Evaluation of Insulin receptor (INSR), Leptin receptor (LEPR) and β_3-adrenergic receptor (ADRB3) gene polymorphisms in Diabetes Mellitus patients of Kashmir valley

Dissertation Submitted for the Award of the Degree of Master of Philosophy in Biochemistry

By

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UNDER THE JOINT SUPERVISION OF

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CERTIFICATE

Certified that the work in the dissertation entitled **"Evaluation of Insulin receptor (INSR), Leptin receptor (LEPR) and β_3-adrenergic receptor (ADRB3) gene polymorphisms in Diabetes Mellitus patients of Kashmir valley"** has been carried out by Ms. Iqra Hameed under the joint supervision of Dr. B.A. Ganai (Department of Biochemistry) and Dr. Dil-Afroze (Department of Immunology and Molecular Medicine, SKIMS), and the work is suitable for the award of M.Phil degree in Biochemistry.

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DECLARATION

I, Iqra Hameed, declare that the work embodied in this dissertation entitled **"Evaluation of Insulin receptor (INSR), Leptin receptor (LEPR) and β_3-adrenergic receptor (ADRB3) gene polymorphisms in Diabetes Mellitus patients of Kashmir valley"** has been carried out by me in the Department of Biochemistry, University of Kashmir and is original. The work embodies the results of my observations which are advancement to the previous knowledge in the subject.

Place: SRINAGAR **IQRA HAMEED**

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Dedicated to my Dad...

Who cherished my dreams through sacrifice

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For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely my own.

Iqra Hameed

ABBREVIATIONS

µg	Microgram
µl	Microlitre
µM	Micromolar
APO	Apolipoprotein
BMI	Body mass index
BP	Blood pressure
CAD	Coronary artery disease
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
HDL	High density lipoprotein
hr	Hour
LDL	Low density lipoprotein cholesterol
min	Minute
^{o}C	Degree Celsius
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
SBP	Systolic blood pressure
SD	Standard deviation
sec	Second
SNP	Single nucleotide polymorphism
TG	Triglyceride
UTR	Untranslated region
WHR	Waist-to-hip ratio

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ABSTRACT

The prevalence of type 2 diabetes mellitus has reached epidemic proportions worldwide. This complex disorder is characterized by defects in insulin secretion and insulin resistance. Type 2 diabetes is a consequence of complex interactions among multiple genetic variants and environmental risk factors. The genetic basis of type 2 diabetes is still poorly understood. The overarching aim of this study was to investigate various polymorphisms in Insulin receptor (INSR), leptin receptor (LEPR) and β_3-adrenergic receptor (ADRB3) genes and evaluate their role in the incidence and progression of type 2 diabetes. Three single nucleotide polymorphisms (233, 234 and 276) in the exon 3 of INSR gene, a CTTTA pentanucleotide repeat Del/Ins polymorphism in the 3□ UTR of LEPR gene and a single nucleotide polymorphism (Trp64Arg) in the ADRB3 gene were analysed. The study comprised of 500 local subjects including 200 type 2 diabetes patients and 300 age and gender matched healthy controls. Restriction fragment length polymorphism and single stranded conformation polymorphism techniques were used for genotyping and the results were validated by direct sequencing assay. We observed that codon 233, 234 and 276 of INSR gene were monomorphic in our population thereby ruling out any possible association with type 2 diabetes. However we found an association of LEPR and ADRB3 gene polymorphisms with risk factors and severity of type 2 diabetes, such as obesity, abnormal lipid profile, hypertension, elevated HbA1c levels and serum creatinine levels. The frequency of Del allele was 89.7% and 59.3% in cases and controls respectively ($p<0.001$; odds ratio 6.0; 95% confidence interval 3.61 - 9.97). The wild allele (Del) in the LEPR gene showed a positive association with the disease as well as associated risk factors. The genotypes for Del/Ins polymorphism did not satisfy Hardy and Weinberg equilibrium (χ^2=57.22). In ADRB3 gene, frequency of variant C (Arg) allele was observed to be 40% and 10.2% in cases and controls respectively ($p<0.001$; odds ratio 5.89; 95% confidence interval 3.69-9.39). Presence of C (Arg) allele was observed to be a risk factor, where as the wild T (Trp) allele exerted a protective effect against type 2 diabetes in our population. Presence of C allele was directly related to higher BMI, WHR, dyslipidemia and uncontrolled diabetes. Genotypes for Trp64Arg polymorphism were in Hardy and Weinberg equilibrium (χ^2=0.48). The study signifies that *Del* allele in LEPR gene and *Arg* allele in ADRB3 are genotypic risk factors that confer susceptibility to type 2 Diabetes Mellitus in ethnic Kashmiri population.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a heterogeneous disorder characterized by two interrelated metabolic defects: insulin resistance coupled with impaired insulin secretion by β-cells in the pancreas (Kahn, 2008). An array of dysfunctions is associated with T2DM that are characterized by hyperglycemia and associated with microvascular, macrovascular and neuropathic complications. T2DM is regarded as a multifactorial disease with both a genetic component and an important non-genetic component(s) which undoubtly interacts in order to precipitate the diabetic phenotype (Beck *et al.,* 1994). The number of people developing T2DM has increased rapidly in the last thirty years, at first in America and Europe, and more recently in Asia where it is said to be reaching epidemic proportions. Age, physical activity patterns, dietary habits, lifestyle and obesity play a crucial role in the modulation of the disease occurrence. In the past few years, major advances relating to the genetic basis of T2DM have been made. This has cumulated in the discovery and confirmation of around twenty common predisposing loci (Lyssenko, 2009) but the variance in disease risk explained by these variants is much lower than predicted based on heritability studies. It follows that the genetic associations discovered to date represent the tip of the iceberg with respect to the genetic landscape of T2DM risk. In contrast to the genetic basis to T2DM, a great deal of robust evidence exists which documents the impact of lifestyle behaviors on the development of T2DM. Epidemiological studies have identified strong T2DM risk relationships for obesity, sedentary behaviours (Hu *et al.,* 2001, 2003; Dunstan *et al.,* 2007) and diets rich in energy (Davis *et al.,* 2009), processed carbohydrates (Hu *et al.,* 2001) and animal fats (Winzell and Ahren, 2004). The strongest evidence comes from clinical trials, which show that intensive lifestyle interventions targeting weightloss through diet modification and physical activity have a major beneficial impact on diabetes incidence in high-risk individuals (Hammam *et al.,* 2006; Lindstrom *et al.,* 2006). The pattern of disease occurrence within and between populations that differ in their genetic and environmental reinforcement suggests that T2DM is caused by the interaction between adverse lifestyle behaviors and in part by the genetic profile of an individual. The predisposition to T2DM is thought to be conferred by a number of different genes that in isolation may have only minor effects, but in combination lead to the characteristic pathophysiological effects (Morton and Lio, 1997; McCarthy *et al.,* 2002). This genetic susceptibility may be conferred by an unfavorable combination of individual polymorphisms in the genes involved, each one controlling part of the pathogenic process (De Meyts, 1997). These genes are defined as “diabetogenes” (Hoban *et al.,* 1990; Taylor *et al.,*

1990; Moller *et al.,* 1996) many of which encode proteins of a functional complex that play a key role in glucose metabolism and others involved in the signal transduction characterizing insulin's biological activity (Taylor *et al.,* 2001). The investigation into the molecular mechanism for insulin action has clarified how insulin resistance is related to genetic factors and environmental factors (hyperglycemia, free fatty acids, inflammatory mechanism, etc.). Known genetic factors, include not only insulin receptor and insulin receptor substrate (IRS)-1 gene polymorphisms that directly affect insulin signals but also polymorphisms of thrifty genes such as the β_3-adrenergic receptor gene and the uncoupling protein (UCP) gene, associated with visceral obesity and promote insulin resistance. Glucolipotoxicity and inflammatory mediators are also important as the mechanisms for impaired insulin secretion and insulin signalling impairment. Recent attention has focused on the involvement of adipocyte-derived bioactive substances (adipokines) in insulin resistance. While TNF-α, leptin, resistin, and free fatty acids act to increase resistance, adiponectin improves resistance. Several single-nucleotide polymorphisms (SNPs) have been described in these genes that have been associated with T2DM. Studies have investigated various SNPs associated with T2DM and found that among heterogeneous populations throughout the world, there are similar polymorphisms that convey genetic risks for T2DM in subgroups of main populations, i.e. Mexican Americans, Pima Indians, gypsies of southern Slovakia, African Americans, to name a few. This feature has been reported in cases of isolated ethnic groups (Gharbi *et al.,* 2002; Vasarova and Hanson, 2003; Sánchez-Corona *et al.,* 2004; Arfa *et al.,* 2007; Knowler *et al.,* 2007) and among underprivileged groups in developed countries (Arroyo *et al.,* 1999).

Kashmir valley is a unique geographical area located in the north Indian state of Jammu and Kashmir. It differs from other areas in terms of its location, climate, amount of ultraviolet rays received, socio-demographic as well as lifestyle habits. The prevalence of T2DM in Kashmir valley was 6% in subjects aged ≥ 40 years (Zargar *et al.,* 2000). In the young adults (20-40 years), a prevalence rate of 2.4% was observed (Zargar *et al.,* 2008). According to a recent hospital based retrospective study (Zargar *et al.,* 2009), mortality trends among people with diabetes admitted to the tertiary care hospital (Sher-I-Kashmir Institute of Medical Sciences, Srinagar) were reviewed by screening the hospital records of all people with diabetes who died over the last nine years. Deaths associated with diabetes accounted for 7.11% (Zargar *et al.,* 2009). The mean age at death for people with diabetes was 60.07 years in men and 57.36 years in women. These studies show an increasing trend in the prevalence of the diabetes mellitus in Kashmir valley owing to increased susceptibility to the contributing factors of the disease and the changing lifestyle. However, apart from cross-sectional and retrospective studies, no

research has been done at genetic or molecular level to evaluate the causal genetic factors or polymorphic studies in key diabetogenes which would provide an insight to the susceptibility, control and treatment of the disease. In light of these observations this study is intended to evaluate various polymorphisms in key genes related to diabetes like Insulin receptor (*INSR)*, Leptin receptor (*LEPR*) and β_3-adrenergic receptor (*ADRB3*) genes: their prevalence, possible role in pathogenesis and correlation with risk factors.

REVIEW OF LITERATURE

2.1. History of Diabetes Mellitus

The first document known to mention descriptions of polyuric state resembling diabetes is an Egyptian papyrus dating from approximately 1500 BC. The term "diabetes" was first used by the Greek physician Aretaeus of Cappadocia in the 2nd century AD. His writings contain a description that is accurate and clinical: "diabetes is an awful disease melting the body and limbs of the patient into urine. Life is short and painful and sooner rather than later the patient will expire" (Mogensen, 2011). During the same period, Galen, the Roman physician, spoke of two cases of a rare disease characterized by polyuria and polydipsia. Pre-Aretaeus there are hints of diabetes in certain hieroglyphs and in ancient accounts from India, where ants were drawn to sweet urine (almost a biological test for diabetes). In the 5th and 6th centuries, two notable Indian physicians, Susruta and Sharuka, described for the first time the association of polyuria with a sweet tasting substance in the urine (Fernandez, 2006**)**. In the 17th century, Thomas Willis made several clever observations about the disease which still hold true today. He wrote that diabetes had been rare in classical times (Galen) "but in our age, given to good fellowship and gusling down chiefly of unllayed wine, we meet with examples and instances enough, I may say daily, of this disease..... As to what belongs the cure, it seems a most hard thing of this disease to draw propositions for curing, for this cause lies so deeply hid, and hath its origin so deep and remote". Since then, plentiful efforts have been made to unravel the deep causes of this frequent disease. The idea that lifestyle contributes to the development of T2DM was proposed in France over a century ago by Étienne Lancereaux (1829-1910). He classified diabetes into *diabète maigre* ("lean diabetes") and *diabète gras* ("fat diabetes"), equivalent to diabetes types 1 and 2. Increased affluence and decreased physical activity have since created a near-epidemic of T2DM, which began mainly in the post-war US, but spread worldwide to China, India, and Japan and many countries in the Middle and Far East (Mogensen, 2011).

2.2. Definition and classification of Diabetes Mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycaemia (elevated levels of glucose in the blood) resulting from defects in insulin secretion, insulin action, or both. There are two major types of diabetes mellitus: type 1 and type 2 diabetes (Diabetes Care, 2004)**.** Type 1 diabetes is an autoimmune disease that usually occurs in childhood but the onset may occur at any age; this type of diabetes results from a cellular-mediated autoimmune

destruction of the beta-cells in the pancreatic islets which usually leads to absolute insulin secretion deficiency. Type 2 diabetes on the other hand is a metabolic disorder that generally appears later in life but may occur in childhood and is characterized by the combination of insulin resistance and relative insulin secretion deficiency (Diagnosis and classification of diabetes mellitus, Diabetes Care, 2009). This type of diabetes usually begins predominantly with insulin resistance, which is a condition characterized by the inability of cells to respond to the action of insulin in transporting glucose from the bloodstream into muscle, fat, and liver cells (Lillioja *et al.,* 1998). This condition causes a compensatory increase in the secretion of insulin from the pancreatic beta cells (hyperinsulinemia) in order to overcome the state of insulin resistance and thus help glucose enter the cells. However, in the long term, beta-cell mass and function progressively declines (Festa *et al.,* 2006). The natural history of T2DM in many individuals involves years of insulin resistance balanced by elevated insulin secretion. The pivotal point is when the beta-cells begin to fail, and insulin production declines. Thus T2DM is characterized by both defects in insulin secretion and by cellular insulin resistance. Beside type 1 and type 2 diabetes there are several other classes of diabetes which are characterized by genetic defects of beta-cell function (Maturity Onset Diabetes of the Young: MODY1-6), transient neonatal diabetes, genetic defects in insulin action, disorders of the exocrine pancreas, endocrinopathies, drug- or chemical-induced diabetes, infections induced diabetes, uncommon forms of immune-mediated diabetes, and gestational diabetes mellitus (Diabetes Care, 2009). However, approximately 90–95% of all diabetes cases are T2DM (National Diabetes Fact Sheet, 2005).

2.3. Diagnosis of type 2 Diabetes Mellitus

T2DM is diagnosed using either repeat fasting or two hour plasma glucose concentrations following oral glucose challenge i.e. fasting blood glucose levels >126 mg/dl (>7.0 mmol/l) without symptoms, 2-hour glucose levels >200 mg/dl (>11.1 mmol/l) after an oral glucose tolerance test (OGTT) without symptoms, or random blood glucose levels >200 mg/dl [>11.1 mmol/l] with symptoms (Diabetes Care, 2003). Because the progression from normoglycemia to hyperglycemia is slow and gradual, there are intermediate stages. These are defined as impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) where glucose values are considered to be above “normal” glucose tolerance but below those used to diagnose diabetes. As a result, many individuals have ‘intermediate hyperglycemia’ (WHO, 1999) sometimes referred as ‘impaired glucose regulation’, ‘prediabetes’ or, ‘non-diabetic hyperglycemia’

(Forouhi *et al.,* 2006). According to the criteria of World Health Organization (WHO, 1999) IFG and IGT are diagnosed when a person presents with fasting venous plasma glucose levels between 100–125 mg/dl (6.1 to 6.9 mmol/l) and 2-hour blood glucose level between 140–199 mg/dl (7.8 –11.1 mmol/l) during a 75-g OGTT. In 2003, the American Diabetes Association (ADA) recommended that the IFG threshold should be lowered to 100 mg/dl (5.6 mmol/l) (Genuth *et al.,* 2003). However, not all agencies, including WHO, have accepted this recommendation.

The majority of those diagnosed with IFG and IGT (around 60%) do subsequently develop T2DM (Unwin *et al.,* 2002). It is for this reason that IFG and IGT are commonly used to identify high-risk groups. For example, all-cause mortality rates in individuals with IFG or IGT are almost twice those of persons with normal glucose levels (Saydah *et al.,* 2001), justifying early intervention. Although the etiology of T2DM has not been established in full, a number of risk factors are well defined. According to the ADA (Screening for type 2 diabetes: Diabetes Care 2004), the risk of developing T2DM is associated with age (increased risk at ≥45 years), overweight/obesity, and lack of physical activity (PA). T2DM is more common in individuals with a family history of the disease, in certain ethnic groups (e.g. African-Americans, Hispanic-Americans, Native Americans, Asian-Americans, and Pacific Islanders), and in individuals with hypertension (≥140/90 mmHg in adults), dyslipidaemia (HDL cholesterol ≤35 mg/dl [0.90 mmol/l] and/or a triglyceride level ≥250 mg/dl [2.82 mmol/l]), IFG, IGT, a history of vascular disease or gestational diabetes, or polycystic ovary syndrome. In addition, a range of common genetic variants are also known to raise the risk of T2DM (Lyssenko *et al.,* 2005; 2008; Zeggini *et al.,* 2008), of which some may interact with lifestyle factors to modify the risk of the disease (Franks *et al.,* 2007).

2.4. Epidemiology of type 2 Diabetes Mellitus

2.4.1 The global burden of type 2 Diabetes Mellitus

Over recent decades, the progressively increasing global prevalence of T2DM (Zimmet, 1999) has created a major public health challenge. This is because T2DM is a major cause of premature morbidity and mortality, and as such it imposes a heavy burden on affected individuals and society as a whole. Furthermore, the disease is associated with long-term microvascular and macrovascular injury, such as retinopathy (eye disease), nephropathy (kidney disease), neuropathy (damaged nerves), peripheral vascular disease, cerebrovascular disease (including hemorrhagic stroke), and atherosclerotic disease (Report of the expert

committee on the diagnosis and classification of diabetes mellitus (WHO, 1999; Diabetes Care, 2003). Mortality rates in adults with T2DM are 2-4 folds higher than those observed in non-diabetic individuals, with many premature deaths in people with diabetes being attributable to cardiovascular disease (CVD) (Haffner *et al.,* 1998; Fox *et al.,* 2007). According to the WHO, the number of people with diabetes of all ages worldwide increased from 30 million to 171 million between 1985 and 2000 (WHO, 2006). These numbers are expected to increase to 366 million in 2030. The estimated prevalence of diabetes approximated 2.8% in 2000 and is predicted to be around 5.8% in 2030 (Wild *et al.,* 2004). In Sweden, it is estimated that diabetes affects ~350,000 people (2.2–4.5% of the population) (Berger *et al.,* 1998; Eliasson *et al.,* 2002; Jansson *et al.,* 2007). Costs incurred from diabetes complications make up 1.6–6.6% of total health care spending in eight European countries, including Sweden (~5%) (Jonsson, 2002).

WHO estimates that between 2000 and 2030 the most striking increase in T2DM prevalence will be among persons aged 65 years and older. By 2030, it is estimated that more than 48 and 82 million older adults (>65 yrs) in developed and developing countries, respectively, will be afflicted with T2DM (Wild *et al.,* 2004). The DECODE Study Group, which is comprised of nine European countries (including Sweden) estimates that the prevalence of T2DM will be <10% in persons younger than 60 years of age and 10–20% in persons aged 60–79 years (Diabetes Care, 2003). The reason for this shift in the demographic distribution of affected individuals is threefold: firstly, global populations are aging; secondly the complications of T2DM can be treated more efficiently than ever before, which means that people are living longer with diabetes; and thirdly, lifestyle behaviours that increase diabetes risk are becoming more common in all age groups (Colagiuri *et al.,* 2005; Hussain *et al.,* 2007).

The “Top 10” countries in the world, in terms of the number of people with diabetes, for 2011 and 2030, are shown in Table 1. At both time points, the three countries with the largest number of people with diabetes are China, India and United States.

India is one of the leading countries in the world with largest number of diabetic subjects earning the dubious distinction of being termed the “diabetes capital of the world”. Nowhere is the diabetes epidemic more pronounced than in India as the World Health Organization (WHO) reports show that 32 million people had diabetes in the year 2000 (Wild *et al.,* 2004). The International Diabetes Federation (IDF) estimates the total number of diabetic subjects to be around 61 million in India and this is further set to rise to 100 million by the year 2030. Other “hot spots” for diabetes include the Gulf region in the Middle East, another area where the epidemic is taking hold (Qiao *et al.,* 2004).

2.4.2 Type 2 Diabetes Mellitus in Kashmir valley

Major differences in the prevalence rates of Diabetes Mellitus have been observed in migrant Indians in different countries and even in different parts of India (Ramiya *et al.,* 1990). Most of the studies consider Indians as one homogenous group, which is inappropriate. Indians differ in ethnicity, religion, place of origin (different parts of India) and diet. The Valley of Kashmir lies in the northern region of the Indian subcontinent, in the state of Jammu and Kashmir. The population is predominantly Muslim and of uniform ethnicity. A cross-sectional population survey was undertaken to determine the prevalence of T2DM and impaired glucose tolerance in subjects aged 40 years or more in Kashmir Valley (Zargar *et al.,* 2001). 6091 subjects aged 40 years or older were randomly selected. Abnormalities of carbohydrate intolerance were determined as recommended by WHO. 12.34% subjects had an abnormal Glucose tolerance test (GTT), 8.09% were having impaired glucose tolerance (IGT) and 4.25% suffered from undiagnosed diabetes mellitus. The prevalence of IGT as well as of diabetes was significantly more in females as compared to males (P<0.001) (Zargar *et al.,* 2001). Prevalence of known diabetes as well as that of abnormal GTT steadily increased with age. Subjects with diabetes on GTT had a higher waist: hip ratio. Obese subjects showed significantly higher basal as well as 2 hr blood glucose. The prevalence of diabetes as well as IGT was significantly higher in the urban population. The study concluded that 1.89% of the general population had known diabetes, 4.25% had undiagnosed diabetes and 8.09% had impaired glucose tolerance test; making the total load of abnormal glucose tolerance 14.23% in Kashmir Valley. In subjects greater than 40 years of age having a family history of diabetes, obesity, higher age (50 years or above), female sex, and urban origin had more chances of developing abnormal glucose tolerance (Zaragar *et al.,* 2001). In another study the burden of T2DM and other abnormalities of glucose tolerance were assessed in young-adult (20-40 years) men and non-pregnant women in 3032 subjects from Kashmir Valley (Zargar *et al.,* 2008). The study included a questionnaire, anthropological measurements, blood sampling, and a standard OGTT. Out of 3024 subjects screened, prevalence of diabetes, impaired glucose tolerance (IGT), and impaired fasting glycemia [IFG, World Health Organization Geneva, 1999], IFG [American Diabetes Association, 2004] was 2.5%, 2.0%, 11.9% and 26.7%, respectively. Overall, age-adjusted prevalence of T2DM (known plus unknown), IGT, IFG (WHO) and IFG (ADA) was 2.4%, 1.6%, 11.1% and 25.2% respectively (Zargar *et al.,* 2008). According to this study the difference in diabetes prevalence was significant by age, habitat, family history of diabetes and BMI. The ratio of known-to-unknown diabetes was 1:10.

Table 1: Top 10 countries for estimated numbers of adults with diabetes, 2011 and 2030

Rank	Country/Territory	2011 (Millions)	Country/Territory	2030 (Millions)
1	China	90.0	China	129.7
2	India	61.3	India	101.2
3	United States	23.7	United States	29.6
4	Russian Federation	12.6	Brazil	19.6
5	Brazil	12.4	Bangladesh	16.8
6	Japan	10.7	Mexico	16.4
7	Mexico	10.3	Russian Federation	14.1
8	Bangladesh	8.4	Egypt	12.4
9	Egypt	7.3	Indonesia	11.8
10	Indonesia	7.3	Pakistan	11.4

(Courtesy: International diabetes federation, 2011)

2.4.3 Type 2 Diabetes in children–the emerging threat

There is now a major emerging global phenomenon that reveals a new perspective of the global diabetes epidemic. This is the younger age of onset being seen in T2DM, which was formerly considered a disease of adults (Zimmet *et al.,* 2001). However, in recent years, T2DM is appearing at a younger age, not only in the young adult population, but also in adolescents and, occasionally, in children (Zimmet *et al.,* 2001; Shaw *et al.,* 2008). Although T2DM has traditionally been considered a disease of adult onset, in the past decade T2DM incidence has increased rapidly in the young; in some aboriginal groups such as Pima Indians, T2DM is as common in children as it is middle-aged adults of lower risk ethnic groups (Franks *et al.,* 2006). The explanations for the rising trends in paediatric T2DM are likely to be attributable to changing lifestyles and the high prevalence of obesity in contemporary children (Wiegand *et al.,* 2004; Singh *et al.,* 2004). Data on T2DM incidence in European children are scarce. Nevertheless, the proportion of children of European descent diagnosed with T2DM appears to remain low.

T2DM has already been reported in children from Japan and other Asian nations, the USA, the Pacific Islands, Hong Kong, Australia, and the United Kingdom (Zimmet *et al.,* 2001). A French study (Ortega *et al.,* 2001) indicated a relatively low, but increasing, number of children with glucose levels exceeding the thresholds for T2DM and an Austrian population-based study (Rami *et al.,* 2003) reported an incidence of 0.25/100,000/year. In the U.K., the incidence of T2DM was substantially higher in children from ethnic minority groups: 3.9 and 1.25/100,000/year for children of African and South Asian origin, respectively, compared to 0.35/100,000/year in ethnically European children (Haines *et al.,* 2007). In Japan, T2DM accounts for 80 percent of cases of diabetes in childhood in that country (Kitagawa *et al.,* 1998). This is certainly an emerging public health problem of significant proportions as the fall in the age of onset of T2DM is an important factor influencing the future burden of the disease. Onset in childhood heralds many years of disease and an accumulation of the full range of both micro- and macrovascular complications particularly as compliance to hypoglycemic medications is often an issue (Dabelea *et al.,* 1999). The risk determinants for T2DM in children and adolescents are similar to those seen in adults, with obesity almost always being present (Alberti *et al.,* 2004). In-utero exposure to hyperglycemia now appears to be an additional risk factor to having a family history of diabetes and suggests that better management of diabetes in pregnancy and prevention of gestational diabetes may reduce the risk of diabetes developing in the offspring (Pettitt *et al.,* 1988).

2.5. Pathophysiology of type 2 Diabetes Mellitus

2.5.1 Regulation of insulin secretion

Insulin is secreted by pancreatic β-cells in response to elevated blood glucose. Glucose is taken into the β-cells via high capacity facilitated insulin-independant glucose transporter 2 (GLUT 2). Intracellular glucose is phosphorylated by glucokinase, and glucose-6-phosphate is metabolized in the mitochondria, resulting in an increase of ATP/ADP ratio, and the closure of ATP-sensitive potassium channels (K_{ATP}). An increase in intracellular potassium (K^+) concentration leads to the depolarization of the cell membrane, and voltage –sensitive calcium (Ca^{2+}) are opened. Influx of Ca^{2+} into the β-cells triggers the translocation and exocytosis of insulin granules (Barg, 2003). Glucose stimulated insulin secretion is biphasic. The first 5-10 minutes is a rapid phase, followed by a prolonged phase as long as glucose stimulation persists (Del Prato *et al.,* 2002). Insulin secretion is influenced by a variety of other factors. Nutrients and metabolites, e.g. FFAs, amino acids, ketone bodies, as well as incretins, such as glucose dependant insulinotropic polypeptide and glucagon-like polypeptide-1 are well known regulators of the first-phase insulin secretion (Henquin and Meissner, 1986; Haber *et al.,* 2003; Vilsbøll and Holst, 2004). Pancreatic hormones and peptides such as insulin and C-peptide, glucagon and pancreastatin regulate insulin secretion in paracrine and intra-islet endocrine manner (Leibiger *et al.,* 1998). Other hormones (e.g. growth hormone, cortisol, epinephrine, thyroid hormones) also play an important role in the regulation of insulin secretion. Pancreatic islets are rich in autonomic nerves, and sympathetic neuro-mediators inhibit insulin secretion, whereas parasympathetic stimulation leads to an increase of insulin secretion (Kiba, 2004). Leptin inhibits insulin secretion by the activation of K_{ATP} channels (Kieffer *et al.,* 1997).

2.5.2 Insulin resistance and type 2 Diabetes Mellitus

T2DM is said to be a "2-hit" disease in which Insulin Resistance (IR) is accompanied by β-cell defects preventing the compensatory upregulation of insulin secretion (Bergman *et al.,* 2002). The genetic and molecular bases for the reduction in insulin sensitivity and β-cell function are not fully understood, but it seems that body fat distribution and especially visceral fat are major determinants of IR while reductions in β-cell mass contribute to β-cell dysfunction (Kahn, 2003). Hitherto, T2DM had been attributed solely to the development and worsening of IR (Donath and Halban, 2004). Reduced glucose metabolism due to sedentary lifestyles, increased glucose availability due to overfeeding, and progressive reduction in glucose storage as insulin-sensitive tissues "resist" plasma glucose transport were presumed to worsen glycemia and lead

to T2DM (Gerich, 1999; Donath and Halban, 2004). A number of clinical data points support IR as a critically important etiologic factor in type 2 diabetes: (1) T2DM and IR are highly correlated; more than 80% of individuals with T2DM also manifest IR (Gerich, 1999). IR is also common in obese individuals who have not yet developed T2DM (Balkau *et al.,* 2002). IR may thus provide the pathophysiologic linkage between increasing weight and increasing glycemia. (2) IR appears to precede (is detectable earlier than) beta-cell dysfunction in the pathogenesis of type 2 diabetes (Gerich, 1999).(3) IR appears to cause beta-cell dysfunction via the mechanism of beta-cell exhaustion; i.e., increased secretory demand, due to peripheral tissue resistance to insulin signalling, results in continual beta-cell hyperstimulation and eventual failure (Kahn, 2001; Leahy, 2005). While T2DM is indeed highly correlated with IR, what requires explanation—if IR is indeed causative—is the absence of correlation between IR and T2DM. Prevalence rates of IR in various populations are often far greater than those of T2DM; clearly, large majorities of individuals with IR do not go on to develop the disease (Porte and Kahn, 1995; Bonora *et al.,* 1998; Weyer *et al.,* 1999; Zavaroni *et al.,* 1999; DeFronzo, 2004). Temporal priority of IR in the pathogenesis of T2DM has been disputed by a number of studies wherein beta-cell function has been carefully assessed and potentially confounding factors, such as obesity and adipose tissue distribution, accounted for (Pimenta *et al.,* 1995; Gerich, 1999). Not only may beta-cell dysfunction pathogenically precede IR, but there is also some evidence that the former may contribute to the latter. For example, loss of pulsatile insulin release, a beta-cell secretory dysfunction characteristic of type 2 diabetes, has been associated with the development of IR (Matthews *et al.,* 1983; Kahn, 2001). To consider IR as causative of beta-cell dysfunction, and thus of T2DM, seems counterintuitive if only because so many individuals with IR do not develop the disease (Weyer *et al.,* 1999; Leahy, 2005). Studies seeking to distinguish carefully between the relative contributions of IR and insulin secretory deficits to T2DM (both pathogenesis and pathophysiology) suggest that IR is neither a necessary nor a sufficient condition (Nesher *et al.,* 1987; Arner *et al.,* 1991; Byrne *et al.,* 1996). The key defect, in both lean and obese individuals with diabetes, is impaired insulin secretion. By contrast, lean patients with T2DM were not insulin resistant, and obese controls, while insulin resistant, did not have diabetes because their insulin secretory capacity was sufficient to maintain gluco-homeostasis (Nesher *et al.,* 1987).

2.5.3 Beta-cell dysfunction as primary defect

While not the primary cause of T2DM, IR remains an important treatment target; it is itself a risk factor for atherosclerosis and cardiovascular disease and, if untreated, promotes hyperglycemia and the glucotoxic environment to which beta-cells in susceptible individuals may be particularly vulnerable (Buchanan *et al.,* 2002; Bertoni *et al.,* 2007; Abbasi *et al.,* 2008). Nevertheless, a consensus of investigators and clinicians have determined that beta-cell dysfunction, commonly but not necessarily occurring in a subset of individuals with IR, is primarily responsible (both a necessary and sufficient condition) for T2DM. A functional beta-cell can compensate for IR indefinitely, perhaps for the lifetime of the individual; progressive loss of betacell function, on the other hand, is crucial to the development of T2DM and to the worsening of hyperglycemia even under treatment (Porte and Kahn, 1995; Del Prato *et al.,* 2007; Bonora *et al.,* 2008; Lencioni *et al.,* 2008). Loss of beta-cell function is likely to be accompanied by reduction in beta-cell mass. Autopsy studies have confirmed that functional decrements are associated with such reductions. Beta-cell apoptosis begins early in the disease process and at diagnosis between 40% and 60% of normal beta-cell volume may already have been lost (Butler *et al.,* 2003).

2.5.4 Mechanisms of beta-cell dysfunction

Contenders for the sources of beta-cell dysfunction include glucotoxicity, which may mean nothing more, at least initially, than that genetically susceptible beta-cells are extremely sensitive to even modestly elevated glucose levels. For these beta-cells, chronic glucose elevations even slightly above the normal range inaugurate a toxic environment characterized by deleterious changes in function, mass, and phenotype. Worsening beta-cell response then exacerbates the glucotoxic environment, which worsens beta-cell response in a vicious spiral of deterioration. Acute phase insulin response is lost; a number of stress response processes are activated, indicating an increase in cellular stress; and an upregulation of apoptotic genes has been noted (Weir and Bonner, 2004). Lipotoxicity associated with elevated levels of free fatty acids—commonly observed in the obese, the insulin resistant, and individuals with T2DM — has been linked to decreased insulin synthesis and increased metabolic stress in beta-cells (Standl, 2007; Poitout and Robertson, 2008). Human amyloid, expressed as islet amyloid polypeptide, is cytotoxic (Janson *et al.,* 1999). Amyloid deposition in beta-cells is a characteristic histopathology of T2DM, and has been observed in up to 90% of patients (Lencioni *et al.,* 2008; Finegood and Topp, 2001). Severity of amyloidosis correlates with

duration and severity of diabetes (Lorenzo *et al.,* 1994; Hayden and Tyagi, 2002; Hoppener and Lips, 2006; Lencioni *et al.,* 2008).

2.6 Causes of the rise in prevalence of diabetes

2.6.1. Genetic predisposition

Several studies on migrant Indians across the globe have shown that Asian Indians have an increased risk for developing T2DM and related metabolic abnormalities compared to other ethnic groups (Mohan *et al.,* 1986; McKeigue *et al.,* 1991; Abate and Chandalia, 2001). Although the exact reasons are still not clear, certain unique clinical and biochemical characteristics of this ethnic group collectively called as the "Asian Indian phenotype" is considered to be one of the major factors contributing to the increased predilection towards diabetes (Joshi, 2003; Deepa *et al.,* 2006). Despite having lower prevalence of obesity as defined by body mass index (BMI), Asian Indians tend to have greater waist circumference and waist to hip ratios (Ramachandran *et al.,* 1997) thus having a greater degree of central obesity. Again, Asian Indians have more total abdominal and visceral fat for any given BMI (Raji *et al.,* 2001) and for any given body fat they have increased insulin resistance (Chandalia *et al.,* 1999). Moreover, they have lower levels of the protective adipokine-adiponectin and have increased levels of adipose tissue metabolites (Abate *et al.,* 2004). Studies on neonates suggested that Indian babies are born smaller but relatively fatter compared to Caucasian babies and are referred to as "the thin fat Indian baby" (Yajnik, 2002; Yajnik *et al.,* 2003). Another study confirmed this finding and suggested that the "thin fat phenotype" in neonates persisted in childhood and could be a forerunner of the diabetogenic adult phenotype (Krishnaveni *et al.,* 2005). These findings suggest that Asian Indians are more prone to diabetes and related metabolic abnormalities. Genetic factors that determine body fat distribution and glucose metabolism have to be fully elucidated for the better understanding of the biochemical and molecular mechanisms behind the etiopathogenesis of diabetes. Studies have shown that while some genes seem to confer increased susceptibility to diabetes in Indians (Abate *et al.,* 2005; Vimaleswaran *et al.,* 2005), some protective genes in Europeans do not appear to protect Indians (Radha *et al.,* 2006).

2.6.2 Inherited factors in type 2 Diabetes Mellitus

Heritability estimates provide an indication of the extent to which genetic and environmental factors influence the variance of specific traits (phenotypes) within populations. Heritability is

formally defined as a ratio of variances, specifically as the proportion of total variance in a population for a particular measurement, taken at a particular time or age that is attributable to variation in additive genetic or total genetic values, termed narrow-sense heritability (h2) and broad-sense heritability (H2), respectively (Visscher *et al.,* 2008). The H2 for T2DM ranges from 26% to 75% (Kaprio *et al.,* 1992; Poulsen *et al.,* 1999; Barroso, 2005). The offspring risk ratio is often used to express the heritable risk of developing a disease. In the Framingham Offspring Study the RR in offspring with one diabetic parent was ~3.5, and when both parents had diabetes the risk ratio (RR) increased to ~6.0, compared with the risk in offspring of non-diabetic parents (Meigs *et al.,* 2000). The heritable risk of diabetes extends beyond the influence of the parents to other family members. In the Framingham study, a history of diabetes in any biologic ancestral family member or sibling independently and progressively increased diabetes risk in the proband. Multiple studies of twins also provide compelling evidence for a genetic component for T2DM. Estimates for concordance rates range from 0.29 to 1.00 in monozygotic (MZ) twins, while in dizygotic (DZ) twins the range was 0.10– 0.43 (Gottlieb and Root, 1968; Barnett *et al.,* 1981; Newman *et al.,* 1987; Kaprio *et al.,* 1992; Poulsen *et al.,* 1999; Medici *et al.,* 1999). Lastly, the high levels of heritability for insulin sensitivity and insulin secretion also supports a genetic component to diabetes (Gerich, 1998; Elbein *et al.,* 1999; Elbein *et al.,* 2000).

2.6.3 Ethnicity

Evidence for a genetic component of T2DM comes in part from ethnic-specific differences in prevalence rates for T2DM, which range from 1% in Chile Mapuche Indian, 2% among Caucasians in Europe, to frequencies as high as 41% in the Nauru (Pacific Island) and 50% among Pima Indians in Arizona (Diamond, 2003). A 2004–2006 U.S. national survey including people aged 20 years or older indicated that 11.8% of African-Americans, 10.4% of Hispanics-Americans, 7.5% of Asian- Americans, and 6.6% of non-Hispanic whites Americans had clinically manifest diabetes. Among Hispanics, rates were 12.6% for Puerto Rican Americans, 11.9% for Mexican Americans, and 8.2% for Cuban Americans (CDC, 2008). All these findings are age-adjusted. Ethnic variability can be partially attributed to non-genetic environmental and cultural factors. However, some studies show that diabetes prevalence differs markedly across ethnic groups, even when environmental exposures are similar. For example, Asians living in the UK have a prevalence of diabetes 3.8-fold higher than that in whites in the UK (Mather and Keen, 1985). According to the WHO, between 2000 and 2030,

Asia and Africa are likely to experience a 2–3-fold increased prevalence of T2DM (Wild *et al.,* 2004). The organisation further predicts that the most new cases of T2DM will emerge from India, China, and the USA, partly because these countries have some of the world's largest populations, but also because these are ethnically at risk populations that are rapidly adopting obesogenic lifestyles. According to the WHO, Bangladesh, Brazil, Indonesia, Japan, and Pakistan will also be heavily burdened by T2DM in the future.

2.7 Genetic Studies associated with type 2 Diabetes Mellitus

In 2001, the draft sequence of the human nuclear genome was published (Lander *et al.,* 2001; Venter *et al.,* 2001). The human genome consists of approximately 2.85 billion base pairs encoding about 20,000–25,000 genes. Although 99.9% of the human DNA sequence is thought to be identical between unrelated individuals, about 0.1% of coded DNA differs between the two chromosomal strands at the same base (Kruglyak and Nickerson, 2001). It is these differences that account for the diversity in human phenotypes and their responsiveness to environmental exposures including, but by no means limited to, diet and physical activity. DNA variation occurs in several known forms. Sequence variations occurring in less than 1% of the population are defined as mutations whereas more common variants are defined as polymorphisms (Strachan and Reed, 1999). SNPs are the most commonly studied form of genetic variant. Other well known polymorphisms are variable number of tandem repeats (VNTRs) that include mini-satellites (repeat sequences of several nucleotides) that tend to cluster near ends of chromosomes and microsatellites (usually as di-, tri-, and tetra-nucleotide repeats) that are distributed throughout the genome (Strachan and Reed, 1999), inversions, insertions, deletions, copy number variants (CNVs), and other complex rearrangements (Feuk *et al.,* 2006).

Polymorphisms are thought to occur because of selective pressures or randomly (genetic drift). Random variations eventually disappear as the contributing alleles become either fixed or extinct. Irrespective of the specific type of polymorphism, there is a plethora of common DNA sequence variants that can (and have) been used as disease predisposing markers in human population-based studies. Although many *bona fide* examples of disease associated polymorphisms have been identified, by and large, these are non-functional variants, which merely 'tag' the (often) unobserved causal variant. Approximately 12 million SNPs have been identified (Sachidanandam *et al.,* 2001). More than 90% of the genomic variability between individuals is thought to be attributable to SNPs (Palmer and Cardon, 2005). The majority of

these SNPs are biallelic and can be transition (purine-purine A↔G or pyramidine-pyramidine C↔T) or transversion (purinepyramidine or pyramidine-purine) substitutions (Kimura, 1980). Approximately two thirds of SNPs are transition substitutions between double ring structured purines (A↔G) or between single ring structured pyrimidines (T↔C), whereas one third of SNPs are transversion substitutions between a purine and a pyrimidine nucleotide (Guo and Jamison, 2005). The classification of SNPs is dependent on their genomic location. Coding SNPs (cSNP) are located in exons. Individual exons may contain coding DNA and/or non-coding DNA (untranslated sequences) and may be either synonymous or non-synonymous (Burton *et al.,* 2005). Synonymous SNPs are typically silent and alter the DNA sequence, but do not change the amino acid coding sequence. Non-synonymous cSNPs alter the DNA sequence in a coding region such that the amino acid coding sequence of the protein is changed. These cSNPs are prioritized as genetic markers because a change in the amino acid structure and function may impact the formation of the target protein. The majority of SNPs are located in the non-coding region of the genome (Strachan and Read, 1999). However, some of these intronic SNPs have no known function but may play a regulatory role in modulating gene expression of coding regions. These SNPs are termed regulatory or rSNPs. rSNPs located in the promoter region may affect transcription factor sites and rSNPs located in the 5□ UTR and 3□ UTR (untranslated regions) may also affect protein-binding sites by changing sequence motifs. rSNPs may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA. Intronic SNPs and intergenic SNPs (regions between genes) lie in the non-coding regions. It is generally thought that non-synonymous SNPs in a coding sequence are more likely to affect the function or availability of a protein than other SNP classes (Carlson *et al.,* 2004). However, all SNP types can cause disease, for example by altering the regulation of transcription of a critical protein. The true distribution of disease associated variants between non-coding and coding sequences is unknown (Carlson *et al.,* 2004).

There are at least six established ways in which SNP genotyping might help advance our understanding of the molecular basis to human disease (Schork *et al.,* 2000; Palmer *et al.,* 2005). These include:

1) Hypothesis-free gene discovery and mapping

2) Association-based candidate polymorphism testing

3) Diagnostics and risk profiling

4) Prediction of response to environmental stimuli

5) Pharmacogenetics

6) Homogeneity testing and epidemiological study design

2.7.1 Different types of polymorphic studies on type 2 diabetes

The most common polymorphism in the human genome and which has been most extensively used in genetic studies is the single-nucleotide polymorphism. Polymorphisms are terminologically distinguished from mutations by a frequency criterion meaning that different forms of the polymorphism termed alleles are observed more often in the general population than mutations, with a population frequency of < 1% often used as a cut-off value. Currently, more than 9 million SNP are described in databases. SNPs occur on average once every 200 base pairs in the human genome, many of those however being very rare (Crawford and Nickerson, 2005). Currently, the modest effect sizes of the known T2DM susceptibility variants limit their use, individually or in combination, in the prediction of disease risk (Lango *et al.,* 2008). It is not yet known how many the epigenetic effects, such as the maternal uterine environment impact on the risk of diabetes in the offsprings and thus inflates the estimates of heritability (Dabalea and Pettitt, 2001). In addition, not only the SNPs used in most studies but also the copy number variants in genome may associate with risk of T2DM. Gene-gene interactions and gene-environmental interactions could be contributory factors in the difficulties in finding T2DM genes (McCarthy and Zeggini, 2009). Three aspects on the study of T2DM genes are presented in the following paragraphs.

Linkage scans

In typical genetic linkage studies, correlations between inheritance of a trait and chromosomal regions within family units, such as sibling pairs or multigenerational pedigrees, are investigated. Linkage studies have had some success in identifying the molecular basis of monogenic diseases, but fewer successes with common, more complex phenotypes, such as T2DM or atherosclerosis (Hegele, 2002). In the late 1980 and 1990s, genome mapping provided genome wide collections of markers and technologies for typing linkages in hundreds of individuals. Multiple genome wide linkage scans for type 2 diabetes have been done, but no single region in the genome has been widely replicated in these studies. Recently, a large study combining 83 linkage reports was published and it provided some evidence that there might be major genes for type 2 diabetes in chromosomal locations 6q, 1q,1p, 2q, 20q, 17pq, 8p, 19q and 9q (Lillioja and Wilton, 2009). However, there has been no clear path for progressing from linkage to gene identification (Florez *et al.,* 2003). Studies are variable between populations. If only a subset of type 2 susceptibility genes is required for the disease and the frequencies of

these genes differ between populations, the results are variable. When the risk allele is present at a high frequency and has only a modest impact, linkage suffers a loss of power. This is because allele sharing of used markers is only observed if the risk allele is inherited from only one side of the pedigree (Florez *et al.,* 2003.)

Candidate gene studies

Candidate genes, either positional or functional, can be studied by linkage based methods or association studies. The typical association study is a case-control study, where the difference in allele frequency of candidate gene polymorphisms is examined between affected individuals and unrelated controls, for example between type 2 diabetes patients and healthy individuals. There are three possible explanations for a positive association between disease and allele. First, there might be a true association so that the gene variant actually has a causative role in the development of the disease. Second, the positive association can arise if the marker studied is in linkage disequilibrium with another truly causal locus. Linkage disequilibrium takes place, when two genetic markers occur together more frequently than would be expected from random association. This means that marker allele is so close to the other allele that these alleles are inherited together over many generations. This can happen because of a variety of reasons, including recent admixture of populations with different allele frequencies, selection in favour of a specific allele, genetic drift or population bottlenecks or new mutations (Weeks and Lathrop, 1995.) The linkage disequilibrium between alleles can be measured with some parameters. D is the most common measure, and can be calculated as $PAB - PA \times PB$, where PA is a frequency of allele A in the first loci and PB the frequency of allele B in the second loci. The value of D is dependent on allele frequencies, but another measure r^2 is the correlation of alleles at the two sites, and is formed by dividing D^2 by the product of the four allele frequencies at the two loci. The case of $r^2 = 1$ is known as perfect LD. In perfect LD, observations at one marker provide complete information about the other marker (Ardlie *et al.,* 2002.) Selection bias or another bias can also lead to false positive association. For instance, some population substrata may be more susceptible to develop T2DM because of an unmeasured factor, such as ethnicity or genetic background, for which the genotype is merely an indirect marker (Hegele, 2002). Therefore, genetic isolates, such as Finns with a history of a small founder population, long-lasting isolation and population bottlenecks represent exceptional resources in the identification of disease genes. It can also be assumed that the vast majority of cases are caused by the same mutation, and disease alleles reveal linkage disequilibrium (LD) with markers over significant genetic intervals compared to older populations (Peltonen, 2000.)

Candidate gene studies have had disappointing outcomes in the field of T2DM. Many studies have been limited in power; the small sample sizes are inadequate to detect the kinds of effects that are known to be realistic in complex traits. It might also be that the candidate genes are selected on the basis of limited knowledge of the etiology of the disease. In addition, there might have been a poor understanding of the architecture of genetic variation. The genotyping methods have been time-consuming and also the irreproducibility of results may have been further reinforced by liberal thresholds for defining significance and the tendency to over-interpret the results (McCarthy and Zeggini, 2009.)

Genome wide association studies

Over the last few years, genome-wide association studies (GWAS) have been extremely successful in the detection of loci for complex disease traits such as obesity and T2DM. The GWAS method involves testing associations with disease traits for a large number of genetic markers (usually more than 1,000,000 SNPs) over the whole genome. The method differs from the traditional biologic candidate gene approach, in that no specific hypothesis is tested; the approach instead relies heavily on replication of association signals across multiple populations and generally requires very large sample sizes to overcome the problems (related mainly to diminished power) inherent in conducting so many association tests (Hardy and Singleton, 2009). This finding highlights the relative importance of inherited defects in insulin secretion on beta-cells rather than insulin resistance in the etiology of T2DM (Florez, 2008; Prokopenko *et al.,* 2008). With the commercially available chips, a very large number of SNPs (105 -106) in the genome can nowadays be detected from an individual (Grant and Hakonarson, 2008). At least 30 GWA scans for T2DM have been performed (McCarthy and Zeggini, 2009). GWAs have proved to be successful in identifying susceptibility variants, but the power of individual studies to detect small or modest effects at common SNPs has been limited (McCarthy and Zeggini, 2009). It is very disconcerting that the GWA studies and linkage studies detect different genes; GWA studies may detect genes that linkage studies have been insufficiently powered to detect but linkage studies may identify genes that have multiple variants within the same gene and variants rare enough individually will be undetected by current GWA studies (Lillioja and Wilton, 2009). Continuous efforts through GWA meta-analysis and fine mapping may represent the way to explain the "missing heritability" of T2DM, since it is claimed that about 20 known variants explain only 5–10% of the inherited predisposition of T2DM (McCarthy and Zeggini, 2009).

2.8 Review of literature on candidate genes of the present study

2.8.1 Insulin Receptor (INSR) gene

The insulin receptor is a complex multifunctional protein subserving various biological effects. The receptor's organizational structure was firmly established, based largely on the amino acid sequence deduced after cloning of the insulin receptor cDNA (Ebina *et al.,* 1985; Ullrich et al.1985). Subsequently it was shown that the cDNA was derived from a gene composed of 22 exons located on chromosome 19 (Seino *et al.,* 1989). The insulin receptor is synthesized as a single-chain precursor polypeptide containing 1343 (Sudhof *et al.,* 1985) or 1355 (Williams *et al.,* 1989) amino acids preceded by a 27-residue NH_2-terminal signal sequence. During processing and transport to the cell surface, the 27-amino acid leader sequence is cleaved, and oligosaccharide side chains are added at specific glycosylation sites. Two monomers associate to form a dimeric structure, and a cleavage site consisting of four basic amino acids (Arg-Lys-Arg-Arg; positions 720-723) is removed, resulting in discrete α and β subunits. These subunits are held together by αβ-disulfide bonds to form the mature heterotetrameric αβ-insulin receptor. The two identical α-subunits are entirely extracellular and contain either 719 or 731 residues, depending on the presence or absence of a 12-amino acid insert (positions 720-731), which arises by alternate splicing of exon 11 into the insulin-receptor mRNA transcript in a tissue-specific manner (McClain *et al.,* 1989; Seino and Bell, 1989). The β-subunit is a 620-residue membrane-spanning protein containing an extracellular region of 194 residues, a transmembrane anchoring domain of 23 amino acids, and a 403-residue cytoplasmic extension. Activation of the insulin receptor on the plasma membrane of cells by binding of insulin is the initial event that triggers the insulin receptor-signalling cascade, leading to the multiple cellular responses induced by insulin (Ullrich *et al.,* 1985). Homozygous or compound-heterozygous mutations in the insulin receptor gene are found in patients with syndromes of severe insulin resistance (Williams *et al.,* 1989). Moreover, in patients whose insulin receptor mutations do not lead to a complete loss of insulin receptor function, milder syndromes of insulin resistance are reported, such as the Rabson-Mendenhall syndrome (OMIM 262190) and type A insulin resistance (OMIM147670) (Williams *et al.,* 1989; Yarden and Ullrich, 1989; Yarden and Ullrich, 1988). The less severe phenotype of these patients is believed to result from the retention of some functionality by these mutant insulin receptors. In 1988, the query of whether mutations in the insulin receptor (INSR) gene account for the insulin resistance in patients with non-insulin dependent diabetes mellitus (NIDDM) was raised (Kadowaki *et al.,* 1988).

Subsequently reports of NIDDM cases carrying mutations at INSR were observed (Taira *et al.,* 1989). These mutations end in receptors with slightly decreased kinase activity or affinity for insulin. With such cases, environmental factors including obesity may trigger the onset of diabetes. A reduction in the number of insulin receptors in obese people is also now known to cause resistance to insulin (Taira *et al.,* 1989). Proper diet and normal blood sugar levels may in turn improve the insulin excretion capacity by improving the function of the receptors (Taira *et al.,* 1989). A study on tyrosine kinase domain of insulin receptor gene of 103 patients revealed Arg 1152 Gln mutation (Cocozza *et al.,* 1992). Another study detected Gly 1008 Val in tyrosine kinase domain of insulin receptor gene which related to insulin resistance (Nozaki *et al.,* 1993). A Leu 1193 Trp mutation, causing defective tyrosine kinase activity was detected (Iwanishi *et al.,* 1993). A report on leu 1153Met mutation in tyrosine kinase domain of insulin receptor gene was observed in another study (Cama *et al.,*1991) Presence of Thr 831 Aal and Try 1334 Cys mutations (Kan *et al.,*1995); and Met 958 Val mutation were also reported (Elbein *et al.,*1993). Imano and Kawamori detected homozygote mutations in tyrosine kinase domain of insulin receptor gene, and proposed the more severity of the disease compared to those harbouring heterozygote mutations in tyrosine kinase domain of insulin receptor gene (Imano and Kawamori, 1994). A missense Arg 981 Gln and nonsense mutations in tyrosine kinase domain of insulin receptor gene were reported (Kusari *et al.,* 1991). Missense mutation (Arg 1174 Gln) was screened in tyrosine kinase domain of insulin receptor gene related to NIDDM (Hojlund *et al.,* 2004). Review of the literature and sequences of exon 3 identified four possible regions subject to genetic variation with a potential of five polymorphisms: a nonsynonymous transition (T↔C) in codon 233 (Seino *et al.,* 1990), a synonymous substitution at codon 234 (GAC to GAT) (Kadowaki *et al.,* 1990), a synonymous change at codon 276 (CAG to CAA), and two possible repeats just 5□ of exon 3 (Seino *et al.,* 1990a). Each of the three substitutions predicts a restriction site difference: *MspI*, *FokI*, and *RsaI* (3313C allele, 3317T and 3443T), respectively. These polymorphisms are located within exon 3, close to exon 2, which is the region that codes for the insulin binding site on the receptor (Hanis and Bertin, 1992). Another polymorphism, the NsiI RFLP at exon 8, has been associated with arterial hypertension (Schrader *et al.,* 1996). Very few studies have been carried out on the polymorphic sites in exon 3 of INSR gene; however the documented results are conflicting (Kadowaki *et al.,* 1990; Seino *et al.,* 1990; Hanis and Bertin, 1992; Kazemi *et al.,* 2009). Biochemical analyses of the various mutations/polymorphisms seen in patients with insulin-resistant syndromes provide insight into the residues of the insulin receptor that are critical for correct functioning and processing. Furthermore, by studying multiple patients with

the same mutation/polymorphism, insight can be obtained into the extent of genetic background an important modulator of phenotypic expression of insulin receptor gene.

2.8.2. Leptin receptor (LEPR) gene

The "*obese*" locus was first described 6 decades ago (Ingalls *et al.,* 1950) and was later shown by positional cloning to be the *LEP* gene that encodes a secreted protein, leptin (Zhang *et al.,* 1994). Mice and humans homozygous for a leptin gene mutation (*Lep ob/ob*) develop hyperphagia, early-onset obesity, severe IR, steatosis, hypothalamic hypogonadism, deficits of the thyroid and growth hormone axes, and immunosuppression (Montague and Farooqi, 1997; Ahima *et al.,* 2006). Circulating concentrations of leptin reflect fat cell stores – increasing with overfeeding and decreasing with starvation. Leptin administration to obese leptin-deficient animals has reversed their hyperphagia, hypothermia, decreased locomotor activity, and all neuroendocrine and immunological abnormalities (Friedman and Halaas, 1998). Leptin therapy also dramatically reduced body weight (mostly fat mass) in four children (Farooqi *et al.,* 1999; 2002; Gibson *et al.,* 2004) and three adults (Licinio *et al.,* 2004) with leptin deficiency. Leptin is expressed mainly in WAT adipocytes, though low levels are produced in the stomach, mammary gland, placenta, and skeletal muscle (Ahima *et al.,* 2006).

The diverse nature of leptin is supported by the universal distribution of leptin receptor (LEPR). *LEPR* was cloned in 1995 (Tartaglia *et al.,* 1995). It was identified to be the same gene which was mutated in diabetic *db/db* mouse (Lee *et al.,* 1996), which had earlier been shown to have a similar phenotype to *ob/ob* mouse, but to be resistant to leptin treatment (Leibel *et al.,* 1997). Also the leptin resistant Zucker *fatty* rat (*fa/fa*) carries a mutation in the *LEPR* gene (Iida *et al.,* 1996). *LEPR* localises to chromosome 1p31 (Chung *et al.,* 1996a), and the long form has 18 exons (Chung *et al.,* 1996b). LEPR is a single transmembrane-domain receptor, which displays a structural similarity to the class I cytokine receptor family (Fruhbeck, 2006). The LEPR is produced in several alternatively spliced forms, designated LEPRa–LEPRf, that share the common extracellular domain (>800 aa) and the transmembrane domain (34 aa), and have a variable intracellular domain, characteristic of each of the 52 isoforms (Fruhbeck, 2006). "Short forms" of LEPR may have roles in binding (LEPRe), transport (LEPRa), and clearance (LEPRc and LEPRd) of leptin (Margetic *et al.,* 2002), whereas only LEPRb, the "long form" (1162 aa), encodes all protein motifs capable of activating the janus kinase – signal transduction and transcription (JAK-STAT) pathway which in turn stimulates transcription of target genes (Gullicksen *et al.,* 2003). Although regulation of

hypothalamic neuropeptide gene expression by leptin is well described, the role of JAK–STAT signalling in this response remains uncertain, as leptin can affect neuronal firing rate independently of its transcriptional effects. For example, a subset of 'glucose-responsive' neurons in the hypothalamus become hyperpolarized (and therefore decrease their firing rate) within minutes of leptin application (Spanswick *et al.,* 1997). Glucose influences the membrane potential of glucose-responsive neurons indirectly through its oxidation to generate ATP, which in turn controls the activity of ATP-sensitive potassium channels (K_{ATP}) in the plasma membrane (Dunn-Meynell *et al.,* 1998). Closure of K_{ATP} channels in response to increasing intracellular concentrations of ATP (relative to ADP) raises intracellular [K^+], which depolarizes the cell and increases its firing rate. Because leptin maintains K_{ATP} channels in the open configuration (Spanswick *et al.,* 1997), positively charged K^+ ions diffuse out of the cell and lower its membrane potential. This effect on K_{ATP} channels can be detected even in isolated patches of plasma membrane, excluding a mechanism involving transcriptional effects of leptin.

Leptin resistance is common in obesity, and the mechanisms responsible for this phenomenon may include defects of the leptin receptor, leptin transport, genes involved in leptin signal transduction, or defective transport of leptin across the blood-brain barrier (Gullicksen *et al.,* 2003). At the level of pancreatic β-cell, leptin resistance may contribute to dysregulation of the adipo-insular axis and promote the development of hyperinsulinaemia and manifest as T2DM in overweight patients (Seufert, 2004). Mutations in *LEPR* are rare; in a recent study, subjects with hyperphagia and severe early-onset obesity were sequenced for *LEPR* mutations, and only 8 nonsense or missense mutations were found, each resulting in impaired receptor signalling (Farooqi *et al.,* 2007). However, the *LEPR* gene is highly polymorphic, and associates with obesity traits in several studies. The long form of the *LEPR* gene is expressed in pancreatic beta-cell (Kieffer *et al.,* 1996), where it is proposed to mediate leptin-induced inhibition of insulin secretion (Kieffer *et al.,* 1997; Emilsson *et al.,* 1997). This mechanism has been suggested to explain the occurrence of hyperinsulinaemia in ob/ob and db/db mice (Kieffer *et al.,* 1997). Furthermore, linkage between the acute insulin response and the microsatellite marker D1S198 flanking the leptin receptor gene, has also been reported in Pima Indians (Thompson *et al.,* 1995). Several studies thus favour the idea that the leptin receptor may play a role in regulation of insulin secretion.

A number of sequence determinants are known to affect mammalian mRNA abundance (Ross, 1995). Occurrence of multiple copies of the core sequence UUAUUUA(U/A)(U/A), designated as the A+U destabilizing element (AUDE), is known to destabilize a variety of mRNA species

(Brown *et al.,* 1996). A closely reminiscent sequence of 5□-UUACUUUAUA-3□ is created in the 3□-UTR of the insertion allele; whether the extra C present in this sequence eliminates the destabilizing effect of the core sequence, is currently not known. A common CTTTA-pentanucleotide *Del/Ins* variant of the 3'-UTR part of the *LEPR* gene is believed to generate an A+U-rich sequence, that should be able to form a stemloop structure, which may affect mRNA stability in the cell (Oksanen *et al.,* 1998). The *Del/Ins* variant has been found to be associated with serum insulin levels (Oksanen *et al.,* 1998; Lakka *et al.,* 2000), serum high density lipoprotein (HDL)-cholesterol and apolipoprotein A (apoA)-I levels (Nishikai *et al.,* 2004), and susceptibility to T2DM (Lakka *et al.,* 2000). It is, potentially, of greater importance that a motif compatible with stem-loop formation, with eight complementary nucleotides forming the stem and ten nucleotides the loop, is generated in the 3□-UTR of the pentanucleotide insertion-containing mRNA molecule (Fig 1).

Stem-loop sequences have been detected in a number of transcripts, including mRNAs for histones, transferrin receptor, insulin-like growth factor II and cytokines (Ross, 1995; Brown *et al.,* 1996). Although the mechanisms by which stem-loops affect mRNA stability is not completely understood, in many cases these motifs have been shown to bind to regulatory proteins, which in turn could affect the rate of degradation and/or translation of mRNA (Ross, 1995). Associations of *LEPR* Ins/Del variants with diabetes and related traits are listed in the Table 2.

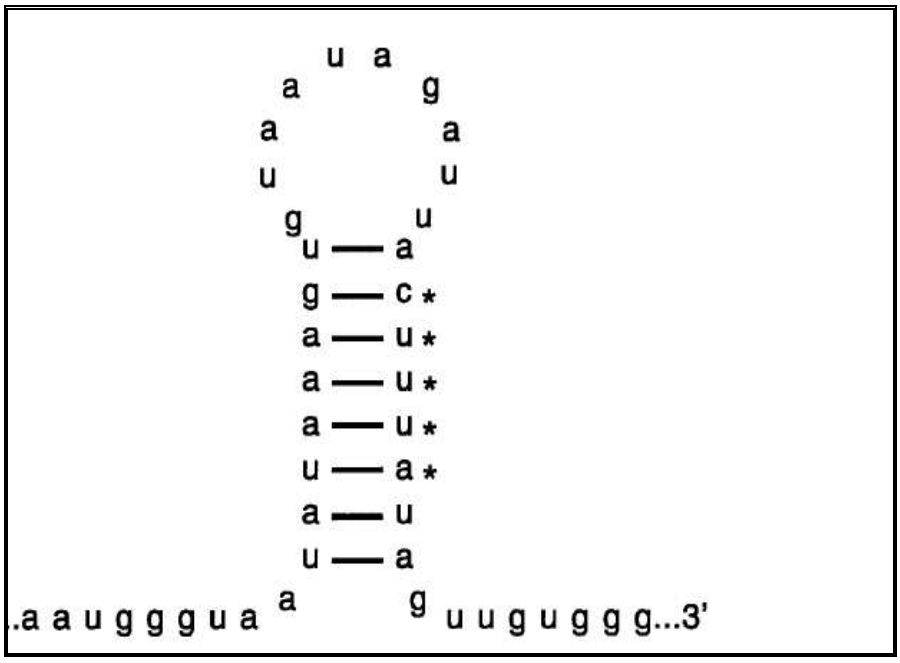

Figure 1: Putative stem-loop structure generated by the presence of the CUUUA insertion (marked with asterisks) in the leptin receptor mRNA

Table 2: Associations of *LEPR* Ins/Del variants with diabetes and related traits

Subjects	Variant	Risk allele	Association	Reference
Finnish Caucasoid	*3'UTR del/ins*	*Del*	Higher serum insulin	Oksanen *et al.,* 1998
Caucasoid (men)	*3'UTR del/ins*	*Del*	Higher serum insulin Higher risk of diabetes	Lakka *et al.,* 2000
Japanese	*3'UTR del/ins*	*Ins*	Lower HDL-cholesterol and apoA-I Levels	Nishikai *et al.,* 2004
Finnish	*3'UTR del/ins*	*Del*	Higher body weight	Salopuro *et al.,* 2005
Multicentre study	*3'UTR del/ins*	*Del*	Lower weight and WC reduction	Zacharova *et al.,* 2005
Italian	*3'UTR del/ins*	*Del*	Higher risk of diabetes Higher serum Insulin levels	Nannipieri *et al.,* 2006

HDL: high density lipoprotein, apoA: apolipoprotein A, WC: waist circumference
Del: Deletion, Ins: Insertion

2.8.3 β_3-adrenergic receptor (ADRB3) gene

The β_3-adrenergic receptor crosses the cell membrane seven times, is coupled to guanine-nucleotide–binding (G) proteins, and is localized in adipose tissue (Figure 2.5). Stimulation of the receptor by β_3-adrenergic agonists activates adenylate cyclase, which increases intracellular concentrations of cyclic AMP (cAMP) and results in increased lipolysis and thermogenesis (Emorine *et al.,* 1989; Krief *et al.,* 1993; Spronsen *et al.,* 1993). There is evidence that molecular abnormalities in the β_3-adrenergic receptor may lead to obesity and T2DM. Expression of the receptor is markedly decreased in rodent models of obesity (Muzzin *et al.,* 1991; Collins *et al.,* 1994); mice with knockout (disruption) of the gene for the receptor have marked reductions in lipolysis stimulated by β_3-agonists (Susulic *et al.,* 1995) and β_3-specific agonists have potent antiobesity and antidiabetic effects in both animals (Mitchell *et al.,* 1992; Himms *et al.,* 1994) and humans (Mitchell *et al.,* 1989; 1992).

The β_3-adrenergic receptor is expressed in visceral fat in humans 11 and is considered responsible for increases in lipolysis and the delivery of free fatty acid into the portal vein (Lönnqvist *et al.,* 1995). An increase in visceral fat mass, in turn, correlates with resistance to insulin in skeletal muscle (Colberg *et al.,* 1995). Any abnormality in the β_3-adrenergic receptor could therefore explain the link between abdominal obesity and insulin resistance.

The β_3-adrenergic receptor (ADRB3) is mainly expressed in adipose tissue, and contributes to variations in energy expenditure and body fat distribution (Clement *et al.,* 1995; Shuldiner and Sabra, 2001; Lowell and Bachman, 2003). Polymorphisms of the ADRB3 have been suggested to participate in the etiopathogenesis of obesity (Arner, 1995; Shuldiner and Sabra, 2001). In particular, a single nucleotide polymorphism in the ADRB3 gene (substitution of tryptophan 64 with arginine (Trp64Arg)) has been associated with obesity, insulin resistance, abnormal lipid profile and arterial hypertension (Walston *et al.,* 1995; Widen *et al.,* 1995; Urhammer *et al.,* 1996; Strazzullo *et al.,* 2001; Hallman *et al.,* 2004; Hao *et al.,* 2004). However, since the prevalence of the Trp64Arg mutation differs among ethnic groups (Shuldiner and Sabra, 2001), other studies have failed to show any relationship between this polymorphism and obesity (Kurokawa *et al.,* 2001; Frederiksen *et al.,* 2003). Furthermore, it has been suggested that the association of this ADRB3 polymorphism with body weight and obesity related phenotypes may be dependent upon the presence of other susceptibility genes and/or exposure to other environmental factors (Shuldiner and Sabra, 2001). Several case-control studies revealed that the polymorphism of the β_3-adrenergic receptor gene (Trp64Arg) is associated with the development of NIDDM, insulin resistance, obesity and syndrome X (Reaven, 1988). It was

also reported that the Trp64Arg polymorphism is significantly associated with the development of NIDDM and obesity in Pima Indians (Wallston *et al.,* 1995), Finns (Widen *et al.,* 1995) and French Caucasians (Clement *et al.,* 1995). Moreover, the locus of Trp64Arg mutation was reported to be associated with the development of obesity and hyperinsulinemia in Japanese (Kadowaki *et al.,* 1995; Yoshida *et al.,* 1995; Fujisawa *et al.,* 1996). However, other studies suggested that this polymorphism is not associated with the development of NIDDM and obesity (Li *et al.,* 1996; Oksanen *et al.,* 1996; Urhammer *et al.,* 1996; Nagase *et al.,* 1997; Ueda *et al.,* 1997; Azuma *et al.,* 1998). Various studies showing associations of Trp64Arg variant in ADRB3 gene with diabetes and related traits are listed in chronological order in Table 3.

Table 3: Associations of *Trp64Arg* variant in ADRB3 gene with diabetes and related traits

Subjects	Risk allele	Association	Reference
Pima Indian	*Arg*	Lower age at onset of T2DM	Walston *et al.,* 1995
French Caucasoid	*Arg*	Higher weight gain during adult life	Clement *et al.,* 1995
Finnish Caucasoid	*Arg*	Lower age at onset of T2DM Higher WHR, insulin response in OGTT, dBP, and lower rate of glucose disposal (in NGT)	Widen *et al.,* 1995
Japanese	*Arg*	Higher BMI, fasting and 2h insulin in OGTT (in NGT)	Kadowaki *et al.,* 1995
Finnish Caucasoid	*Arg*	Earlier onset of obesity	Oksanen *et al.,* 1996
Japanese	*Arg*	Greater VAT area, higher body fat, sBP, glucose, insulin, cholesterol, and TG levels,Lower HDL-cholesterol	Sakane *et al.,* 1997
Mexican American	*Arg*	Lower age at onset of T2DM Higher 2h insulin in OGTT	Silver *et al.,* 1997
Mexican American	*Arg*	Higher BMI, fat mass, and WC	Mitchell *et al.,* 1998
Swedish Caucasoid	*Arg*	Higher BMI, lower sensitivity for receptor agonist in VAT adipocytes	Hoffstedt *et al.,* 1999
Mexican American	*Arg*	Interaction with *PPARγ2 Pro12Ala* on higher BMI, insulin, and leptin levels	Hsueh *et al.,* 2001
Chinese (neonates)	*Arg*	Higher insulin and insulin-to-glucose ratio	Wang *et al.,* 2004
Hungarian children	*Arg*	Higher weight, body fat, sBP, and insulin levels	Erhardt *et al.,* 2005
Japanese	*Trp*	Higher frequency of BP elevation	Masuo *et al.,* 2005
Turkish	*Trp*	Type 2 Diabetes	Turgay, 2007
Kyrgyz	*Arg*	Metabolic syndrome	Mirrakhimov *et al.,* 2011

T2DM, Type 2 diabetes mellitus; NGT, normal glucose tolerance; WHR, waist-to-hip ratio; OGTT, oral glucose tolerance test; dBP, diastolic blood pressure; BMI, body mass index; VAT, visceral adipose tissue; sBP, systolic blood pressure; TG, triglyceride; HDL, high density lipoprotein; WC, waist circumference.

AIM & OBJECTIVES

The aim of the research is to determine for the first time the prevalence of various polymorphisms in Insulin receptor (INSR), leptin receptor (LEPR) and β_3-adrenergic receptor (ADRB3) genes in the Type 2 Diabetes Mellitus patients of Kashmir valley and appraise their possible associations with the risk factors and severity of disease.

- To verify a non-synonymous transition Leu233Pro (T↔C), and two synonymous substitutions Asp234 (C↔T) and Gln276 (G↔A) in the exon 3 of *INSR* gene in patients with Type 2 Diabetes Mellitus.
- To evaluate the prevalence of 3′ UTR *Ins/Del* polymorphism of *LEPR* gene in Type 2 Diabetes Mellitus.
- To assess the prevalence and association of Trp64Arg (T↔C) polymorphism in *ADRB3* gene in Type 2 Diabetes Mellitus.
- To correlate the genotypes with risk factors and severity of disease.

MATERIAL & METHODS

The research entitled "Evaluation of Insulin receptor (INSR), Leptin receptor (LEPR) and β_3-adrenergic receptor (ADRB3) gene polymorphisms in Diabetes Mellitus patients of Kashmir valley" is a prospective research undertaken in the department of Biochemistry, University of Kashmir in collaboration with Sheri-I-Kashmir Institute of Medical Sciences (SKIMS), Srinagar Kashmir.

4.1 Sample Size

A total of 500 ethnic Kashmiri subjects were selected for the study, which includes 200 type 2 Diabetes mellitus patients and 300 healthy controls.

4.2 Definition of Case

Selection of cases was based on American Diabetes Association (ADA) and/or World Health Organisation (WHO) criteria for type 2 Diabetes Mellitus. The controls comprise of age and gender matched healthy non-diabetic subjects.

4.2.1 Inclusion Criteria

- Subjects with symptoms of diabetes (thirst, increased urination, unexplained weight loss) plus a random plasma glucose concentration > 200 mg/dL (11.1 mmol/L)
- Subjects with fasting plasma glucose > 125 mg/dL (7.0 mmol/L) after an overnight (at least 8-hr) fast
- Subjects with two-hour plasma glucose > 200 mg/dL (11.1 mmol/L) during a standard 75 g oral glucose tolerance test
- Subjects with HbA1c level >6.5 %
- Documented cases of diabetes mellitus

4.2.2 Exclusion Criteria

- Unwilling patients
- HIV positive patients
- Drug induced diabetes patients
- Stress induced diabetes patients
- Gestational diabetes patients
- Genetic syndromes associated with diabetes
- Subjects not complying with the requisite patient proforma

4.3 Anthropometric and Systemic measurements

Weight and height were measured using standard anthropometric techniques with subjects in light-weight clothing without shoes. Waist circumference was measured midway between the lower rib margin and the iliac crest at the end of gentle expiration using a metal anthropometric tape. Anthropometric measurements, including Body Mass Index (BMI) and Waist to Hip ratio (WHR) were calculated for each subject. BMI was expressed as kg/m^2. BMI is used to reflect the total body fat, while WHR is an indirect measurement of body fat centralization. Hypertension was defined as a systolic pressure >140 mmHg or diastolic pressure >90 mmHg.

4.4 Sample Collection

Collection of samples was conducted in the Endocrinology Department of SKIMS, Srinagar Kashmir. After proper consent from each subject, 3-4 ml of blood was collected in EDTA vacutainer. Plasma was separated and immediately sent for biochemical analysis. The samples were stored at -20º C until processed. DNA was extracted from peripheral leukocytes according to Phenol-Chloroform method (Sambrook and Russel, 2001).

Note: Chemicals used and preparation of reagents are illustrated in *Apendix I* and *Apendix II* respectively.

4.5 DNA extraction from Blood

1. To 3 ml of blood, 9 ml of erythrocyte lysing buffer was added.
2. The contents were shaken gently, incubated for 30 min on ice, and centrifuged at 4000 rpm for 10 min.
3. The supernatant (blood waste) was removed and the pellet was resuspended in 9 ml of erythrocyte lysis buffer and centrifuged for 10 min at 4000 rpm.
4. The supernatant was discarded. 5 ml of SE-buffer, 20 µl proteinase K (10mg/ml) and 100 µl 20% SDS were added to the pellet. The contents were shaken gently and incubated overnight at 37°C in a water bath. During this step the white blood cells' membranes are denatured and DNA goes out in solution.
5. Next day, equal volume of Tris-equilibrated (TE) phenol was added to the contents and shaken slightly for 10 min. The tubes were centrifuged at 3000 rpm for 5 minutes.
6. The supernatant was aspirated and transferred into a new tube. Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the contents. The emulsion was then centrifuged for 10 min at 2000 rpm with minimal breaking force. After centrifugation

two phases were observed: DNA is extracted into the supernatant and proteins separated into the lower phase.

7. The aqueous phase was removed using a Pasteur pipette without disturbing the protein interface and was transferred into a new tube to which equal volume of chloroform/isoamylalcohol (24:1) were added. The tubes were shaken for 10 min and centrifuged at 3000 rpm for 5 min.
8. The upper aqueous phase (containing the DNA) was transferred into a clean and sterile conical centrifuge tube using a sterile Pasteur pipette, followed by the addition of 300µl of chilled 3M sodium acetate (pH 5.2) and an equal volume of isopropanol along the sides of the tube.
9. DNA was precipitated by gentle swirling of the tube and observed visually as a white thread like strand that was spooled out using a cut-tip and transferred into a 1.5 ml eppendorf tube.
10. The DNA was washed twice in 70% ethanol by inversion to clean it from any remaining salts and the tube was centrifuged at 11000g for 4 min. The supernatant was discarded without disturbing the DNA pellet.
11. After discarding the supernatant the pellet was air dried from excess ethanol by leaving the tubes open and inverted.
12. The dried pellet was re-suspended in 300 µl of DNA storage buffer and left overnight at 37º C for complete dissolution.
13. DNA purity and concentration was determined spectrophotometerically (Pharmacia, Gene Quant) by measuring the absorbance at 260 nm. Purity was assessed by the $A_{260/280}$ ratio.
14. The quality of extracted DNA was determined by electrophoresis, on 1% agarose gel.
15. DNA was dissolved in TE-storage buffer at -20ºC for longer storage and shelf life.

4.6 Genotyping

4.6.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR), a powerful technique developed by a team headed by Kary Mullis at Cetus Corporation, is used to amplify a segment of DNA in vitro (Saiki *et al.,* 1985; Mullis *et al.,* 1987). This method utilizes a DNA polymerase and two oligonucleotide primers to synthesize a specific DNA from a single stranded template sequence. The oligonucleotides typically have different sequences and are complementary to sequences that lie on opposite strands of the template DNA and flank the segment of DNA that is to be amplified. The length of the primers usually 20 bases or more must be sufficient to overcome the statistical likelihood

that their sequence would occur randomly in the overwhelmingly large number of non-target DNA sequences in the sample. PCR is carried out in a series of cycles. Each cycle begins with a denaturation step to render the target DNA single stranded. This is followed by an annealing step during which the primers anneal to their complementary sequences so that their 3' hydroxyl ends face the target. Finally each primer is extended through the target region by the action of DNA polymerase. Since the products of one round of amplification serve as templates for the next cycle, the three step cycles are repeated until a sufficient amount of the product is produced. The major product of this exponential reaction is a segment of double-stranded DNA whose termini are defined by the 5' termini of the oligonucleotide primers and whose length is defined by the distance between the primers. The earliest PCR experiments utilised the Klenow fragment of Escherichia coli DNA polymerase I at a temperature of 37°C and often produced incompletely pure target products as judged by gel electrophoresis. However, the isolation of a heat-resistant DNA polymerase from Thermus-aquaticus (Taq) (Chien *et al.,* 1976) allows primer annealing and extension to be carried out at an elevated temperature, thereby reducing mismatched annealing to non-target sequences. This added selectivity results in the production of large amounts of virtually pure target DNA. Another important advantage of Taq polymerase is that it escapes inactivation at higher temperatures and need not be replaced after every denaturation step. This has allowed automation of PCR using machines that have controlled heating and cooling capability.

4.6.1.1 Procedure for PCR amplification of INSR, LEPR and ADRB3 genes

All precautions were taken to ensure contamination free amplification of DNA. The primer sequence, annealing temperatures and amplicon size is given in Table 4. For INSR gene, a 533 bp segment of exon 3 encompassing the 233, 234 and 276 SNPs was amplified using a common primer pair. A 114/119 bp sequence (depending upon the Del or Ins allele present) in 3 UTR of LEPR gene was amplified. In ADRB3 gene, a 250 bp region was amplified encompassing the Trp64Arg polymorphism.

Protocol

The amplification reaction was carried out in 25µl reaction volume in 0.2ml PCR tubes. The reaction contained the following reagent volumes:-

⇒	10X PCR buffer	2.5µl
⇒	10mM dNTP mix	0.5µl
⇒	Forward Primer	0.5µl
⇒	Reverse Primer	0.5µl

⇒	*Taq* DNA polymerase (1U)	0.2µl
⇒	Genomic DNA (250 ng)	1.0µl
⇒	Deionized water	19.8µl
	Total volume	**25.0µl**

For 50µl reaction (especially for sequencing) the reagent volumes along with template DNA were simply doubled. The following temperature profile was used for amplification:-

1. Initial denaturation	95°C for 4 min
2. Denaturation	95°C for 30 sec
3. Annealing	x°C for 30 sec*
4. Extension	72°C for 30 sec
5. Final Extension	72°C for 7 min.

* x was 3-5°C lower than the melting temperature (T_m) of the primers.

Temperature profile from step 2-4 was used for 35 cycles before final extension.

Table 4: Primer sequence, Annealing Temperature and Product Size of *INSR*, *LEPR* and *ADRB3* gene

Gene	*SNP*	*Primers*	*Annealing Temperature*	*Product Size (bp)*
INSR	***233 (T↔C)*** ***234 (C↔T)*** ***276 (G↔A)***	Forward: 5′-ACAGGAATTGGACAAAGCCAT-3′ Reverse: 5′-AGCAGAGACCTCACTCATAGCCAA-3′	***58ºC***	***533***
LEPR	***Del/Ins***	Forward: (5′-CATGCCTCAATTCCAAAC-3′) Reverse: (5′-GAGAGAACAAACAGACAACATT-3′).	***55 ºC***	***114/119***
ADRB3	***Trp64Arg***	Forward: 5'-CCAGTGGGCTGCCAGGGG-3' Reverse: 5'-GCCAGTGGCGCCAACGG-3'	***65ºC***	***255***

4.6.1.2 Detection of PCR products

The amplified products were detected by electrophoresis on 2% agarose in a mini gel system. The fidelity of amplicons was confirmed by running a 100bp DNA marker along with the PCR products. Electrophoresis was carried out at 100 volts until the loading dye had migrated to a distance of two thirds from the wells. The gel was examined on a Gel-documentation system and photographed.

4.6.2 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences that result from differing locations of restriction enzyme sites. The basic technique for detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digestion. The resulting DNA fragments are then separated by length through agarose gel electrophoresis.

The SNPs in INSR and ADRB3 genes were analyzed by RFLP. The enzyme characteristics, RFLP programme of INSR and ADRB3 genes are shown in Table 5a, 5b and 5c respectively.

The PCR products of INSR gene were simultaneously digested with *MspI*, *FokI* and *RsaI*. Each enzyme is characteristic of the restriction site created by the 233, 234 and 276 SNPs respectively. The wild alleles remain uncut upon digestion with these restriction enzymes where as the variant allele is digested giving a fragment characteristic of the variant present at the polymorphic site. The Trp64Arg (T↔C) polymorphism in ADRB3 gene was assessed using *MspI* restriction enzyme. The wild allele (TT) remains uncut where as the variant allele (CC) is digested into 158 bp and 97 bp fragments. The restriction digestion products were analysed on 2.5% agarose gel electrophoresis.

Table 5a: ***Characteristics of restriction enzymes***

Enzyme	Restriction site	Source	Conc.
MspI	5□...C↓CGG…3□ 3□…GGC↑C…5□	*Moraxella species*	10U/µl
RsaI	5□…GT↓AC…3□ 3□…CA↑TG…5□	*Rhodobacter sphaeroides*	10U/µl
FokI	5□…GGATG(N)$_9$↓NNNN…3□ 3□…CCTAC(N)$_{13}$↑…5□	*Flavobacterium okeanokoites*	10U/µl

Table 5b: ***RFLP programme for INSR gene***

Reagents	**Volume**	**Incubation**
Nuclease free Water	15µl	Incubation was carried at 37°C for 12 hours
10x Buffer	2µl	
PCR Product	10µl	
MspI *RsaI* *FokI*	1µl 1µl 1µl	

Table 5c: ***RFLP programme for ADRB3 gene***

Reagents	**Volume**	**Incubation**
Nuclease free Water	17µl	Incubation was carried at 37°C for 12 hours
10x Buffer	2µl	
PCR Product	10µl	
MspI	1µl	

4.6.3 Single-strand conformation polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) technique is a simple and efficient means to detect any small alteration in PCR-amplified product. It is based on the assumption that slight nucleotide change affects the migration of single-stranded DNA fragment and, therefore, results in visible mobility shifts across a non-denaturing polyacrylamide gel (Orita *et al.,* 1989). Polyacrylamide gel is used for analysis of DNA with specialized buffer systems and without urea. In nondenaturing PAGE the components used to synthesize matrix are acrylamide monomers, N, N-methylene bisacrylamide (bis), ammonium persulphate (APS) and N,N,N□,N□-tetramethylenediamine (TEMED). APS when dissolved in water generates free radicals, which activate acrylamide monomers inducing them to react with other acrylamide molecules forming long chains.

4.6.3.1 Procedure for *SSCP to detect Del/Ins polymorphism in LEPR gene*

A 5 bp Del/Ins polymorphism in LEPR gene was screened using PCR-SSCP. The 114/199 bp region was amplified using PCR technique. Del/Ins polymorphism was assessed by SSCP. The SSCP technique involved the following steps:

4.6.3.1.1. Preparation of glass plates

A pair of glass plates one notched and the other unnotched (21.5x30.0) cm^2 and spacers were thoroughly rinsed with deionised water. To assemble the plates, two spacers (0.4mm thick) were placed on the edges of the plate. The plates were clamped together on one side with bull dog binder clips, and the other side and the bottom were sealed with gel sealing tape.

4.6.3.1.2. Preparation of gel solution

12% non-denaturing polyacrylamide gel was used to analyse the PCR products. The gel was prepared as follows:-

⇒	30% acrylamide:bis-acrylamide stock solution	40 ml
⇒	5x TBE	20 ml
⇒	50% Glycerol	20 ml
⇒	10% Ammonium persulphate	0.7 ml
⇒	Distilled water	19.3ml
	Total volume of the gel solution	**100 ml**

50µl of TEMED was added to the gel solution just before pouring it into the mould of glass plates.

4.6.3.1.3. Casting of the gel

The glass plate mould was placed at a ~10° angle to the working table. 30ml of gel solution was drawn into a 50 ml hypodermic syringe ensuring no air bubble goes into the syringe. A thin stream of gel solution was allowed to flow at the top corner between the plates. Immediately a comb was inserted into the gel solution to form the sample wells. The gel was allowed to polymerize for about 45 min. After polymerization the comb was removed and the wells were washed with 1x TBE buffer to remove any un-polymerized gel solution.

4.6.3.1.4. Denaturation of PCR products

5µl of each PCR product of LEPR gene was mixed with 15µl of formamide gel-loading buffer (see appendix 1), heat denatured at 95°C in thermocycler for 5 min and immediately chilled on ice for 10-15 min.

4.6.3.1.5. Electrophoresis

The glass plate mould with polymerized gel was assembled into an electrophoresis apparatus. The lower buffer reservoir was fitted to the electrophoresis apparatus and filled with 0.5x TBE made from the same stock as gel solution. With the help of Hamilton syringe 5-6µl of sample (denatured PCR products+ formamide gel-loading buffer) was loaded on the gel. The upper buffer reservoir was then placed properly on the plates and filled with 0.5x TBE buffer. The electrodes were fitted to a high voltage power supply. Electrophoresis was carried out at a constant voltage of 3-4 V/cm of gel for nearly 16-18 hours. After electrophoresis was completed, the plates were removed from the electrophoresis apparatus and separated. The plate with the gel stuck to it was placed in a gel staining tray for silver staining.

4.6.3.1.6. Silver staining

Silver staining was used to detect the DNA bands (mobility shift) in the SSCP gel. Silver staining was performed as follows:

1. To the gel in staining tray, gel fixing solution was added with volume sufficient to cover the gel by ~2-3mm. The gel was allowed to fix for 30 minutes.
2. Gel fixing solution was removed by suction and the gel was rinsed twice with distilled water.
3. Silver staining solution was added to stain the gel for 15-20 minutes.
4. The silver staining solution was removed by suction and the gel was rinsed three times with distilled water.
5. Developer was added and the gel was kept in the developer until brown to deep brown DNA bands appeared. Upon the appearance of bands, the developer was immediately removed and distilled water was added to wash the gel for 2 minutes.

6. Distilled water was removed by suction and the developing was stopped by adding stopping solution for 10 minutes.
7. After giving the stop solution treatment, the gel was immediately photographed and labelled for documentation.

4.7 DNA sequencing

10% of the PCR products of INSR and ADRB3 gene, and bands showing mobility shift in the LEPR gene were sent for sequencing. The services of Macrogen (South Korea) were availed for sequences. Results were obtained in the portable document format.

4.8 Statistical analysis

Statistical Package for Social Sciences (SPSS, version 16.0) and Java Stat software was used for statistical analysis. Data was described as Mean ± SD and percentage, unless stated otherwise. Inter group variants of metric data was measured by Students t-test at 95% confidence interval. The non metric data was analyzed by Mann Whitney-U test. Fischer's exact test and odds ratio analysis was used for genotyping analysis where *P* value of <0.05 was considered to be significant. Hardy and Weinberg equilibrium was determined by χ^2 analysis.

RESULTS & DISCUSSION

5.1 Anthropometric, Systemic and Clinical Parameters of study and control population

This study comprised of 500 subjects ethnic Kashmiri subjects. 200 cases of T2DM and 300 non-diabetic controls were evaluated. The comparison of anthropometric, systemic and clinical parameters between study and control population is shown in Table 6. The mean age of cases and controls was 50.4 ± 11.1years and 49.2 ± 12.4 years respectively. Subjects were matched for age and gender. In both cases and controls, the predominant age group was 51-60 years (Fig 2). Mean BMI of cases and controls was 24.2±5.3 kg/m^2 and 21.2±3.3 kg/m^2, respectively (Fig 3). The cases presented with higher BMI and WHR as compared to controls ($p<0.001$). This is indicative of the role of central obesity in the etiology of T2DM. The systemic examination showed that 54% of cases were hypertensive which was significantly higher than the control population ($p<0.001$). Overall, 85.5% of cases had abnormal levels of fasting blood sugar (>125 mg/dl) and 75.5% had abnormal post prandial glucose levels (>200 mg/dl). The status of disease was determined by HbA1c levels (Fig 4). Only 22% of cases were in the controlled state (<7%), 29.5% of cases had mild status of diabetes (7-8%) whereas 48.5% of cases suffered from uncontrolled diabetes (>8%). Deranged lipid profile was observed in study population (Fig 5). TG levels (>150 mg/dl) was observed in 55.5% of cases and 35% of control subjects ($p<0.001$). High LDL levels (>130) were seen in 18% of cases and only 8.7% of controls ($p=0.002$) where as 72.9% of cases had significantly lower HDL levels than in controls ($p=0.003$). Higher cholesterol levels (>200 mg/dl) was observed in cases than in controls ($p<0.001$). Elevated serum Creatinine levels (>1.5 mg/dl) were observed in 20.5% of cases as compared to 1.3% of controls ($p<0.001$). Based on the type of treatment regimes, patients were categorized into three groups, viz. Insulin, oral antidiabetic drugs (OADs) and a combination of both. Out of these three groups, 40.5 % of the cases were on OADs, 31.5% were using insulin and 28% were undergoing both the treatments (Insulin + OADs). OADs was the most common form of treatment since majority of the patients recruited for the study were newly detected cases of type 2 diabetes mellitus.

Table 6: Anthropometric, Systemic and Clinical parameters in Study and Control subjects

Parameter	Cases	Controls	*P* value
Age (years)	50.4 ± 11.1	49.2 ± 12.4	0.9
BMI (kg/m^2)	24.2 ± 5.3	21.2 ± 3.3	< 0.001
WHR	0.9 ± 0.1	0.8 ± 0.1	< 0.001
BP (Systolic)	130.5 ± 20.7	124.0 ± 14.6	< 0.001
BP (Diastolic)	81.8 ± 10.5	79.8 ± 8.9	0.040
BG-F (mg/dl)	201.1 ± 86.8	78.9 ± 8.1	< 0.001
BG-PP (mg/dl)	286.2 ± 116.9	100.3 ± 12.4	< 0.001
HbA1c (%)	8.8 ± 2.5	5.0 ± 0.7	< 0.001
TGs (mg/dl)	205.1 ± 123.2	152.5 ± 82.6	< 0.001
LDL (mg/dl)	103.6 ± 27.6	88.1 ± 25.7	0.002
HDL (mg/dl)	38.5 ± 9.2	41.9 ± 7.7	0.003
Cholesterol (mg/dl)	159.7 ± 57.3	133.7 ± 46.3	< 0.001
Sr Cr (mg/dl)	1.1 ± 0.5	0.7 ± 0.2	< 0.001

BMI: Body Mass Index, WHR: Waist to Hip ratio, BP: Blood Pressure, BG-F: Blood Glucose Fasting, BG-PP: Blood Glucose Post Prandial, TGs: Triglycerides, LDL: Low Density Lipoproteins, HDL: High Density Lipoproteins, Chol: Cholesterol, Sr Cr: Serum creatinine.

(Data expressed as Mean ± SD, Non metric data was analyzed by Mann Whitney-U test, *P* value for inter group variants measured by Student's t-test at 95% confidence interval).

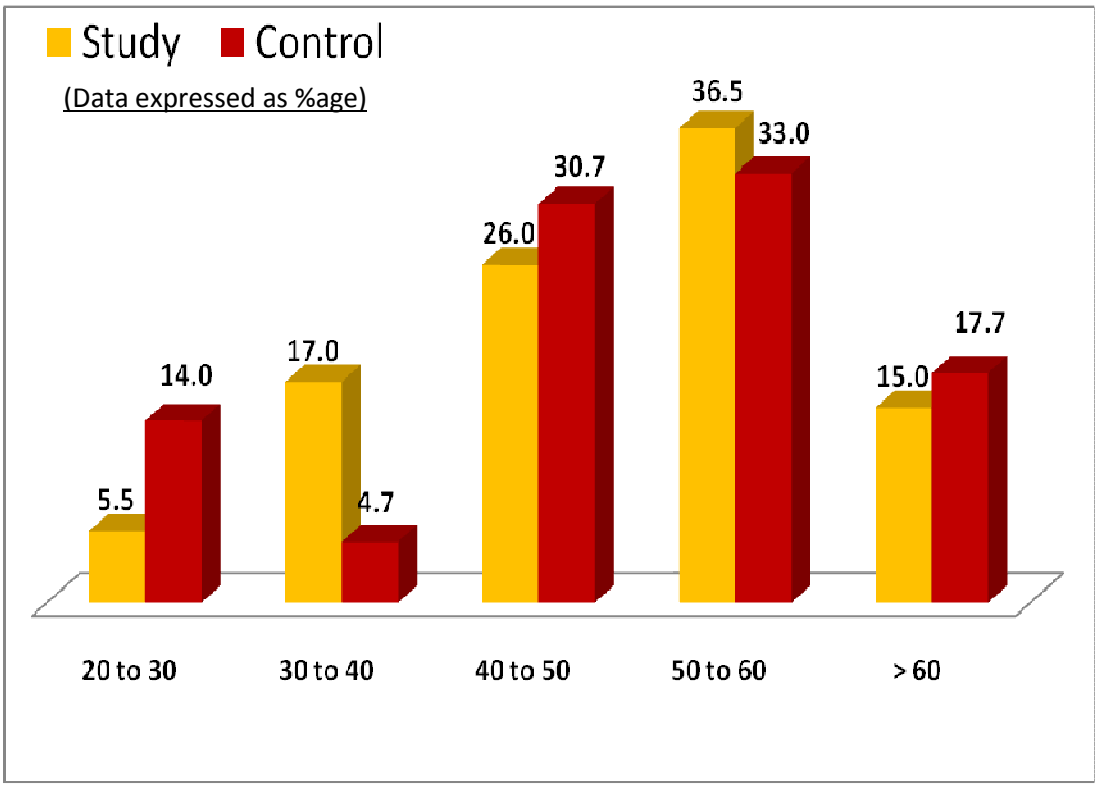

Figure 2: Age distribution of the study and control Subjects in years

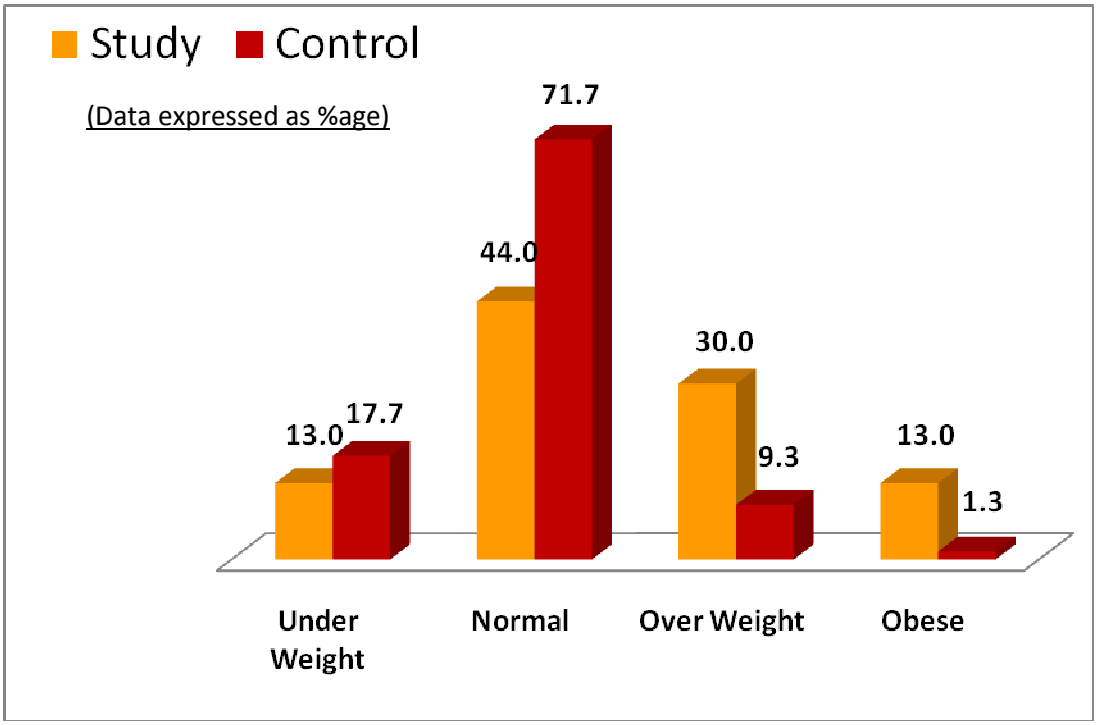

Figure 3: Body Mass Index (kg/m^2) of study and control subjects

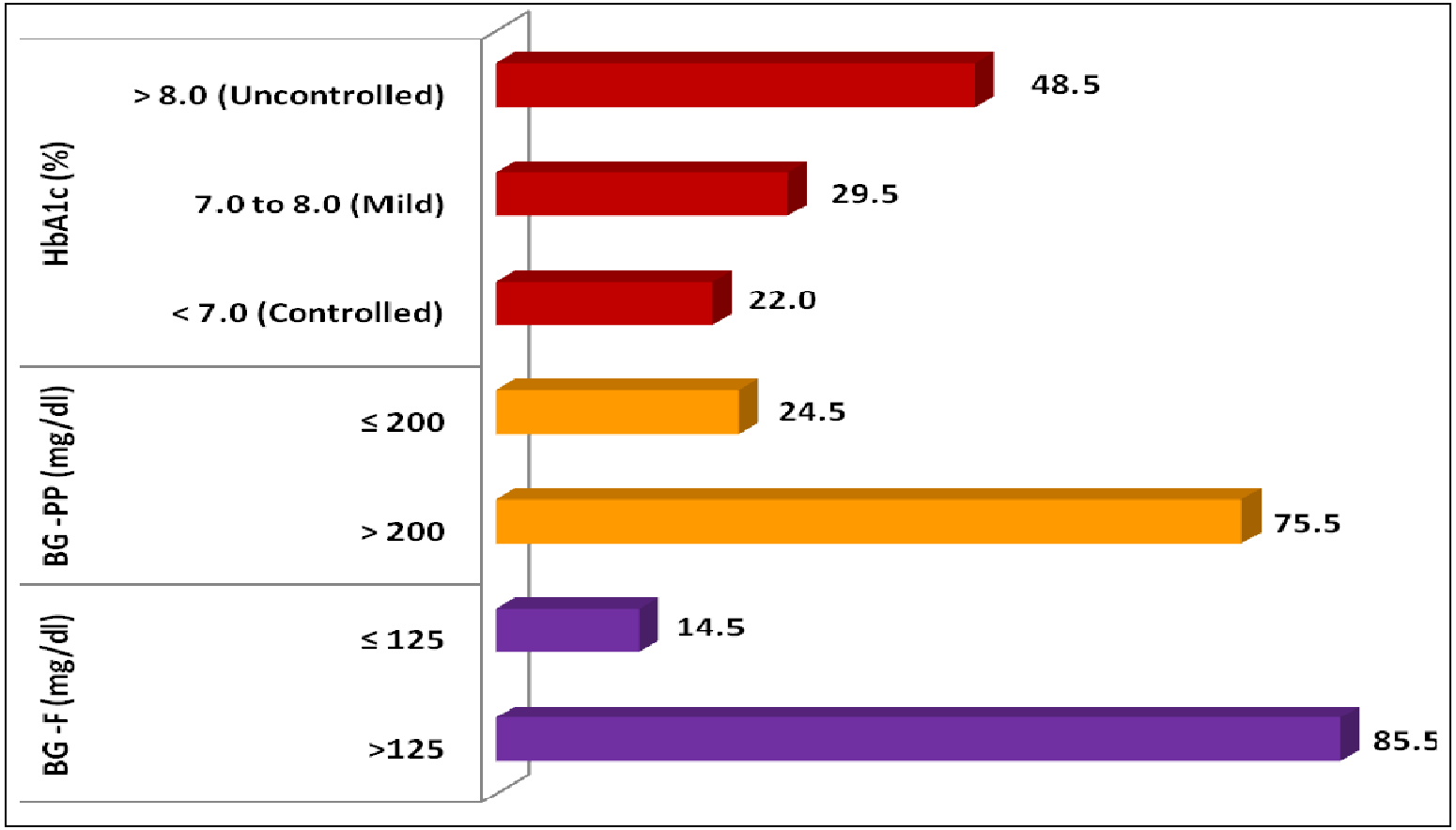

Figure 4: Blood Glucose and HbA1c levels in the Study Group (Data expressed as %age)

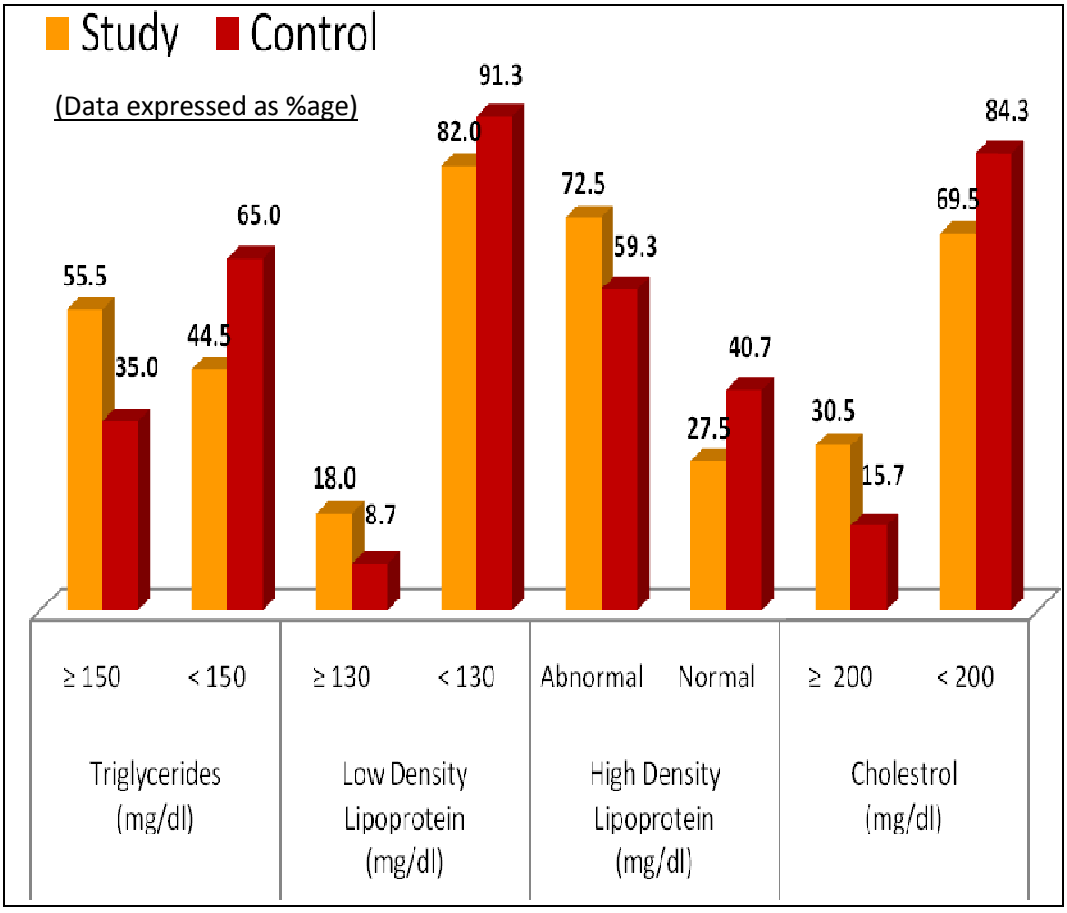

Figure 5: Lipid Profile of the study and control subjects

5.2 Qualitative and Quantitative analysis of genomic DNA

The purity of extracted genomic DNA was assessed using relative absorbance values ($A_{260/280}$), the ratio ranged from 1.8-2. Concentration of extracted DNA was determined using the formula:

Concentration = A_{260} x Absorbance factor (50µg/ml) x Dilution Factor.

Integrity of genomic DNA was checked using 1% agarose gel electrophoresis. DNA was intact as shown by the presence of bands in the wells of the gel.

5.3 Genotyping Analysis of INSR gene polymorphism

In INSR gene, three SNPs located in the exon 3 were evaluated: a T↔C at codon 233, a C↔T at codon 234 and G↔A transition at codon 276. Only homozygous wild alleles were present and the frequency of T (233), C (234) and G (276) allele was same in both cases and controls. The 533 bp amplicon of INSR gene generated using polymerase chain reaction was digested individually as well as simultaneously with *MspI*, *RsaI* and *FokI* restriction enzymes. The digestion products were resolved on 2% agarose gel. No variation was observed in the exon 3 of INSR gene as the amplicons remained uncut after restriction digestion (Fig 6). The fidelity of restriction enzymes was cross checked in order to rule out the possibility of enzymatic inactivity. The results thus obtained were further validated by the sequencing report of samples that showed no variation (Fig 7a and 7b). The INSR gene was monomorphic for all three suspected polymorphisms in our population as no variation was observed in either cases or controls.

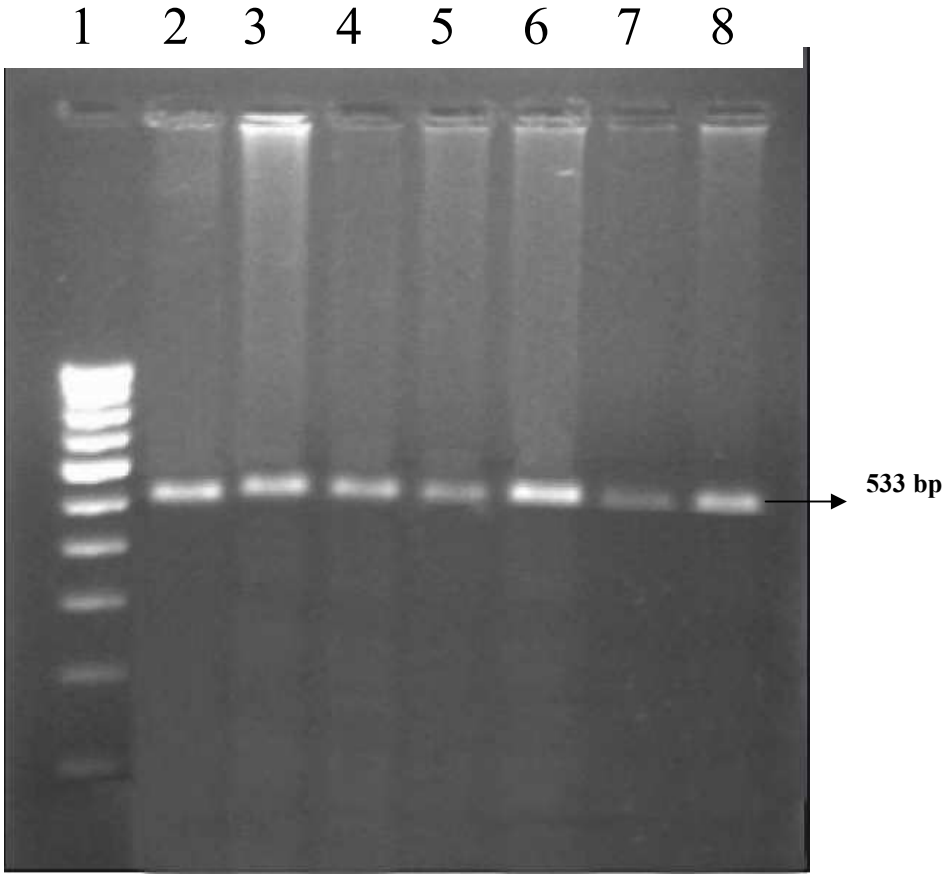

Figure 6: PCR-RFLP gel picture of INSR gene

Lane 1: 100 bp DNA marker
Lane 2 and 6: Undigested samples (533 bp Amplicons)
Lane 3, 4 and 5: Amplicons digested with restriction enzymes *MspI*, *RsaI* and *FokI* respectively
Lane 7 and 8: Amplicons digested simultaneously with restriction enzymes *MspI*, *RsaI* and *FokI*

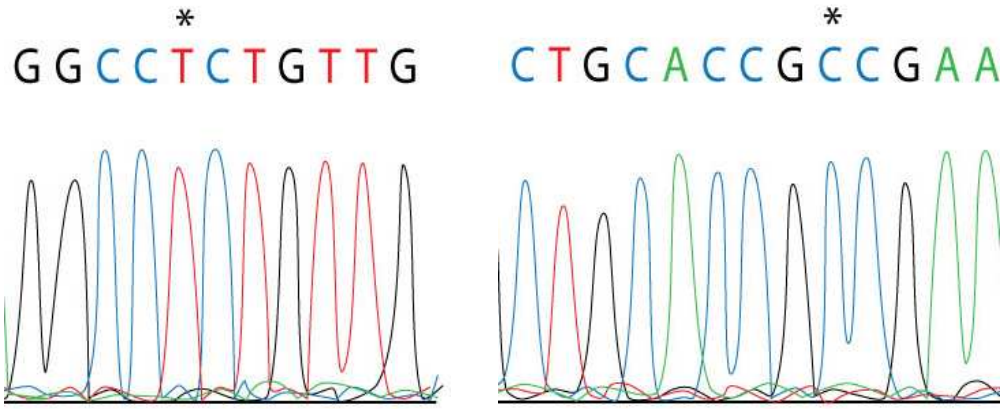

Figure 7a: Partial sequence electropherogram of INSR gene encompassing codon 233 and 234

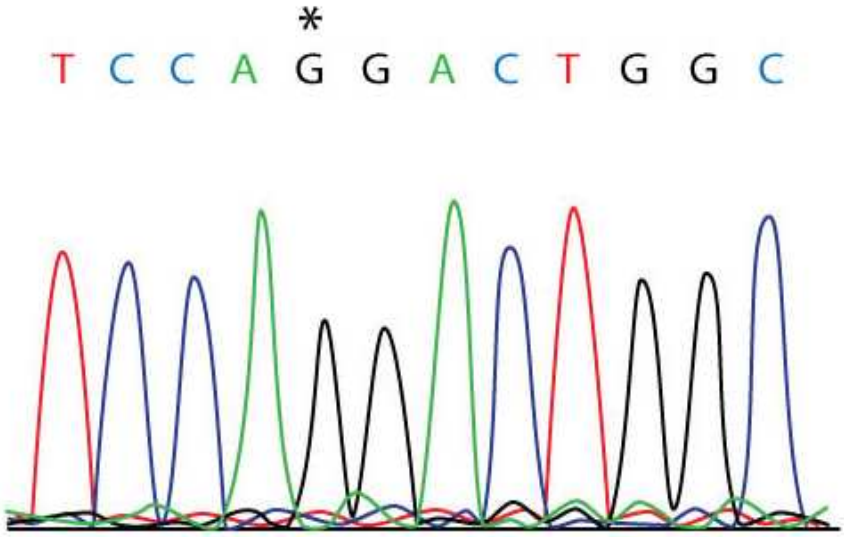

Figure 7b: Partial sequence electropherogram of INSR gene encompassing codon 276

(*Homozygous wild alleles)

5.4 Genotyping Analysis of LEPR gene polymorphism

The 3□ UTR Del/Ins polymorphism was assessed using PCR-SSCP technique. A 114/119 bp del/ins amplicon was generated using PCR (Fig 8a). The PCR products were subjected to SSCP on 12% non-denaturing polyacrylamide gel. Amplicons subjected to PAGE in double stranded state did not differ in their relative mobility thereby resolved as single conspicuous bands (Fig 8b). The amplicons maintained in single stranded state gave unique band patterns corresponding to each particular genotype. The relative mobility shift relies on intra-strand looping. Three genotypes were observed D/D, D/I and I/I: each with its unique band pattern (Fig 8c). The homozygous D/D genotype was present in 79.5% of cases and 24.7% of controls. 20.5% of cases and 69.3% of controls exhibited the D/I genotype (Fig 9). The rare I/I genotype was present in only 6% of controls and absent in cases. The D/D genotype was significantly higher in the cases than in controls ($p<0.001$). The frequency of Del allele was 89.7% and 59.3%, while as the frequency of Ins allele was 10.3% and 40.7% in cases and controls respectively (Table 7). The frequency of Del allele corresponds directly to the severity of disease as shown by the intra-group genotype analysis. The Del allele showed high significance with the severity of disease (Table 8). Among the cases, except for age and HDL levels, there was a significant association between the presence of Del allele and the severity of disease as indicated by intra group genotype analysis. The association also showed gender specificity with more number of females having the Del allele than the males ($p<0.05$). Del allele seems to be directly associated with higher BMI, WHR, HbA1c levels and various other risk factors of T2DM as well as metabolic syndrome. Frequency of Ins allele was predominant in control population than in cases suggesting a protective role imparted by Ins genotype against the prevalence and severity of T2DM in our population. The results were validated by the sequencing analysis of the 3□ UTR of LEPR gene (Fig 10a, 10b and 1c). Sequencing report was in conformity with the genotyping results.

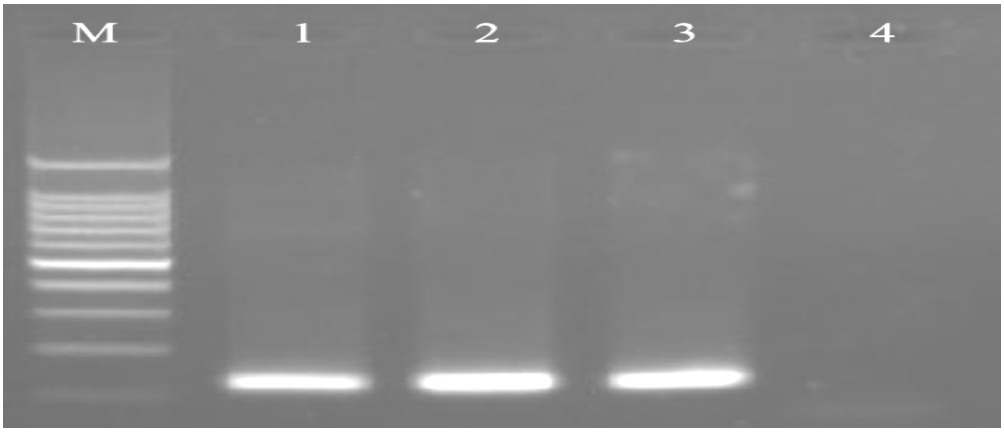

Figure 8a: Amplified product of LEPR gene (114/119bp)

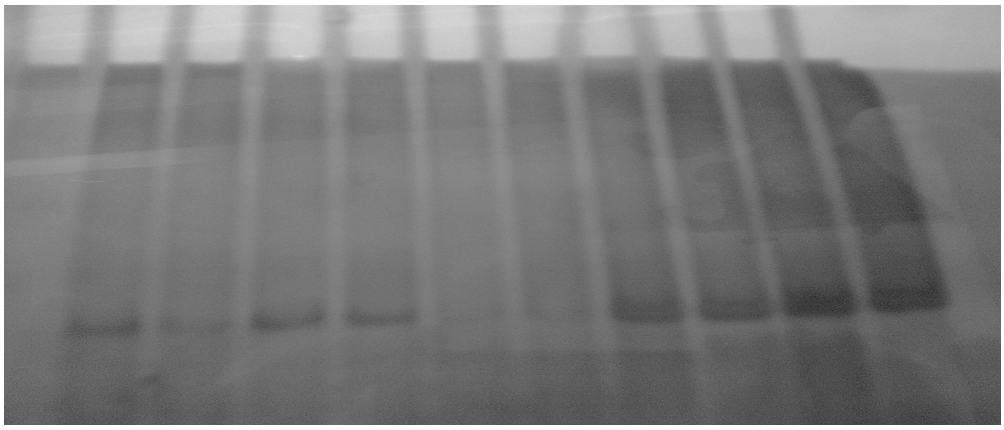

Figure 8b: Gel picture of amplicons in double stranded state

1 2 3 4 5 6 7 8 9

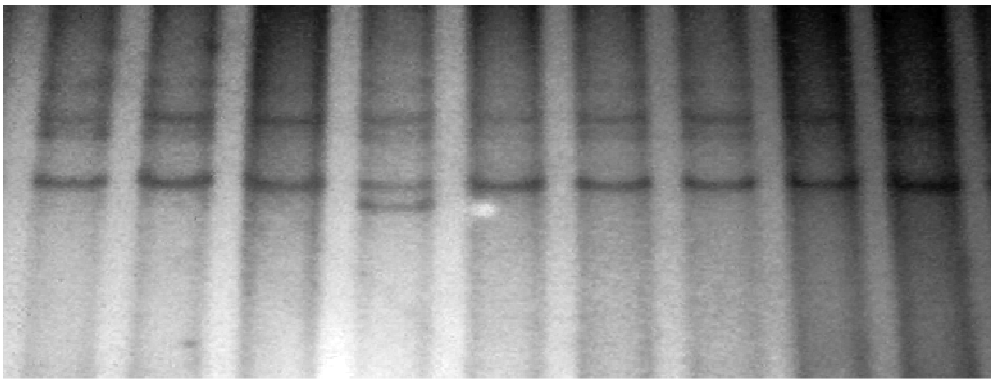

Figure 8c: PCR-SSCP picture of amplicons in single stranded state

Lane 1, 2, 6 and 7: Ins/Ins genotype (119 bp)

Lane 3, 5, 8 and 9: Del/Del genotype (114 bp)

Lane 4: Del/Ins genotype (114/119 bp)

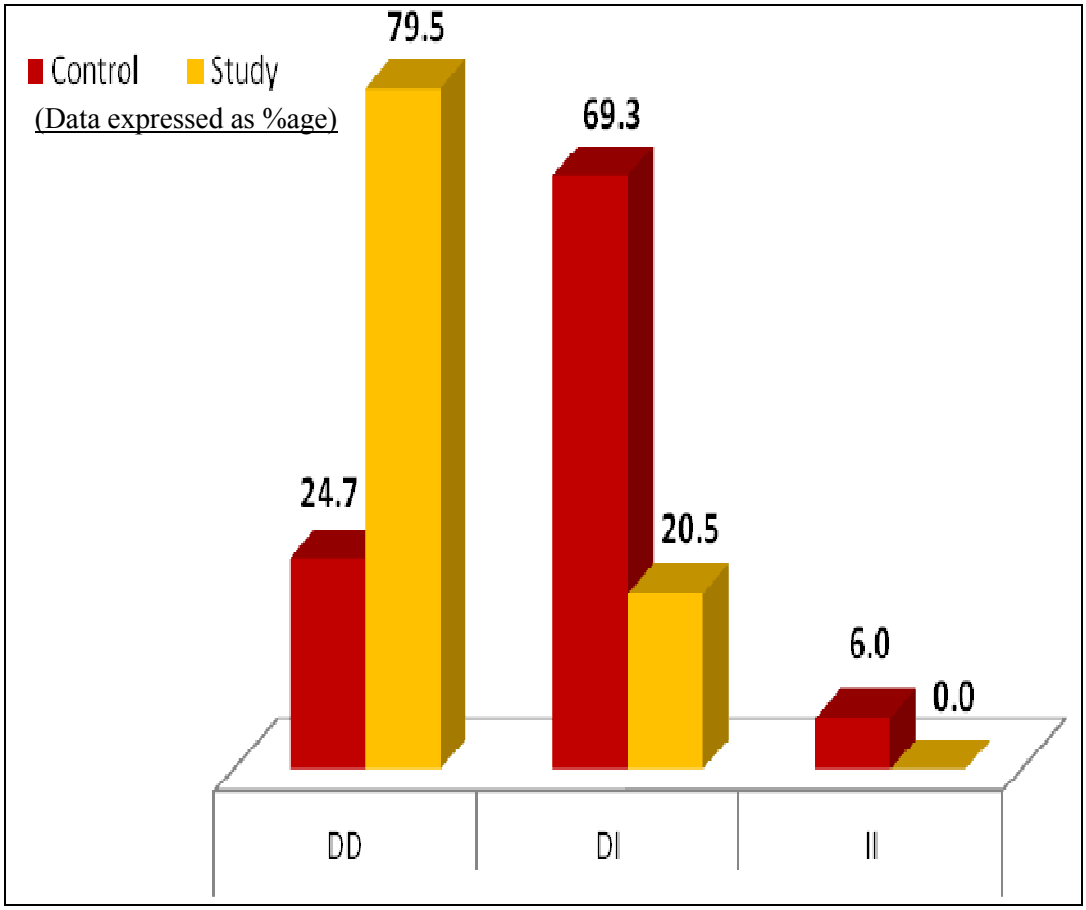

Figure 9: Histogram showing the distribution of *LEPR* (Del/Ins) alleles in study and control population

(DD: Del homozygous, DI: Del/Ins heterozygous, II: Ins homozygous)

Table 7: Genotype analysis and Allele frequency of 3□ UTR Del/Ins polymorphism in LEPR gene

3□ UTR Del/Ins Polymorphism		Cases (%)	Controls (%)	OR (95 % CI)	*P* value	χ^2
Genotype	DD	79.5	24.7			
	DI	20.5	69.3	10.90 (7.07 -16.80)	<0.001	60.4
	II	0.0	6.0	77.35 (7.75 - 63.72)	<0.001	33.51
	DI+II	20.5	75.3	11.84 (7.70 - 18.23)	<0.001	6.5
DD (Risk Allele)	Present	79.5	24.7	11.84 (7.70 - 18.23)	<0.001	
	Absent	20.5	74.3			
Allele Frequency	Del	89.7	59.3	6.00 (3.61 - 9.97)	<0.001	
	Ins	10.3	40.7			

Fischer's exact test and odds ratio analysis was used for genotyping analysis where *P* value of <0.05 at 95% confidence interval was considered to be significant.
DD (Deletion homozygous): Reference allele

Table 8: Genotypic correlation of 3□ UTR Del/Ins polymorphism of LEPR gene with various clinical and laboratory features among the study population

Parameter	DD	DI	*P* value
Age (years)	50.7 ±10.7	49.0 ±12.6	0.392
BMI (kg/m^2)	25.0 ±5.4	21.1 ±3.8	<0.001
WHR	0.92 ±0.10	0.85 ±0.09	<0.001
BP (Systolic)	132.5 ±20.7	122.7 ±19.0	0.006
BP (Diastolic)	83.0 ±10.4	77.1 ±9.6	0.001
BG-F (mg/dl)	212.7 ±91.4	156.1 ±43.9	<0.001
BG-PP (mg/dl)	303.1 ±121.7	220.6 ±62.0	<0.001
HbA1c (%)	9.4 ±2.5	6.6 ±0.6	<0.001
TGs (mg/dl)	225.7 ±128.6	125.0 ±43.9	<0.001
LDL (mg/dl)	108.8 ±27.1	83.3 ±18.9	<0.001
HDL (mg/dl)	38.3 ±9.7	39.3 ±7.5	0.515
Chol (mg/dl)	170.1 ±57.7	119.2 ±32.6	<0.001
Sr Cr (mg/dl)	1.2 ±0.5	0.7 ±0.2	<0.001

BMI: Body Mass Index, WHR: Waist to Hip ratio, BP: Blood Pressure, BG-F: Blood Glucose Fasting, BG-PP: Blood Glucose Post Prandial, TGs: Triglycerides, LDL: Low Density Lipoproteins, HDL: High Density Lipoproteins, Chol: Cholesterol, Sr Cr: Serum creatinine.

(Data expressed as Mean ± SD, Non metric data was analyzed by Mann Whitney-U test, *P* value for inter group variants measured by Student's t-test at 95% confidence interval).

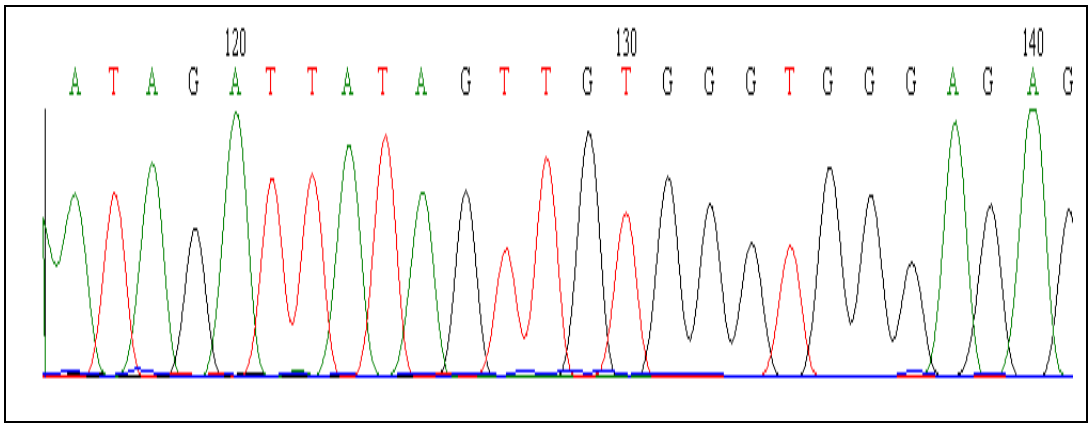

Figure 10a: Partial sequence electropherogram of 3□ UTR Del/Ins polymorphism of LEPR gene (Homozygous wild Del/Del)

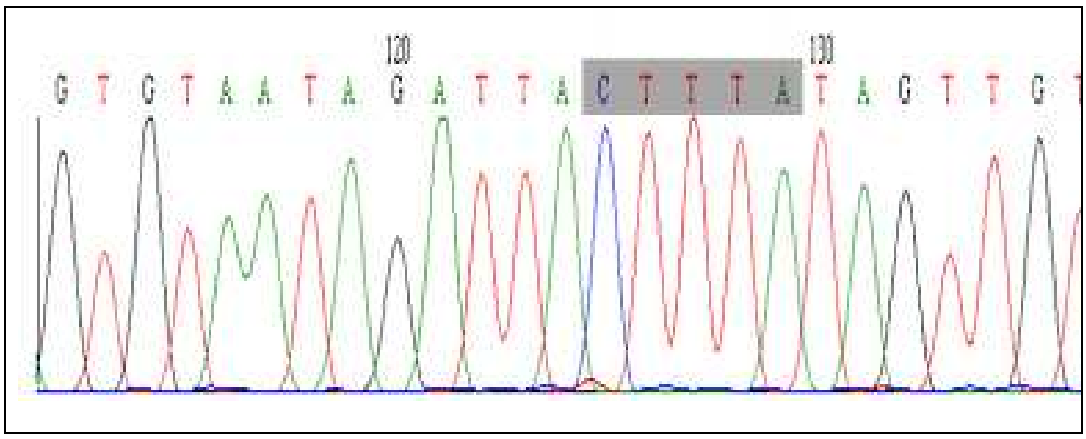

Figure 10b. Partial sequence electropherogram of 3□ UTR Del/Ins polymorphism of LEPR gene (Homozygous variant Ins/Ins)

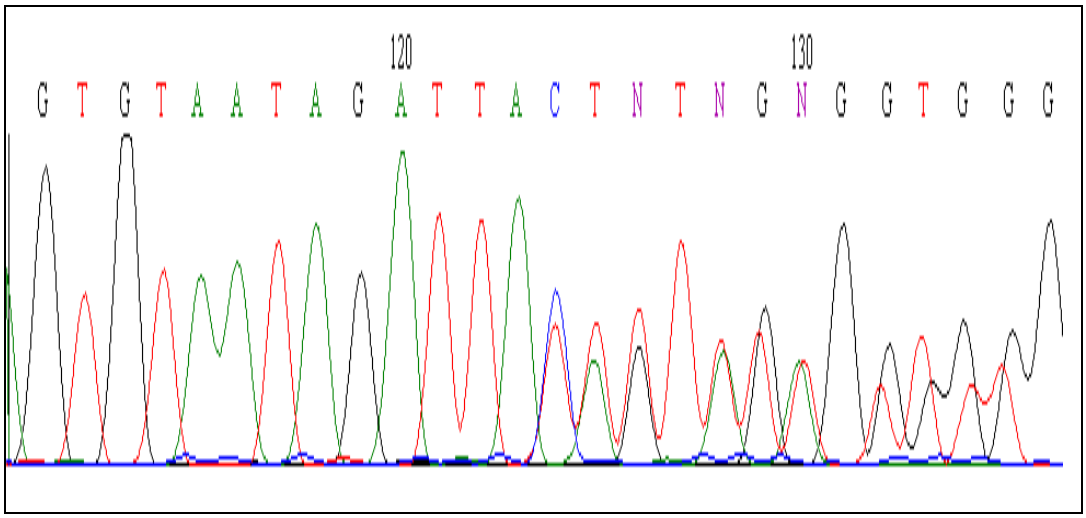

Figure 10c. Partial sequence electropherogram of 3□ UTR Del/Ins polymorphism of LEPR gene (Heterozygous Del/Ins)

5.5 Genotyping Analysis of ADRB3 gene polymorphism

In ADRB3 gene, Trp64Arg (T↔C) polymorphism was evaluated in T2DM patients and non-diabetic controls. A 255 bp amplicon was generated using polymerase chain reaction. Genotyping was done using RFLP method. The amplicons were digested using *MspI* restriction enzyme that differentiates the three genotypes: a 255 bp uncut TT (Trp64Trp) wild homozygous, a 158 + 97 bp variant homozygous (CC) and a 255 + 158 + 97 bp heterozygous (CT) genotype (Fig 11). Homozygous wild allele (TT) was present in 80.3% of controls and 24.5% of cases. Control population exhibited high frequency of TT genotype as compared to cases in which the predominant genotype was CT allele (Fig 12). 71% of cases and 19% of controls carried the heterozygous CT genotype respectively. The CC homozygous variant genotype was present in 9% of cases and 0.7% of controls respectively. The frequency of C allele was observed to be 40% and 10.2% in cases and controls respectively (Table 9). This frequency distribution elucidates a correlation between the presence of C allele and the risk of T2DM, where as the T allele exerts a protective effect against T2DM in our population ($p<0.05$). On intra-group analysis of ADRB3 genotypes with clinical parameters and diabetic risk factors, a significant association was observed $(p<0.05)$ that was suggestive of the deranged status and severity of T2DM with respect to the presence of C allele (Table 10). However the age of the subjects and HDL levels did not vary statistically across the genotypes. The highest association was observed in anthropometric measurements and serum creatinine levels. T allele frequency was observed to be 89.8% and 60% in controls and cases respectively which elucidates a protective effect conferred by the wild T allele and predisposition to disease and high risk factors by the variant C allele. Partial sequence electropherogram of homozygous wild allele and homozygous variant allele is shown in Figure 13a and Figure 13b respectively.

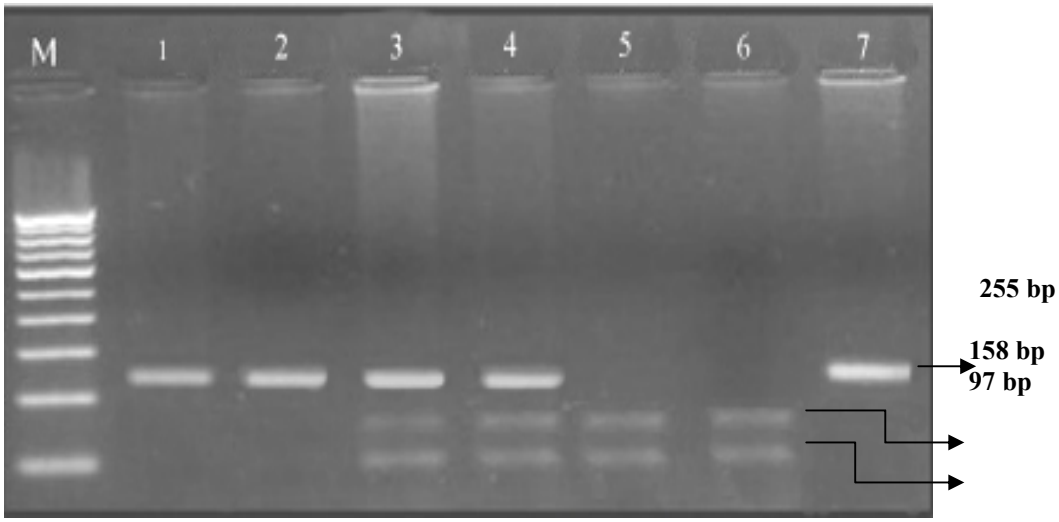

Figure 11: PCR-RFLP picture of Trp64Arg (T↔C) polymorphism

Lane 1and 2: Homozygous wild genotype (TT)
Lane 3and 4: Heterozygous genotype (CT)
Lane 5 and 6: Homozygous variant genotype (CC)
Lane 7: Undigested amplicon
Lane M: 100 bp DNA marker

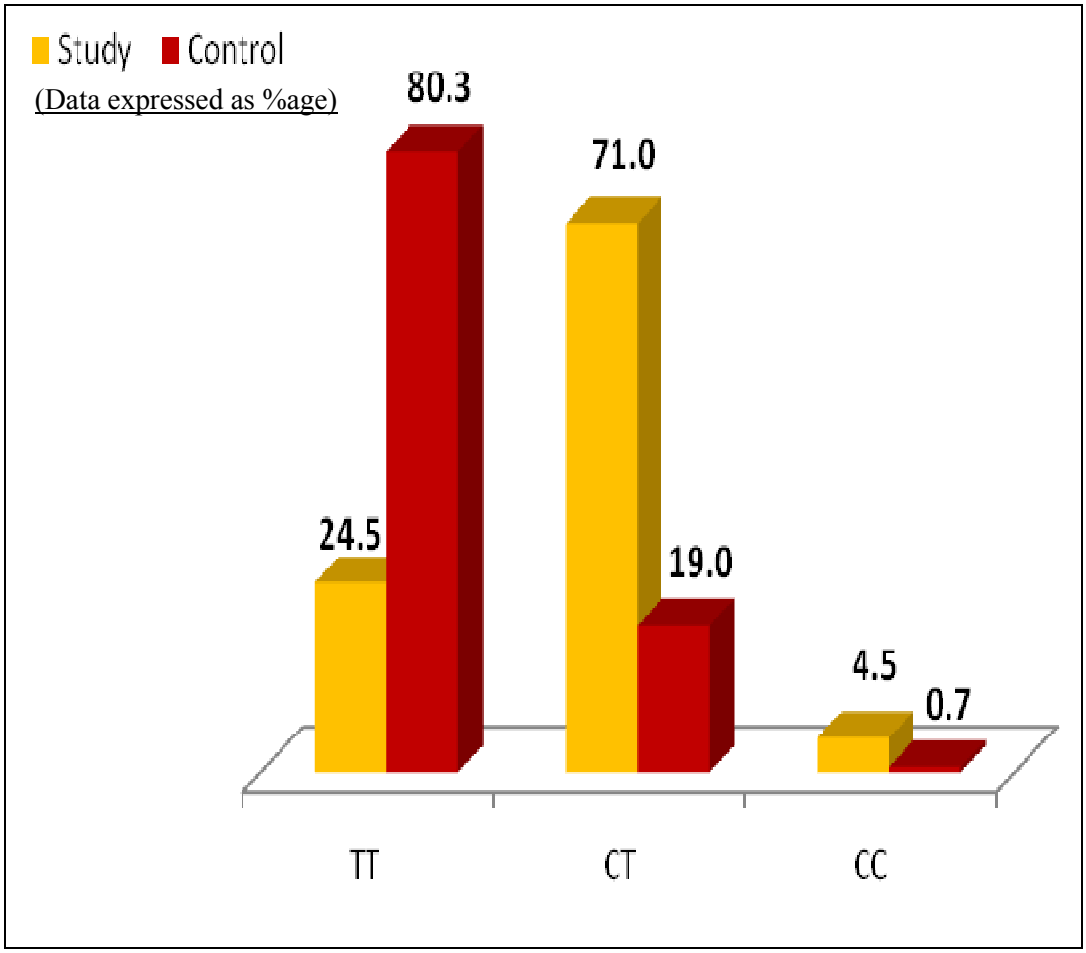

Figure 12: Histogram showing the distribution of ADRB3 Trp64Arg (T↔C) alleles in study and control population

(TT: Wild homozygous, CT: Heterozygous, CC: Variant homozygous)

Table 9: Genotype analysis and Allele frequency of Trp64Arg (T↔C) polymorphism in ADRB3 gene

Trp64Arg (T↔C) Polymorphism		Cases n (%)	Controls n (%)	OR (95% CI)	*P* value	χ^2
Genotypes	TT	49 (24.5)	241 (80.3)			
	CC	9 (4.5)	2 (0.7)	0.04 (0.01-0.19)	<0.001	28.71
	CT	142 (71.0)	57 (19.0)	0.08 (0.05-0.12)	<0.001	147
	CC+CT	151 (75.5)	59 (19.7)	0.08 (0.05-0.12)	<0.001	153.5
CC (Risk Allele)	Present	4.5	0.7	7.02 (1.69-9.09)	<0.05	
	Absent	95.5	99.3			
Allele Frequency	C	40	10.2	5.89 (3.69-9.39)	<0.001	
	T	60	89.8			

Fischer's exact test and odds ratio analysis was used for genotyping analysis where *P* value of <0.05 at 95% confidence interval was considered to be significant.
TT (Wild homozygous): Reference allele

Table 10: Genotypic correlation of Trp64Arg (T↔C) polymorphism of ADRB3 gene with various clinical and laboratory features among the study population

Parameter	TT (n=49)	CT (n=142)	CC (n=9)	*P* value
Age (years)	50.3 ±12.2	50.5 ±10.9	49.6 ±9.3	0.969
BMI (kg/m^2)	20.9 ±3.7	24.9 ±5.3	30.5 ±3.3	<0.001
WHR	0.85 ±0.09	0.91 ±0.09	1.01 ±0.06	<0.001
BP (Systolic)	166.2 ±60.0	213.4 ±93.5	196.4 ±42.5	0.004
BP (Diastolic)	238.6 ±95.9	303.4 ±121.9	273.2 ±56.9	0.003
BG-F (mg/dl)	166.2 ±60.0	213.4 ±93.5	196.4 ±42.5	0.004
BG-PP (mg/dl)	238.6 ±95.9	303.4 ±121.9	273.2 ±56.9	0.003
HbA1c (%)	6.8 ±1.0	9.4 ±2.5	11.0 ±1.3	<0.001
TGs (mg/dl)	129.6 ±51.9	226.5 ±131.0	277.9 ±99.6	<0.001
LDL (mg/dl)	84.6 ±19.6	108.4 ±26.7	130.1 ±25.3	<0.001
HDL (mg/dl)	40.2 ±7.5	38.3 ±9.8	33.0 ±6.6	0.086
Chol (mg/dl)	121.2 ±32.0	168.6 ±57.1	228.1 ±47.6	<0.001
Sr Cr (mg/dl)	0.8 ±0.3	1.2 ±0.5	1.4 ±0.3	<0.001

BMI: Body Mass Index, WHR: Waist to Hip ratio, BP: Blood Pressure, BG-F: Blood Glucose Fasting, BG-PP: Blood Glucose Post Prandial, TGs: Triglycerides, LDL: Low Density Lipoproteins, HDL: High Density Lipoproteins, Chol: Cholesterol, Sr Cr: Serum creatinine.

(Data expressed as Mean ± SD, Non metric data was analyzed by Mann Whitney-U test, *P* value for inter group variants measured by Student's t-test at 95% confidence interval).

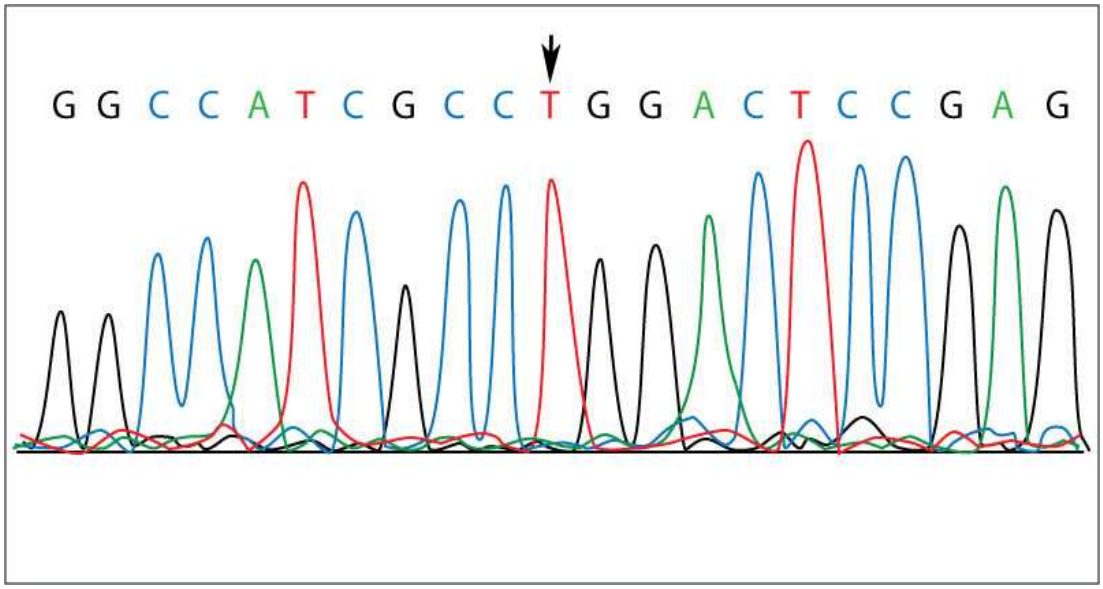

Figure 13a: Partial sequence electropherogram of Trp64Arg (T↔C) polymorphism (wild homozygous)

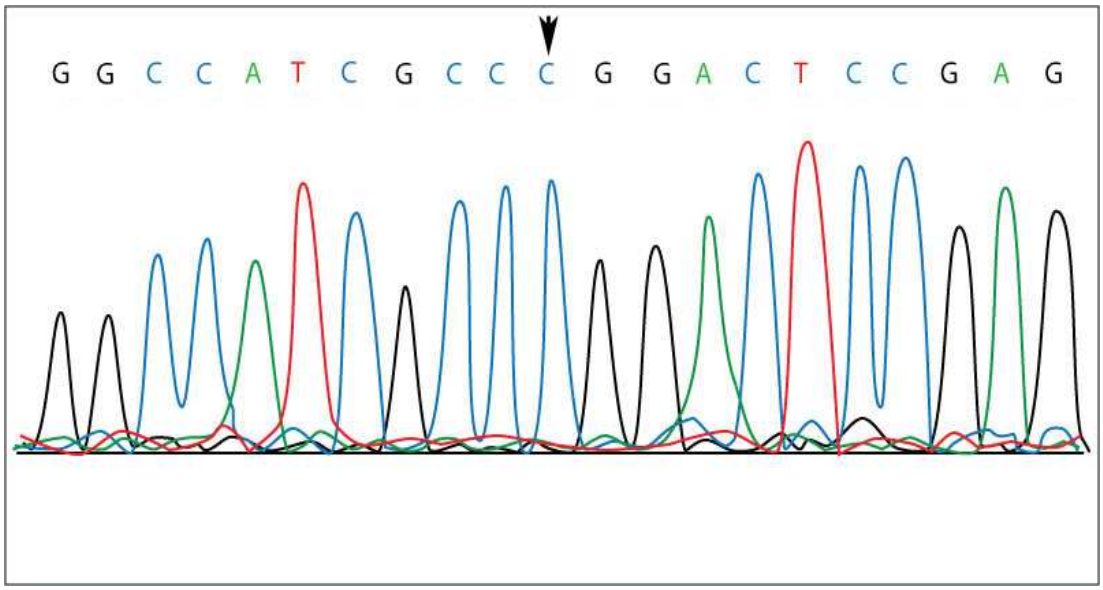

Figure 13b: Partial sequence electropherogram of Trp64Arg (T↔C) polymorphism (variant homozygous)

DISCUSSION

T2DM is an emerging epidemic in countries worldwide. Owing to the heterogeneity of this disorder, the risk factors and sequence of events remain to be elusive. Various factors are involved in its pathogenesis, progression and status. Both genetic and environmental factors are involved in its etiology. Ethnic variability can be partially attributed to non-genetic environmental and cultural factors. However, some studies show that diabetes prevalence differs markedly across ethnic groups, even when environmental exposures are similar. According to the WHO, between 2000 and 2030 most new cases of T2DM will emerge from India, China, and the USA, partly because these countries have some of the world's largest populations, but also because these are ethnically at risk populations that are rapidly adopting obesogenic lifestyles. As originally hypothesized (Neel, 1962) natural selection may have favoured a metabolically efficient phenotype, based on a 'thrifty genotype' that increased the chances of surviving periods when food was scarce. The resulting thrifty phenotype could be characterized by a lower rate of energy expenditure and/or hyperphagia. Either or both of these characteristics would result in positive energy balance and increase fat stores during 'times of plenty' which would be advantageous for survival during periods of drought and limited food supplies. However, in westernized societies, where food is plentiful, and typically high fat, this 'thrifty genotype' would be dis-advantageous, resulting in marked obesity and increased risk of T2DM. The "common-disease-common-variant" hypothesis states that the genetic predisposition is a result of multiple, relatively common genetic variants with small or modest effects. It has been estimated that ~20 genes are needed to explain 50% of the burden of a disease in the population if the predisposing genotypes are common (≥25%), even though the individual risk ratios are relatively small (RR=1.2-1.5) (Yang *et al.,* 2005). Identification of the genetic elements of obesity and T2DM is one of the most important areas of research because discovery of the risk genes would certainly facilitate understanding of the disease, its complications and its treatment, cure, and prevention.

This study was sought in view of the alarming rise in T2DM rates in Kashmir valley. The aim of this study was to evaluate for the first time various polymorphisms in INSR, LEPR and ADRB3 genes. Correlation between various genotypes and clinical parameters was also determined in order to assess the role of genetic factors in the disease presentation and progression. A total of 200 cases of T2DM and 300 controls were analysed. The subjects were matched for age and gender. Highest prevalence of diabetes was observed in the age group of 51-60 years. Higher BMI and WHR were seen in cases as compared to controls. Our

results are in accordance with the data showing that people with T2DM are overweight or obese primarily with central obesity (Astrup, 2001). This type of obesity is strongly associated with insulin resistance. In our study, the patients presented with dyslipidemia and characteristic pattern of lipid abnormalities commonly referred to as "diabetic dyslipidaemia". Epidemiologic data suggest that for every unit of increase (i.e., between 2.7 and 3.6 kg) in BMI, risk of developing diabetes increases by 12% (Ford *et al.,* 1997). Obesity is associated with at least 45 co-morbidities (Jung, 1997) including T2DM (Prentice, 1997). Many epidemiological studies have documented the strong association between obesity and T2DM. For example, the 14-year follow-up Nurses' Health Study of 114,281 women aged 30–55 years at baseline observed that BMI was the most important risk factor for T2DM (Colditz *et al.,* 1995). Although many studies focus on body mass *per se*, body fat distribution is also an important independent risk factor for diabetes. A cross-sectional study of 5,080 individuals from different ethnic populations (Hindu and Muslim Asian Indians, African-origin Creoles, and Chinese Mauritians) showed that waist-to-hip ratio (WHR) is positively associated with T2DM independently of BMI (Dowse *et al.,* 1991). A 13.5-year follow-up of 792 Swedish men aged 54 years confirmed the previous findings, suggesting that WHR is significantly associated with T2DM risk even after adjustment for BMI (Ohlson *et al.,* 1985).

Elevated levels of TGs, Cholesterol and LDL-C, and depressed HDL levels in cases differ significantly from non-diabetic controls. Diabetic dyslipidaemia results from lipoprotein dysmetabolism combined with abnormalities in insulin action. Hyperinsulinaemia is also associated with low HDL-C levels. In a 20- year follow-up of the Framingham Heart Study, hypertriglyceridemia and low HDL-C were associated with increased T2DM risk in both men and women (Kannel, 1985). We observed similar associations in our study, however dyslipidemia was observed in greater proportion of our control population, especially the depressed HDL levels and elevated TG levels. High blood pressure is not generally considered a causal factor in the development of T2DM. However, arterial endothelial dysfunction has been proposed as a causal link between elevated blood pressure and insulin resistance, which clearly could forge a causal link between blood pressure and diabetes (Meigs *et al.,* 2004; 2006). T2DM and hypertension are strongly correlated, largely owing to their shared relationships with obesity and other lifestyle factors. Thus, the relationship between high blood pressure and T2DM may not be completely causal. Our study indicates a 54% prevalence rate of hypertension in diabetic patients, which is higher than other documented reports (Arauz *et al.,* 2003; Hu *et al.,* 2005). However, a greater percentage of

the controls in our study were also hypertensive. These facts are elusive of the presence of predisposing factors related to metabolic syndrome in our population. Dyslipidaemia and hypertension are independent risk factors for cardiovascular diseases. These observations specify that our population is prone to cardiovascular and atherosclerotic diseases. The changing dietary habits and sedentary lifestyle in our population due to western acculturation leads to the accumulation of predisposing risk factors that in association with genetic components precipitates the diabetic phenotype.

HbA1c levels >8% were observed in nearly half (48.5%) of the cases which is suggestive of the extent of uncontrolled diabetes in our population. This discrepancy may be due to several reasons including unawareness, late detection and unresponsiveness and non-compliance to treatment. A study demonstrated that the ratio of diagnosed to undiagnosed diabetes in our population is 1:10 (Zargar *et al.,* 2008). The study reflects the meagre familiarity with diabetes care and management in patients from Kashmir valley.

Serum creatinine is used to estimate glomerular filtration rate and to stage the level of chronic renal disease. Serum creatinine levels were elevated in 20.5% of the cases, suggesting impaired renal function secondary to the microvascular changes due to diabetes.

We analysed three genes for polymorphic alleles and their association with T2DM. Genotyping analysis and sequencing reports were used to analyze these variants. In INSR gene, three polymorphisms (T↔C at codon 233, C↔T at codon 234 and G↔A transition at codon 276) located in exon 3 were evaluated. The results indicate that our population is monomorphic for these three polymorphisms as no variant allele was observed in either case or control. Similar results were observed in Mexican-American population (Craig and Hanis, 1991). French and mixed ethnicity samples were also screened in another study that demonstrated monomorphic results for these three SNPs (Hanis and Bertin, 1992). Based on these replicative results by various studies, the previously documented study has been attributed to sequencing errors and cloning artifacts (Taylor *et al.,* 1990). In a study on ethnic Iranian population, all 22 exons of the INSR gene were screened for variation. Several changes were reported including some novel polymorphisms (Kazemi *et al.,* 2009). However, there was no report of the polymorphisms at codon 233, 234 and 276 (Kazemi *et al.,* 2009). Thus, our observations on INSR gene polymorphism are in conformity with the studies conducted on several other populations (Hanis and Bertin, 1992; Kazemi *et al.,* 2009).

The insertion allele of the 3□-UTR Del/Ins polymorphism has been associated with lower serum insulin levels in obese individuals (Oksanen *et al.,* 1998). This finding has been confirmed in subsequent studies (Francke *et al.,* 1997; Lakka *et al.,* 2000). The 3□-UTR Del/Ins polymorphism of the LEPR gene generates an AU-rich sequence that should be able to form a stem-loop structure, which may affect mRNA stability in the cell (Oksanen *et al.,* 1998). We screened this pentanucleotide (CTTTA) Del/Ins in 3□ UTR of LEPR gene. In our population the genotypes of this polymorphism were not in Hardy Weinberg equilibrium (χ^2=57.22). This can be due to genetic mutation (drift) or natural selection for the locus in consideration. The Del/Ins genotype was predominantly found in control population. Frequency of Del allele was 89.7% and 59.3% and frequency of Ins allele was 10.3% and 40.7% in cases and controls respectively. Del allele showed a linear association with the prevalence of diabetes as well as with the associated risk factors. According to our observations, Ins allele conferred a protective effect. The genotype and allele frequency of this polymorphism in our population is comparable to another study (Lakka *et al.,* 2000) in which the carriers of the insertion allele had a 79% reduced risk of T2DM compared with non-carriers in the 4-year follow-up (Lakka *et al.,* 2000). Previously a genetic linkage between the acute insulin response (AIR) and the microsatellite marker *D1S198* was reported (Thompson *et al.,* 1996). Due to the close physical distance between the leptin receptor locus and a putative genetic element controlling the AIR, the leptin receptor is also a candidate for the AIR genetic element. The earliest observable diabetic phenotype in the *db/db* mouse is hyperinsulinemia followed by obesity and β-cell failure (Hummel *et al.,* 1996). Another study reported that the leptin receptor is expressed on β-cells, indicating that leptin may directly affect insulin secretion (Kieffer *et al.,* 1996).

Functional alterations in ADRB3 may promote the development of obesity and insulin resistance (Li *et al.,* 1996; Clement *et al.,* 1997). A change in the ADRB3 gene causes the substitution of the coding sequences from tryptophan into arginine in 64th position, and this is able to influence receptor's affinity to nor-epinephrine and its interaction with Gs proteins in adipocytes (Walston *et al.,* 1995). We analyzed the Trp64Arg polymorphism (T↔C) and ADRB3 genotypes were found to be in Hardy-Weinberg equilibrium in our population (χ^2=0.48). Homozygous wild allele (TT) was present in 80.3% of controls and 24.5% of cases. 71% of cases and 19% of controls carried the heterozygous CT genotype respectively. The CC homozygous variant genotype was present in 9% of cases and 0.7% of controls respectively. Frequency of C allele was 40% and 10.2% in cases and controls respectively.

Presence of C allele was observed to be a risk factor for T2DM, where as the T allele exerted a protective effect against T2DM in our population ($p<0.05$). Presence of C allele was directly related to higher BMI, WHR, dyslipidemia and uncontrolled diabetes. The Arg64Arg was rarely present in our study. Similar results were observed in Kyrgyz population (Mirrakhimov *et al.,* 2011). Our study is in accord with some studies that show associations between Arg64 variant of the ADRB3 gene and early development of T2DM and decreased early insulin response to glucose load (Walston *et al.,* 2000). Some studies with cellular transfection had shown an association with decreased glucose dependent insulin secretion (Perfetti *et al.,* 2001). Approximately half of the studies found a positive association of the Trp64Arg polymorphism of the ADRB3 gene with obesity and IR (Kadowaki *et al.,* 1995; Walston *et al.,* 1995; Widen *et al.,* 1995; Kurabayashi *et al.,* 1996; Proenza *et al.,* 2000), whereas the other studies did not find any relationships between the presence of such polymorphism and obesity, T2DM (Awata and Katayama, 1996; Gagnon *et al.,* 1996; Oeveren *et al.,* 2001). Such discordant results may be partially explained by ethnicity, age, or population differences in studied samples.

Almost invariably, discussion of the current diabetes epidemic engulfing both the developed and now the developing world includes reference to the modern "toxic" environment. For many of us, food has become so plentiful (if nutritionally suspect) that overfeeding on empty calories is all but inescapable. The preponderance of sedentary activities has transformed physical exercise into something that must be scheduled as opposed to an unavoidable burden of daily life. The result is an obesity epidemic paralleling, and contributing to the diabetes epidemic. The "thrifty gene" hypothesis has proposed a survival advantage to a fat-storing phenotype among Paleolithic populations. Persistence of that phenotype could predispose certain groups and individuals to weight gain and T2DM (Prentice *et al.,* 2008). This study and its findings may be useful and provide some basic information regarding our understanding of complex pathological interconnections between metabolic heritage and modern environment.

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Appendix I

Materials used	**Source**
Acrylamide	Sigma Aldrich, USA
Bis-acrylamide	Sigma Aldrich, USA
Agarose	Sigma Aldrich, USA
Ammonium acetate	Sigma Aldrich, USA
Ammonium persulphate	Sigma Aldrich, USA
Chloroform	Qualigens, USA
dNTP Mix	Fermentas, USA
EDTA	Sigma Aldrich, USA
Ethidium bromide	Sigma Aldrich, USA
Ethanol	Merck
Glycerol	Qualigens, USA
Isopropanol	Qualigens, USA
Isoamyl alcohol	Qualigens, USA
Markers (100bp)	Fermentas, USA
PCR buffer	Fermentas, USA
Phenol	Merck
Proteinase K	Genei, USA
Restriction enzymes	Fermentas, USA
Sodium acetate	Sigma Aldrich, USA
Sodium hydroxide	Merck
Taq DNA polymerase	Biotools, Spain
TEMED	Sigma Aldrich, USA

APPENDIX II

Preparation of reagents

1. Tris buffer (1M)

Tris base	121.14 g
Deionised water	800 ml

The solution was vigorously stirred on a magnetic stirrer to dissolve the contents and the pH of the solution was adjusted to 8.0 by adding - 42 ml of concentrated HCl. The volume of the solution was made up to 1 litre with deionised water.

2. Na_2EDTA (0.5 M: pH 8.0)

Na_2EDTA	186.12g
NaOH	~10 g
Deionised water	800 ml

The solution was vigorously stirred on a magnetic stirrer to dissolve the contents. The pH of the solution was adjusted to 8.0 by adding NaOH pellets. The volume of the solution was made up to 1 litre with deionised water.

3. Erythrocyte Lysing buffer

1M NH_4Cl	15.5ml
1M $KHCO_3$	1ml
0.5 M Na_2EDTA	20µl
Deionised water	80ml

The volume of solution was made upto 100ml with deionised water.

4. Saline EDTA (SE BUFFER)

5 mM NaCl	3ml
0.5 M Na_2EDTA	8ml
Deionised water	80ml

The volume of solution was made upto 100ml with deionised water.

5. Proteinase K (10 mg/ml)

10 mg of Proteinase K was dissolved in 1 ml of deionised water and stored at -20^{o}C in aliquots of 1 ml each.

6. Tris-saturated Phenol

500 g of phenol was dissolved in 500 ml of 0.5M Tris (pH 8.0) and 8-Hydroxyquinoline was added to a final concentration of 0.1 % and stirred on a magnetic stirrer for 15 minutes. The phases were allowed to separate and the upper aqueous phase was removed.

The procedure was repeated thrice with 0.1M Tris (pH 8.0). Then equal volume of 100mM Tris (pH 8.0) was added and stirred to emulsify, phases were allowed to separate and the upper aqueous phase was removed. The procedure was repeated with 1X TE until pH of upper, aqueous phase was less than pH 7.2. The phenol was stored at 4°C in a dark bottle.

7. **Phenol:Chloroform:Isoamyl alcohol**

Phenol: Chloroform: Isoamyl alcohol (25:24:1) reagent was prepared by mixing 25 volumes of Tris-saturated phenol, 24 volumes of chloroform and 1 volume of isoamyl alcohol. The solution was stored at 4°C in a dark bottle.

8. Sodium acetate (3M: pH 5.2)

Sodium acetate.$3H_2O$	408.1 g
Deionised water	800 ml

The solution was stirred on a magnetic stirrer to dissolve the contents. The pH of the solution was adjusted to 5.2 with glacial acetic acid and final volume was made up to 1 litre. The solution was sterilized by autoclaving and stored at 4°C.

9. DNA storage buffer

1 M Tris	1ml
0.5 M Na_2EDTA	0.02 ml
Deionised water	80 ml

The volume of the solution was made up to 100 ml with deionised water.

10. TE (lX)

1 M Tris base	10ml
0.5 M Na_2EDTA	2ml
Deionised water	800 ml

The volume of the solution was made up to 1 litre with deionised water.

11. 50 X TAE (pH 8.0)

Tris base	242 g
0.5 M Na_2EDTA	100 ml
Glacial acetic acid	57.1 ml
Deionised water	500 ml

The volume of the solution was made up to 1 litre with deionised water. The solution was sterilized by autoclaving and stored at room temperature.

12. 1X TAE (pH 8.0)

50X TAE	20ml
Deionised water	980 ml

13. Ethidium bromide

10 mg/ml Ethidium bromide was prepared by dissolving 0.1 g of Ethidium bromide in 10 ml of deionised water. The solution was stored in a dark bottle at 4°C.

14. 6X gel loading buffer

Bromophenol blue was dissolved to a final concentration of 0.25% in 40% sucrose solution in deionised water and stored at 4°C.

15. 30% acrylamide

Acrylamide	29 g
Bis-acrylamide	1 g
Deionised water	50 ml

The solution was stirred on a magnetic stirrer to dissolve the contents. The volume of the solution was made up to 100 ml with deionised water and stored at 4°C.

16. 10% ammonium persulphate

Ammonium persulphate	0.1 g
Deionised water	1ml

The solution was stored at 4°C.

17. 10X TBE (pH 8.0)

Tris base	108g
Boric acid	55 g
0.5 M Na_2EDTA	40 ml
Deionised water	600 ml

The solution was stirred on a magnetic stirrer to dissolve the contents. The volume of the solution was made up to 1 litre with deionised water. The solution was sterilized by autoclaving and stored at room temperature.

18. Formamide gel loading buffer

Deionised formamide	4ml
0.5 M Na_2EDTA	0.01 ml
1.5 NaOH	0.033 ml
1% bromophenol blue	1.25 ml
1 % xylene cyanol FF	1.25ml

19. Gel fixing solution

Ethanol	100 ml
Glacial acetic acid	50ml

The volume of the solution was made up to 1 litre with deionised water.

20. Staining solution

Silver nitrate	2g
37% formaldehyde	0.75 ml
Deionised water	600 ml

The volume of the solution was made up to 1 litre with deionised water.

21. Developer

Sodium carbonate	30 g
37% formaldehyde	0.5 ml
$Na_2S_2O_3.5H_2O$	1 mg
Deionised water	600 ml

The solution was stirred on a magnetic stirrer to dissolve the contents. The volume of the solution was made up to 1 litre with deionised water.

22. Stop solution

Methanol	500 ml
Glacial acetic acid	120 ml

The volume of the solution was made up to 1 litre with deionised water.

23. 5X TBE

Tris base	54 g
Boric acid	27.5 g
0.5 M Na_2EDTA	20 ml

The solution was stirred on magnetic stirrer to dissolve the contents and final volume made upto 1litre with deionised water.

PATIENT PROFORMA

Code No.....

Name.....

Age/Sex.....

Height.....

Weight.....

BMI (kg/m^2).....

Waist/Hip Ratio.....

Blood Sugar (F).....

Blood Sugar (PP).....

HbA_1C.....

Lipid Profile.....

.....

.....

Serum Creatinine.....

Date of Detection.....

Type of Therapy.....

Familial History.....

Other Remarks.....