL) >100 mg/dL (Bakris, 2011 and Moorehead *et al.,* 2005).

3.4.2 Genetic Risk Factors

Many of the studies have described that, genetic components play an important role in the pathogenesis of T2DM (Harrison *et al.,* 2013 and Amini & Janghorbani, 2007). Some of the studies reported that individuals who have a family history have an increased risk of developing T2DM and the risk increases when both parents are affected (Amini & Janghorbani, 2007 and Bjornholt *et al.,* 2000). Specially, monozygotic or twin individuals are highly affected with T2DM compared to dizygotic individuals (Medici *et al.,* 1999).

The prevalence of diabetes varies among different ethnic groups and this variation across the different ethnic groups share a similar environment, which supports the idea of genetic factors contribute to the predisposing of disease (Elbein, 2006). Genetic factors predispose to the development of T2DM by reducing insulin sensitivity and insulin secretion (Gerich, 2007 and Elbein, 2006). The study by Lyssenko *et al* (2007) identifying 11 genetic variants (TCF7L2, PPARG, FTO, KCNJ11, NOTCH2, WFS1, CDKAL1, IGF2BP2, SLC30A8, JAZF1, and HHEX) and all are associated with the risk of T2DM due to impaired beta-cell function but those genes are not associated with clinical risk factors (Lyssenko *et al.,* 2007). However, among the 11 genes, *ACE* gene was not included in the study. And the gene transcription factors 7-like 2 (TCF7L2) is the highest risk of type 2 diabetes which results impairment of insulin secretion (Lyssenko *et al.,* 2007).

These days there are many genes associated for the occurrence T2DM and progress of diabetic complications. such as genes in the Renin-Angiotensin aldosterone system (RAAS), Endothelial nitric oxide synthesis (ENOS) (Neugebauer & Baba, 2000), Glucose metabolism (Ricci *et al.,* 2006), Cytoskeleton genes (Barry *et al.,* 2009), Inflammation (Ahluwalia *et al.,* 2009) , Growth factors, Oxidative stress (Yamagishi & Matsui, 2010), and lipid metabolism (Wu, 2014). However, this study focuses *ACE* gene polymorphism in RAAS, because this gene and other genes in the RAAS such as *angiotensinogen* and *angiotensin II* type 1 receptor gene plays a vital role in regulating glucose metabolism and blood pressure, electrolyte and fluid homeostasis those leads to pathogenesis of DM and facilitate the progress of diabetic complications (Zhou and Schulman, 2009).

3.4.2.1 Gene polymorphisms in the Renin-Angiotensin-Aldosterone System

Genes involved in this system are the major concern of this paper because genes of the Renin-Angiotensin-Aldosterone System (RAAS) have many important roles than the above genes which is reported by many of the researcher, some of its role includes in glucose metabolism, and the regulation of blood pressure (Al-rubeaan *et al.,* 2014), electrolytes and fluid homeostasisd (O*hshigel.,et al.* 2010). The RAAS genes consist of renin (Liu *et al.,* 2013), *angiotensinogen* (*AGT*) (Fogarty *et al.,* 1996), *angiotensin-1 converting enzyme* (*ACE*) (Haque1 *et al.,* 2010); *Angiotensin converting enzyme* 2 (*ACE2*), *Angiotensin II type 1 receptor* (*AT1R*) (Wysocki *et al.,* 2006) and *Angiotensin II* type 2 receptor (*AT2R*) (Bindom *et al.,* 2010) all of these genes or genetic loci responsible for excess *Ang II* production or availability are potential candidates for development of diabetic complications. Because *Angiotensin II* (*Ang II*) is a powerful vasoconstrictor and increase in intraglomerular pressure results in proteinuria and glomerulosclerosis (Ha *et al.,* 2015). Among those genes, this study will be focus on *Angiotensin converting enzyme* (*ACE*) gene *insertion/deletion* polymorphism because most of the studies were reported of this gene was highly associated with T2DM, diabetic with hypertension, diabetic retinopathy and diabetic nephropathy.

3.4.2.1.1 Angiotensin Converting Enzyme

In the 1950s Skeggs and colleagues, discovered an enzyme that converts *ANG I* to *ANG II* (named as hypertension I and hypertension II) (Leonard *et al.,* 1956). Called "*converting enzyme*". After a number of years later recognized an enzyme in human blood, called *kininase II*, which was able to degrade bradykinin (Kuoppala *et al.,* 2000). *Converting enzyme and kininase II* is later shown to be the same enzyme (Dorer *et al.,* 1974) and today the enzyme is referred to as *Angiotensin-converting enzyme*.

ACE gene is located at chromosome 17q23 and spans approximately 21kb DNA, consists of 26 exons and 25 introns (Erdos, 1990). The amino acid domain of the *ACE* coded by exon 1 to exon 12, while the carboxyl domain coded by exon 13 to exon 26. The gene encodes 2 isoforms of *ACE*. The somatic form *(sACE)* a glycoprotein composed of a single large polypeptide chain expressed in somatic tissue of vascular endothelial cells at the brush border of renal proximal convoluted tubule, epididymal duct epithelia and the jejunal villus (Sibony *et al.,* 1993). The

other is testicular form (testis *ACE*, germinal *ACE*), expressed uniquely in germinal cells with a precise stage-specific pattern, starting in round spermatids and finishing in spermatozoa (Sibony *et al.,* 1993).

Angiotensin is produced by the action of renin on *angiotensinogen* to form inactive decapeptide *Angiotensin I* and its subsequent conversion to the biologically active octapeptide (*Angiotensin II*) by *Angiotensin -converting enzyme* which is mediated via the *Angiotensin* type 1 receptor (Carey & Siragy, 2003). *Angiotensin converting enzyme* (*ACE*) is a zinc metallopeptidase membrane-bound enzyme widely distributed in several types of cells including vascular endothelial cells, various absorptive epithelial cells, neurons, macrophages, T-lymphocyte and other mononuclear cells, male germinal cells, and is also present in a circulating form in biological fluids such as plasma, amniotic fluid, seminal fluids (Joyce-tan *et al.,* 2016 and Erdos, 1990).

In plasma and on the surface of endothelial cells, it converts the inactive decapeptide *Angiotensin I* into the vasopressor- and aldosterone-stimulating octapeptide *Angiotensin II* (Leonard *et al.,* 1956). It also inactivates bradykinin (BK) (Kuoppala *et al.,* 2000), a vasodilator nonapeptide involved in the control of vascular tone and implicated in inflammatory responses and finally the enzyme is involved in the metabolism of several other biologically active peptides because of its broad enzyme specificity and wide distribution in the body (Erdos, 1990).

Angiotensin I converting enzyme (ACE) gene is one of the most enormously studied genes and the main part of this paper. Because it has the key role in the rennin-Angiotensin system (RAS) and its physiological application in different parts of the body (Moynahan *et al.,* 2001). The polymorphism of this gene was first explained by Rigat and his coworkers, typically refers as the *insertion (I)* or *deletion (D)* of an *Alu* repetitive sequence of 287-bp in intron 16 of the gene , found in three forms: *D/D* and *I/I* homozygote and *I/D* heterozygote (Rigat *et al.,* 1990). *Alu ACE I/D* polymorphisms are also suitable markers for studying genetic variation in human populations (Skipworth *et al.,* 2011and Yoshida *et al.,* 1996).

3.4.2.1.2 Gender and *ACE* gene polymorphism

Glucose utilization is higher in women with the *DD* genotype than in women with the *II* genotype, homozygous *D* allele women of the *ACE* gene are more insulin sensitive, whereas homozygous *I* allele women of the *ACE* gene have greater insulin resistance and potential risk for T2DM (Kaleemullah *et al.,* 2011). A higher insulin level is an indirect indicator for insulin résistance, subjects with *I* allele are more insulin-resistance than non *I* allele individuals (Chiu & McCarthy, 1997). Study in south Indian population revealed that, the *DD* genotype is associated with the female population where as individual with *I* allele (homozygous *II* and heterozygous *ID*) in unaffected males which act as a protective role in male population (Nagamani *et al.,* 2015). However, the other study conducted in China indicated that male subjects with *DD* genotype had higher serum *ACE* activity than female subjects with *DD* genotype (Zhang *et al.,* 2014)

3.4.2.1.3 Metabolic syndrome and *ACE* gene polymorphism

Metabolic syndrome is a collective effect of genetics and certain environmental factors which results glucose intolerance, hypertension, abdominal obesity and dyslipidemia (Reaven,1988) Study in china related to association of the *ACE I/D* polymorphism with metabolic syndrome in patients with T2DM have a higher prevalence of dyslipidemia, albuminuria, serum uric acid and serum triglyceride levels it provides genetic information of the renin-Angiotensin system involved in the pathophysiology of metabolic derangement (Lee & Tsai, 2002).

Study in USA about glucose metabolism notify that, fasting glucose and insulin are similar among genotypes of *ACE* gene, but 2-hour glucose levels were higher in *DD* genotype which has lower insulin sensitivity than in *ID* and *II* genotype subjects (Bonnet *et al.,* 2008). The other study by Ali-Bahara *et al.,* (2014) reported that a decrease in *DD* genotype of *ACE* and increases in *ID* and *II* genotypes are associated with changes in fasting blood sugar, triglyceride, total cholesterol and blood pressure (Ali-Bahar *et al*., 2014). However, the other study conducted in Iranians T2DM patients indicated that metabolic syndrome is not associated with *ACE* gene polymorphism (Nikzamir *et al.,* 2008).

3.4.2.1.4 *ACE Insertion/Deletion* and Susceptibility to Diabetes Mellitus

There are may candidate genes associated to the ourrence of T2DM such as glucokinase (GCK), insulin receptor substrate-1 (IRS-1), potassium inwardly rectifying channel subfamily J member 11 (KCNJ11), peroxisome proliferator-activated receptor gamma, hepatocyte nuclear factor- 1A (HNF1A) and hepatocyte nuclear factor-4A (HNF4A) (Singh, 2011). However, genetic variants in the RAAS are highly associated with metabolic syndrome, especially hypertension, T2DM and diabetic complications (Al-Rubeaan *et al.,* 2013). Activation of the RAAS and enhanced production of *Ang II* has an inhibitory effect on insulin signal transduction pathway of insulin IRS-1 phosphorylation and reduce glucose uptake through *GLUT4* that result for insulin resistance (Zhou and Schulman, 2009). Additionally, *Ang II* increases reactive oxygen species which leads to damaging the pancreatic β-cells and causes impair insulin secretion from the pancreas through vasoconstriction and reduction in islet blood flow. All these effects lead to the development of DM (Zhou and Schulman, 2009) and activation of RAAS leads to the development of hypertension, macrovascular and microvascular complication in patients with diabetes mellitus (Hsueh and Wyne, 2011).

The role of *ACE I/D* polymorphism in the pathogenesis of diabetes mellitus and its complications were reported in various populations. *Angiotensin II* (*AngII*) has been confirmed in the pathogenesis of T2DM by inhibiting the secretion of insulin or producing of insulin resistance, promoting the generation of inflammation and fibrosis (Yusuf *et al*., 2001 and Danser *et al*., 1995). The *DD* genotype of the *ACE* gene is mostly associated in the pathogenesis of T2DM and individuals having *DD* genotype are highly susceptibility to T2DM (Yang *et al*., 2006 and Ergen *et al*., 2004). The study in Egypt also indicated *ACE* gene *I/D* polymorphism are associated with T2DM patients and the *D* allele is high risk for T2DM (Zarouk *et al*., 2009). However, a meta-analysis study indicated that there was no association between *ACE gene* polymorphism and T2DM in Chinese population (Zhou *et al*., 2012). Similarly, other studies reported that *ACE* gene polymorphism (*I/D*) cannot be considered as a risk factor for T2DM in the Lebanese population (Chmaisse *et al*., 2009), in Japan (Ichikawa *et al*., 2014). Moreover, the relationship between *ACE* gene polymorphism in T2DM, diabetic complications and diabetic hypertension in the diabetic population has not any reported data in the Ethiopian population.

3.4.2.1.5 *ACE* gene Polymorphism in Patients with Diabetic Nephropathy

ACE is a component of the renin–Angiotensin system and catalyses the conversion of the inactive precursor decapeptide *Angiotensin I* to active octapeptide *Angiotensin II*. *Angiotensin II* leads to the release of catecholamines from the adrenal medulla and prejunctional nerve, induces vasoconstriction, sodium retention and renal hemodynamic changes that result in intraglomerular hypertension and causes glomerulosclerosis (Hsueh and Wyne*.,* 2011 and Leehey *et al*., 2000). In T2DM, patients there are conflict regarding the association between the *ACE* genotype and diabetic nephropathy (Schmidt *et al*., 1995 and Fujisawa *et al*., 1995). The homozygous *DD* genotype is an independent risk factor and has a high prognostic value for the onset and progression of diabetic nephropathy in T2DM. The study in India showed that haplotypes, deletion allele *D* and *DD* genotype of the *ACE* gene are associated with greater risk of diabetic nephropathy than the haplotypes *insertion*(*I*) allele (Haque *et al.,* 2010 and Naresh *et al.,* 2009). However study in Turkish (Mustafa *et al.,* 2001), Switzerland (Walder *et al.,* 1998), and Germany (Schmidt *et al.,* 1995) were revealed that, type 2 diabetic patients of *ACE I/D* polymorphism genome distribution and allele frequencies (*DD, ID, II* and *D* and *I*) doesn't seem to be associated with diabetic nephropathy.

Studies in Iran shows that, the frequency of *DD* genotypes and *D* alleles are increased in DN patients than the normal individuals (30.6% vs 14.3%) (Nikzamir *et al.,* 2006). The other follow-up study in Iraq reported that, the genotype and allele frequencies of *ACE* gene polymorphism of the homozygous *DD* genotype are at high risk of DN which is two folds than wild type *II* and the minor allele frequency *D* allele is higher in DN than the normal individuals (Hussein *et al.,* 2015). In addition to this during their follow-up studies, they also concluded that, the frequency of *DD* genotype was not differing between DN patients and patients without nephropathy (30.6% vs. 20%) (Hussein *et al.,* 2015). In contrast, the *I/D* polymorphism (*DD, ID* and *II*) of *ACE* genotype has not associated in patients with and without nephropathy with the duration of diabetes ≥20 years Arfa *et al.,* 2008 and (Schmidt *et al.,* 1995). Also the other study in Japanese reveal that the *ACE* gene polymorphism is associated with myocardial infarction, but not with nephropathy patients of NIDDM (Fujisawa *et al.,* 1995).

The *ACE DD* genotype polymorphism is associated with more rapid decline GFR which increases mortality rate in type 2 diabetic patients with established renal disease, and the D allele

has also a co- dominant effect on the development of diabetic nephropathy but not associated with non-diabetic nephropathy (Zarouk *et al.,* 2009 and Fava *et al.,* 2001).

3.4.2.1.6 *ACE* Gene Polymorphism in Patients with Diabetic Hypertensive

The product of *ACE* (*Angiotensin II*) induces vasoconstriction through inactivation of the vasodilator peptide bradykinin, theses leads to the occurrence hypertension (Hsueh and Wyne, 2011 and Leehey *et al*., 2000). The association between *ACE I/D* polymorphism and hypertension is controversial. Some of the studies have proposed that the *DD* genotypes are increase numbers of critical hypertension in the diabetic population (Pasquale *et al*., 2005 and O'Donnell *et al.,* 1998) and *D* allele with hypertension in a south Indian population (Anbazhagan *et al*., 2009) and in a south China population (Jiang *et al*., 2009). Others have not established as a significant relationship between *ACE DD* genotype and *D* allele among hypertensive individuals (Tascilar *et al*., 2009 and Chuang *et al*., 1997). The study in Iranian patients with the frequency of *DD* genotype (27 cases in T2DM with hypertension Vs 11 cases in T2DM without hypertension $P < 0.026$) indicates that *DD* genotype individuals are independently associated with hypertension in the diabetic population (Nakhjavani *et al*., 2007).

ACE gene *DD* genotype and *D* allele is a risk factor for hypertension and higher lipid levels but is not a risk factor for diabetes in elderly population than those in patients with *II* genotype without association of their blood glucose level (Zhou *et al*., 2013). However, the other study indicate that *I* allele 63% and *I/D* genotype 37% in patients and 48% *I* allele and 52% *I/D* genotype for healthy controls of *ACE* gene which indicates *I* allele and *I/D* genotype is associated with T2DM and hypertension (Panjaliya *et al*., 2013).

3.4.2.1.7 *ACE* Gene Polymorphism in Patients with Diabetic Retinopathy

Genetic factors are contributed in the developmental role of diabetic retinopathy (Abhary *et al*., 2009). A number of gene polymorphism studies on the components of retinal RAS such as *Ang I, Ang II, renin, ACE, AT-1* and *AT-2* leads to increase levels of prorenin, rennin and *angiotensin II* are associated with proliferative DR and diabetic macular edema telling the connection of RAS in pathogenesis of diabetic retinopathy (Lu *et al*., 2012; Noma, 2009; and Nagai N, 2005). However, the biological mechanism through which the *ACE I/D* polymorphism related to an increased

risk of proliferative diabetic retinopathy is unclear. It is reported that *Ang II* participates in the progress of retinopathy through inducing the synthesis of growth factors in the vascular endothelial and connective tissue (Wilkinson-Berka, 2006).

A meta-analysis study includes 2,224 Chinese patients showed reasonable evidence of an association between the *ACE I/D* polymorphism and proliferative diabetic retinopathy (PDR) (Lu *et al*., 2012). The study in Pakistan also shows that the *ACE* genotype *I/D* is associated with PDR ($p = 0.009$) and its sub-clinical class non-proliferative diabetic retinopathy (NPDR) ($p = 0.006$) (Saleem *et al*., 2015). The other study in Turkey revealed that *ACE DD* genotype is an independent risk factor for retinal vein occlusion (second most common retinal vascular disease after diabetic retinopathy in the elderly and it leads to serious visual loss and blindness) (Kutluturk *et al*., 2014).

Numerous studies have addressed the molecular epidemiology of *ACE I/D* polymorphism among DR (Thomas *et al*., 2003 and Marre *et al.,* 1994). A recent study in china indicated that *ACE* gene polymorphism have no difference between DR males and females compared to respective controls other than used for a risk factor and prognostic marker for DR and T2DM (Khan *et al*., 2015). Treatment with blockage of the RAS provides beneficial effects in the provisions of delaying the development and progression of diabetes and DR (Sjolie *et al*., 2008).

4. MATERIAL AND METHODS

4.1 Study Area

This study was conducted in Bahir Dar Felegehiwot Referral Hospital. Bahir Dar town is located in the Amhara region, Northwest of Ethiopia served by Felegehiwot Referral Hospital, a tertiary referral hospital serving over 7 million people from the surrounding area including Debretabor hospital, Woreta hospital, Dangla hospital, Durbetie hospital, Merayi hospital, Adiet hospital, Enjibara hospital, Gemjabet hospital, Burie hospital, Chagni hospital, Pawi hospital and Mota hospital. For this study, samples were collected from the diabetic clinic of Bahir Dar Felegehiwot Referral Hospital. The biochemical analysis of the study was done in Gondar University Referral Hospital, clinical diagnostic laboratory. However, the molecular part of the experimental analysis was conducted in Gondar University Atse Tewodros Campus, College of Natural and Computational Sciences, Department of Biotechnology.

4.2 Study Design and Period

A case control comparative study was carried out from January – October, 2016 in Bahir Dar Felegehiwot Referral Hospital.

4.3 Population

During the study period, all patients who were diagnosed by physicians according to the WHO criteria for diabetes mellitus and healthy control subjects were the source population. Whereas T2DM without complication, type 2 diabetic hypertension, diabetic retinopathy and diabetic nephropathy patients who were diagnosed by physicians were the study population.

4.4 Inclusion and Exclusion Criteria

Patients diagnosed by physicians having type 2 diabetes, diabetic hypertension, diabetic retinopathies, diabetic nephropathies and all age groups were recruited in the study. Patients were diagnosed with T1DM, patients suffering from acute and chronic infection, malignancies, congestive heart failure, HIV, urinary tract infection and acute febrile illness were excluded from

this study. Healthy controls having hypertension at the time of data collection and healthy controls having hyperglycemia during biochemical analysis were excluded in the study and replaced by other healthy individuals.

4.5 Variables

The dependent variables in these studies were age, sex, duration of diabetics and status of diabetes. Whereas the independent variables include serum creatinine, glucose level, cholesterol, triglycerol, blood urea, *ACE* gene *I/D* polymorphism, and obesity.

4.6 Sample Size and Sampling Technique

A total of 222 subjects (111 T2DM and 111 healthy controls) were selected by non probability purposive sampling technique. Among 111 T2DM, 35 had T2DM without complications, 35 had diabetic hypertension, 30 had diabetic retinopathy and 11 had diabetic nephropathy. Among T2DM, 55 were men and 56 were women and healthy control groups consisted, 55 men and 56 women.

4.7 Data Collection, Clinical Measurements and Laboratory Method

4.7.1 Questionnaire

The socio- demographic characteristics and clinical parameters of both patients and healthy control subjects such as smoking, gender, age, duration of diabetes, diabetic complications and family history were taken through semi-structured questioner.

4.7.2 Anthropometric Measurements

Body weight was measured using a portable digital scale while height was measured using a portable stadiometer. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters and participants were categorized as underweight < 18.5, healthy (BMI 18.5 - 25 kg/m2), overweight (BMI 25.0–29.9 kg/m2) or obese (BMI ≥ 30 kg/m2) (Ferguson *et al.*, 2011). Blood pressure was recorded in the sitting position after 5 min of rest by using a digital device and systolic and diastolic blood pressures were calculated from mean value

after three readings. Patients were considered as hypertension, if mean systolic blood pressure (SBP) ≥ 140mmHg and mean diastolic blood pressure (DBP) ≥90mmHg Or if they used antihypertensive medication.

4.7.3 Sample Collection

Five milliliters of blood sample were collected from the vein of all subjects of an overnight fasting patients and healthy controls by laboratory personnel. From 5ml blood sample, 3ml was kept in plain tube without anticoagulants for biochemical tests. The remaining 2ml of the blood was kept in Ethylene Diamine Tetra Acetic Acid (EDTA) tube for isolation of DNA and stored at -21ºC.

4.7.4 Laboratory Methods

4.7.4.1 Biochemical Analysis

A non –anticoagulated blood (3ml in plain tube) sample was centrifuged at 8000 rpm for 10 minutes, the sera were aspirated and transferred in to sterilized eppendorf tube and stored at –20 ^{0}C until processing. Each test was subjected for an enzymatic analysis of Glucose, total cholesterol, triglycerides, urea, and creatinine by Mindary fully automated analyzer in the clinical chemistry diagnostic laboratory of college of medicine and health sciences. Fasting blood glucose (FBG) level was used to assess the glycemic control. For a non diabetic individuals (healthy control group), when FBG was > 110 mg/dl, the glycemic control was considered as unsatisfactory; when FBG ≤ 110 mg/dl the glycemic control was considered as satisfactory (American College of Endocrinology, 2002). However, in the case of diabetic patients when FBG >130mg/dl, the glycemic control was considered poor glycemic control. When FBG <130mg/dl, the glycemic control was considered good glycemic control (Kassahun *et al*., 2016). If total cholesterol > 200 mg/dl and triglycerides > 150 mg/dl, were taken as abnormal (NCEP 2001). If serum creatinine > 1 mg/dl, it was taken as abnormal result (Colagiuri *et al*., 2015). Estimated glomerular filtration rate (eGFR) was calculated online by using the Chronic Kidney Disease Epidemiology Program equation (CKD-EPI) and individuals having eGFR < 60 ml/min/1.73m^2 were considered as having a chronic kidney disease (Levey *et al*., 2009). Moreover, based on eGFR, individuals were classified as risk categories (low, moderate,

high or very high) by the Kidney Disease Improving Global Outcomes (KDIGO) 2012 prognostic grids (KIDGO, 2013).

4.7.4.2 Genomic DNA Isolation

For DNA extraction, 300µl of EDTA anticoagulated bloods of both patients and healthy controls was transferred in to sterilized 1.5ml eppendorf tube. A non-enzymatic salting out method was used for DNA extraction to get high yield genomic DNA with a small amount of blood sample (Suguna, *et al*., 2014). Red blood cells (RBCs) were lysised and removed by using RBC lysis buffer solution. Similarly, white blood cells were lysised using a nuclear lysis buffer solution. The proteins were precipitated with a high concentrated salt (6M NaCl) and DNA was precipitated by chilled absolute ethanol, followed by washing of genomic DNA with 70% ice-cold ethanol and finally the DNA was dissolved with Tris-EDTA buffer (TE) and this sample was stored at -21^{0}c till used.

The quality of isolated genomic DNA was confirmed by using 1.5 % agarose gel electrophoresis. Finally via following the above mentioned procedures a pure genomic DNA was obtained as shown in the Figure 1. Then the patients and respective control samples were genotyped for *ACE Insertion/Deletion* (*I/D*) polymorphism using sets of primers and appropriate PCR conditions.

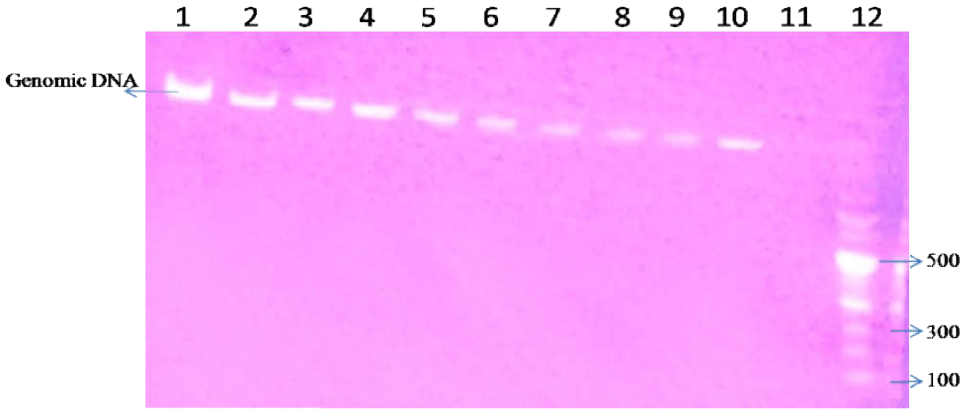

Figure 1: Genomic DNA purity determination by using 1.5 % agarose gel electrophoresis. Note: 12, 100bp DNA ladder; 11 indicate control water and loading dye; 1 up to 10 indicate isolated genomic DNA.

4.7.4.3 Polymerase Chain Reaction (PCR)

The *Insertion*/*Deletion* (*I/D*) alleles of *ACE* gene polymorphisms were identified by TC 412 PCR amplification of using specific primers. A final volume (25 µl) of PCR reaction mixture were prepared using 6pmol of forward and reverse primers (forward primer 5′-CTGGAGACCACTCCCATCCTTTCT-3′ and reverse primer 5′GATGTGGCCATCACATTCGTCAGAT-3′) (Patel *et al*. 2011 and Rigat *et al.*1992), 5 mM of MgCl2, 0.5 mM of each dNTP, 0.75 U of hot start *Taq* polymerase, 3 µl of template DNA and water were used.

PCR amplification was set with an initial denaturation at 95°C for two minutes. Hot start Taq polymerase was kept for 15 minutes at 95°C to activate the enzyme. Then, the DNA was amplified for 30 cycles. The cycle steps were denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 45 seconds followed by a final extension at 72°C for nine minutes. Finally the PCR product was held at 4^0c until it was analyzed by agarose gel electrophoresis.

4.7.4.4 Agarose Gel Electrophoresis

PCR amplified products of *ACE I/D* genotypes were electrophoretically separated for 50min at 50 V on a 2% agarose gel. To stain and visualize DNA upon UV transillumination in gel, 3 µl of 2% Ethidium Bromide was also added. The PCR amplified products (12µl) mixed with 3 µl loading dye then loaded into wells of agarose gel. Electrophoresis was carried out in 1X trice acetate EDTA (TAE) buffer and the gel was visualized by UV transilluminator.

After electrophoresis, band sizes of 190bp (*Deletion*) and 490bp (*Insertion*) polymorphisms fragments were obtained and image was captured with the help of a smart phone camera and digital camera. Therefore, there were three genotypes after electrophoresis: A 490bp band (genotype *II*), a 190bp band (genotype *DD*) and both 490bp and 190bp band (*ID* genotype). The genotypes were calculated to determine the association of *ACE* (*I/D*) gene polymorphisms in patients with T2DM, DR, DN, diabetic with hypertension and with other clinical and socio-demographic characteristics.

4.8 Statistical Analysis

Data were analyzed by using SPSS version 20. Quantitative data were presented as mean and standard deviation ($x \pm s$). To compare continuous variables between T2DM group and healthy control groups, T2DM and T2DM complication, *t*-test for independent samples was used. Distribution of the genotype and allele frequencies in T2DM and healthy control groups, T2DM and T2DM with complications were compared using chi-square test. The risk associations of *DD* genotype and *D* allele with T2DM were assessed by the odd ratio at 95% confidence interval (CI). Relationships between the *ACE* genotypes and clinical variables were compared with one-way analysis of variance (ANOVA).

4.9 Ethical Consideration

The study protocol was approved by the college of natural and computational sciences ethical committee of the University of Gondar on its meeting held on 11/08/08 ethical clearance. To take care and keep confidentiality of participants result, written informed consent was obtained from all study participants before collecting of blood sample.

5. RESULTS

5.1 Demographic, Clinical and Biochemical Characteristics between T2DM and Healthy Controls.

The demographic parameter of study subjects is given in Table 1. There was no statistically significant variation between cases and controls in respect to age and sex. Among cases, 55 were men and 56 were women, (*P* 0.789), their mean age was 54.6±12.2 years. The control group consisted, 55 men and 56 women their mean age was 54.0±14.4 years, (*P* 0.811). The clinical characteristics systolic blood pressure (SBP), diastolic blood pressure (DBP), glucose, total cholesterol, triglycerol and urea have shown a significant difference ($P < 0.001$), BMI ($P < 0.05$) and creatinine ($P < 0.02$) higher in T2DM than control group. However, eGFR was not significantly differed between the two groups (*P* 0.052).

Table 1: Demographic, Clinical and biochemical characteristics of T2DM patients and healthy controls

Variables	T2DM	Control	*P* value
Subject (n)	111	111	------
Gender (M/F)	55/56	55/56	0.789
Age (yr)	54.7±12.3	54.3±14.4	0.811
BMI(kg/m^2)	24.9±4.4	23.6±5.4	0.049
SBP(mmHg)	133.7±19.5	121.2±6.9	0.000
DBP(mmHg)	82.6±13.6	76.4±5.5	0.000
Glucose(mg/dl)	206.3 ±72.3	78.2 ±15.6	0.000
TC(mg/dl)	194.81±47.6	130.4 ±48.9	0.000
TG(mg/dl)	177.5 ±135.3	99.6 ±56.7	0.000
Creatinine(mg/dl)	0.7 ±0.2	0.6 ±0.1	0.010
Urea(mg/dl)	22.2 ±8.3	18.8± 4.6	0.000
eGFR	118.4 ±27.1	124.7 ±20.2	0.052

Data expressed as means ± SD*P<0.05* were considered as significant and *P*> 0.05 (not significant) **Note**: BMI: Body mass index. SBP: Systolic blood pressure. DBP: Diastolic blood Pressure. TC: Total cholesterol, TG: Triglycerol, eGFR: Estimated glomerular filtration rate.

5.2 Demographic, Clinical and Biochemical Characteristics between T2DM, Diabetic Complications and Diabetic Hypertension.

Gender, total cholesterol and triglycerol had not any significant association among the groups (Table 2). However, age ($P < 0.02$); DBP ($P < 0.001$) and urea ($P < 0.05$) have shown a significant association in hypertensive diabetic population compared with T2DM and other complications. SBP was significant for both diabetic hypertension ($P < 0.001$) and diabetic nephropathy ($P < 0.05$) patients. Creatinine ($P < 0.01$) and eGFR ($P < 0.01$) had shown statistically significant association only in diabetic nephropathy patients. Glucose ($P < 0.02$) was significant only in diabetic retinopathy patients.

Table 2: Comparison of demographic, clinical and biochemical variables between T2DM and its complications**.**

Variable	Group 1	Group 2	Group 3	Group 4
Gender (M/F)	20/16	19/15	11/19	5/ 6
	P 0.896		*P* 0.128	*P* 0.569
Age(yr)	50.6± 13.3	57.6±10.4	55.9 ± 11.5	55.3 ± 16.0
	*P** 0 .018		*P* 0.089	*P* 0.397
BMI(kg/m^2)	24.7± 3.7	25.1 ± 5.8	24.7 ± 3.9	24.8 ± 5.2
	P 0.662		*P* 0.990	*P* 0.962
SBP(mmHg)	121.8±10.2	153.3 ± 16.3	126.3 ± 16.8	130.5 ± 17.1
	*P** 0.000		*P* 0.209	*P** 0.047
DBP (mmHg)	75.6±7	96.4 ± 9.6	73.5 ± 9.7	79.1 ± 12.2
	*P** .000		*P* 0.333	*P* 0.383
Glucose(mg/dl)	192.9 ± 65.2	194.0 ± 75.8	239.9 ± 82.3	194.2 ± 59.8
	P 0.952		*P** 0.014	*P* 0.955
Cholesterol(mg/dl)	190 ± 50.7	205.3 ± 50.2	186.9 ± 50.8	198.6 ± 51.4
	P 0.210		*P* 0.801	*P* 0.638
Triglycerol(mg/dl)	156.6 ± 65.4	191.6± 182.1	165.7± 107.7	169.0± 148.9
	P 0.291		*P* 0.689	*P* 0.793
Creatinine(mg/dl)	0.7 ± 0.2	0.77 ± 0.3	0.64 ± 0.24	0.9 ± 0.3
	P 0.235		*P* 0.225	*P** 0.009
Urea(mg/dl)	22 ± 7.5	26.4 ± 8.4	18.0 ± 8.2	23.3± 6.97
	*P**0.028		*P* 0.044	*P* 0.612
eGFR	122.6 ± 19.7	112.7 ± 28.3	124.3 ± 29.3	99.6 ± 27.8
	P 0.096		*P* 0.765	*P** 0.004

Data expressed as means ± SD, *P<0.05* were considered as significant and *P*> 0.05 (not significant). **Note**: Group 1 , type 2 diabetes; group 2, type 2 diabetes with hypertension; group 3, type 2 diabetes with retinopathy and group 4, type 2 diabetes with nephropathy. Group 1, was compared with group 2, 3 and 4.

5.3 *ACE* Genotype distribution in T2DM Patients and Healthy Controls

The *ACE* genotypes frequency distribution in patients with type 2 diabetes and healthy control is given in table 3. The *ACE DD* genotype and *D* allele were more frequent in diabetic patients (64.2% and 79.3 %) as compared to controls (35.6% and 59.9%). The frequency of homozygous *DD* genotype in type 2 diabetic patients was twofold higher than healthy control group (OR, 2.984: CI 1.332 – 6.689). *D* allele was five times than *I* allele in diabetic patients (OR, *D* 2.178; CI: 1.168 – 3.232 $P < 0.001$) compared to healthy controls (*I* OR, 0.459; CI: 0.309 – 0.681 $P < 0.001$). However, *ACE* genotype *I/D* and *II* were less frequent in T2DM patients (31.4% and 37.5%) in comparison to healthy controls (69.0% and 62.5 %).

Table 3: Distribution of *ACE* genotypes and allele frequencies between T2DM and Healthy Control participants.

Genotype	**T2DM**	**Control**	OR	OR 95% CI	*P*
DD	77 (64.2%)	42 (35.6%)	2.984	1.332 - 6.689	0.008
ID	22 (31.4%)	49 (69.0%)	0.7641	0.318 - 0.834	0.547
II	12 (37.5%)	20 (62.5 %)			
***P*-value .000**	**X^2 21.290a**				
Allele frequencies					
D	176 (79.3 %)	133(59.9%)	2.178	1.468 - 3.232	0.000
I	46 (20.7 %)	89 (40%)	0.459	0.309 - 0.681	0.000
***P*-value 0.000**	**X^2 22.562a**				

$P < 0.001$ (statistically significant)

5.4 Association of *ACE I/D* Genotype with Clinical and Biochemical Characteristics of T2DM and Healthy Controls.

The *ACE DD* genotype was highly associated with the clinical variables than *I/D* and *II* genotypes. SBP (131.5±18.9 VS 123.2±8.3 and 121.3±10.7), glucose (168.2±86.8 Vs 110.5±61.9 and 114.7±75.6), total cholesterol (179.2±56.8 Vs 141.2±57.1 and 145.0±42.7), triglycerol (167.5±136.3 Vs 102.4±51.8 and 109.3±53.9) and urea (22.7±7.6 Vs 17.9±5.1 and 17.8±4.3) had $P < 0.001$, DBP (81.9±12.4 Vs 77.4±6.5 and 75.2±9.6) ($P < 0.01$) and BMI (24.9±4.4 Vs 24.2±5.8 and 22.0±4.3) ($P < 0.02$) respectively (Table 4).

Table 4: One-way ANOVA analysis of clinical and biochemical characteristics according to *ACE* genotype in T2DM and healthy controls.

Genotype result	Age	SBP	DBP	Glu	TC	TG	CRT	Urea	eGFR	BMI
DD	54.9± 12.8	131.5± 18.9	81.9± 12.4	168.2± 86.8	179.2± 56.8	167.5± 136.3	.697±. 24	22.7± 7.6	120.2± 27.8	24.9±4 .4
ID	54.6± 14.2	123.2± 8.3	77.4± 6.5	110.5± 61.9	141.2± 57.1	102.4± 51.8	.644±. 2	17.9± 5.1	122.9± 18.4	24.2±5 .8
II	52.3± 13.9	121.3± 10.7	75.2± 9.6	114.7± 75.6	145.0± 42.7	109.3± 53.9	.699±. 18	17.8± 4.3	123.6± 19.5	22.0±4 .3
p-value	.610	.000	.001	.000	.000	.000	.257	.000	.663	.014

Data expressed as means ± SD, *P<0.05* were considered as significant and *P*> 0.05 (not significant) **Note:** SBP, systolic blood pressure; DBP, diastolic blood pressure; Glu, glucose, TC, total cholesterol; TG, Triglycerol; CRT, creatinine; eGFR, estimated glomerular filtration rate; BMI, body mass index.

5.5 *ACE* Genotype Distribution in T2DM, Diabetic Complications and Diabetes with Hypertension.

As shown in Table 5, the frequency distribution of *ACE* genotypes and alleles among T2DM and its complications were indicated. The frequency of *ACE DD* genotype (41.6%, 28.6%, 19.5%, and 10.4%, $P < 0.01$) and *D* allele (60.4%, 44.1%, and 37.8% and 18.0%, $P < 0.001$) was more frequent in T2DM patients with hypertensive and diabetic retinopathy complication than T2DM without complication and diabetic nephropathy patients respectively.

Table 5: Frequency of *ACE I/D* genotype and allele polymorphism T2DM and diabetic complications.

Genotype	**T2DM without complication**	**Diabetic hypertension**	**Diabetic Retinopathy**	**Diabetic with Nephropathy**
DD	15 (19.5.0%)	32 (41.6%)	22 (28.6%)	8(10.4%)
ID	12 (54.5%)	3 (13.6 %)	5 (22.7%)	2(9.3%)
II	7 (58.3%)	0(.0%)	3 (25.0%)	1(8.3%)
P-value 0.002	**X^{2-} 20.713[a]**			
***D* allele**	42 (37.8%)	67(60.4%)	49 (44.1%)	20 (18.0%)
***I* allele**	26 (23.4%)	3 (2.7%)	14 (12.6%)	4 (3.6 %)
p-value 0.000	**X^2** 34.892a			

P< 0.05 (Statistically significant)

5.6 One way ANOVA of *ACE* Genotype Distribution to the Associated Risk Factors among T2DM and Diabetic Complications

The companied one way ANOVA of clinical characteristics of T2DM and its complication with respect to *ACE* genotype indicated in Table 6. There had no any significance difference in glucose, diabetic duration, BMI, triglycerol, creatinine and eGFR among type 2 diabetes and its complications. Whereas age (56.6±11.2, $P < 0.04$), SBP (138.4±21.2, $P < 0.01$), DBP (84.2 ± 14.0, $P < 0.01$), total cholesterol (203.7±45.3, $P < 0.01$), urea (24.5 ± 8.5, $P < 0.001$) were significance different among the groups.

Table 6: Companied one- way ANOVA distributions of *ACE* I/D genotype and clinical characteristics among T2DM, DR, DN and diabetic with hypertension.

Genotype result	Age	BP	DBP	Glucose	Total cholestrol	Triglycerol	Creatinine	Urea	eGFR	BMI	Duration of diabetes
DD	56.6± 11.2	138.4± 21.2	84.2 ± 14.0	215.3±71.5	203.7±45.3	186.1± 149.2	0.74 ± 0.26	24.5 ± 8.5	115.2 ± 27.1	24.7 ± 4.9	8.1 ± 4.7
ID	52.9± 12.5	125.0± 12.2	79.1± 12.1	178.2±60.5	186.0 ± 55.8	144.9 ± 51.8	0.72± 0.23	17.8 ± 6.8	116.6 ± 19.5	25.3 ± 3.7	8.5 ± 4.3
II	47.3± 17.0	121.0 ±9.8	72.3± 5.8	193.9 ±108.1	148.8 ± 53.5	123.4 ±63.2	0.69 ± 0.26	16.9 ± 6.4	133.7 ±32.3	25.5 ± 4.3	6.8 ± 3.6
p-value	0 .037	0.001	.008	0.105	0.001	0.171	0.842	0.000	0.081	0.777	0.596

Data expressed as means ± SD, *P<0.05* were considered as significant and *P*> 0.05 (not significant) **Note:** SBP, systolic blood pressure. DBP, diastolic blood pressure. eGFR estimated glomerular filtration rate.

Table 7: Shows *ACE* genotype distribution regarding to clinical variables with a comparison of T2DM to its complications. eGFR, creatinine, triglycerol, body mass index, and gender did not show statistically significance variation between T2DM and its complication respect to *ACE* genotype ($P > 0.05$). Age has shown statistically significant association in patients having diabetic hypertension *DD* genotype (56.8 ± 11.0, $P < 0.05$). Similarly, systolic and diastolic blood pressure indicated statistically significant association in diabetic hypertension of *DD* genotype ($P < 0.001$ and $P < 0.01$) respectively. Urea was significant in all complication and had highest x±s was recorded among *DD* genotype (26.6 ± 8, $P <0.01$), (22.4 ± 8.6 $P < 0.02$), (25.6 ± 7.3 $P < 0.01$) in diabetic with hypertension, retinopathy and nephropathy. There was no statistically significant association between glucose level and *ACE I/D* genotypes among diabetic hypertension individuals. However, *DD* genotype individuals among diabetic retinopathy ($P < 0.02$) and nephropathy ($P < 0.05$) showed statistically significant variations to the level of glucose. Total cholesterol level indicated statistically significant association in diabetic retinopathy patients *DD* genotype (DD 200.6 ± 42.6, ID 184.3 ± 53.3, II 147.8 ± 56, $P < 0.01$).

Table 7: One- way ANOVA *ACE* genotype distribution and clinical characteristics in comparison to T2DM to its complications.

Variable	T2DM Vs Diabetic Hypertension	T2DM Vs Diabetic Retinopathy	T2DM Vs Diabetic Nephropathy
Sex (F/M)	15/20	16/19	6/ 5
	P 0.147	*P 0* .178	*P* 0.064
Age	*DD* (56.8 ± 11.0)	*DD* (55.2 ± 11.2)	*DD* (55.2 ± 12.2)
	ID (49.1 ± 11.7)	*ID* (51.9 ± 12.9)	*ID* (50.3 ± 13.7)
	II (49.2 ± 17.3)	*II* (49.1 ± 16.6)	*II* (47.1 ± 17.9)
	P 0.049	*P* 0.329	*P* 0.277
BMI	*DD* (24.8 ± 5.2)	*DD* (24.5 ± 3.7)	*DD* (24.9 ± 4.4)
	ID (26.2 ± 4.2)	*ID*(24.7 ± 3.7)	*ID* (25.0 ± 3.9)
	II (24.6 ± 3.9)	*II* (25.8 ± 4.4)	*II* (24.4 ± 3.7)
	P 0.593	*P* 0.647	*P* 0.940
Systolic	*DD* (144.4 ± 20.3)	*DD* (125.7 ± 16.4)	*DD* (125.9 ± 14.7)
	ID (126.3 ± 14.3)	*ID*(122.9 ± 8.5)	*ID* (123.2 ± 8.9)
	II (118.4 ± 10.2)	*II* (119.7 ± 9.2)	*II* (120.2 ± 11.0)
	*P** 0 *.000*	*P* 0.428	*P* 0 .508

Diastolic	*DD* (89.3 ± 13.2)	*DD* (74.5 ± 9.2)	*DD* (76.7 ± 9.4)
	ID (83.7 ± 11.3)	*ID* (76.8 ± 7.5)	*ID* (78.6 ± 7.4)
	II (70.9 ± 6.2)	*II* (71.6 ± 5.5)	*II* (72.0 ± 6.6)
	*P** 0.001	*P* 0.270	*P* 0.177
Glucose	*DD* (202.6 ± 73.7)	*DD* (237.8 ± 66.2)	*DD* (214.4 ± 58.9)
	ID (189.8 ± 61.3)	*ID* (180.2 ± 57.5)	*ID* (177.8 ± 60.1)
	II (139.9± 35.2)	*II* (184.6 ± 108.2)	*II* (157.2 ± 61.6)
	P 0.061	*P** 0.012	*P** 0.038
Cholesterol	*DD* (206.15 47.532)	*DD* (200.6 ± 42.6)	*DD* (201.9 ± 53.0)
	ID (183.00 62.761)	*ID* (184.3 ± 53.3)	*ID* (187.4 ± 54.8)
	II (166.50 35.765)	*II* (147.8 ± 56)	*II* (165.7 ± 33.5)
	P 0.065	*P** 0.008	*P* 0 .195
Triglycerol	*DD* (185.8 ± 161.1)	*DD*(179.2 ± 100.5)	*DD*(172.0 ± 115.8)
	ID (155.7 ± 58.2)	*ID* (145.9 ± 49.3)	*ID* (150.6 ± 50.9)
	II (137.3 ± 62.9)	*II* (120.6 ± 65.3)	*II* (139.4 ± 59.2)
	P 0.557	*P* 0.101	*P* 0.609
Creatinine	*DD* (0 .7 ± 0.3)	*DD* (0.7 ± 0.3)	*DD* (0.7 ± 0.2)
	ID (0.71 ± 0.2)	*ID* (0.7 ± 0.2)	*ID* (0.8 ± 0.2)
	II (0.8 ± 0.16)	*II* (0.8 ± 0.8)	*II* (0.8 ±0.2)
	P 0.762	*P* 0.869	*P* 0.856
Urea	*DD* (26.6 ± 8)	*DD* (22.4 ± 8.6)	*DD* (25.6 ± 7.3)
	ID (19.1 ± 7.6)	*ID* (17.2 ± 5.8)	*ID* (18.4 ± 6.2)
	II (18.5 ±5.1)	*II* (16.3 ± 6.2)	*II* (19.1± 5.0)
	*P**0.001	*P** 0.018	*P** *0.004*
eGFR	*DD* (115.8 ± 27.5)	DD (122.2 ± 25.3)	DD (114.3 ± 27.4)
	ID (119.8 ± 15.9)	ID (118.312 ± 11)	ID (115.6 ± 20.1)
	II (122.1 ± 19.7)	II (133.4 ± 33.9)	II (123.8 ± 19.1)
	P 0.729	*P* 0.271	*P* 0.593
Diabetic duration	*DD* (7.6 ± 4.5)	*DD* (7.5 ± 4.7)	*DD* (6.7 ± 3.9)
	ID (7.07 ± 3.8)	*ID* (8.9 ± 4.3)	*ID* (8.4± 3.7)
	II (7.3 ± 3.5)	*II* (7.5 ± 3.2)	*II* (6.6 ± 3.9)
	P 0.909	*P* 0.506	*P* 0 .376

Data expressed as means ± SD, *P<0.05* were considered as significant and *P*> 0.05 (not significant) **Note:** T2DM, type 2 diabetes mellitus. BMI, body mass index**.** eGFR, estimated glomerular filtration rate.T2DM was compared with T2DM with hypertension, retinopathy and nephropathy.

Agarose gel result of *ACE* genotype distribution in T2DM Patients, Healthy Control and diabetic complications.

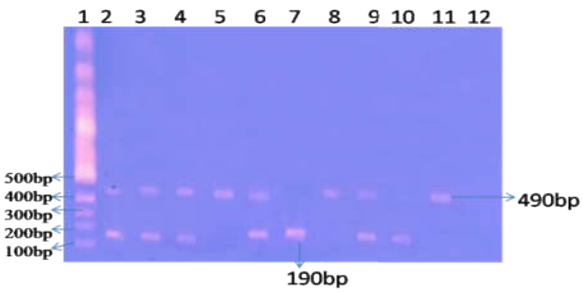

Figure 2: Detection of *ACE* gene on 2% agarose gel electrophoresis with 2% Ethidium bromide visualized under UV light.
Lane 1: 100bp DNA Marker. Lanes 2, 3, 4, 6 and 9 are heterozygous *ID* genotype 490, and 190 bp. Lanes 5, 8 and 11 homozygous II genotype 490 bp. Lane 7 and 10 homozygous *DD* genotype 190 bp: Lane 12 : Control.

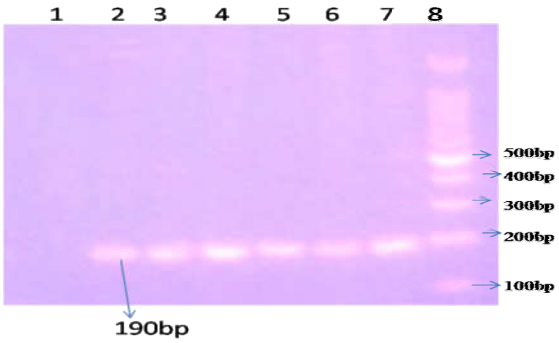

Figure 3. Detection of *ACE* on 2% agarose gel electrophoresis having diabetic hypertension patients.
Lane 1: Control: Lanes 2 , 3, 4, 5, 6 and 7: homozygous DD genotype 190bp; Lane 8: 100bp DNA marker.

ACE Genotype distribution between T2DM and healthy control groups

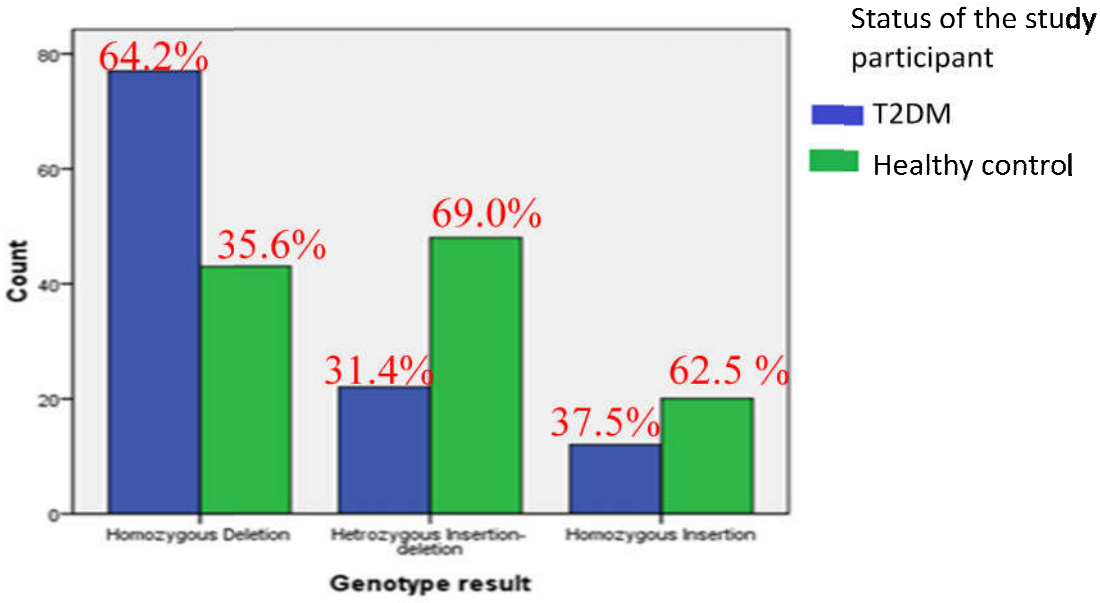

Figure 4: Chi-Square *ACE* genotype distribution between T2DM and control

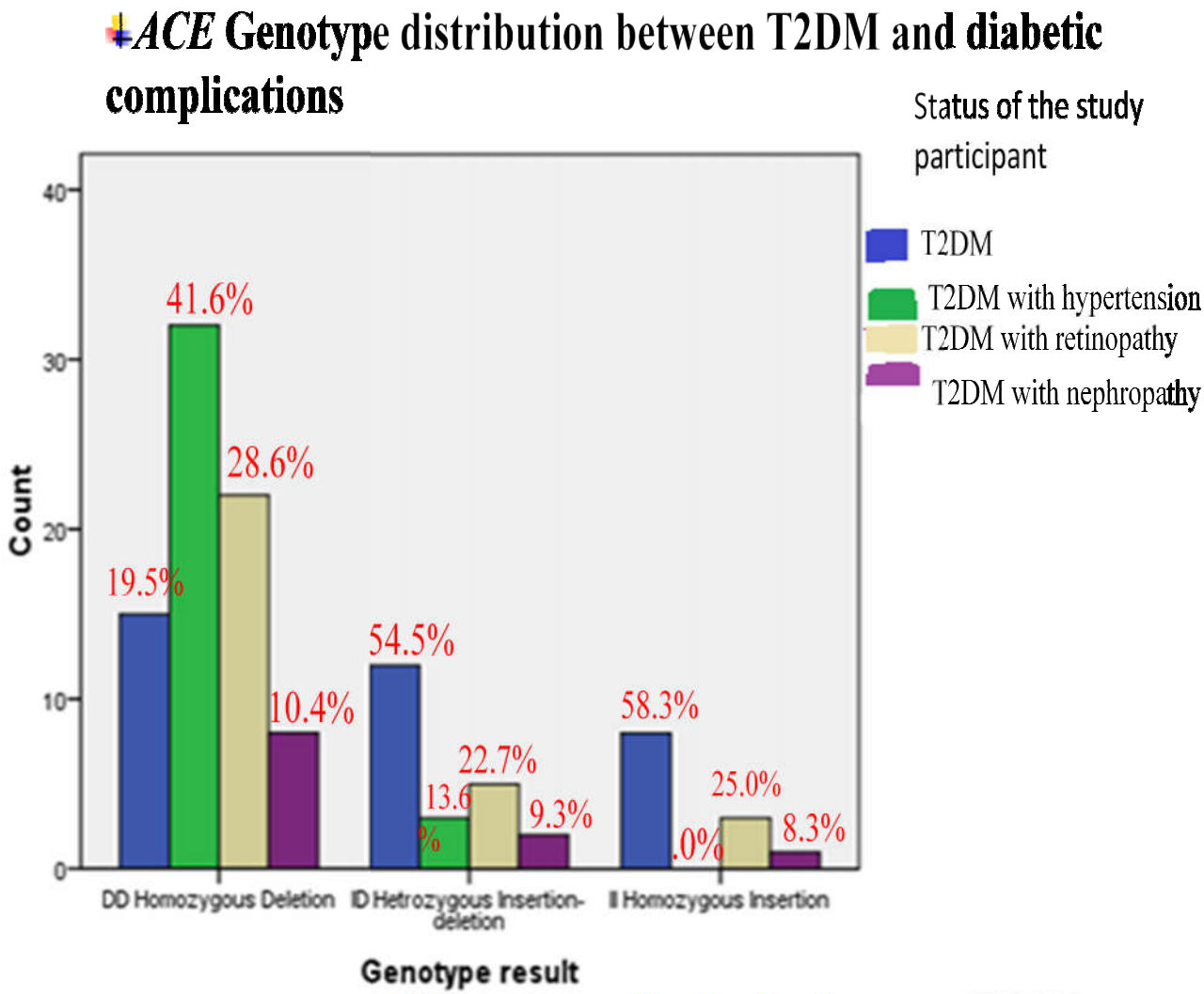

Figure 5: Chi-Square *ACE* genotype distribution between T2DM and diabetic complications.

6. DISCUSSION

Type 2 diabetes mellitus is a metabolic disorder of chronic disease that leads to the development of micro and macrovascular complications such as DR, DN, diabetic neuropathy and cardiovascular disease (Tesfaye & Gill, 2011; Al-Khawlani *et al*., 2010 and Michael, 2008). Hypertension is the most common risk factor of diabetes mellitus. Genetic or non-genetic factors are able to predispose to diabetes and facilitate the progress of diabetic complications, as reported by different authors (Vivian *et al.,* 2010; Holt, 2004 and Strojek *et al.,* 1997). The non-genetic risk factors such as BMI, age (oldest age), life style, smoking, dyslipidemia, and environmental factors are the most common risk factors for T2DM, diabetic complications and diabetic hypertentions (Viswanathan *et al.*, 2012; Bakris, 2011; Roaeid and Kadiki. 2011; Haque *et al.,* 2010 and Hribal *et al.,* 2002). There are many candidate genetic variants associated to the occurrence of T2DM (Singh, 2011). However, genes of the RAAS such as *renin*, *angiotensinogen* (*AGT*), *angiotensin-1 converting enzyme* (*ACE*), *angiotensin converting enzyme 2* (*ACE2*), *aldosterone synthase* (*CYP11B2*), *angiotensin II type 1*(*AT1R*) *and type 2* (*AT2R*) receptors all of these genes or genetic loci responsible for excess *Ang II* production and *Ang II* has the most common physiological effect on T2DM, diabetic complication and diabetic hypertension (Hsueh and Wyne, 2011 and Zhou and Schulman, 2009).

The *ACE I/D* polymorphism has a potential link to diabetes and able to assist the progross of diabetes to microvascular complications, macrovascular complications and hypertention (Saleem *et al*., 2015; Zhou *et al*., 2013 and Schmidt *et al*., 1995). However, the *ACE* gene polymorphism and risk of T2DM has conflicting reports. In this study, *ACE* gene polymorphism and associated risk factors in patients with T2DM, diabetic complication and diabetic hypertension with a healthy control group were studied. It was found that the frequency of *DD* genotype as well as *D* allele has shown statistically significant increase in diabetic patients in comparison to healthy control groups (64.2% and 79.3% Vs 35.6 and 59.9% $P < 0.001$) respectively as shown in Table 3 and Figure 4. *DD* genotype carriers have shown more than twofold increased risk of developing T2DM as compared to *II* genotype carriers (OR: 2.984, CI: 1.332 - 6.689, $P < 0.02$). *D* allele was five times higher than *I* allele associated to diabetic patients than healthy controls (OR, *D* 2.178; CI: 1.168 – 3.232 $P < 0.001$ Vs *I* OR, 0.459; CI: 0.309 – 0.681 $P < 0.001$). This finding is in agreement with some other studies, which indicated *DD* genotype is associated with

high increased risk of T2DM and *D* allele has high relative risk towards developing type 2 diabetes mellitus (Ali-Bahar *et al.,* 2014; Zarouk *et al*., 2009; Nikzamir *et al*., 2008; Yang *et al*., 2006; Stephens *et al.,* 2005; Ergen *et al*., 2004 and Hsieh *et al.,* 2000) and while others reported that there was no association between *ACE DD* genotype and T2DM (Choudhry *et al.,* 2012; Chmaisse *et al.,* 2009 and Degirmenci *et al.,* 2005). The risk factors such as mean SBP, DBP, total cholesterol (TC), triglycerol (TG), fasting blood glucose (FBG), creatinine and urea ($P < 0.001$), and BMI ($P < 0.05$) has shown statistically significant difference in cases as compared to healthy control groups (Table 1). This is similarity with other studies where SBP, DBP, TC, TG, FBG level, creatinine and urea contents showed statistically significant association to T2DM patients than healthy control study groups (Nikzamir *et al.,* 2008; Yang *et al.,* 2006 and Ergen *et al.,* 2004). The study in Brazil is also similar with the current study indicated that FBG, dyslipidemia (TC and TG) are highly prevalence in T2DM patients (Carolino *et al.,* 2008). Whenever the genotype distribution and these clinical parameters were compared, there were statistically significant association between the *DD* genotype holders and the amount of SBP, TC, TG and urea ($P < 0.001$), DBP ($P < 0.01$), and BMI ($P < 0.02$) among T2DM patients compared to healthy control groups having the same genotype holders (Table 4). This is disagree with some other reports by Choudhry *et al.,* (2012); Degirmenci *et al.,* (2005) and Hsieh *et al.,* (2000) indicated that clinical characteristic of T2DM patients such as age, BMI, SBP, DBP, hemoglobin A1C (HbA1C), TC, TG, HDL cholesterol levels, duration of diabetes, FBG and random blood sugar (RBS) level according to *ACE I/D* genotypes are no significance difference (Choudhry *et al.,* 2012; Degirmenci *et al.,* 2005 and Hsieh *et al.,* 2000). Similarly the study in USA showed that BMI, waist, fat-free mass, and physical activity did not differ by *ACE* genotype (Bonnet *et al*., 2008). On the other hand, in this study some of the clinical variables such as age, FBG, creatinine and eGFR did not showed statistically significant association towards *ACE* genotype holders between study case groups and healthy controls (Table 4). This is harmony with the study in USA, age and fasting glucose are not associated with *ACE* gene polymorphism, but 2-h glucose levels are higher in *DD* than *ID* and II subjects of T2DM (Bonnet *et al*., 2008). Cigarette smoking is a risk factor for T2DM, diabetic complications and *DD* genotype are greater risk (Sairenchi *et al.,*2004). However, in this study there were no any cases that are smoking cigarettes.

Whenever the associations of *ACE* gene polymorphism with type 2 diabetic hypertension patients were evaluated, in this study there were existed statistically significant association between *DD* genotype and type 2 diabetic hypertension patients (41.6% Vs 19.5%) Table 5 and Figure 5. This is in agreement with the previous study conducted in a south Indian population (Anbazhagan *et al*., 2009), in south china population (Jiang *et al*., 2009), in Malaysian population (Ramachandran *et al.,* 2008) and in Iranian population (Nakhjavani *et al*., 2007) which have indicated the existence of statistically significant association between the *DD* genotype and *D* allele among diabetic hypertension patients whereas others did not found a positive correlation between the *DD* genotype of *ACE* gene polymorphism and diabetic hypertension patients (Tascilar *et al*., 2009 and Chuang *et al*., 1997). Another study conducted on Turkish population also indicated no significant association between *ACE* gene polymorphism and hypertension (Gunes *et al.,* 2004). This variation is may be due to ethnic factors (Saab *et al.,* 2007) and environmental background (Ono *et al.,* 2003). Among the clinical/biochemical risk factors, including SBP ($P < 0.001$), DBP ($P < 0.01$), age and urea ($P < 0.05$) were significantly associated in diabetic hypertension patients compared with T2DM patients. This is in agreement with other reports found that age and SBP is high in the affected population (Alsafar *et al.,* 2015 and Roaeid and Kadiki, 2011). The other risk factors including BMI, TC, TG, creatinine, FBG and duration of diabetes were no significance association in diabetic hypertension compared with T2DM. This is in agreement with the studies by Ramachandran *et al.,* (2008) and Nakhjavani *et al.,* (2007) indicated that BMI, TC, TG, LDL, HDL and FBG level are no significance difference between type 2 diabetic hypertension and T2DM (Ramachandran *et al.,* 2008 and Nakhjavani *et al.,* 2007). The other report also indicated that BMI and TC are no significance variation between diabetic hypertension and non diabetic hypertension group (Mengesha, 2007). On the other hand, the present finding is dissimilar with other reports found that TG, creatinine and duration of diabetes are associated with type 2 diabetic hypertension compared to non hypertension T2DM (Mengesha, 2007 and Nakhjavani *et al.,* 2007). When comparing the association of risk factors and *ACE* gene polymorphism; *DD* genotypes of diabetic hypertension patients were highly affected by SBP, DBP and age compared with T2DM patients. This result is in agreement with other study, which showed diabetic patients >50 years of age have had double risk of developing diabetic complication than younger counter parts < 50) (Roaeid and Kadiki, 2011). While other risk factors in the present

study such as BMI, TC, TG, FBG, creatinine and urea regarding to *ACE* gene polymorphism did not show significance variation between diabetic hypertension and T2DM. This is dissimilar with the report of Zhou *et al.* (2013) found that TG level is higher in the *ID* and *DD* genotype than II genotype carriers, but level of FBG is similar with the present finding indicated that FBG level had no different on *ACE* gene polymorphism between type 2 diabetic and diabetic hypertension (Zhou *et al.* 2013).

Regarding to diabetic retinopathy, *Angiotensin II* gene has been reported to play a role in the progress of retinopathy by stimulation of growth factors such as vascular endothelial growth factor, platelet-derived growth factor and connective tissue growth factor (Wilkinson-Berka., 2006). In the present study, the frequency of *DD* genotype was high in diabetic retinopathy patients when computed against T2DM patients without complications (28.6% Vs 19.5%) as shown in Table 5 and Figure 5. This result is in complete harmony with other similar studies conducted elsewhere like in Turkey, where *DD* genotype was an independent risk factor in DR patients (Kutluturk *et al*., 2014), and in Chaina, a Meta analysis study; *DD* genotypes increase the risk of developing DR (Lu *et al.,* 2012). While the other report in china indicated that *ACE* gene polymorphism has no difference in diabetic retinopathy patients (Khan *et al*., 2015). On the other hand the recent report in Pakistan opposed the present study and indicated that *ID* genotype individuals are at risk for DR (Saleem *et al*., 2015). Whenever in the present study the role of two genotypes (*ID* and *II*) of *ACE* gene polymorphism were analyzed towards risk of DR among T2DM patients, they did not show significant association. The clinical/biochemical characteristics glucose ($P < 0.014$) and urea ($P < 0.004$) were associated in DR patients as compared to T2DM shown in Table 2. This result is dissimilar with reports from China; fasting plasma glucose has no significant difference between DR and diabetic non retinopathy groups (DNR), but blood urea nitrogen is associated with DR than in DNR group (Meng *et al*., 2015). Other clinical/biochemical characteristics such as gender, age, BMI, SBP, DBP, creatinine, eGFR, total cholesterol and triglycerol did not show statistically significant association in DR patients as compared to T2DM shown in Table 2. This is harmony with the report of Meng *et al*., (2015) showed that age, BMI and triglycerol no statistically significant differences between the DR and DNR groups (Meng *et al*., 2015). However, the other studies shown a significant association of BMI, SBP, triglycerides cholesterol, total cholesterol and duration of diabetes in patients with DR (Meng *et al*., 2015; Rani *et al.,* 2009 and Al-Shammari *et al.,* 2005). When the

genotype distribution and these clinical parameters were compared, there were statistically significant association between the *DD* genotype holders and the amount of glucose, total cholesterol and urea in DR patients compared with DNR patients with the same genotype holders. This is in agreement with other report; high total cholesterol is associated risk factor for the development of DR (Hussain *et al., 2013*). While, the other parameters did not show statistically significant association towards *DD* genotype holders between DR and T2DM patients Table 7.

In diabetic nephropathy, Sample size was not enough in patients having DN, to assess the association of clinical characteristics and *ACE* gene polymorphism with the severity of nephropathy in the current study. This is not surprise, because, similarly the report in the northern part of Ethiopia, diabetic clinic at Mekelle Hospital indicated that among 105 diabetic patients only 2% of them had diabetic nephropathy (Gill *et al*., 2008). However, within the existing sample size of the current study, the risk factors including SBP ($P < 0.05$), creatinine ($P < 0.01$) and eGFR ($P < 0.01$) were associated with DN patients. This result is harmony with other reports showed that high blood pressure (BP) associated with DN is known to worsen renal function, that initiate and accelerate DN When a patient develops persistent proteinuria and elevated arterial BP, kidney function starts to decline as a result of decreased eGFR among progressors with elevated SBP compared to non progressors (Viswanathan *et al*., 2012). Serum creatinine is a risk factor among progressors DN compared to non-progressors of T2DM which leads to the occurrence of DN (Viswanathan *et al*., 2012). Similarly another study revealed that elevated BP, SBP but not DBP was a risk factor for the development of diabetic nephropathy (Kasper *et al*., 2004). The other studies also indicated that SBP, creatinine and eGFR are associated with the development of diabetic nephropathy (Fontela *et al.,* 2014, Shlomo *et al*., 2011 and Degirmenci *et al*., 2005). However, these risk factors did not associated with *ACE* gene polymorphism in DN patients. On the other hand, risk factors such as glucose and urea has statistically significant association with *DD* genotype of DN patients (*DD*: 214.4 ± 58.9, *ID*: 177.8 ± 60.1 and II: 157.2 ± 61.6 $P < 0.05$) and (*DD*: 25.6 ± 7.3, *ID*: 18.4 ± 6.2 and *II* 19.1± 5.0 *$P < 0.01$)* respectively. This is similar with the study in Austria; poor glycemic control is associated with *ACE*-D allele, a major risk factor for the development of diabetic nephropathy (Barnas *et al*., 1997). The other study also indicated that fasting glucose, post-prandial glucose levels and serum urea are higher in progressors of diabetic kidney complication compared to

non-progressors of T2DM patients (Viswanathan *et al*., 2012). On the other hand, blood glucose has no direct effect upon kidney disease but has an indirect effect through serum creatinine (Ahmad *et al*., 2014). The other risk factors such as age, TC and TG were not associated with DN either independently as a risk factor or associated with *ACE* gene polymorphism. This is harmony with other studies age, serum total cholesterol and LDL cholesterol are not associated with diabetic nephropathy (Shen *et al*., 2008). However, TG is an independent risk factor for diabetic nephropathy (Shen *et al*., 2008).

7. CONCLUSION

In conclusion, the present study indicated that *ACE* gene of *DD* genotype and *D* allele have been associated with high risk of diabetes and diabetic complications in diabetes patients (OR:*DD* 2.984, CI: 1.332 - 6.689, $P < 0.02$ and *D* 2.178; CI: 1.168 – 3.232 $P < 0.001$ Vs *I*, 0.459; CI: 0.309 – 0.681 $P < 0.00$). Moreover, this genotype and allele was associated with some of the clinical Parameters such as age, SBP, DBP, glucose, total cholesterol and urea. Type 2 diabetes mellitus patients having *DD* genotype have had high relative risk of getting diabetic complication as compared to *ID* and *II* genotype holders. The frequency of *DD* genotype was high in diabetic hypertension and diabetic retinopathy patients than T2DM without complication and diabetic nephropathy. The genotype (*ID* and *II*) and *I* allele in the healthy control group were high as compared to the cases. Similarly, *ID* and *II* genotype in the study subjects (cases) had less risk of developing diabetic complications as compared to cases with the *DD* genotype. Some of the associated risk factors, *DD* genotype and both risk factors and *DD* genotype were associated to T2DM and its complications, which indicates the need for doing a similar study with a larger sample size to unravel the independent associated risk factors and *ACE* gene between T2DM and diabetic complication.

8. LIMITATIONS OF THE STUDY

This study has some limitations:

Sample size was not enough in patients having diabetic nephropathy, to assess the relation of clinical characteristics and *ACE* gene polymorphism with the severity of nephropathy. The assay for LDL and HDL could not done, those tests could have given a better estimate of lipoproteins. The tests were used only one primer for the detection *ACE* genotype *I/D*, it is better to use a second primer after electrophoresis to increase the specificity of *DD* genotyping.

9. RECOMMENDATIONS

Based on the present result of this study the following recommendations are forwarded

- ✓ Early diagnosis is very much essential for better treatment out come and avoid complications, thus it is advisable to the general population to conduct periodic diabetic test. Because during sample collection from healthy control groups, 5 individuals had hypertension and 9 individuals had hyperglycemia
- ✓ To use *DD* genotype as a marker for risk of Diabetic similar studies on the same and different population are warranted with large sample size.
- ✓ However, depending on the present finding all diabetes patients are best treated with *ACE* inhibitor drugs to prevent the progress of diabetes individuals for further diabetic complication.

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APPENDIX

Appendix 1 Consent form

I, the undersigned (full name) ----------who is living at ----------Zone, wereda---------- kebele ---------- and urban/rural ---------- willingly agree to provide 5ml of my blood sample for the principal investigator of this project. The investigator should ethically bind and use this blood sample only for the said research and will completely be responsible if they don't keep the result and status of the sample confidential.

Name and signature of the participant date

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

Appendix 2 Questionnaire for associated risk factors for all diabetes patients

Demographic information

Question		Response
1	Sex (record male/ female as observed)	Male , female
2	How old are you?	Years ----------
	Behavioral measurements	
6	Do you smoke any tobacco product? Such as cigarettes, cigars or pipes?	Yes No
7	Do you currently smoke tobacco daily? This question is only for current smoker of tobacco product.	Age(years)-------
8	How old were you first started smoking daily?	Age(years)----------
9	On average, how many cigarettes per day do you use?	----------
10	Did someone smoke in closed areas in your work place?(in the building, in a work area or a specific office)	Yes No
11	In question 10 if the answer is Yes asked as how many days did someone smoke in closed areas in your work place?	Number of days ----- Don't know Work in a closed

	History of diabetes	
1	How old are you? (you age 1st diagnosis) When you ever been told by a doctor or other health worker that you have raised blood sugar or diabetes?	Age (years)--------
2	Are (were) any family member suffering from DM disease?	Yes No
3	Are (were) you suffering from other disease? Like kidney, retinopathy, hypertension and neuropathy.	Yes No
4	Does the doctor told you; other disease are due to diabetic?	Yes No
5	How old are you?(you age 1st diagnosis with other disease)	Age(year)----------
6	Are (were) any family member suffering from other disease? Like kidney, retinopathy, hypertension and neuropathy	Yes No
	Physical measurement	
1	Height (in cm)	In centimeter ----------
2	Weight (in kg)	In kilogram----------
3	Blood pressure-record three heart rate readings	SBP/ DBP (mmHg) 1.----------2.-------- 3.------- Average----------beats per minute

Appendix 3 *P* value of clinical characteristics between T2DM and Control

Group Statistics

	Status of the study participants	N	Mean	Std. Deviation	Std. Error Mean
Gender	T2DM	111	.50	.502	.048
	Controls	111	.48	.502	.048
systolic	T2DM	111	133.66	19.504	1.851
	Controls	111	121.26	6.891	.654
diastolic	T2DM	111	82.61	13.571	1.288
	Controls	111	76.44	5.452	.517
BMI3	T2DM	111	24.915	4.3862	.4163
	Controls	111	23.604	5.4189	.5143
glucose	T2DM	111	206.37	72.398	6.872
	Controls	111	78.23	15.672	1.488
cholestrol	T2DM	111	194.16	47.871	4.544
	Controls	111	130.47	48.934	4.645
triglycerol	T2DM	111	177.54	135.330	12.845
	Controls	111	99.67	56.761	5.387
ceratinine	T2DM	111	.720	.2484	.0236
	Controls	111	.642	.1899	.0180
Urea	T2DM	111	22.23	8.326	.790
	Controls	111	18.77	4.600	.437
EGFR	T2DM	111	118.411	27.1582	2.5777
	Controls	111	124.702	20.2433	1.9214

Age	T2DM	111	54.70	12.340	1.171
	Controls	111	54.27	14.425	1.369

Appendix 4 *P* value of clinical characteristics between T2DM and Control

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Gender	Equal variances assumed	.198	.657	.267	220	.789	.018	.067	-.115	.151
	Equal variances not assumed			.267	220.000	.789	.018	.067	-.115	.151
systolic	Equal variances assumed	63.869	.000	6.314	220	.000	12.396	1.963	8.527	16.266
	Equal variances not assumed			6.314	137.041	.000	12.396	1.963	8.514	16.279
diastolic	Equal variances assumed	79.461	.000	4.446	220	.000	6.171	1.388	3.435	8.907
	Equal variances not assumed			4.446	144.603	.000	6.171	1.388	3.428	8.915
BMI3	Equal variances assumed	.043	.835	1.980	220	.049	1.3101	.6617	.0060	2.6142
	Equal variances not assumed			1.980	210.849	.049	1.3101	.6617	.0057	2.6145
glucose	Equal variances assumed	152.816	.000	18.225	220	.000	128.135	7.031	114.279	141.992
	Equal variances not assumed			18.225	120.287	.000	128.135	7.031	114.215	142.055

cholestrol	Equal variances assumed	.000	.983	9.803	220	.000	63.694	6.497	50.888	76.499
	Equal variances not assumed			9.803	219.894	.000	63.694	6.497	50.888	76.499
triglycerol	Equal variances assumed	13.623	.000	5.591	220	.000	77.874	13.929	50.422	105.325
	Equal variances not assumed			5.591	147.540	.000	77.874	13.929	50.348	105.400
ceratinine	Equal variances assumed	1.234	.268	2.610	220	.010	.0775	.0297	.0190	.1360
	Equal variances not assumed			2.610	205.858	.010	.0775	.0297	.0190	.1360
Urea	Equal variances assumed	43.732	.000	3.832	220	.000	3.459	.903	1.680	5.239
	Equal variances not assumed			3.832	171.421	.000	3.459	.903	1.677	5.242
EGFR	Equal variances assumed	3.167	.077	-1.956	220	.052	-6.2902	3.2151	-12.6265	.0460
	Equal variances not assumed			-1.956	203.400	.052	-6.2902	3.2151	-12.6293	.0489
Age	Equal variances assumed	2.604	.108	.240	220	.811	.432	1.802	-3.119	3.983
	Equal variances not assumed		.657	.240	214.851	.811	.432	1.802	-3.119	3.984

Appendix 5 Chi-Square *ACE* genotype distribution between type 2 diabetic and control.

Genotype result * Status of the study participants Crosstabulation

			Status of the study participants		
			T2DM	Controls	Total
Genotype result	Homozygous Deletion	Count	77	43	120
		% within Genotype result	64.2%	35.8%	100.0%
		% within Status of the study participants	69.4%	38.7%	54.1%

		% of Total	34.7%	19.4%	54.1%
	Hetrozygous Insertion-deletion	Count	22	48	70
		% within Genotype result	31.4%	68.6%	100.0%
		% within Status of the study participants	19.8%	43.2%	31.5%
		% of Total	9.9%	21.6%	31.5%
	Homozygous Insertion	Count	12	20	32
		% within Genotype result	37.5%	62.5%	100.0%
		% within Status of the study participants	10.8%	18.0%	14.4%
		% of Total	5.4%	9.0%	14.4%
	Total	Count	111	111	222
		% within Genotype result	50.0%	50.0%	100.0%
		% within Status of the study participants	100.0%	100.0%	100.0%
		% of Total	50.0%	50.0%	100.0%

Appendix 6 Chi-Square *ACE* genotype *P* value between T2DM and Control

Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	21.290[a]	2	.000
Likelihood Ratio	21.680	2	.000
N of Valid Cases	222		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 16.00.

Appendix 7 Chi-Square *ACE* genotype distribution among type 2 diabetic Complications.

Genotype result * Status of the study participants Crosstabulation

			Status of the study participants				
			T2DM	t2dm with hypertention	t2dm with retinopathy	t2dm with nephropathy	Total
Genotype result	DD Homozygous Deletion	Count	15	32	22	8	77
		% within Genotype result	19.5%	41.6%	28.6%	10.4%	100.0%
		% within Status of the study participants	42.9%	91.4%	73.3%	72.7%	69.4%
		% of Total	13.5%	28.8%	19.8%	7.2%	69.4%
	ID Hetrozygous Insertion-deletion	Count	12	3	5	2	22
		% within Genotype result	54.5%	13.6%	22.7%	9.1%	100.0%
		% within Status of the study participants	34.3%	8.6%	16.7%	18.2%	19.8%
		% of Total	10.8%	2.7%	4.5%	1.8%	19.8%
	II Homozygous Insertion	Count	8	0	3	1	12
		% within Genotype result	66.7%	.0%	25.0%	8.3%	100.0%
		% within Status of the study participants	22.9%	.0%	10.0%	9.1%	10.8%
		% of Total	7.2%	.0%	2.7%	.9%	10.8%
Total		Count	35	35	30	11	111
		% within Genotype result	31.5%	31.5%	27.0%	9.9%	100.0%
		% within Status of the study participants	100.0%	100.0%	100.0%	100.0%	100.0%
		% of Total	31.5%	31.5%	27.0%	9.9%	100.0%

Appendix 8 Chi-Square *ACE* genotype *P* value between T2DM and Control.

Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	20.713[a]	6	.002
Likelihood Ratio	23.636	6	.001
N of Valid Cases	111		

a. 5 cells (41.7%) have expected count less than 5. The minimum expected count is 1.19.

Appendix 9 Multivariate logistic regression of genotypes among type 2 diabetes and control.

Parameter Estimates

Status of the study participants[a]		B	Std. Error	Wald	df	Sig.	Exp(B)	95% Confidence Interval for Exp(B)	
								Lower Bound	Upper Bound
T2DM	Intercept	-.511	.365	1.957	1	.162			
	[genotype=DD]	1.093	.412	7.050	1	.008	2.984	1.332	6.689
	[genotype=ID]	-.269	.447	.363	1	.547	.764	.318	1.834
	[genotype=II]	0[b]	.	.	0	.	.	.	.

a. The reference category is: Controls.

b. This parameter is set to zero because it is redundant.

Appendix 10 logistic regression of I/D allele among type 2 diabetes and control.

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I.for EXP(B)	
								Lower	Upper
Step 1[a]	D	.778	.201	14.945	1	.000	2.178	1.468	3.232
	Constant	-1.094	.320	11.689	1	.001	.335		

a. Variable(s) entered on step 1: D.

		B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I.for EXP(B)	
								Lower	Upper
Step 1[a]	I	-.778	.201	14.945	1	.000	.459	.309	.681
	Constant	.463	.180	6.582	1	.010	1.589		

a. Variable(s) entered on step 1: I.

Appendix 11: Online calculating formula of eGFR by using CKD-EPI equation.

CKD-EPI equation calculator should be used when Scr reported in mg/dL. This equation is recommended when eGFR values above 60 mL/min/1.73 m2 are desired.

GFR = 141 × min (Scr /κ, 1)α × max(Scr /κ, 1)-1.209 × 0.993Age × 1.018 [if female] × 1.159 [if black]
where:
Scr is serum creatinine in mg/dL,
κ is 0.7 for females and 0.9 for males,
α is -0.329 for females and -0.411 for males,
min indicates the minimum of Scr /κ or 1, and
max indicates the maximum of Scr /κ or 1.

The equation does not require weight because the results are reported normalized to 1.73 m2 body surface area, which is an accepted average adult surface area.

Serum creatinine ☐ (mg/dL)

Age* ☐

African American ☐ Yes ☐ No

Gender ☐ Male ☐ Female

Calculate Clear

GFR value: ☐ mL/ min/1.73 m2**

*This equation should only be used for patients 18 and older.

The National Kidney Disease Epidemiology Program presently recommends reporting estimated GFR values greater than or equal to 60 mL/min/1.73 m2 simply as "≥60 mL/min/1.73 m2", not an exact number.