

**ASSOCIATION OF *INS VNTR* VARIANTS WITH TYPE
2 DIABETES MELLITUS IN OVERWEIGHT TO OBESE
SUBJECTS IN A BANGLADESHI POPULATION**



MS (BIOTECHNOLOGY) THESIS

SUBMITTED BY

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STUDENT ID NO: 07176002

SEMESTER: SUMMER, 2008

SESSION: 2007-2008

**DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCE
BRAC UNIVERSITY
DHAKA, BANGLADESH
SEPTEMBER 2008**

ASSOCIATION OF *INS VNTR* VARIANTS WITH TYPE 2 DIABETES MELLITUS IN OVERWEIGHT TO OBESE SUBJECTS IN A BANGLADESHI POPULATION

(A DISSERTATION SUBMITTED TO THE BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE MASTERS OF SCIENCE DEGREE IN BIOTECHNOLOGY)



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ABSTRACT

Background and Aim: Diabetes is regarded as a public health problem all over the world including Bangladesh. Type 2 diabetes variety constitutes the major bulk of total diabetic patients. Obesity and insulin resistance were found to be associated with T2DM, which is now a day considered as component of metabolic syndrome. The present study was undertaken to determine INS VNTR variant genotype frequencies in type 2 diabetes mellitus in a Bangladeshi population and explore its association with obesity and insulin secretion. **Subjects and Methods:** A total number of 111 type 2 diabetic subjects and 251 healthy controls were recruited. Detailed clinical and anthropometric measurements were recorded. Oral glucose tolerance test was performed. Blood glucose was measured by glucose-oxidase, triglyceride, total cholesterol and HDL-cholesterol, and SGPT by enzymatic colorimetric, creatinine by alkaline picrate method. Insulin was estimated by enzyme linked immunosorbent assay (ELISA). DNA was extracted using commercially available Kits from QIAGEN which utilizes Silica gel DNA separation. INS VNTR A>T variant was determined by PCR-RFLP using restriction endonuclease HphI. Data were managed using Statistical Program for Social Science (SPSS). Unpaired Student's 't' test and Chi-squared tests were performed where appropriate. **Results:** The INS VNTR A>T genotype frequencies in the controls were 0.690, 0.254 and 0.056 for homozygous wild, heterozygous variants and homozygous variants respectively and, 0.731, 0.250 and 0.019 in the T2DM subjects which appeared to be of almost similar frequency. Absolute insulin level (mean $\pm$ SD) between the Controls and T2DM did not show significant difference. Anthropometric measurements, BMI and body fat mass did not show statistical significant difference between the Control and T2DM. Subjects with wild and variant genotype either in the Controls and T2DM did not show significant difference regarding circulating insulin, BMI and BFM. However, WHR was found to be significantly higher among subjects with variant INS VNTR genotype compared to those with wild in the Controls ($p=0.003$). The difference in the T2DM did not reach to the level of significance ($p=0.08$). **Conclusions:** It was concluded that INS VNTR variant genotype: (i) frequencies are substantially different in Bangladeshi population compared to that of the Caucasoids; (ii) is not associated with circulating insulin in the controls and T2DM; (iii) is not associated with type 2 diabetes in Bangladeshi population; and (iv) seem to have an association with central obesity in our population.

DEDICATED
TO
MY BELOVED PARENTS

WHO HAS GIVEN ME, THE HEARTIEST SUPPORT

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LIST OF ABBREVIATIONS

ADRB3	β_3 -adrenergic receptor gene
BFM	Body fat mass
BMI	Body Mass Index
CHD	Coronary Heart Disease
DBP	Diastolic blood pressure
FCPD	Fibrocalculus pancreatic diabetes
FSG	Fasting serum glucose
HDL-c	High density lipoprotein cholesterol
Hm	homozygous
HOMA B	Homeostasis model assessment B-cell Function
HOMA S	Homeostasis model assessment insulin sensitivity
Ht	heterozygous
HW	Hardy-Weinberg
Hz	homozygous (Hz) variant
IDDM2	Insulin dependent diabetes mellitus-2
IDF	International Diabetes Federation
IGF2-INS-TH	Insulin-like growth factor II-insulin-tyrosine hydroxylase
<i>INS</i>	Insulin gene
MAC	Mid arm circumference
mRNA	Messenger Ribonucleic Acid
MS	Metabolic Syndrome
NCEP	National Cholesterol Education Program
NHANES	National Health and Nutrition Examination Survey
NHLBI	National, Heart, Lung and Blood Institute.
NIDDM	Non-insulin dependent diabetes mellitus

NIH	National Institute of Health
PCOS	Polycystic ovary syndrome
PPARG	Peroxisome proliferative activated receptor, gamma gene
PPG	Postprandial glucose
SBP	Systolic blood pressure
SGPT	Serum glutamic pyruvic transaminase
SNP	Single Nucleotide Polymorphism
SSF	Sub-scapular skin fold
T Chol	Total cholesterol
T2DM	Type 2 diabetes mellitus
T1DM	Type 1 diabetes mellitus
TG	Triglycerides
TSF	Triceps skin fold
UTR	Untranslated region
VNTR	Variable number of tandem repeats
WHO	World Health Organization
WHO-MONICA	WHO-Multinational Monitoring of trends and determinants in Cardiovascular disease
WHR	Waist hip ratio
YDM	Young-onset Diabetes Mellitus

Chapter 1

INTRODUCTION

1. Introduction

Obesity is characterized by accumulation of excess body fat which leads to disorder of body weight regulatory systems. Overweight refers to an excessive amount of body weight or more body fat (adipose tissue) than is optimally healthy. The excess weight may come from muscle, bone, fat, and/ or body water. However, overweight and obesity are not mutually exclusive; since obese persons are also overweight (NIH, NHLBI, 1998). Obesity is associated with combination of metabolic abnormalities including glucose intolerance, insulin resistance, hyperinsulinemia, dyslipidemia, hypertension with central adiposity. The clustering of these metabolic abnormalities is termed as metabolic syndrome. The syndrome was first described by Reaven in 1988 (Reaven, 1988). It has since been realized that obesity is often the cause of insulin resistance that leads to the metabolic abnormalities (Ferrannini *et al.*, 1997).

Obesity is a major component of metabolic syndrome (MS). In adults, MS is defined according to its cut-off level and varies in terms of its indicators. The World Health Organization (WHO Consultation, 1999) and the European Groups for the Study of Insulin Resistance (Balkau and Charles, 1999) include measures of insulin resistance, but the US National Cholesterol Education Program (NCEP, 2002) in its definition of obesity included any three of the following as causative factors: glucose level, triglyceride level, high density lipoprotein cholesterol (HDL-c) level, systolic blood pressure, and waist circumference (insulin is not included). International Diabetes Federation (IDF) has defined MS on presence of central obesity plus two of the following four additional factors: raised triglyceride level, reduced HDL-c level, raised blood pressure, and raised fasting plasma glucose level. Sex and, for the first time, ethnicity-specific cut-points for central obesity, as measured by waist circumference, are included (Ford, 2005).

For clinical practice and epidemiologic studies, overweight and obesity are assessed by means of indicators based on weight and height measurements. (WHO, 1995). According to WHO, a BMI of 25.0–29.9 is overweight and BMI more than 30 defined as obesity (WHO, 1997). This definition varies widely in

population to population especially in Europe and USA. In 2004, a WHO Expert Consultation (WHO, 2004) identified potential public health action points along the continuum of the BMI for Asian populations. Cut-offs value of 23 and 27.5 were suggested to identify 'increased risk' and 'high risk' for overweight, respectively.

The United States has the highest rates of obesity among the developed world (OECD, 2005). From 1980 to 2002, obesity has doubled in adults and prevalence of overweight has tripled in children and adolescents (Ogden *et al.*, 2006). According to the WHO classification scheme (WHO, 1998), 54% of U.S. adults are overweight (BMI ≥ 25) and 22% are obese (BMI ≥ 30) (Flegal *et al.*, 1998). Data from the most recent National Health and Nutrition Examination Survey (NHANES) (Ogden *et al.*, 2006) showed that among adult men the prevalence of obesity was 31.1% in 2003-2004, and 33.3% in 2005-2006. Among adult women, the prevalence of obesity in 2003-2004 was 33.2%, and in 2005-2006 was 35.3%. The study of WHO-MONICA revealed different obesity prevalence in several European countries. The lowest prevalence of obesity was found in Sweden and Northern France (7-14%) whereas the highest prevalence (40%) was reported from East-European countries. The results of the DHP-survey suggest that 50% of the adult German population is overweight and 20% are obese. Data from the MONICA-study show lower prevalence for Switzerland, where 38% of adult population is considered to have overweight and 11% are obese. Several studies in Southeast Asia also reported an increase in overweight (Gillespie and Haddad, 2003). In 1999 in 11% of urban northern Indian women were found to be obese in contrast to 3.7% of rural women. In China overweight or obesity increased from 12.9% to 27.3% of adults from 1991 to 2004, because of sedentary life lead (Popkin, 2007). In Bangladesh, 9.1% of urban poor and 4.1% of rural women were 'overweight or obese' (BMI ≥ 25.0). But only 0.4% of rural and 1.1% of urban poor women was found obese (BMI ≥ 30.0). On the other hand, 9.8% of urban poor and 5.5% of rural women were found to be 'at risk of overweight' (BMI 23.0 - <25) (Shafique *et al.*, 2007).

Obesity is linked with a large range of medical complications and it suggested to play a central role in 'insulin resistance' or 'metabolic syndrome', which

includes hyperinsulinemia, hypertension, hyperlipidemia, non-insulin dependent diabetes mellitus (NIDDM) or type 2 diabetes mellitus (T2DM) (Ford *et al.*, 1997), increased risk of atherosclerotic cardiovascular disease (Willett *et al.*, 1995), stroke (Rexrode *et al.*, 1997), gallbladder diseases (Khare *et al.*, 1995), obstructive sleep apnea and respiratory problem (Millman *et al.*, 1995), osteoarthritis (Cicuttini *et al.*, 1996), some types of cancer (endometrial, breast, prostate, and colon) (Huang *et al.*, 1997) and polycystic ovary syndrome (Dunaif, 1992).

In human body, obesity creates resistance to insulin which leads to hyperglycemia and develops T2DM. Insulin resistance alone will not lead to T2DM, but individuals with insulin-resistance also show impaired B-cell function. However, B-cell adaptation eventually fails in the subset of obese individuals who develop T2DM (Butler *et al.*, 2003; Unger and Orci, 2001). Indeed, most individuals with T2DM, whether obese or lean, show a net decrease in B-cell mass. Thus, T2DM is a disease of relative insulin deficiency (Butler *et al.*, 2003). Although insulin resistance is characteristic of obesity and T2DM, it is not established that all of insulin's actions are impaired in individuals with both conditions (Kahn and Flier, 2000).

The primary determinant of insulin resistance is suggested to be the free fatty acid accumulation in the liver, fat cells and particularly, skeletal muscle of obese patients which interferes with the normal insulin signaling (McGarry, 2002). As a consequence of insulin resistance, the pancreas needs to increase its insulin production to maintain normal values of glycemia. Under these conditions, hyperinsulinemia turns the liver into a fat-producing factory with all of its negative downstream effects (McGarry, 2002; Berenson *et al.*, 1998). The development of insulin resistance contributes to the modulation of regulatory substances by adipocytes which included leptin, resistin and adiponectin in diabetic patients (Champe *et al.*, 2005). The effect of insulin resistance in the development of early-onset metabolic syndrome has been described by Weiss *et al.* (2004), who observed that the prevalence of metabolic syndrome among obese children increases with increasing insulin resistance.

It is suggested that overweight and obesity are influenced by various environmental and genetic factor. Environment is related to obesity, dietary patterns and practices, health outcomes, and health-related behaviors (Lee and Cubbin, 2002; Mainous *et al.*, 2008) and have independent effects on disease risk (Henderson *et al.*, 2005). The effects of each of these dietary components on obesity are complex and controversial (Barry *et al.*, 2005). Human obesity arises from the interactions of multiple genes, environmental factors, and behavior. While a genetic basis for obesity exists, defining the genetic contribution has proven to be a difficult task (Yang *et al.*, 2007).

Obesity develops predominantly from a polygenic multifactorial trait. Polymorphisms in various candidate genes controlling appetite, metabolism, and adipokine release may predispose to obesity when sufficient calories are present. These genes seem to operate through modifying susceptibility; the occurrence of the gene increases the risk of developing a characteristic but it is not essential for its expression, nor is it itself sufficient to explain the development of the disease (Yang *et al.*, 2007). Twin, adoption, and family studies have now established that an individual's risk of obesity is increased when he or she has relatives who are obese (Meyer, 1995; Maes *et al.*, 1997). Other studies have shown consistently that 40% to 70% of the variation in obesity-related phenotypes, such as body mass index (BMI), sum of skin fold thickness, fat mass, and leptin levels, is heritable (Allison *et al.*, 1996; Comuzzie *et al.*, 1996). In fact, anonymous major genes accounting for as much as 40% of the variation in BMI (Moll *et al.*, 1991; Price, 1996) and 40% of the variation in fat mass (Comuzzie *et al.*, 1995; Rice *et al.*, 1993) have been reported, along with major genes influencing specific measures of adipose tissue distribution (Borecki *et al.*, 1995; Hasstedt *et al.*, 1989).

Obesity does not show a typical Mendelian transmission pattern and may depend on several susceptibility genes with low or moderate effects. There is firm evidence that genes influencing energy homeostasis and thermogenesis, adipogenesis, leptin-insulin signaling transduction, and hormonal signaling peptides play a role in the development of obesity (Loktionov, 2003). To date, 176 human obesity cases due to single-gene mutations in 11 different genes have been reported, 50 loci related to Mendelian syndromes relevant to human

obesity have been mapped to a genomic region, and causal genes or strong candidates have been identified for most of these syndromes (Rankinen *et al.*, 2006).

The number of studies reporting associations between DNA sequence variation in specific genes and obesity phenotypes has increased considerably, with 426 findings of positive associations in 127 candidate genes. In some studies it was found that candidate genes such as SNP of the peroxisome proliferative activated receptor, gamma gene (PPARG) (Cole *et al.*, 2000; Ek, *et al.*, 2001) and the β_3 -adrenergic receptor gene (ADRB3) (Emorine *et al.*, 1994; Kurokawa *et al.*, 2001) are associated with body mass index and obesity in different population. The insulin gene (*INS*) is usually considered as a candidate gene in the genetic analysis for animal complex traits like growth rate, body composition, and fat deposition (Qiu *et al.*, 2006). Previous studies demonstrate that *INS* gene is associated with obesity (Weaver *et al.*, 1992; Le Stunff *et al.*, 2001).

INS gene encodes Insulin, a peptide hormone produced by the B-cells of the islets of Langerhans of the pancreas. The effects of insulin on glucose metabolism are most prominent in three tissues: liver or hepatic tissue, muscle tissue and adipose tissue. In each case, the effect of insulin is to enhance the uptake of glucose into the cells, where it is metabolized and stored as glycogen or used as an energy substrate in the synthesis of proteins or fats. A previous study shows that Insulin increases the production and storage of fatty acids in adipose tissue, thereby exacerbating obesity (Unger, 2003).

INS gene is located in the IGF2-*INS*-TH region on short arm of human chromosome 11. The locus is designated as Ch 11p15.1. Genes in this region are insulin-like growth factor II, a major fetal growth factor also expressed in adult life; insulin, the best known regulator of intermediary metabolism, and tyrosine hydroxylase, the rate-limiting enzyme in biogenic amine synthesis. Previous genetic epidemiological studies, mainly case studies, mostly of single polymorphic sites within these genes, have suggested that inter-individual variation of this region influences risk of CHD, hypertension, hypertriglyceridemia, glucose tolerance, early and late life diabetes mellitus,

polycystic ovary syndrome (PCOS), longevity, growth in early and late life and body mass. The variable number of tandem repeat (VNTR) that lies immediately adjacent to the 5' promoter region of *INS* is believed to have a direct effect on *INS* expression regulation (Kennedy *et al.*, 1995). The *INS*-VNTR, comprising a variable number of repeats of an oligonucleotide sequence (ACAGGGGT(G/C)(T/C)GGGG) (Bell *et al.*, 1982).

The polymorphism of the VNTR can be classified into two main groups: small class I alleles (28–44 repeats) and large class III alleles (138–159 repeats) at frequencies of about 70% and 30%, respectively, and class II alleles of intermediate size are rare (Stead and Jeffreys, 2000). Different degrees of association have been reported between the *INS* VNTR class I/III genotype and insulin-related traits or diseases. The class I allele is associated with increased expression of insulin mRNA and insulin levels (Lucassen *et al.*, 1995; Bennett and Todd, 1996; Le Stunff *et al.*, 2000). The allelic variation of VNTR is also associated with the risk of diabetes. It has been found consistently that the class I allele increases the risk of type I diabetes (Julier *et al.*, 1991; Lucassen *et al.*, 1993; Bennett and Todd, 1996). Associations were found between the class III allele and T2DM in most (Bennett and Todd, 1996; Huxtable *et al.*, 2000; Ong *et al.*, 1999) but not all (Hansen *et al.*, 2004) studies. Associations of the *INS* VNTR allele with fetal growth (Bennett *et al.*, 2004) or polycystic ovaries (Powell *et al.*, 2005) are still debated. In fact, class I and class III are composed of a number of different alleles.

The entire *INS* gene and its flanking intergenic regions contain a VNTR and 10 non-coding SNP markers that span the 4.1 kb segment. It has been shown in Caucasian populations that these 10 SNPs are in tight linkage disequilibrium with each other and with the VNTR such that they constitute two major haplotypes (Cox *et al.*, 1988; Julier *et al.*, 1991; Lucassen *et al.*, 1993). In such a region of tight linkage disequilibrium, assaying for one marker would generally provide genotype information of all the others. According to Julier *et al.* (1991), candidate *INS* polymorphisms were determined by comparison of independent sequences from individuals. Restriction site polymorphisms are designated by their position, followed by the restriction enzyme; other polymorphisms are designated by base pair change.

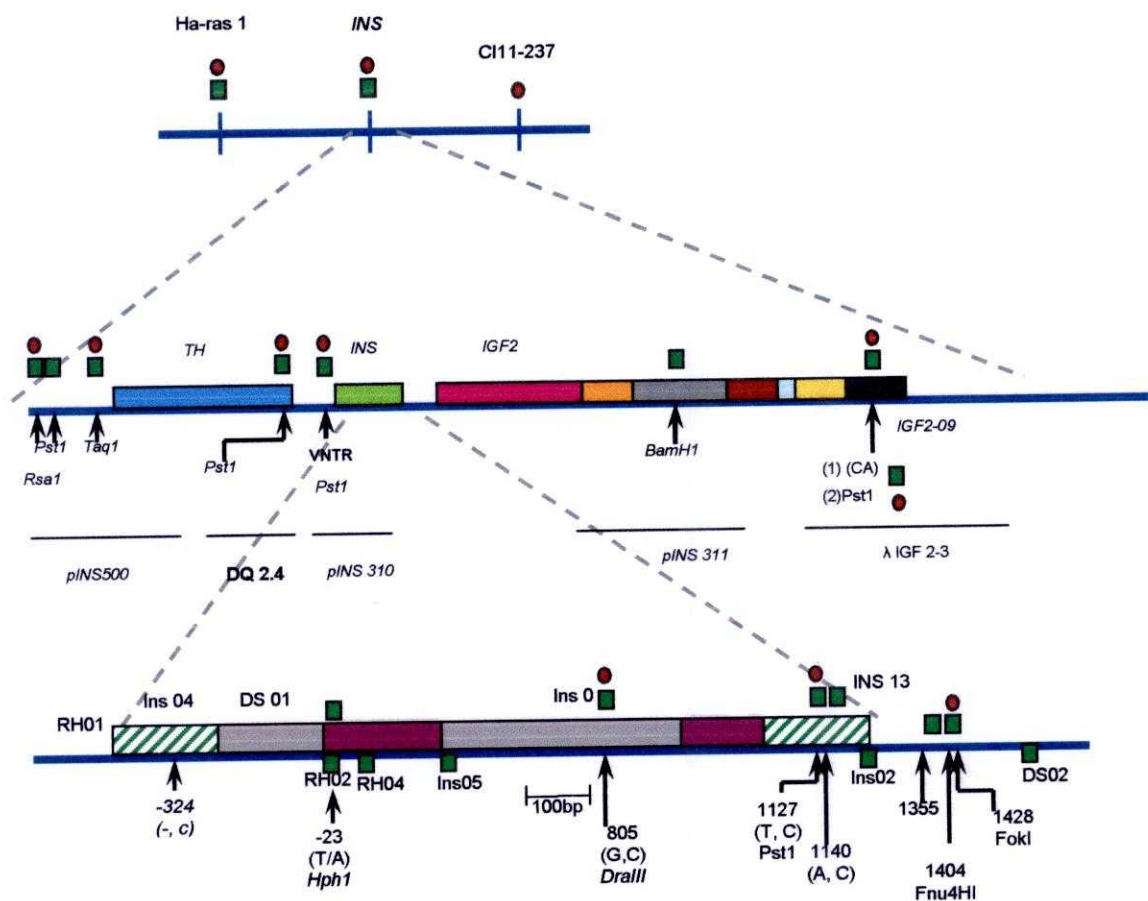


Figure 1: Map of polymorphisms of INS and surrounding loci (●, characterized in isolated diabetics and control; ■, characterized in multiplex families), and primers used for sequencing (Julier *et al.*, 1991)

Several potential polymorphisms in *INS* were at, -23/*hph1*, 805/*DraIII*, 1127/*Pst1*, 1140 (A,C), identified by base pair change between two insulin alleles of one individual (Ulrich *et al.*, 1980), other four new polymorphisms were at -324 (insertion) in 5' region and 1355 (C,T), 1404/*Fnu4HI*, 1428/*FokI* in the 3' flanking sequences (Figure 1). Owerbach and Gabbay (1993) hypothesized that the VNTR was the most likely candidate for Insulin dependent diabetes mellitus-2 (IDDM2) or type 2 diabetes (T2DM). The only polymorphisms that were now candidates for IDDM2 were the -2733 A/C, VNTR, -23/*HphI* and +1140 A/C loci, because both protective haplotypes alleles are negatively associated at each of these four loci (Bennett and Todd, 1996).

The +1127*Pst*I marker is located on 3' untranslated region (UTR) and play a crucial role in regulating insulin production (Bagadi *et al.*, 2005). The Polymorphism at +1127*Pst*I in *INS* is tightly linked with its VNTR.

It has been reported to be associated with diabetes (Lucassen *et al.*, 1993). The *Pst*I (+) allele is always transmitted with the class I alleles, whereas the *Pst*I (-) allele is most often transmitted with class III alleles (Bennett *et al.*, 1995; Lucassen *et al.*, 1995). A recent study by Sanchez-Corona *et al* (2004), analyzed eight different polymorphisms and the *Pst*I polymorphism in *INS* was significantly associated with hypertriglyceridemia and components of MS like obesity.

The -23*Hph*I 'A' allele is in complete linkage disequilibrium with class I alleles of the neighboring VNTR and the -23*Hph*I 'T' allele, with class III alleles, in European population (Bennett and Todd, 1996). The -23*Hph*I "T" allele was found to be associated with increased levels of insulin gene transcription (Bennett and Todd, 1996; Kennedy *et al.*, 1995; Vafiadis *et al.*, 1996) and circulating insulin levels (Dos Santos *et al.*, 2004; Le Stunff *et al.*, 2000). In Pima Indian, -23*Hph*I 'T' allele found to be associated with lower birth weight and T2DM (Lindsay *et al.*, 2003). In a study with children, Le Stunff *et al* (2000) found this polymorphism to be associated with different effects of body fatness on insulin secretion. Moreover, they that insulin VNTR and *Hph*I polymorphisms are possible genetic markers to predict future B-cell failure in obese patients.

There huge inconsistencies regarding the wild nucleotide at the site of interest, A and T have shown interchangeably in different articles (Julier *et al.*, 1991). In this script A has been taken as wild allele (class I allele). The role of insulin VNTR and *Hph*I A>T polymorphisms in T2DM and insulin secretion is still controversial. Reports have shown increased level of insulin level with those having *INS* VNTR wild allele (Dos Santos *et al.*, 2004). On the other hand Vafiadis *et al* (1997) and Pugliese *et al* (1997) observed higher insulin levels in subjects with variant genotypes. Though there are reports which, however, failed to reflect any effect of the VNTR genotype on insulin secretion (Heude *et al.*, 2004).

The debate also yet to be clearly addressed regarding the role of polymorphisms allele in obesity, as in a population based study Manjinder et al (2005) observed lack of association for INS VNTR variant allele with important determinants of body size and adult obesity in middle-aged men and women. On these regards, to settle down the controversy, it is important to design a study on INS VNTR variants addressing overweight to obese diabetes subjects.

Numbers of studies have done involving overweight and obese Bangladeshi subjects to explore the etopathogenesis of type 2 diabetes in the population. Compromised insulin secretory capacity found to be the major feature both in nonobese and obese type 2 diabetic subjects in Bangladeshi origin (Junaid, 2000; Zinnat *et al.*, 2003; Al-Mahmud, 2000; Roy *et al.*, 2007). Factors, possibly genetic components, influencing insulin secretion in these subjects have not yet been looked into among these subjects. Series of studies have also been undertaken to explore etiopathogenesis in the young onset diabetic patients as well. In one of these studies role of INS VNTR polymorphic marker genotype was determined to explore its association with young onset diabetes (Hassan, 2006). However, the candidate marker has yet to be investigated in type 2 diabetes subjects. Since there is strong indication that INS VNTR variant genotype, class III allele, is associated with obesity and higher *in vivo* insulin expression it is important to explore the association of INS VNTR variant on overweight to obese Bangladeshi T2DM subjects.

Hypothesis

The hypothesis of the present study is that *INS VNTR* polymorphism is associated with obesity and this association increases the risk of T2DM in Bangladeshi population.

Objective

General objectives

The general objective of the present study was to find the role of *INS VNTR* A>T variant in the development of overweight, obesity and T2DM in Bangladeshi population.

Specific objectives

The specific objectives of the present study were to:

- Explore the association of *INS VNTR* A>T variant with overweight and obesity in a Bangladeshi population
- Identify whether the *INS VNTR* A>T variant genotype associated with some of this feature of metabolic syndrome apart from obesity
- Examine the possibility that *INS VNTR* variant genotype allele is associated with BMI or T2DM.

Chapter 2

REVIEW OF LITERATURE

2. Review of Literature

2.1 Metabolic Syndrome

Metabolic syndrome is a combination of medical disorders that increase the risk of developing cardiovascular disease, diabetes and obesity together. Although it doesn't have a coconscious definition, most health professionals would include the some other principal components for metabolic syndrome.

The first international definition was given by the World Health Organization in 1999 (WHO, 1999). This was focusing on the glucose intolerance and insulin resistance. Other components as elevated blood pressure ($>140/90$ mmHg), raised plasma triglycerides (>1.7 mmol/l), decreased HDL cholesterol (for men <0.9 mmol/l, for women <1.0 mmol/l), obesity (waist-hip ratio for men >0.9 , for women >0.85 ; and/or BMI >30 kg/m²), and microalbuminuria (urinary albumin excretion rate >20 g/min or albumin: creatinine ratio >30 mg/g) were also involved (WHO, 1999).

Subsequently In 2002, the National Cholesterol Education Program-Third Adult Treatment Panel (ATP III), however, proposed a new definition concentrating rather on the obesity and dyslipidaemia. Three or more of the following risk factors are needed for the diagnosis of metabolic syndrome: fasting plasma glucose >6.1 mmol/l, blood pressure $>130/85$ mmHg, triglycerides >1.7 mmol/l, HDL cholesterol for men <1.03 mmol/l, for women <1.29 mmol/l, waist circumference for men >102 cm, for women >88 cm (NCEP, 2002).

The National Cholesterol Education Program's Adult Treatment Panel III (ATP III) identified the metabolic syndrome as a multiplex risk factor for cardiovascular disease (CVD). ATP III criteria for metabolic syndrome differ somewhat from those of other organizations. The scientific evidence related to definition of metabolic syndrome (MS) was reviewed and considered from several perspectives: (i) major clinical outcomes, (ii) metabolic components, (iii) pathogenesis, (iv) clinical criteria for diagnosis, (v) risk for clinical outcomes, and (vi) therapeutic interventions (NCEP, 2002).

In 2005, the International Diabetes Federation (IDF) put forward a consensus worldwide definition of the metabolic syndrome. The definition for use in clinical practice is similar to the ATP III criteria focusing on the central obesity, but still

keeping type 2 diabetes mellitus (T2DM) in the definition of the syndrome and strongly recommends the oral glucose tolerance test (OGTT). On the other hand, central obesity and insulin resistance were acknowledged equally important causative factors (IDF, 2003).

2.2 Components of Metabolic Syndrome

ATP III identified 6 components of the metabolic syndrome that relate to CVD:

- Abdominal obesity
- Atherogenic dyslipidemia
- Raised blood pressure
- Insulin resistance - glucose intolerance
- Proinflammatory state
- Prothrombotic state

2.3 Obesity

Overweight refers to an excess of body weight compared to set standards. The excess weight may come from muscle, bone, fat, and/or body water. Obesity refers specifically to having an abnormally high proportion of body fat (NIH, NHLBI, 1998). One can be overweight without being obese, however many people who are overweight are also obese. The definition of overweight and obesity is still a matter of debate for two main reasons: the lack of a simple, low-cost, accurate, and reproducible method to measure fat mass in infants, children, young or adults and the lack of cutoffs of fat mass for children to identify individuals at moderate or high cardiovascular and metabolic risk in childhood and adulthood.

2.3.1 Measures of Obesity

A number of methods are used to determine if an individual is overweight or obese. Some of them are based on mathematical calculations of the relation

between height and weight others are based on measurements of body fat. These methods are described below.

Body Mass Index

Body Mass Index (BMI) can be used to measure both overweight and obesity in adults. BMI is a direct calculation based on height and weight, and it is not gender-specific. BMI is found by dividing a person's weight in kilograms by height in meters squared.

The National Institutes of Health (NIH) identify overweight as a BMI of 25-29.9, and obesity as a BMI of 30 or greater. However, overweight and obesity are not mutually exclusive; since obese persons are also overweight (NIH, NHLBI, 1998). Defining overweight as a BMI of 25 or greater is consistent with the recommendations of the World Health Organization (WHO, 1998) and most other countries. In 2004, a WHO expert consultation suggested using cut-offs of 23 kg/m^2 and 27.5 kg/m^2 to identify 'increased risk' and 'high risk' for overweight among Asian populations (WHO, 2004). According to these criteria, for many Asian populations, the suggested category for overweight was classified and recommended as follows: (i) less than 18.5 underweight; (ii) 18.5–23 increasing but acceptable risk; (iii) 23–27.5 increased risk; and (iv) 27.5 or higher high risk.

Weight-for-height Chart

Weight-for-height charts are another measure used to determine if a person is overweight (although they do not measure body fat). These charts, which have been used by doctors and other health care workers for decades, usually give a range of acceptable weights for a person of a given height. Many versions of weight-for-height charts exist, some showing different acceptable weight ranges for men and women.

Measurements of Body Fat

There are a number of ways to measure body fat. Historically, the standard method is to weigh a person underwater; this procedure is limited to laboratories with specialized equipment. Other simpler methods for measuring body fat include *skinfold thickness* measurements and *bioelectrical impedance analysis* (BIA). Skin fold thicknesses are measures of the subcutaneous (lying just

beneath the skin) fat at specific sites of a person's body, such as the triceps (the back of the upper arm).

Waist hip Ratio

Health professionals may also rely on a person's waist measurement to determine the location of excess body fat and the corresponding health risks for body weight and height measurements. Studies indicate that increased abdominal or upper body fat is related to the risk of developing heart disease, diabetes, high blood pressure, gallbladder disease, stroke, and certain cancers, and is associated with overall mortality. Body fat concentrated in the lower body (around the hips, for example) may be less harmful in terms of mortality and morbidity (likelihood of disease), with the exception of varicose veins and orthopedic problems (Bouchard *et al.*, 1990; Peiris *et al.*, 1989).

2.3.2 Classification of Obesity

According to BMI

The primary classification of obesity is based on the measurement of BMI, designed for related BMI to risk of disease. The relation between BMI and disease risk varies among individuals and among different populations. The classification can be applied generally to adults. The basis for this BMI classification scheme stems from observational and epidemiologic studies which relate BMI to risk of morbidity and mortality. Classification by BMI for overweight and obesity according to WHO 1997 given below

Obesity Class		BMI (kg/m ²)
Underweight		<18.5
Normal		18.5-24.9
Overweight		25.0-29.9
Obesity	I	30.0-34.9
	II	35.0-39.9
Extreme Obesity	III	≥40

According to waist circumference

Although waist circumference and BMI are interrelated, waist circumference provides an independent prediction of risk over and above that of BMI. Waist circumference measurement is particularly useful in patients who are categorized as normal or overweight on the BMI scale.

HIGH RISKS

Men	>102 cm (> 40 inch)
Women	> 88 cm (> 35 inch)

In some populations (Asian-Americans or persons of Asian descent), waist circumference is a better indicator of relative disease risk than is BMI. Waist circumference also assumes greater value for estimating risk for obesity-related disease at older ages (NIH, NHLIB, 1998).

2.3.3 Prevalence of Obesity

Overweight and obesity are found worldwide, and the prevalence of these conditions in the United States ranks high along with other developed nations. Approximately 280,000 adult deaths in the United States each year are attributable to obesity (Allison *et al.*, 1996). Below are some frequently asked questions and answers about overweight and obesity statistics. Unless otherwise specified, the figures given represent total (not age-adjusted) numbers.

Overweight and obesity in the U.S. occur at higher rates in racial / ethnic minority populations such as African American and Hispanic Americans, compared with White Americans. During 1988-1994, the prevalence of overweight (BMI $\geq$ 25) was 62.5%, and the prevalence of obesity (BMI $\geq$ 30) was 30.2% in non-Hispanic Black population. This prevalence is increased 69.6% and 39.9% respectively in 1999 to 2000.

World Health Organization (WHO) Expert Consultation proposed a new BMI cutoff of 23.0 for public health action in Asia (WHO Expert Consultation, 2004). The use of this cutoff, however, was not directly supported by data on mortality

(Stevens, 2003). Indeed, deaths from any cause were lowest among men with a BMI of 24.0 to 24.9 and women with a BMI of 25.0 to 26.9 in a representative group of Chinese subjects (Gu *et al.*, 2006). The prevalence of CED (BMI <18.5 kg/m²) was 38.8 and 29.7% among rural and urban poor women, respectively. On the other hand, 9.1% of urban poor and 4.1% of rural women were 'overweight or obese' (BMI ≥25.0 kg/m²). Obesity (BMI ≥30.0 kg/m²) was found among 0.4% of rural and 1.1% of urban poor women. In addition, a larger proportion of women were 'at risk of overweight' (BMI 23.0-25.0 kg/m²) (9.8% of urban poor and 5.5% of rural women). The prevalence of 'at risk of overweight or overweight' (BMI ≥23.0 kg/m²) was higher among urban poor compared with rural women (18.9 vs 9.6%). Similarly, the prevalence of CED was higher among rural women than among urban poor women (38.8 vs 29.7%) (Shafique *et al.*, 2007).

Among urban poor women, prevalence of overweight and obesity increased considerably from 6.6% in 2000 to 10.1% in 2001, but did not increase afterwards. In 2004, the prevalence of overweight among urban poor women was quite high (9.1%) and almost double that among rural women (5.5%). In addition, the overall prevalence of women being at risk of or actually being overweight or obese was 18.9% in urban poor areas and 9.6% in rural areas. These findings concur with those of a recently published report based on Bangladesh Demographic Health Survey (BDHS) (Talukder *et al.*, 2006). According to this study, from 1996/97 to 2004, the proportion of women with at-risk BMI (≥23 kg/m²) increased from 5.1 to 10.2% in rural areas and 24 to 26% in urban areas.

2.4 Risks of Obesity

2.4.1 Morbidity

Above a BMI of 20 kg/m², morbidity for a number of health conditions increases as BMI increases. Higher morbidity in association with overweight and obesity has been observed for hypertension (Brown *et al.*, 1998), type 2 diabetes (Ford *et al.*, 1997), coronary heart disease (CHD) (Willett *et al.*, 1995), stroke (Rexrode *et al.*, 1997), gallbladder disease (Khare *et al.*, 1995), osteoarthritis (Cicuttini *et al.*, 1996), sleep apnea and respiratory problems (Chua and Chediak, 1994) and

some types of cancer (endometrial, breast, prostate, and colon) (Giovannucci *et al.*, 1996; Huang *et al.*, 1997). Obesity is also associated with complications of pregnancy, menstrual irregularities, hirsutism, stress incontinence, and psychological disorders (depression) (Faith and Allison, 1996). The nature of obesity-related health risks is similar in all populations, although the specific level of risk associated with a given level of overweight or obesity may vary with race/ethnicity, and also with age, gender, and social conditions.

2.4.1.1 Hypertension

Data from NHANES III show that the age adjusted prevalence of high blood pressure (SBP ≥ 140 mm Hg, DBP ≥ 90 mm Hg) increases progressively with higher levels of BMI in men and women (Brown *et al.*, 1998). The direct and independent association between blood pressure and BMI or weight has been shown in numerous cross-sectional studies (Criqui *et al.*, 1982), including the large international study of salt (INTERSALT) carried out in more than 10,000 men and women (Dyer and Elliott, 1989).

2.4.1.2 Dyslipidemia

High total cholesterol

The relationship of the age-adjusted prevalence of high total cholesterol (≥ 240 mg/dL), to BMI from NHANES III is shown in Figure 02 (Brown *et al.*, 1998). At each BMI level, the prevalence of high blood cholesterol is greater in women than in men. In a smaller sample, higher body weight is associated with higher levels of total serum cholesterol in both men and women (Denke *et al.*, 1994) at levels of BMI > 25 . Several large longitudinal studies also provide evidence that overweight; obesity and weight gain are associated with increased cholesterol levels. In addition, the pattern of fat distribution appears to affect cholesterol levels independently of total weight.

High triglycerides

The strong association of triglyceride levels with BMI has been shown in both cross-sectional and longitudinal studies, for both sexes and all age groups. In three adult age groups, namely 20 to 44 years, 45 to 59 years, and 60 to 74 years, higher levels of BMI, ranging from 21 or less to more than 30, have been associated with increasing triglyceride levels; the difference in triglycerides

ranged from 61 to 65 mg/dL (0.68 to 0.74 mmol/L) in women and 62 to 118 mg/dL (0.70 to 1.33 mmol/L) in men.

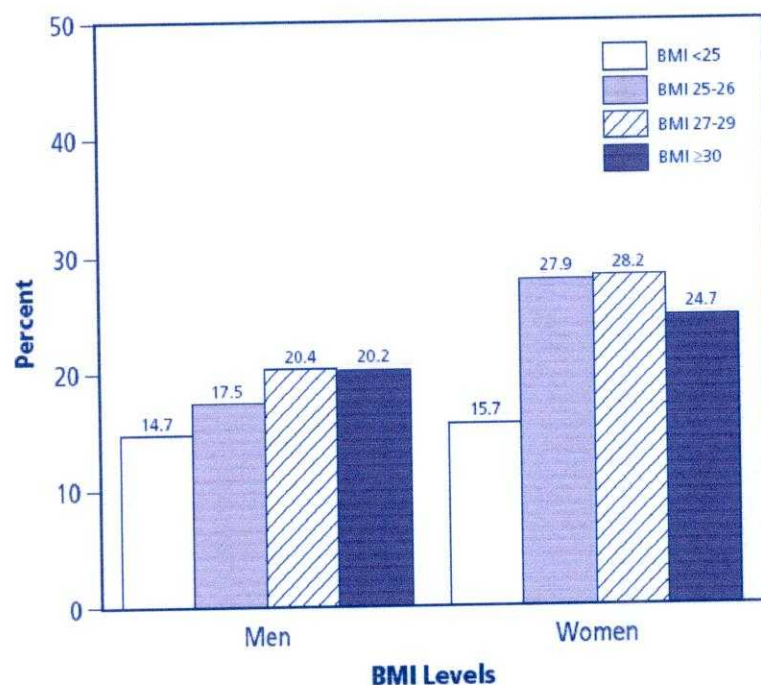


Figure 2: NHANES III age adjusted prevalence of High blood cholesterol according to Body Mass Index (Brown *et al.*, 1998)

HDL and LDL cholesterol

The age-adjusted prevalence of low high-density lipoprotein (HDL)-cholesterol in relation to BMI levels, based on NHANES III data. HDL-cholesterol levels at all ages and weights are lower in men than in women. Although low HDL-cholesterol in this study was defined as < 35 mg/dL (0.91 mmol/L) in men and < 45 mg/dL (1.16 mmol/L) in women, the panel accepts the definition of low HDL-cholesterol as < 35 mg/dL for men and women used by the National Cholesterol Education Program's (NCEP, 2002). The link between total serum cholesterol and CHD is largely due to low-density lipoprotein (LDL). A high-risk LDL-cholesterol is defined as a serum concentration of ≥160 mg/dL. Cross-sectional studies have reported that HDL-cholesterol levels are lower in men and women with higher BMI. Longitudinal studies have found that changes in BMI are associated with changes in HDL-cholesterol. On the other hand LDL-cholesterol

levels are higher by 10 to 20 mg/dL in relation to a 10 unit difference in BMI, from levels of 20 to 30 kg/m². (Denke *et al.*, 1994). According to extensive epidemiological data, a 10 mg/dL rise in LDL-cholesterol corresponds to approximately a 10 percent increase in CHD risk over a period of 5 to 10 years (Law *et al.*, 1994).

2.4.1.3 Diabetes Mellitus

The increased risk of diabetes as weight increases has been shown by prospective studies in Norway (Westlund *et al.*, 1972), the United States (Lew and Garfinkel, 1979), Sweden (Larsson *et al.*, 1981) and Israel (Medalie *et al.*, 1974). More recently, the Nurses' Health Study, using data based on self-reported weights, found that the risk of developing type 2 diabetes increases as BMI increases from a BMI as low as 22 (Colditz *et al.*, 1990). Since women in particular tend to under report weight, the actual BMI values associated with these risks are likely to be higher than the Nurses' Health Study data would suggest. An association between type 2 diabetes and increasing relative weight is also observed in populations at high risk for obesity and diabetes, such as in American Indians (Lee *et al.*, 2002). In recent studies, the development of type 2 diabetes has been found to be associated with weight gain after age 18 in both men (Chan *et al.*, 1994) and women (Colditz *et al.*, 1990). The relative risk of diabetes increases by approximately 25% for each additional unit of BMI over 22 kg/m² (Colditz *et al.*, 1990). In addition, in a prospective study representative of the U.S. population, it was recently estimated that 27% of new cases of diabetes was attributable to weight gain in adulthood of 5 kg (11 lb) or more (Ford *et al.*, 1997). Both cross-sectional and longitudinal studies show that abdominal obesity is a major risk factor for type 2 diabetes (Chan *et al.*, 1994)

Overweight and obese people are at an increased risk for developing the following conditions also, cardiovascular disease, stroke, cancer, congestive heart failure, angina pectoris, cholecystitis, cholelithiasis, osteoarthritis, gout, fatty liver disease, sleep apnea and other respiratory problems, Polycystic ovary syndrome (PCOS), Fertility complications, Psychological disorders, Uric acid nephrolithiasis (kidney stones), Stress urinary incontinence, endometrium *etc.*

2.4.2 Mortality

As stated in the introduction to the guidelines, in the majority of epidemiologic studies, mortality begins to increase with BMIs above 25 kg/m². The increase in mortality generally tends to be modest until a BMI of 30 kg/m² is reached. For persons with a BMI of 30 kg/m² or above, mortality rates from all causes, and especially from cardiovascular disease, are generally increased by 50 to 100 percent above that of persons with BMIs in the range of 20 to 25 kg/m². (WHO Expert Committee, 1995). Three aspects of the association between obesity and mortality remain unresolved.

2.5 Causes of Obesity

Most researchers agree that a combination of excessive calorie consumption and a sedentary lifestyle are the primary causes of obesity (Bleich *et al.*, 2007). In a minority of cases, increased food consumption can be attributed to genetic, medical, or psychiatric illness, but in general the rising prevalence of obesity is attributed to the availability of an easily accessible and palatable diet (Pool, 2001), car culture, and mechanized manufacturing. A 2006 review identifies ten other possible contributors to the recent increase in the rate of obesity: (1) insufficient sleep, (2) endocrine disruptors - food substances that interfere with lipid metabolism, (3) decreased variability in ambient temperature, (4) decreased rates of smoking, which suppresses appetite, (5) increased use of medication that leads to weight gain, (6) increased distribution of ethnic and age groups that tend to be heavier, (7) pregnancy at a later age, (8) intrauterine and intergenerational effects, (9) positive natural selection of people with a higher BMI, (10) assortative mating, heavier people tending to form relationships with each other (Keith *et al.*, 2006).

2.5.1 Dietary habit

Despite the widespread availability of nutritional information in schools, doctors' offices, on the internet and on product packaging, it is evident that overeating remains a substantial problem. In the period of 1971-2000, obesity rates in the United States increased from 14.5% to 30.9% (Flegal *et al.*, 2002). During the same time period, an increase occurred in the average amount of calories consumed. For women, the average increase was 335 calories per day (1542

calories in 1971 and 1877 calories in 2004), while for men the average increase was 168 calories per day (2450 calories in 1971 and 2618 calories in 2004). Most of these extra calories came from an increase in carbohydrate consumption rather than an increase in fat consumption (Wright *et al.*, 2004). Agricultural policy and techniques in the United States and Europe have led to lower food prices. In the United States, subsidization of corn, soy, wheat, and rice through the U.S. farm bill has made the main sources of processed food relatively cheap compared to fruits and vegetables (Pollan, 2007).

2.5.2 Lifestyle

A sedentary lifestyle plays a significant role in obesity. In 2000 the CDC estimated that more than 40% of the US population was sedentary, another 30% was active but not sufficiently and less than 30% had an adequate level of physical activity (Caballero, 2007). There has been a trend toward decreased physical activity in part due to increasingly mechanized forms of work, changing modes of transportation, and increasing urbanization. Studies in children and adults have found a association between the number of hours of television watched and the prevalence of obesity (Gortmaker *et al.*, 1996; Vioque *et al.*, 2000). Obese people are less active than those of normal weight. For example in Canada, 27.0% of sedentary men are obese as opposed to 19.6% of active men (Tjepkema, 2005).

2.5.3 Clinical syndromes

Certain physical and mental illnesses and the pharmaceutical substances used to treat them can increase one's risk of obesity. Medical illnesses that increase obesity risk include several rare genetic syndromes (listed above) as well as some congenital or acquired conditions: (1) hypothyroidism, (2) Cushing's syndrome, (3) growth hormone deficiency (Rosén *et al.*, 1993) and (4) eating disorders such bulimia nervosa, binge eating disorder and compulsive overeating. Certain medications may cause weight gain and or negative changes in body composition, such as insulin, atypical antipsychotics, steroids, sulfonylureas, and some fertility medication.

2.5.4 Genetics

Like many other medical conditions, obesity is the result of interplay between genetic and environmental factors. Polymorphisms in various genes controlling appetite, metabolism, and adipokine release may predispose to obesity when sufficient calories are present. Obesity is a major feature in a number of rare genetic conditions: Prader-Willi syndrome, Bardet-Biedl syndrome, MOMO syndrome, leptin receptor mutations, melanocortin receptor mutations. In a people with early-onset severe obesity (defined by an onset before ten years of age and body mass index over three standard deviations above normal), 7% harbor a single locus mutation (Farooqi and O'Rahilly, 2006). Apart from the above syndromes, an association has been found between an *FTO* gene polymorphism and weight. The 16% of adults in the study who were homozygous for this allele weighed about 3 kilograms more than those who had not inherited this trait and subsequently had a 1.6 fold greater rate of obesity (Frayling *et al.*, 2007). The percentage of obesity that can be attributed to genetics varies from 6% to 85% depending on the population examined (Yang *et al.*, 2007). On a population level, the *thrifty gene hypothesis* postulates that certain ethnic groups may be more prone to obesity when exposed to an equivalent environment. Individuals with greater adipose reserves would be more likely survive famine. This tendency to store fat however would be maladaptive in societies with stable food supplies (Chakravarthy and Booth, 2004). This is the presumed reason why Pima Indians, who evolved in a desert ecosystem, developed some of the highest rates of obesity when exposed to a Western lifestyle (Pool, 2001).

2.6 Genetics of Obesity

The more common genetic disorder, such as diabetes and obesity result from the combined contributions of many genes. These are called polygenic disorders. To make things even more complicated, a disease can be polygenic in some families and monogenic in others. This is true for both diabetes and obesity, and makes it very difficult to determine the contributions of distinct genes. Because of the heterogeneous nature of T2D, and its multifactorial and polygenic inheritance, there has been very limited success in identifying the genes that contribute to the disease in the vast majority of affected patients.

There are two main reasons for a person to be obese; one is that the person may consume more calories than they should every day. The other reason is because of genetics. Genes do play a major part in obesity. Although, genes are not destiny, obesity can be prevented or managed with a combination of diet, physical activity, and medication. Like T2D, obesity is a multifactorial disorder. As food has become available in unlimited supply in Western countries, and people have become more sedentary, underlying genetic risks for obesity have become more obvious. Both twin and adoption studies have demonstrated a significant genetic contribution to the risk of developing obesity, and estimates of the relative genetic contribution to the etiology of T2D are as high as 90%. The area identified by the researchers contains molecular markers that are highly statistically associated with a number of features fundamental to the form of obesity that commonly triggers related health problems. Studies have shown that if one or both parents are obese, the child has a much greater risk at becoming obese himself (Myers & Vargas, 2002). The genetics may not directly link the children to obesity, but it does create an environment that allows the child to have a higher chance of being overweight (Laing, 2002). As with T2D initial success in identifying obesity genes has been limited to less common monogenic forms of obesity. By focusing on individuals with severe (BMI > 40) and early onset obesity, several genes have been identified. To date, several candidate genes have proven to cause diabetes in individual families. Homozygous mutations in the leptin and leptin receptor genes cause severe obesity in humans just as they do in mice.

The gene-environment interaction is known to play a role in multiple diseases. Obesity is associated with many comorbidities, and an interaction between obesity and a positive family history has been shown in several of these associated illnesses. In individuals with a strong family history of diabetes and increased BMI, reduced beta-cell compensation to the insulin resistance associated with obesity was found, increasing the risk for developing type 2 diabetes. BMI was strongly associated with breast cancer risk among women with a strong family history of breast cancer, but only weakly associated in those women without a family history. In women with a family history of premature coronary artery disease, BMI was an independent predictor of coronary artery

calcification. Further studies are needed to elucidate the possible interactions between obesity and genetic background, but obtaining a family history may be helpful in assessing the risk of the individual obese patient.

2.6.1 Insulin and *INS* gene

INS gene in human chromosome encoded a hormone named insulin. Insulin is secreted uniquely from the islet B-cells of the pancreas and plays a major role in the maintenance of energy homeostasis. Insulin secretion is tightly regulated to maintain blood glucose levels within a narrow physiological range. Insufficient secretion of insulin from B-cells contributes to the chronic hyperglycemia characteristic of diabetes. Short-term regulation of insulin secretion, such as in response to a meal, occurs mainly at the level of exocytosis. In adult mammals, expression of the insulin gene is essentially restricted to the pancreatic B-cell.

Glucose is the major nutrient regulator of pancreatic B-cell functions and coordinately regulates insulin gene expression, insulin biosynthesis, and insulin secretion. Glucose controls all steps of insulin gene expression, including transcription, pre-RNA splicing, and mRNA stability. A more distal glucose-responsive element appears to bind a glucose-sensitive complex that is specifically present in primary islets but remains to be identified (Sander *et al.*, 1998). Glucose enhances insulin gene transcription by a number of complementary mechanisms that include recruitment of transcription factors to regulatory sites, histone modifications, and initiation of transcription. Glucose is also the major physiologic regulator of the insulin gene. When B-cells are exposed to elevated levels of glucose for prolonged periods of time, glucose becomes toxic to insulin secretion, gene expression, and B-cell survival. The relevance of nutrient-induced B-cell dysfunction in humans comes from the fact that chronic hyperglycemia and associated disorders of lipid metabolism are thought to contribute to the deterioration of pancreatic B-cell function observed in patients with T2D (Poitout and Robertson, 2002).

The entire sequence of the human insulin gene (*INS*) was first published in 1980 (Ullrich *et al.*, 1980). *INS* was mapped to the short arm of chromosome 11 using human-mouse somatic cell hybrid lines (Owerbach *et al.*, 1981) and subsequently by *in situ* hybridization to 11p15 (Ullrich *et al.*, 1982). *INS* has been

located between the genes for tyrosine hydroxylase (*TH*) and insulin-like growth factor II (*IGF2*) on 11p15.5 (Figure 3) (Junien and Heyningen, 1991). Restriction mapping studies of the *INS* region detected a polymorphism that appeared to be due to an insertion-deletion of DNA sequences upstream of the gene (Bell *et al.*, 1981). This polymorphic region was later shown to be composed of tandemly repeated 14–15 bp sequences related to the most common repeat ACAGGGGTGTGGGG. This minisatellite, or variable number of tandem repeats (VNTR) locus was positioned at 363 bp from the insulin gene transcription starting site. However, to allow direct comparison with the positional assignment of other polymorphisms in the region (Figure 3), using the 30 (ACAG-30) boundary of the VNTR (Bell *et al.*, 1982) and the published sequence of the *TH-INS-IGF2* region (GenBank accession number J00265), the VNTR locus starts -596 bp from the *INS* translation starting (ATG) codon. Polymorphisms are designated their position with respects to the first base of the initiating ATG (+1) codon of *INS*.

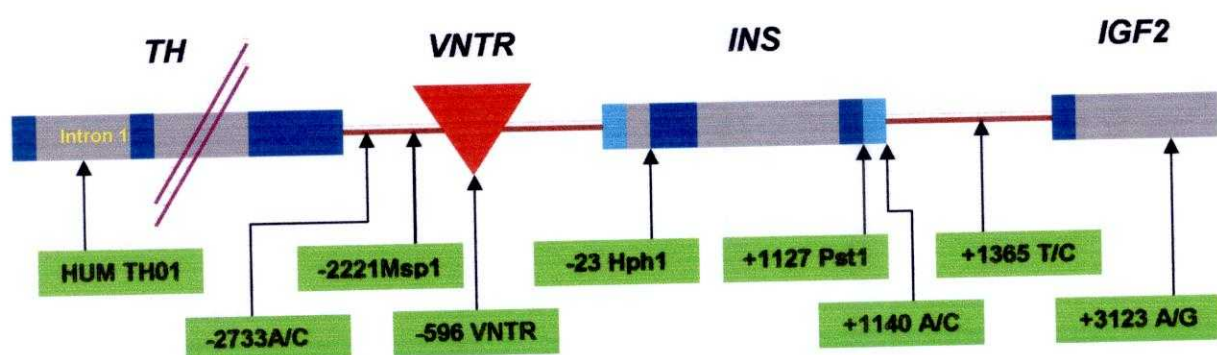


Figure 3: Diagrammatic Representation of *TH-INS-IGF2* region of Chromosome 11 showing the various polymorphisms (Bennette and Todd, 1996).

Variable numbers of tandem repeat polymorphisms (VNTRs) are widely dispersed throughout the genome. They consist of repeats of stereotypic DNA sequences and are generally found in non-coding regions of DNA. For many VNTRs the number of repeat units displays large inter-individual variation, which has made them useful tools in forensic medicine and gene mapping studies as well as the findings of various genetic disorders. In the majority of cases, the function of these VNTR polymorphisms remains a mystery (Undlien and Berg,

2001). The numbers of repeats of the variable oligonucleotide range from about 26 to over 200, but differences in length are not randomly distributed. Instead, VNTR alleles occur in three discrete size classes. Class I, the shorter polymorphic region, is composed of alleles with 26 to 63 repeat units, and the longer class III polymorphic region has alleles ranging from 141 to 209 repeat units. An intermediate class II polymorphic region that is rare in Caucasians has an average allele length of about 80 repeat units (Bell *et al.*, 1982).

The VNTR locus is originally regarded as being part of the insulin gene (Bell *et al.*, 1981), but its role in insulin gene expression, if any, was unclear. It was therefore unlikely that the VNTR itself was the susceptibility locus, but rather a marker for a linked gene(s) that influences IDDM susceptibility, as originally suggested (Bell *et al.*, 1984). Identification of *IDDM2* therefore required further genetic analysis of the region surrounding the *INS* VNTR locus, first to detect all candidate etiologic mutations in the *TH-INS-IGF2* region by comparing the sequences from several chromosomes (Julier *et al.*, 1991; Lucassen *et al.*, 1993; Owerbach and Gabbay, 1993), and second to delineate the minimal region associated (due to linkage disequilibrium) significantly with type 1 diabetes (T1D). A combination of published and newly derived polymorphic RFLP markers was used to systematically define the minimal region of strongest association to a 4.1 kb encompassing the insulin gene and 50 VNTR locus, and other polymorphisms (Julier *et al.*, 1991). Strongest evidence for association was provided by the VNTR, -23/*HphI* and +1140 A/C loci (Lucassen *et al.*, 1993). However, because the all loci were in tight linkage disequilibrium, it was difficult to determine which was the primary etiologic mutation (or mutations), and which were only hitchhiking polymorphisms.

2.7 *INS* VNTR and disease association

Several reports on associations between particular *INS*-VNTR alleles and diseases and phenotypic traits have been published, some of which are listed in Table 1.

Table 1: Various disease and phenotypic traits associated with INS-VNTR alleles.

Disease/Phenotypic trait	Associated INS-VNTR allele	References
Type 1 diabetes	Class I alleles confer susceptibility	Bell <i>et al.</i> , 1984
	Class III alleles confer dominant protection	Bennett <i>et al.</i> , 1995
Type 2 diabetes	Class III alleles confer susceptibility	Ong <i>et al.</i> , 1999; Huxtable <i>et al.</i> , 2000
Polycystic ovary syndrome	Class III alleles confer susceptibility	Waterworth <i>et al.</i> , 1997
Birth weight	Class III alleles confer susceptibility	Dunger <i>et al.</i> , 1998
Obesity	Increased class I allele size confer susceptibility	O'Dell <i>et al.</i> , 1999
Thymus expression of insulin	Class III alleles lead to higher insulin expression in the majority of cases	Vafiadis <i>et al.</i> , 1997; Pugliese <i>et al.</i> , 1997

2.7.1 INS VNTR and Obesity

The molecular mechanisms behind the associations with particular *INS-VNTR* alleles are not clear. Furthermore, due to strong linkage disequilibrium between the various polymorphisms in the given region (Figure 3) there is, with the exception of T1D (Bennett *et al.*, 1995) not formal proof to conclude that it is the *INS-VNTR* and not one of the neighbouring polymorphisms that are primarily involved. There is, evidence from both *in vitro* (Lucassen *et al.*, 1995; Kennedy *et al.*, 1995) and *in vivo* studies (Vafiadis *et al.*, 1997) that the *INS-VNTR* has an effect on mRNA transcription of the insulin gene where the various alleles are associated with different levels of transcription. Recently, Le Stunff *et al.* (2000) demonstrated an association between the *INS-VNTR* and fasting plasma insulin levels in obese children. No significant differences in fasting insulin levels were

observed between children homozygous for the VNTR class III marker and class I/III heterozygotes. Allele frequencies were the same as in a large cohort of lean controls, which indicated that the *INS-VNTR* marker was not primarily associated with juvenile obesity, at least not when classifying alleles broadly as VNTR class I and III respectively. Early studies showed that the frequency of class I homozygotes was higher in type 1 diabetic individuals compared with non-diabetic controls (Hitman *et al.*, 1984). Evidence suggested that the polymorphic locus 50 to the insulin gene was associated with an increased risk of developing T1D, and in particular that disease susceptibility was conferred by the VNTR class I homozygous genotype. The class II alleles have been found predominantly in Black populations and are rarely found in Caucasians and little data are available for this particular class of alleles. In addition to length polymorphisms there are sequence differences within the various repeat units. The polymorphisms of the *INS-VNTR* are not randomly distributed and have been grouped into lineages based upon sequence and length (Stead *et al.*, 2000). Moreover, the *INS-VNTR* polymorphism is in strong linkage disequilibrium with several neighbouring single nucleotide polymorphisms (Lucassen *et al.*, 1993). One of these is a T/A polymorphism, which can be identified by the restriction enzyme *HphI*, and is located 23 nucleotides upstream of the insulin gene translation initiation codon. In Caucasian populations the -23 *HphI* T or A alleles can be used as surrogate genetic marker for class I and III VNTR alleles respectively. Increased insulin secretion is associated with increased fat deposition. VNTR class I homozygotes with higher insulin levels also gained weight more rapidly than class I/III heterozygotes and class III homozygotes during adolescence. A putative role of *INS-VNTR* classes as markers of juvenile obesity is nevertheless dubious because there were no differences in allele frequencies between the obese children and a group of lean controls. However, in this study Le Stunff *et al* (2000) did not type the *INS-VNTR* at high resolution and hence could not split the *INS-VNTR* class I and III alleles respectively into lineages or more precisely defined alleles. In a previous study of adults, no differences in BMI between the *INS-VNTR* classes were detected, whereas the length of the class I allele correlated with increasing BMI (O'Dell *et al.*, 1999).

2.7.2 *INS VNTR* and Diabetes

Genomic variation in the regulatory regions of the insulin gene has been associated with increased susceptibility to both type 1 (Ong *et al.*, 2004) and type 2 diabetes (Huxtable *et al.*, 2000). A protective effect of class III VNTR against type 1 diabetes was associated with increased insulin expression in the thymus (Vafiadis *et al.*, 1997; Pugliese *et al.*, 1997). However, Le Stunff *et al.* (2000) have shown, in juvenile obesity class I VNTR homozygotes seemed to have β -cells with an increased capacity to produce insulin and potentially protect against type 2 diabetes. The mechanisms behind these differences have not been resolved. A possible explanation is that transcription factors that regulate insulin gene expression in the thymus and β -cells may differ in their ability to interact with short or long *INS-VNTRs* and partly determine an individual's predisposition to either type 1 or type 2 diabetes.

For the whole cohort of children, Le Stunff *et al.* (2000) demonstrated a stronger influence on the increase in fasting insulin levels secondary to overweight among VNTR class I homozygotes compared with the other genotypes. The authors speculate that class I homozygotes may adjust their insulin secretion to an increase in fat accumulation better than the class I/III heterozygotes and class III homozygotes, and that class III alleles have a dominant negative effect on obesity-induced increase in insulin secretion. Previous studies have shown that the *INS-VNTR* class III allele is associated with a higher risk of type 2 diabetes (Ong *et al.*, 1999; Huxtable *et al.*, 2000). The study indicated that the hyperinsulinaemia in class I homozygous obese children may enhance their capacity to maintain normal blood glucose levels and protect against type 2 diabetes. Identification of a polymorphic locus close to the insulin gene provided a suitable molecular marker for genetic studies. It was therefore not long before several laboratories had evaluated the *INS-VNTR* polymorphism as a genetic marker for diabetes mellitus, though principally for T2D, or non-insulin dependent diabetes mellitus (NIDDM).

Chapter 3

SUBJECTS AND METHODS

3. SUBJECTS AND METHODS

3.1 Place of the study

The study was conducted in the Laboratory of Biomedical Research Group (BMRG), based in Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Dhaka.

3.2 Study period

This study was done during the period of July 2007 to October 2008.

3.3 Study design

It was a case-control type study. The subjects were selected on the availability.

3.4 Study Subjects

A total number of 332 (three hundred thirty two) healthy subjects were selected and purposively recruited from main stream Bangladeshi population irrespective of religion and socioeconomic status. The study subjects comprised of the following groups:

- (i) Healthy control and
- (ii) Type 2 Diabetes mellitus subjects

3.5 Inclusion criteria

- Adult subjects with age range 25-65 yrs
- Overweight and obese subject

3.6 Exclusion criteria

- Subject with acute and chronic disease other than diabetes like, acute infection, hypertension renal disease.
- History of using drugs significantly affecting glucose metabolism (glucocorticoids, oral contraceptives containing levonorgestrel or high-dose estrogen, phenytoin, high-dose thiazide diuretics etc.).

3.7 Recruitment of the subject

Subjects were collected on every working day of the week in the laboratory of BMRG, BIRDEM. Subjects recruited through personal communication. Interested individuals were further cross checked for their weight and informed consent was obtained. Selected subjects were gives a fresh appointment for their glucose tolerant test. Detailed medical and clinical histories were recorded on pre-design case record form (Appendix). They were advised to be on their usual diet and requested to report BMRG Lab in fasting state.

3.8 Anthropometric measurements

Height (m)

Standing height was measured using appropriate scales (Detect-Medic, Detect scales INC, USA) following standard procedure. Height was recorded to the nearest 5 mm.

Weight (kg)

The balance was placed on a hard flat surface and checked for zero balance before measurement. The subjects were in the center of the platform wearing light cloths without shoes. Weight was recorded to the nearest 0.5 kg.

Calculation of BMI (kg/m²)

Body mass indexes (BMI) of the subjects were calculated using standard formula.

$$\text{BMI} = \frac{\text{Weight (kg)}}{[\text{Height (m)}]^2}$$

Mid arm circumference (MAC)

Mid arm circumference was measured at a point mid way between the acromial process of scapula and olecranon process of ulna of right arm hanging relaxed. A measuring tape was used to record the circumference following the above technique at the nearest millimeter.

Skin fold thickness (mm)

Skin fold thickness was measured using Harpenden Skinfold Calipers following standard procedure. Triceps skin fold thickness was measured 1 cm above the mid point between the acromial process of scapula and olecranon process of ulna at mid-line posteriorly with skin fold lying along the long axis of right arm hanging relaxed. Subscapular skin fold thickness was measured 1 cm below the inferior angle of right scapula with skin fold lying at 45 degree. Values were recorded at the nearest millimeter.

Waist circumference (cm)

Waist circumferences were measured to the nearest 0.5 cm with a soft non-elastic measuring tape. The tape was snug, but not so tight as to cause skin indentation or pinching. The waist circumference was taken to the nearest standing horizontal circumference between the lower border of the 12th rib and the highest point of the iliac crest on the mid-axillary line at the end of normal expiration.

Body fat mass (%)

Body fat mass was measured by Omron Body Fat Monitor (Omron Corporation, Japan, Model No: HBF-302). Height in cm, weight in kg, age in yrs and gender of subjects were inputted to the device. Then the subjects held the monitor by both hands with upper limbs horizontal in standing position. The machine was then started and body fat mass (%) was recorded from the monitor.

3.9 Measurement of Blood Pressure

Blood pressure was measured in sitting position, with calf at the level of the heart. After 10 minutes of rest a second reading was taken. Recorded Korotkoff sound I (the first sound) and V (the disappearance of sound) denoted the systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively (according to WHO-IHS).

3.10 Collection of blood samples

Overnight fasting blood was collected between 8.00-9.00 am. Venous blood (10 ml) was obtained by venepuncture following standard procedure. Subjects were

then given glucose to drink (75 g in 300 ml of water). They were requested not to take any food and be rested for two hours. The second blood sample was then obtained. A portion of blood (5 ml) sample was taken into a screw cap tube containing EDTA (1 mg/ml), mixed thoroughly and preserved in at -30°C for future DNA extraction. Fasting and postprandial blood sample were allowed to clot and serum was separated by centrifugation for 10 min at 3000 rpm using refrigerated centrifuge and preserved -25°C for further biochemical analyses.

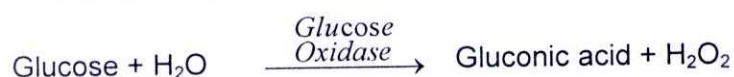
3.11 Biochemical Method

3.11.1 Estimation of Serum Blood Glucose (*Randox laboratories, UK*)

Serum glucose was estimated by Glucose Oxidase (GOD-PAP) method.

Principle

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red violet quinoneimine dye as indicator (Trinder, 1969).



Reagents

Contents	Initial concentration of solution
Buffer	
Phosphate Buffer	0.1 mol/l, pH 7.0
Phenol	11 mol/l
GOD-PAP Reagent	
4-aminophenazone	0.77 mmol/l
Glucose oxidase	≥1.5 kU/l
Peroxidase	≥1.5 kU/l
Standard	
Glucose	5.55 mmol/l (100 mg/dl)

Additional Reagent

Uranyl Acetate 0.16% cat NO DP 647 2x 500 ml

Materials required

- Microcentrifuge tube
- Micropipettes and pipettes tips
- Auto analyzer (Hitachi 704, Hitachi Ltd. Tokyo, Japan)

Procedure

The methods determines glucose without deproteinization. The instrument was calibrated before estimation. Serum and reagent were taken in specific cup. They were arranged serially into the Auto analyzer HITACHI 704, Hitachi Ltd Tokyo, Japan. The Auto analyzer was programmed for the estimation of glucose and allowed to run with following procedure:

5 µl sample and 500 µl reagent were mixed and incubated at 37° C for 10 minutes. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Optical densities or absorbances were fed into a computer and calculation was done using the software program. Values for the unknown samples were calculated by extrapolating the absorbance for the standard using following formula.

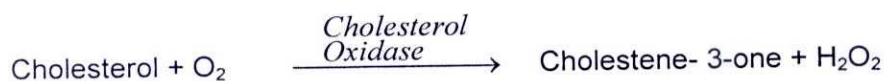
$$\text{Glucose concentration (mmol/l)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times 5.55$$

3.11.2 Estimation of Serum Total Cholesterol

Total cholesterol was measured by enzymatic endpoint method (cholesterol Oxidase/Peroxidase) method in auto analyzer Auto analyzer HITACHI 704, Hitachi Ltd Tokyo, Japan.

Principle

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase (Richmond, 1973).



Reagent composition

Contents

Initial Concentration of Solution

Reagent

4-Aminoantipyrine	0.30 mmol/l
Phenol	6 mmol/l
Peroxidase	≥ 0.5 U/ml
Cholesterol esterase	≥ 0.15 U/ml
Cholesterol oxides	≥ 0.1 U/ml
Pipes Buffer	80 mmol/l; pH 6.8
Standard	5.17 mmol/l (200 mg/dl)

Materials

- Microcentrifuge tube
- Micropipettes and pipettes
- Disposable tips
- Auto analyzer (Hitachi 704, Hitachi Ltd Tokyo, Japan).

Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the Auto analyzer. 5 μ l sample and 500 μ l reagent were mixed and incubated at 37°C for 5 minutes within the Auto lab. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

Concentration of cholesterol in sample was calculated by using software program with the following formula.

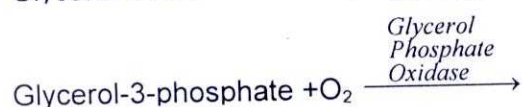
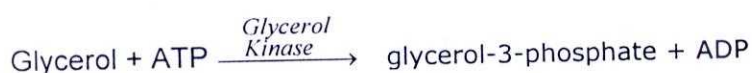
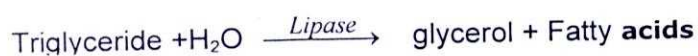
$$\text{Cholesterol concentration (mg/dl)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times \text{concentration of standard.}$$

3.11.3 Estimation of Serum Triglycerides

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of Randox Laboratories Ltd., UK.

Principle

The triglyceride is determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen- peroxide, 4- aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase (Fossati and Prencipe, 1982).



Reagents

Contents	Concentrations in the Test
Buffer	
Pipes Buffer	40 mmol/l, pH 7.6
4-choloro-phenol	5.5 mmol/l
Magnesium-ions	17.5 mmol/l
Enzyme Reagent	
4-aminophenazone	mmol/l
ATP	1.0 mmol/l
Lipases	>150 U/ml
Glycerol-3-phosphate oxidase	1.5 U/ml
Peroxidase	0.5 U/ml
Standard	2.29 mmol/l (200 mg/dl)

Materials

- Micropipettes and pipettes
- Disposable tips
- Auto analyzer

Procedure

Serum and reagents were taken in specific cup. They were arranged serially. Then ID number for test was entered in the analyzer. Five (5) µl sample and 500 µl reagent were mixed and incubated at 37°C for 5 minutes within the cell. Reading was taken at 500 nm.

Calculation of result

Triglyceride concentration was calculated by following formula:

$$\text{Triglyceride concentration (mg/dl)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times \text{Concentration of standard.}$$

3.11.4 Estimation of Serum High Density Lipoprotein (HDL) cholesterol

Serum High density lipoprotein cholesterol (HDLc) was measured by enzymatic colometric method using reagent of Randox laboratories, UK.

Test Principle

HDL (High Density Lipoproteins) is separated from chylomicrons, VLDL (very low density lipoproteins) and LDL (Low density lipoproteins) by the addition of a precipitating reagent (phosphotungstic acid-magnesium chloride) to serum. After centrifugation, the cholesterol contents of HDL fraction, which remains in the supernatant, was determined by the enzymatic colorimetric method using CHOD- PAP (Friedwald *et al.*, 1972).

Reagents composition:

Phosphotungstic Acid: 0.55 mmol/l
Magnesium Chloride: 25 mmol/l

Material Provided

HDL-cholesterol Precipitant.

Materials Required

1. Randox lipid Controls
2. Randox aqueous Cholesterol Standard: 200 mg/dl
3. Reagent solution for cholesterol CHOP-PAP assay.
4. Pipettes (5 μ l –50 μ l, 100 μ l-1000 μ l) and Pipette Tips.
2. Multi-Channel Pipettes and Pipette Tips: 50-300 μ l
3. Buffer and Reagent Reservoirs
4. Vortex Mixture
5. Deionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm 590 nm
7. Orbital Microtiter Plate Shaker
8. Absorbant Paper

Standard Preparation

Dilute Randox aqueous cholesterol standard (200mg/dl) with deionized water by volume of 0, 20, 40, 50, and 100 μ l. The final volume was 200 μ l.

Assay Procedure

1. 100 ml serum samples taken in microcentrifuge tube
2. Add 250 μ l HDL-c Precipitant.

3. Mix well and allow sitting for 10 minutes
4. Vortex the mix components and centrifuged for 15 minutes at 4000 rpm.
5. Transfer 30 µl of each Standard in first six wells.
6. Transfer 30 µl of clear supernatant into the other wells
7. 250 µl of cholesterol reagent was then added into all the 96 wells quickly using Multi-channel pipettes.
8. Incubated for 5 minutes at 37°C on orbital microtiter plate shaker.
9. Absorbance was read at 490 nm.

Calculations

The HDL-c value of each sample was obtained as follows:

The net absorbance value for each level, obtained by subtracting the value for the HDL-c concentration (mg/dl) from the value of individual. The smooth linear curve was drawn and the results of unknown samples were calculated using logistic function.

3.11.5 Estimation of LDL-Cholesterol

The LDL-Cholesterol level in Serum was calculated by using by Friedewald formula (Friedewald *et al.*, 1972).

Formula

$$\text{LDL cholesterol} = \left\{ \text{Total cholesterol} - \left(\text{HDL Cholesterol} + \frac{1}{5} \times \text{Triglyceride} \right) \right\}$$

3.11.6 Estimation of serum creatinine

Estimation of Serum creatinine was done by alkaline-picrate methods using reagents from Randox Laboratories, UK.

Reagents

Standard	177umol/l (2mg/dl)
Picric acid surfactant	35 mmol/L
Sodium hydroxide	0.32 mol/L

Preparation of reagent

All reagents are supplied ready to use, stable to expiry date when stored at +15 to 25°C.

Preparation of working reagent

Mix Equal volumes of Solutions 2+3, Stable for 3 Days At + 15 to+25°C.

Procedure

Wavelength	492 (490-510) nm
Cuvette	1 Cm Light Path
Temperature	25/30/37 C
Measurement	against Air

Pipette into cuvette	Standard	Sample
Working reagent	1.0 ml	1.0 ml
Standard solutions	0.1 ml	---
Sample	---	0.1ml

Mix and after 30 sec, read the absorbance A1 of the standard and sample
Exactly 2 min. later read the absorbance A2 of the standard and sample

Calculation

$$A2-A1 = \Delta A \text{ sample or } \Delta A \text{ standard}$$

$$\text{Concentration of Creatinine in Serum (mg/dl)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 100$$

3.11.7 Estimation of SGPT

SGPT was estimated by UV method using ALT (GPT) opt. kit (RANDOX) (IFCC, 1980).

Principle



Reagents

Contents	Concentration in the test
1. Buffer/Substrate	
Tris biffer	100 mmol/l, pH 7.5
L-alanine	0.6 mol/l
2. Enzyme/Coenzyme/ α -oxoglutarate	
α -oxoglutarate	15 mmol/l
LD	≥ 1.2 U/ml
NADH	0.18 mmol/l

Preparation of Solutions

1. Buffer/Substrate: Buffer/Substrate supplied in the kit was used as it is.
2. Enzyme/Coenzyme/ α -oxoglutarate: One vial of Enzyme/Coenzyme/ α -oxoglutarate² was reconstituted with the appropriate volume of Buffer/Substrate 1:

2 ml	for the	20 x	2 ml kit (AL 1200)
10 ml	for the	20 x	10 ml kit (AL 1205)
20 ml	for the	5 x	20 ml kit (AL 1268)

Cat No AL 2360 5 x 100 ml

One vial of Enzyme/Coenzyme/ α -oxoglutarate 2 was reconstituted with a portion of Buffer/Substrate 1 and then the entire content was transferred to bottle 1 rinsing bottle 2 several times.

Procedure

Wavelength:	340 nm (Hg 334 nm or Hg 365 nm)	
Cuvette:	1 cm light path	
Temperature:	25/30/37°C	
Measurement:	against air	
Pipetted into cuvette:	Macro	Micro
Serum	0.2 ml	0.1 ml
Enzyme/Coenzyme/ α -oxoglu-tarate 2	2.0 ml	1.0 ml

Mixed and initial absorbance was read after 1 minute. Again after 1, 2 and 3 minutes the absorbance was read. The absorbance change per minute was noted and if the value is between

0.11 and 0.16 at 340 nm/Hg 340 nm

0.06 and 0.08 at Hg 365 nm

Only then the values for the first 2 minutes were used for the calculation.

Calculation

To calculate the ALT activity the following formulae was used:

$$\text{U/l} = 1746 \times \Delta A \text{ 340 nm/min}$$

$$\text{U/l} = 1780 \times \Delta A \text{ Hg 334 nm/min}$$

$$\text{U/l} = 3235 \times \Delta A \text{ Hg 365 nm/min}$$

Normal values in serum

	25°C	30°C	37°C
Men	Up to 22 U/l	Up to 29 U/l	Up to 40 U/l
Women	Up to 17 U/l	Up to 22 U/l	Up to 31 U/l

Linearity

If the absorbance change per minute exceeds

0.16 at 340 nm/Hg 334 nm

0.08 at Hg 365 nm

0.1 ml of sample was diluted with 0.9 ml of 0.9% NaCl solution and reassessed.

The result was multiplied by 10.

3.11.8 Estimation of serum insulin

Serum insulin by enzyme linked immunosorbent assay (ELISA) method (Linco Research Inc., USA).

Test Principle

This assay is a sandwich ELISA based, sequentially, on: 1) concurrent capture of human insulin molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of monoclonal anti-human insulin antibodies and the binding of a second biotinylated monoclonal mouse anti- antibody to the captured insulin, 2) washing of unbound materials from samples, 3) binding of horseradish peroxidase to the immobilized biotinylated antibodies, 4) washing of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm after acidification of formed products. Since the increase in absorbance is directly proportional to the amount of captured human insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human insulin.

A. Reagents supplied

Each kit was sufficient to run one 96-well plate and contains the following reagents:

1. Microtiter plate

Coated with mouse monoclonal anti-human insulin antibodies.

2. Adhesive Plate Sealer

3. 10X Concentrate HRP Wash Buffer

Washing solution (buffer with preservatives) 2x50 ml, the vial content was diluted in 1:10 with deionized water.

4. Standards

Human insulin in Buffer: 2, 5, 10, 20, 50, 100 and 200 μ U/ml; 0.5 ml/bottle, ready to use.

5. Quality Controls 1 and 2

Purified Recombinant Human insulin in Assay Buffer, ready to use.

6. Matrix Solution

Heat-treated charcoal stripped off the clot human insulin serum 2 $\times$ 1 ml, ready to use.

7. Assay Buffer

0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% Sodium Azide, 1% BSA, 2 $\times$ 9 ml, ready for use.

8. Human Insulin Detection Antibody

Pre-titered Biotinylated Monoclonal Mouse anti-Human Insulin antibody (2 $\times$ 12 ml), ready to use.

9. Enzyme Solution

Pre-titered Streptavidin-Horseradish Peroxidase

Conjugation Buffer (2 $\times$ 12 ml), ready to use.

10. Substrate

3,3',5,5'-tetramethylbenzidine in Buffer (12 ml), ready for use. Minimize exposure to light.

11. Stop Solution

0.3 M HCl (12 ml), ready for use. Caution: Corrosive Solution

Materials Required

1. Pipettes and Pipette Tips: 10 μ l –20 μ l or 20 μ l-100 μ l
2. Multi-Channel Pipettes and Pipette Tips: 5~50 μ l and 50~300 μ l
3. Buffer and Reagent Reservoirs
4. Vortex Mixture
5. Deionized Water

6. Microtiter Plate Reader capable of reading absorbency at 450 nm 590 nm
7. Orbital Microtiter Plate Shaker
8. Absorbant Paper
9. Standard Refrigerator

Reagent Preparation

HRP Wash Buffer: Diluted 10X HRP washing buffer concentrate 10 fold by mixing the entire content of each bottle with 450 ml deionized water.

Storage and Stability

All components of the kit were stored at 2-8° C. Multiple freeze/thaw cycles of the insulin standards and Matrix solution were avoided.

Assay Procedure

1. Assay Procedure the reagents were allowed to come to room temperature. Then required number of strips from the microtitre assay plate were removed. Strips were assembled in an empty plate holder and filled each well with 300µl of diluted HRP wash buffer and incubated at room temperature for 5 minutes. Then wash buffer was decanted and the residual amount was removed from all wells by inverting the plate and tapping it smartly onto the absorbent towels several times.
2. The microtitre plate was marked. A layout of the plate for blank, standards, QCs and unknown samples were made and recorded on a paper.
3. 20 µl Assay Buffer was added to the NSB (Non Specific Binding) wells and each of the sample wells.
4. 20 µl Matrix Solution was added to the NSB, Standard and Control wells.
5. 20 µl Human Insulin Standard in the order of ascending concentration was added to the appropriate wells.
6. 20 µl of QC 1 and QC 2 was added to the appropriate wells.
7. Then 20 µl of unknown samples were added sequentially in wells according to the plate layout.

8. After that 20 μ l of detection antibody was added to all wells. All procedures were performed within 30 minutes. Plates were covered using plate sealer and incubated at room temperature for 1 hour on an orbital microtitre plate shaker to rotate at moderate speed approximately 400 rpm.
9. At the end of incubation plate sealer was removed and solutions were decanted. Residual solutions were removed by tapping as before. Wells were then washed for 3 times with diluted HRP wash buffer using 300 μ l. After each wash the plate was tapped on a pad of tissue to dry it off.
10. 100 μ l of enzyme solution was added to each well using a multichannel pipette. Plates were covered with sealer and incubated again with moderate shaking at room temperature for 30 minutes on microtitre plate shaker.
11. Then sealer was removed and solutions were decanted from the plate and tapped to remove the residual fluid. Then wells were washed for 5 times using 300 μ l HRP wash buffer per well per wash and after each wash the plate was tapped smartly to remove the residual buffer.
12. 100 μ l of substrate solution was added to each well and covered with sealer and incubated with moderate shaking at room temperature for 10 minutes on microtitre plate shaker. Blue color was formed in the wells of insulin standards with intensity proportional to increasing concentrations of insulin.
13. Finally sealer was removed and 100 μ l of stop solution was added. Plate was placed on a microtitre plate shaker for gentle shaking (400 rpm).
14. Reading was taken using a microplate reader at 450 nm.

Calculation of results for unknown samples

Optical densities of standard and unknown samples were fed in to a computer programme. Results of unknown samples was calculated extrapolating standard four parameter logistic curve using a Software Kinetical 3.

3.11.9 Determination of Insulin secretory capacity and Insulin sensitivity

Homeostasis Model Assessment (HOMA) is a simple widely used method which derives separate indices of B cell secretion (HOMA B) and insulin sensitivity (HOMA S) from the Serum glucose and insulin concentrations under basal conditions by using mathematical formula or software (Levy *et al.*, 1998). The HOMA model has been incorporated in a simple MS-DOS-based computer program (HOMA-CIGMA software) that allows rapid determination of % B (B cell secretion) and % S (insulin sensitivity) from measured values. Although the simple equation gives a qualitatively useful approximation of the model prediction, most authors prefer the computer model. In this study HOMA-CIGMA software was used.

3.12 DNA Extraction

DNA was extracted from whole blood using commercially available DNA extraction kit. All DNA extraction was performed by using QIAamp DNA Mini Kit (250), QIAGEN, Germany. The kit uses the principal at silica gel DNA Isolation from whole blood adopted in spin column.

DNA kit contents

- QIAamp Mini Spin Columns (extraction columns)
- Collection Tubes (2ml)
- Buffer AL (lysis solution C)
- Buffer ATL (lysis solution T)
- Buffer AW1 (pre-wash solution)
- Buffer AW2 (wash solution)
- Buffer AE (elution solution)
- Proteinase K

Equipment and Reagents

- RNase A solution (100 mg/ml)
- Ethanol (96–100%)*
- 1.5 ml microcentrifuge tubes
- Pipet tips with aerosol barrier

- Microcentrifuge (with rotor for 2 ml tubes)
- Vortex mixer
- Temperature control water bath

Procedure

Preparation of reagents

- QIAGEN Protease stock (store at 2–8°C) was diluted by using solvent (5.5 ml) was dispensed into the vial containing lyophilized QIAGEN and mixed thoroughly by gentle swirling movement. The reconstituted protease was aliquoted and storage at –20°C.
- Buffer AL mixed thoroughly by shaking before use.
- Buffer AW1 and AW2 were reconstituted adding recommend amount of absolute ethanol (96–100%) and mixed thoroughly and labeled.

DNA extraction from whole blood

- Frozen blood was thawed at room temperature and made homogenous by brief vortexing.
- Protease solution (20 µl) was taken into a 2 ml microcentrifuge tube.
- Homogenous blood (200 µl) blood was taken to the microcentrifuge tube.
- RNase A solution of 4 µl (100 mg/ml) was added to the tube for RNA-free genomic DNA.
- Mixed thoroughly by swirling movement and very short vortexing then briefly spanned down.
- Buffer AL (200 µl) was added to the sample. For efficient lysis, thorough mix was assured by flip-flop finger movements and by pulse-vortexing. The tube was spanned down briefly.
- The lysate was incubated at 56°C for 10 min. After incubation tubes containing the lysate were left on table to cool down to the room temperature.
- Once the lysate cooled down to room temperature, briefly centrifuged to remove drops from the inside of the lid.

- Absolute ethanol (200 μ l) was added to the tube and mixed through by few gentle pulse of overtaking. The lysate containing tube was then spanned thoroughly.
- QIAGEN Spin Column labeled and placed on a collection tube. Lysate was then transferred to the corresponding column, cap secured and centrifuged.
- Carefully opened the QIAamp spin column and 500 μ l Buffer AW1 buffer was added without wetting the rim. Cap secured and centrifuge at 6000 x g for 1 min. The collection tube containing the filtrate is discarded and placed the spin column into a clean 2 ml collection tube.
- QIAamp Mini spin column was placed on to a new 2 ml collection tube. Spin column cap was carefully opened and 500 μ l Buffer AW2 was added without wetting the rim. Cap was secured and centrifuged at 20,000 x g sfor 3 min.
- The QIAamp Mini spin column was placed on to a clean 1.5 ml microcentrifuge tube. At this stage the microcentrifuge tube was coded that stimulates with the respective extraction column.
- The QIAamp Mini spin column carefully opened and added 200 μ l Buffer AE added. Attention was paid so that elution solution added directly into the center of the column. Incubated at room temperature (15–25°C) for 30 minutes that generally increases DNA yield, and then centrifuge at 6000 x g for 1 min.
- The extraction column was discarded and collection column cap was secured and preserved at –20°C.

3.13 Check for DNA extraction

DNA yield for each sample was checked by agarose gel (1%) electrophoresis.

Agarose gel preparation

Appropriate amount of agarose was taken into polypropylene conical flasks containing required volume of working tris-borate EDTA (TBE). Agarose and buffer solution mixed by swirling of the flasks. It was then heated to boiling point in a microwave oven with intermittent mixing until agarose was properly boiled. The gel was cooled nearer to the gelling point and ethidium bromide (0.5 μ g/ml) was added. The gel was then poured into horizontal gel mould, combs inserted,

and allowed to polymerize. The gel was subsequently placed in horizontal electrophoresis tank filled with working TBE buffer.

3.14 INS VNTR polymorphic marker analysis

INS VNTR polymorphic marker was analyzed by PCR-RFLP. PCR was carried out using following primer set (Julier *et al.*, 1991):

Forward primer: 5'-TCCAGGACAGGCTGCATCAG-3'

Reverse primer: 5'-AGCAATGGGCGGTTGGCTCA -3'

PCR was carried out in 20 µl reaction volume. Product size for the above mentioned primer set is 441 bp.

3.14.1 PCR condition

PCR was carried out by using HotStar Taq Polymerase. PCR condition includes initial step of denaturation 95°C for 15 min followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds and elongation at 72°C for 45 seconds and step of final elongation 72°C for 10 minutes.

3.14.2 PCR reaction components

Final reaction volume of PCR was 20 µl. Composition of reaction mix contained the following

Component	Volume (µl)	Concentration
DNA	4.0	10-50 ng
PCR Buffer	2.0	
dNTPS	0.16	200 µmol/l
Primer Forward	0.80	8 pmoles
Reverse	0.80	8 pmoles
Taq polymerase (HotStar)	0.2	1 units
ddH ₂ O	12.04	
Total volume	20.00 µl	

and allowed to polymerize. The gel was subsequently placed in horizontal electrophoresis tank filled with working TBE buffer.

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3.14.2 PCR reaction components

Final reaction volume of PCR was 20 µl. Composition of reaction mix contained the following

Component	Volume (µl)	Concentration
DNA	4.0	10-50 ng
PCR Buffer	2.0	
dNTPS	0.16	200 µmol/l
Primer Forward	0.80	8 pmoles
Reverse	0.80	8 pmoles
Taq polymerase (HotStar)	0.2	1 units
ddH ₂ O	12.04	
Total volume	20.00 µl	

3.14.3 Check for PCR amplification

5 µl of PCR product was checked for amplification in a 2% agarose gel. The optimum size of the product was ascertained using 100bp DNA ladder. The amplified DNA was visualized using ethidium-bromide under UV light and gel image was documented.

3.15 RFLP analysis of INS VNTR A>T variant

INS VNTR A>T variant polymorphic marker was analyzed using HphI restriction enzyme. Restriction enzyme digestion was performed following standard digestion protocol. The digestion was carried out in a reaction volume of 15 µl. The enzyme digestion protocol was as follows:

Name of the component	Volume (µl)
PCR product	7.0 µl
Buffer	1.5 µl
HphI (5 U/ml)	1 µl
Spermidine	0.25 µl
H ₂ O	5.25 µl
Total reaction volume	15.00 µl

The digestion was carried out for 2 hours at 37°C. The enzyme digest was then resolved in 3% agarose gel.

The INS VNTR A>T polymorphism disrupts the HphI restriction enzyme site. The amplified segment of DNA had two internal cutting sites. Fragments produced by the HphI restriction enzyme of the PCR product were as follows: Wild AA genotype 30, 60, 140 and 211bp; variant AT genotype 30, 60, 140, 211, and 271 bp; and variant TT variant genotype 30, 140 and 271 bp. Fragments 30 and 60 bp could not be resolved. Other fragments were shown in the figure.

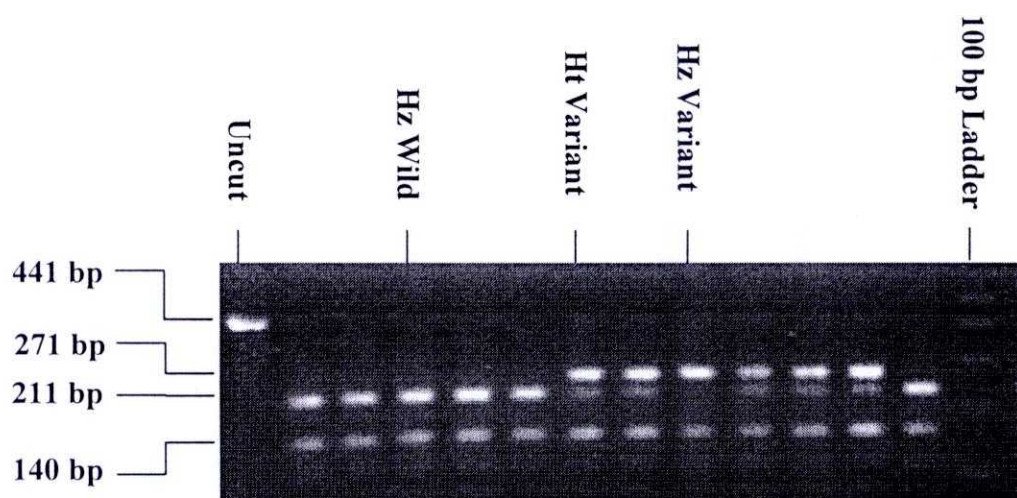


Figure 4: Agarose gel image of *INS* VNTR A>T variant analysis by HphI restriction enzyme digestion.

3.16 Statistical methods

Statistical analysis was performed using Statistical Package for Social Science (SPSS) software for Windows version 10 (SPSS Inc., Chicago, Illinois, USA). Data were expressed as mean $\pm$ SD, number (percentage) as appropriate. The statistical differences between two groups was assessed by students unpaired Student's 't' test. Chi-squared test was also performed in appropriate situation. A two-tailed p value of <0.05 was considered statistically significant.

Chapter 4

RESULTS

4. Results

A total number of 362 unrelated subjects were screened for glycemic status. Of them 251 were healthy controls and 111 were type 2 diabetes mellitus (T2DM). Male female distribution was male 165, female 86 (65.7% vs 34.3% respectively) in control group and male 68, female 43 (61.3% vs 38.7% respectively) in T2DM.

4.1 Age, BMI, blood pressure, serum creatinine and SGPT of the study subjects

Mean ($\pm$ SD) age (yrs) of control and T2DM subjects was 39.7 ± 9.1 and 44.1 ± 7.9 respectively which showed statistical significant difference ($p < 0.001$) between the groups (Table 2).

Mean ($\pm$ SD) body mass index (BMI) of control and T2DM subjects was 24.8 ± 3.8 and 24.9 ± 3.9 respectively which did not show statistical significant difference ($p = 0.706$) (Table 2).

Mean ($\pm$ SD) systolic blood pressure (SBP) of control and T2DM subjects was 111.4 ± 11.7 and 118.7 ± 14.3 respectively which showed significant statistical difference ($p < 0.001$) (Table 2).

Mean ($\pm$ SD) diastolic blood pressure (DBP) of control and T2DM subjects was 75.1 ± 7.9 and 77.0 ± 8.8 respectively which showed statistical significant difference ($p = 0.040$) (Table 2).

Mean ($\pm$ SD) serum creatinine (mg/dl) of control and T2DM subjects was 1.05 ± 0.15 and 0.99 ± 0.18 respectively which showed significant difference ($p = 0.004$) (Table 2).

Mean ($\pm$ SD) serum glutamic pyruvic transaminase (SGPT, U/L) of control and T2DM subjects was 26.6 ± 16.7 and 29.3 ± 25.9 respectively which did not show significant difference ($p = 0.229$) (Table 2).

4.2 Anthropometric measurements of the study subjects

Mean ($\pm$ SD) mid arm circumference (MAC, cm) of control and T2DM subjects was 29.4 ± 3.2 and 29.3 ± 4.6 , respectively which did not show significant difference ($p=0.808$) (Table 3).

Mean ($\pm$ SD) triceps skin fold (TSF) of control and T2DM subjects was 18.5 ± 9.7 and 17.9 ± 8.6 respectively which did not show significant difference ($p=0.582$) (Table 3).

Mean ($\pm$ SD) subscapular skinfold thickness (SSF) of control and T2DM subjects was 25.1 ± 10.0 and 29.4 ± 10.4 respectively which showed statistical significance with $p<0.001$ compared to T2DM (Table 3).

Mean ($\pm$ SD) body fat mass (BFM, %) of control and T2DM subjects was 28.8 ± 0.06 and 28.9 ± 6.00 respectively which did not show significant difference ($p=0.824$) (Table 3).

Mean ($\pm$ SD) waist hip ratio (WHR) of control and T2DM subjects was 0.93 ± 6.1 and 0.95 ± 0.05 respectively. WHR value of T2DM group was significantly higher compared to the Controls ($p=0.004$) (Table 3).

4.3 Serum glucose levels and insulinimic status of the study subjects

Mean ($\pm$ SD) fasting serum glucose (FSG, mmol/dl) of control and T2DM subjects was 4.9 ± 0.5 and 8.1 ± 3.5 respectively ($p<0.001$). Mean ($\pm$ SD) postprandial glucose (PPG, mmol/l) of control and T2DM subjects was 5.8 ± 1.1 and 15.7 ± 4.8 respectively ($p<0.001$) (Table 4).

Mean ($\pm$ SD) fasting serum insulin in control and T2DM was 9.7 ± 2.6 and 13.5 ± 4.8 respectively. Mean serum insulin in the T2DM group was significantly higher compared to the Controls ($p<0.001$) (Table 4).

Mean ($\pm$ SD) of HOMA%S in the Control and T2DM subjects was 82.2 ± 18.6 and 57.7 ± 16.8 respectively. Mean HOMA%B values in T2DM group was significantly lower compared to the Control ($p=0.003$) group (Table 4).

Mean ($\pm$ SD) HOMA% B values in the Control and T2DM subjects was 107.6 ± 26.2 and 86.0 ± 38.86 respectively. Mean HOMA%B value in T2DM group was lower than Control group ($p < 0.001$) (Table 4).

4.4 Lipid levels of study subject

Mean ($\pm$ SD) serum triglycerides (TG, mg/dl) of control and T2DM subjects was 149.1 ± 90.8 and 215.0 ± 147.2 respectively which showed statistical significant difference ($p < 0.001$) (Table 5).

Mean ($\pm$ SD) Serum total cholesterol (mg/dl) of control and T2DM subjects was 196.1 ± 39.1 and 231.4 ± 77.3 respectively which showed significantly higher in the T2DM group compared to the controls ($p < 0.001$) (Table 5).

Mean ($\pm$ SD) high density lipoprotein cholesterol (HDL-c, mg/dl) of control and T2DM subjects was 36.3 ± 11.5 and 32.0 ± 9.2 respectively which showed significant difference ($p = 0.003$) (Table 5).

Mean ($\pm$ SD) low density lipoprotein cholesterol (LDL-c, mg/dl) of control and T2DM subjects were 130.8 ± 38.3 and 148.0 ± 59.2 , respectively which showed significant difference ($p = 0.019$) (Table 5).

Table 2: Age, BMI, BP, S Creatinine and SGPT level of the study subjects

Variables	Control (n=251)	T2DM (n=111)	<i>t/p values</i>
			Control vs T2DM
Age (yrs)	39.7 ± 9.1	44.1 ± 7.9	4.461/<0.001
BMI (kg/m ²)	24.8 ± 3.8	24.9 ± 3.9	0.378/0.706
SBP (mmHg)	111.4 ± 11.7	118.7 ± 14.3	4.593/<0.001
DBP (mmHg)	75.1 ± 7.9	77.0 ± 8.8	2.066/0.040
S creatinine (mg/l)	1.05 ± 0.15	0.99 ± 0.18	2.908/0.004
SGPT (mg/l)	26.6 ± 16.7	29.3 ± 25.9	1.204/0.229

Results were expressed as mean±SD.

Comparison between groups was performed using unpaired Student's 't' test.

BMI, Body mass index; BP, Blood pressure; S creatinine, Serum creatinine; SGPT, Serum glutamic pyruvic transaminase; T2DM, type 2 diabetes.

Table 3: Anthropometric measurements of the study subjects

Variables	Control (n=251)	T2DM (n=111)	<i>t/p values</i>
			Control vs T2DM
MAC (mm)	29.4 ± 3.2	29.3 ± 4.6	0.243/0.808
TSF (mm)	18.5 ± 9.7	17.9 ± 8.6	0.551/0.582
SSF (mm)	25.1 ± 10.0	29.4 ± 10.4	3.593/<0.001
BFM (%)	28.8 ± 6.8	28.9 ± 6.0	0.223/0.824
WHR	0.93 ± 0.06	0.95 ± 0.05	2.895/0.004

Results were expressed as mean±SD.

Comparison between groups was performed using unpaired Student's 't' test.

MAC, mid arm circumferences; TSF, Triceps Skin Fold; SSF, Sub scapular Skin fold; BFM, Body Fat Mass; WHR waist hip ratio; T2DM, type 2 diabetes.

Table 4: Glycemic and Insulinemic status of the study subjects

Variables	Control	T2DM	<i>t/p values</i>
	(n=251)	(n=111)	Control vs T2DM
FSG (mmol/l)	4.9 ± 0.5	8.1 ± 3.5	
PPG (mmol/l)	5.8 ± 1.08	15.7 ± 4.8	
Insulin (pmol/l)	9.7 ± 2.6	13.5 ± 4.8	4.739/<0.001
HOMA % S	82.2 ± 18.2	57.7 ± 16.8	3.102/0.003
HOMA % B	107.6 ± 26.2	86.0 ± 38.9	6.541/<0.001

Results were expressed as mean±SD.

Comparison between groups was performed using unpaired Student's 't' test.

FSG, Fasting Serum Glucose; PPG, Postprandial Glucose; HOMA%S, Insulin sensitivity assessed by homeostatic model assessment; HOMA%B, B-cell function assessed by homeostatic model assessment; T2DM, type 2 diabetes

Table 5: Lipid levels of the study subjects

Variables	Control	T2DM	<i>t/p values</i>
	(n=48)	(n=40)	Control vs T2DM
TG (mg/dl)	149.1 ± 90.8	215.0 ± 147.2	4.377/<0.001
T Chol (mg/dl)	196.1 ± 39.1	231.4 ± 77.3	4.579/<0.001
HDL-c (mg/dl)	36.3 ± 11.5	32.0 ± 9.2	2.973/0.003
LDL-c (mg/dl)	130.8 ± 38.3	148.0 ± 59.2	2.375/0.019

Results were expressed as mean±SD.

Comparison between groups was performed using unpaired Student's 't' test.

TG, Triglycerides; T chol, total cholesterol; HDL-c, High density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol, T2DM, type 2 diabetes.

4.5 Candidate gene marker analysis

INS VNTR genotype in glycaemic groups

Genotype frequencies of the INS VNTR A>T variants in control groups were 0.690 (147), 0.254 (54) and 0.056 (12) for wild type, heterozygous (Ht) variant and homozygous (Hz) variant respectively. In the T2DM group the frequency were 0.731 (76), 0.250 (26) and 0.019 (2), respectively. These genotype frequency distribution in the two groups did not show statistical significant association ($\chi^2=2.346$; $p=0.309$) (Table 6a).

Heterozygous and homozygous variant genotype was figured together. The frequency distribution was as follows: Control 0.690 (147) and 0.310 (66) for wild and variant genotype respectively; T2DM 0.731 (76) and 0.269 (28) respectively. This distribution also did not show significant association ($\chi^2=0.553$; $p=0.457$) (Table 6b).

Hardy-Weinberg distribution

Genotype frequencies for INS VNTR in all (control and T2DM) groups were tested for Hardy-Weinberg (HW) Equilibrium. In all groups there was no significant association found ($\chi^2=3.641$, $p=0.056$).

4.6 INS VNTR genotype on the basis of BMI

Genotype frequencies of the INS VNTR A>T variants in control groups were 0.683 (69), 0.277 (28) and 0.04 (4) for wild type, heterozygous (Ht) variant and homozygous (Hz) variant respectively. In the overweight and obese group the frequency were 0.714 (155), 0.240 (52) and 0.046 (10) respectively. These genotype frequency distribution in the two groups did not show statistical significant association ($\chi^2=0.548$; $p=0.760$) (Table 7a).

Heterozygous and homozygous variant genotype was figured together. The frequency distribution was as follows: Normal group 0.683 (69) and 0.317 (32) wild and variant genotype respectively; overweight and obese group 0.714 (155) and 0.286 (62) respectively. This distribution also did not show significant association ($\chi^2=0.553$; $p=0.457$) (Table 6). This distribution also did not show significant difference ($\chi^2=0.321$; $p=0.571$) (Table 7b).

4.7 INS VNTR genotype of the study on the basis of gender

Genotype frequencies of the INS VNTR A>T variants in male group were 0.686 (140), 0.275 (56) and 0.039 (8) for wild type, heterozygous (Ht) variant and homozygous (Hz) variant respectively. In the female group the frequency were 0.737 (118), 0.212 (25) and 0.051 (6) respectively. These genotype frequency distribution in the two groups did not show statistical significant association ($\chi^2=1.675$; $p=0.433$) (Table 8a).

Heterozygous and homozygous variant genotype was figured together. The frequency distribution was as follows: male group 0.686 (140) and 0.314 (64) wild and variant genotype respectively; female group 0.737 (87) and 0.263 (31) respectively. This distribution also did not show significant association ($\chi^2=0.935$; $p=0.333$) (Table 8b).

Table 6a: INS VNTR genotype frequencies of the study subjects

Genotype	Control	T2DM	Total
	(n=213)	(n=104)	(n=317)
Wild (AA)	0.690 (147)	0.731(76)	0.703 (223)
Ht variant (AT)	0.254 (54)	0.250 (26)	0.252 (80)
Hz (TT)	0.056 (12)	0.019 (2)	0.044 (14)
χ^2/p value	2.346/0.309		

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical differences.

Ht variant, heterozygous variant; Hz variant, homozygous variant; T2DM, type 2 diabetes mellitus; T2DM, type 2 diabetes.

Table 6b: *INS VNTR* genotype frequency of wild and variant (Ht and Hz variant together) in the study subjects

Genotype	Control	T2DM	Total
	(N=213)	(N=104)	(N=317)
Wild type	0.690 (147)	0.731 (76)	0.703 (223)
Variant	0.310 (66)	0.269 (28)	0.297(94)
χ^2/p value	0.553/0.457		

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical differences compare to Controls.

T2DM, type 2 diabetes; Variant, heterozygous and homozygous variant together.

Table 7a: *INS VNTR* genotype frequencies of the study subjects on the basis of BMI

Genotypes	Normal	Overweight and Obese
	(N=101)	(N=217)
Wild (AA)	0.683 (69)	0.714 (155)
Ht Variant (AT)	0.277 (28)	0.240 (52)
Hm Variant (TT)	0.040 (4)	0.046 (10)
χ^2/p value	0.548/0.760	

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical differences.

Ht variant, heterozygous variant; Hz variant, homozygous variant; BMI, body mass index

Table 7b: *INS VNTR* genotype frequency of wild and variant (Ht and Hz variant together) in the study subjects on the basis of BMI

Groups	Normal	Overweight and Obese
	(N=101)	(N=217)
Wild (AA)	0.683 (69)	0.714 (155)
Variant (AT and TT)	0.317 (32)	0.286 (62)
χ^2/p value	0.321/0.571	

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical differences compare to Controls.

Variant, heterozygous and homozygous variant together.

Table 8a: *INS VNTR* genotype frequency of the study subjects on the basis of gender

Genotypes	Male	Female
	(N=204)	(N=118)
Wild (AA)	0.686 (140)	0.737 (87)
Ht Var (AT)	0.275 (56)	0.212 (25)
Hm Var (TT)	0.039 (8)	0.051 (6)
χ^2/p value	1.675/0.433	

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical differences.

Ht variant, heterozygous variant; Hz variant, homozygous variant.

Table 8b: *INS VNTR* genotype frequency of wild and variant (Ht and Hz variant together) in the study subjects on the basis of gender

Groups	Male	Female
	(N=204)	(N=118)
Wild (AA)	0.686 (140)	0.737 (87)
Variant (AT and TT)	0.314 (64)	0.263 (31)
χ^2/p	0.935 /0.333	

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical differences compare to Controls.

Variant, heterozygous and homozygous variant together.

4.8 Anthropometric measurements of the study subjects on the basis of *INS VNTR* genotype

Mean ($\pm$ SD) mid arm circumference (MAC, mm) in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 294.6 ± 30.3 and 293.8 ± 39.7 respectively, which did not show statistical significant difference ($p=0.877$), and in T2DM 295.2 ± 52.7 and 292.2 ± 24.3 respectively, which also did not show significant difference ($p=0.779$) (Table 9).

Mean ($\pm$ SD) triceps skin fold (TSF) in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 18.6 ± 10.8 and 18.8 ± 8.7 respectively, which did not show statistical significant difference ($p=0.903$), and in T2DM 18.6 ± 9.6 and 16.2 ± 6.1 respectively, which also did not show significant difference ($p=0.145$) (Table 9).

Mean ($\pm$ SD) sub scapular skin fold (SSF) in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 25.1 ± 10.6 and 25.4 ± 9.8 respectively, which did not show statistical significant difference ($p=0.865$), and in T2DM 29.9 ± 9.7 and 27.5 ± 8.7 respectively, which also did not show significant difference ($p=0.241$) (Table 9).

Mean ($\pm$ SD) body fat mass (BFM, %) in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 29.3 ± 7.0 and 28.9 ± 6.5 respectively, which did not show statistical significant difference ($p=0.712$), and in T2DM 29.5 ± 5.6 and 28.2 ± 6.2 respectively, which also did not show significant difference ($p=0.321$) (Table 9).

Mean ($\pm$ SD) waist to hip ratio (WHR) in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 0.92 ± 0.056 and 0.95 ± 0.072 respectively, which showed statistical significant difference ($p=0.003$), and in T2DM 0.94 ± 0.052 and 0.96 ± 0.054 respectively, which did not show significant difference ($p=0.085$) (Table 9).

4.9 Glycemic and insulinemic status of study subjects on the basis of *INS VNTR* genotype

Mean ($\pm$ SD) fasting glucose levels in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 4.9 ± 0.5 and 5.0 ± 0.5 respectively, which did not show statistical significant difference ($p=0.265$) (Table 10), and in T2DM 8.1 ± 3.5 and 8.1 ± 3.1 respectively, which showed significant difference ($p=0.959$) (Table 10).

Mean ($\pm$ SD) fasting glucose levels in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 5.6 ± 1.1 and 6.1 ± 0.9 respectively, which showed statistical significant difference ($p=0.004$) (Table 10), and in T2DM 15.9 ± 4.8 and 14.6 ± 4.3 respectively, which showed significant difference ($p=0.222$) (Table 10).

Mean ($\pm$ SD) Insulin levels in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 9.8 ± 2.4 and 9.2 ± 3.3 respectively, which did not show statistical significant difference ($p=0.534$) (Table 10), and in T2DM 12.8 ± 4.6 and 15.0 ± 3.5 respectively, which also did not show significant difference ($p=0.256$) (Table 10).

Mean ($\pm$ SD) HOMA%B in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 112.3 ± 28.1 and 102.5 ± 22.3 respectively, which did not show statistical significant difference ($p=0.325$)

(Table 10), and in T2DM 87.2 ± 39.5 and 88.7 ± 38.3 respectively, which also did not show significant difference ($p=0.927$) (Table 10).

Mean ($\pm$ SD) HOMA%S in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 82.5 ± 17.1296 and 84.9 ± 23.5 respectively, which did not show statistical significant difference ($p=0.737$) (Table 10), and in T2DM 60.4 ± 16.6 and 51.0 ± 10.4 respectively, which also did not show significant difference ($p=0.161$) (Table 10).

4.10 Lipid levels of the study subjects on the basis of INS VNTR A>T variant genotype

Mean ($\pm$ SD) triglycerides (TG, mg/dl) in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 152.4 ± 105.6 and 140.7 ± 60.7 respectively, which did not show statistical significant difference ($p=0.402$), and in T2DM 207.5 ± 136.5 and 215.1 ± 157.3 respectively, which also did not show significant difference ($p=0.810$) (Table 11).

Mean ($\pm$ SD) total cholesterol (mg/dl) in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 196.2 ± 38.4 and 195.1 ± 38.9 respectively, which did not show statistical significant difference ($p=0.841$), and in T2DM 224.5 ± 50.4 and 249.5 ± 124.0 respectively, which also did not show significant difference ($p=0.145$) (Table 11).

Mean ($\pm$ SD) high density lipoprotein cholesterol (HDL-c, mg/dl) in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 36.7 ± 11.5 and 35.9 ± 9.0 respectively, which did not show statistical significant difference ($p=0.662$), and in T2DM 30.9 ± 8.4 and 37.0 ± 11.3 respectively, which showed significant difference ($p=0.017$) (Table 11).

Mean ($\pm$ SD) low density lipoprotein cholesterol (LDL-c, mg/dl) in subjects with wild and variant (heterozygous and homozygous together) genotype in control group was 130.1 ± 33.7 and 131.2 ± 42.7 respectively, which did not show statistical significant difference ($p=0.863$), and in T2DM 149.7 ± 57.3 and 142.8 ± 43.8 respectively, which also did not show significant difference ($p=0.645$) (Table 11).

Table 9: Anthropometric measurements of the study subject on the basis of INS VNTR genotype

	MAC (mm)	TSF (mm)	SSF (mm)	BFM (%)	WHR
Control subjects					
Wild type	294.6±30.3	18.6±10.8	25.1±10.6	29.3±7.0	0.92±0.056
Variant	293.8±39.7	18.8±8.7	25.4±9.8	28.9±6.5	0.95±0.072
<i>t/p values</i>	<i>0.155/0.877</i>	<i>0.122/0.903</i>	<i>0.171/0.865</i>	<i>0.370/0.712</i>	<i>2.964/0.003</i>
T2DM subjects					
Wild type	295.2±52.7	18.6±9.6	29.9±9.7	29.5±5.6	0.94±0.052
Variant	292.2±24.3	16.2±6.1	27.5±8.7	28.2±6.2	0.96±0.054
<i>t/p values</i>	<i>0.281/0.779</i>	<i>1.471/0.145</i>	<i>1.126/0.241</i>	<i>0.998/0.321</i>	<i>1.740/0.085</i>

Results were expressed as mean±SD.

Comparison between groups was performed using unpaired Student's 't' test.

Variant, heterozygous and homozygous variant together; MAC, mid arm circumferences; TSF, triceps skin fold; SSF, sub scapular skin fold, BFM, body fat mass; WHR, waist hip ratio.

Table 10: Glycemic and insulinemic status of the study subjects on the basis of *INS VNTR* genotype

	FSG (mmol/l)	PPG (mmol/l)	Insulin (pmol/l)	HOMA %B	HOMA %S
Control subjects					
Wild type	4.9 ± 0.5	5.6 ± 1.1	9.8 ± 2.4	112 ± 28.1	82 ± 17.1
Variant	5.0 ± 0.5	6.1 ± 0.9	9.2 ± 3.4	102 ± 22.3	85 ± 23.5
<i>t/p values</i>	1.117/0.265	2.930/0.004	0.627/0.534	0.997/0.325	0.339/0.737
T2DM subjects					
Wild type	8.1 ± 3.5	15.9 ± 4.8	12.8 ± 4.6	87.2 ± 39.5	60.4 ± 16.6
Variant	8.1 ± 3.1	14.6 ± 4.3	15.0 ± 3.5	88.7 ± 38.3	51.0 ± 10.4
<i>t/p values</i>	0.051/0.959	1.229/0.222	1.154/0.256	0.09/0.927	1.43/0.161

Results were expressed as mean ± SD.

'T' test was performed to calculate statistical differences.

Variant, heterozygous and homozygous variant together; FSG, fasting serum glucose; PPG, postprandial glucose; HOMA %B, Homeostasis model assessment B-cell Function; HOMA %S, Homeostasis model assessment insulin sensitivity.

Table 11: Lipid levels of the study subject on the basis of INS VNTR genotype

	Triglycerides (mg/dl)	T chol (mg/dl)	HDL-c (mg/dl)	LDL-c (mg/dl)
Control subjects				
Wild type	152.4±105.6	196.2±38.4	36.7±11.5	130.1±33.7
Variant	140.7±60.7	195.1±38.9	35.9±9.0	131.2±42.7
<i>t/p values</i>	<i>0.840/0.402</i>	<i>0.202/0.841</i>	<i>0.439/0.662</i>	<i>0.174/0.863</i>
T2DM subjects				
Wild type	207.5±136.5	224.5±50.4	30.9±8.4	149.7±57.3
Variant	215.1±157.3	249.5±124.0	37.0±11.3	142.8±43.8
<i>t/p values</i>	<i>0.242/0.810</i>	<i>1.469/0.145</i>	<i>2.435/0.017</i>	<i>0.463/0.645</i>

Results were expressed as mean±SD.

'T' test was performed to calculate statistical differences.

Variant, heterozygous and homozygous variant together; T Chol, total cholesterol; HDL-c, High density lipoprotein; LDL-c, Low density lipoprotein.

Chapter 5

DISCUSSION

5. Discussion

Diabetes has become a major health problem all over the world. A recent WHO sponsored estimate suggested a sharp rise in world diabetic population by the year 2030. This estimate also demonstrated that it is the huge rise of diabetic patients in the developing countries, including Bangladesh, will actually be creating the burden (Wild *et al.*, 2004).

Diabetes is not a single disease rather has been recognized as a syndrome. About 90-95% of the total diabetic patients are of type 2 variety (WHO, 1999). Both insulin resistance and/ or secretory defect have been implicated in the pathogenesis of type 2 diabetes mellitus. However, the primacy between the two phenomenon in its pathogenesis of diabetes is still to be clearly understood. Moreover, environmental factors in the presence of genetic susceptibility has shown to be resulting in the heterogeneity of T2DM.

T2DM has been found to be associated with obesity (Chan *et al.*, 1994). However, obesity and type 2 diabetes mellitus are now considered as components of metabolic syndrome (MS) where insulin resistance is one of the main components (Reaven, 1988). The increased expression of insulin gene not only suggested to the gene environment interaction but the variation in the *INS* also implicated. The insulin gene found to be extremely heterogeneous in different race. The variation has been attributed to the -23bp A>T polymorphism in insulin gene resulting in extremely variable number of tandem repeats of a conserved sequence. The resultant alleles are designated as class I, class II and class III allele for small, intermediate and high number repeats respectively. Presence of small number of repeats found to be associated with type 1 diabetes, mainly present in the western developed countries among Caucasoids and the high number of the VNTR, class III allele, with heightened expression of the gene itself among T2DM subjects (Bennet and Todd, 1996; Dunger *et al.*, 1998; Ong *et al.*, 1999). However, the level of *INS* gene expression in the

presence of class III allele also found to show some difference in different studies (Bell *et al.*, 1984; Hitman *et al.*, 1984). The insulin gene -23bp A>T variant has shown to disrupt a restriction enzyme site in DNA sequence. The gene variant has been studied by PCR-RFLP in different studies (Julier *et al.*, 1991; Ong *et al.*, 1999).

It has been earlier mentioned there is an increasing trend in the prevalence of diabetes in Bangladesh and majority of them are of type 2 class. Number of studies, involving both obese and nonobese subjects, have been undertaken to explore the pathophysiology diabetes in this population. Both secretory defect and insulin sensitivity have been observed in Bangladeshi T2DM subjects (Zinnat *et al.*, 2003). There is general view that unlike the western world obesity is not a major problem in this country rather poverty and malnutrition are highlighted most. Very recently it has been shown that overall prevalence of being overweight or obese is about 18.9% in urban areas and 9.6% in rural areas (Shafique *et al.*, 2007). Number of studies prevalence of T2DM is around 5% (Sayeed *et al.*, 2003). Interestingly a recent study involving a rural community demonstrated higher prevalence of impaired glucose tolerance compared to the age adjusted urban population (Sayeed *et al.*, 2003).

The present study aimed to determine INS VNTR variant genotype frequencies in a group of Bangladeshi population and to find the difference in insulin concentration in the presence of variant allele and association of variant allele the development of type 2 diabetes mellitus. The INS VNTR A>T genotype frequencies in controls were 0.690, 0.254 and 0.056 for homozygous wild, heterozygous variants and homozygous variants respectively and, 0.731, 0.250 and 0.019 in the T2DM subjects. The frequencies were found to similar in the two groups. The genotype frequencies in the present attempt the controls found to be consistent with the previous reports involving homogenous Bangladeshi population, the typing were performed in the younger subjects, age ≤ 30 years (Hassan, 2006). A report involving South Indian subjects have shown to have

such higher frequencies of wild genotype for the variant marker which is in sharp contrast to the in the European population (Choong *et al.*, 1999). Wild genotype, class I INS VNTR allele, in the Caucasoids found to vary between 0.446 - 0.636 in different studies (Bell *et al.*, 1984; Owerbach *et al.*, 1982; Hitman *et al.*, 1984). This suggests presence of class I INS VNTR allele in the Asian population.

Circulating level of insulin did not differ between the subjects INS VNTR wild and variant genotype (Control: 9.8 ± 2.4 and 9.2 ± 3.4 ($p=0.534$) with Wild vs variant genotype; DM: 12.83 ± 4.6 and 15.0 ± 3.5 ($p=0.256$) with Wild vs variant genotype). This finding is in contrast to number which demonstrated higher secretion in presence of variant genotype (Bennet and Todd, 1996; Dunger *et al.*, 1998; Ong *et al.*, 1999) but is consistent with studies by report by Heude *et al.* (2004). The absence of this difference may be explained by the insulin secretory defects in the T2DM subjects demonstrated in number of studies. It is interesting to note that though in earlier studies subjects were lean to normal weight contrasting overweight to obese. The absence of difference regarding insulin level in the presence of variant genotype was further substantiated by the lack difference in insulin secretory capacity (as revealed by HOMA%B) or insulin sensitivity (HOMA%S) in the two genotype groups of the Controls and T2DM (Table 9 and 10).

Obesity is one of the major components of metabolic syndrome along with insulin resistance and hyperinsulinemia (Norberg *et al.*, 2007). It has been observed that the Controls and T2DM group did not show significant difference regarding BMI, body fat mass and WHR (Table 12). Although BMI and BFM did not differ among subjects with wild and variant genotype either in the Controls and T2DM but WHR found to differ significantly between the two genotype groups of the Controls (0.920 ± 0.05 and 0.947 ± 0.072 respectively $p=0.003$) and in the T2DM group the difference, however, did not reach to the level of significance (0.942 ± 0.052 and 0.962 ± 0.054 , respectively, $p=0.085$). The finding of the study is consistent with the observation of Le Stunff *et al.* (2000) who

of the study is consistent with the observation of Le Stunff et al (2000) who demonstrated an association between INS VNTR variant allele with childhood and/or juvenile obesity. However, in a population based study Manjinder et al (2005) failed to demonstrate any association between INS VNTR variant allele and important determinants of body size and adult obesity in middle-aged subjects. They also did not find any difference between men and women. The finding in the present study strongly suggests that INS VNTR variant allele may be associated with central obesity, as represented by WHR. This observation appeared to be an important one because waist hip ratio is now regarded as the most relevant measure of central obesity which may be present even in the absence of generalized obesity. Population screening of the INS VNTR variant may be used an important tool to identify the vulnerable groups for central obesity who are at risk for future diabetes and/ or metabolic syndrome.

Chapter 6

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The data from the present study suggest that the INS VNTR variant genotype: (i) frequencies show substantial difference in Bangladeshi population compared to that of the Caucasoids; (ii) is not associated with circulating insulin in the controls and T2DM; (iii) is not associated with type 2 diabetes in Bangladeshi population; and (iv) seem to have an association with central obesity in our population.

Recommendations

A relatively small number of type 2 diabetic patients in the study remained to be one of the most important weaknesses of the study. Since all the study subjects were collected in BIRDEM it appeared that respondents might have come from in and around the Dhaka city. However, much attention was paid to recruit subjects recently moved in Dhaka and just came for a short stay to minimize the concentration of urban based subjects.

The study needs to be expanded with inclusion of substantial number of T2D patients as well as healthy controls to make conclusive remarks regarding findings so reached.

Chapter 7

REFERENCES

REFERENCES

- Allison DB, Kaprio J, Korkeila M, Koskenvuo M, Neale MC, Hayakawa K (1996). The heritability of body mass index among an international sample of monozygotic twins reared apart. *Int J Obes Relat Metab Disord* **20**: 501–506
- Al-Mahmood AK (2000). Role of insulin deficiency and insulin resistance in the pathogenesis of type 2 diabetes in above 40 years Bangladeshi subjects. M Phill Thesis, BIRDEM Academy, University of Dhaka, 56-61.
- Bagadi S, Nagpal JK, Khadapkar R, Das BR (2005). Evaluating the association between type 2 diabetes and polymorphisms at +1127 *INS-PstI* and +3580 *IGF-II-MspI* locus. *Current Science* **89**: 535-539.
- Balkau B, Charles M (1999). Comments on the provisional report from the WHO consultation. European Group for the Study of Insulin Resistance. *Diabet Med* **16**: 442–3.
- Barry JD, Marcello L, Morrison LJ, Read AF, Lythgoe K, Jones N, et al (2005). What the genome sequence is revealing about trypanosome antigenic variation. *Biochem Soc Trans* **33**: 986–989.
- Bell GI, Horita S, Karam JH (1984). Apolymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. *Diabetes* **33**:176–183.
- Bell GI, Karam JH, Rutter WJ (1981).Polymorphic DNA region adjacent to the 50 end of the human insulin gene. *Proc.Natl. Acad. Sci. USA* **78**:5759–63
- Bell GI, Selby MJ, Rutter WJ (1982). The highly polymorphic region near the human insulin gene is composed of simple tandemly repeating sequences. *Nature* **295**: 31–35,
- Bennett AJ, Sovio U, Ruokonen A, Martikainen H, Pouta A, Taponen S, et al (2004). Variation at the insulin gene VNTR (variable number tandem repeat) polymorphism and early growth: studies in a large Finnish birth cohort. *Diabetes* **53**: 2126–2131.
- Bennett ST, Lucassen AM, Gough SC, Powell EE, Undlien DE, Pritchard LE, et al (1995). Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. *Nat Genet* **9**: 284–292
- Bennett ST, Todd JA (1996). Human type 1 diabetes and the insulin gene: principles of mapping polygenes. *Annu Rev Genet* **30**: 343–70.
- Berenson GS, Srinivasan SR, Bao W, Newman 3rd WP, Tracy RE, Wattigney WA (1998). Association between multiple cardiovascular risk factors and atherosclerosis in children and young adults. The Bogalusa Heart Study. *N Engl J Med* **338**:1650–1656
- Bleich S, Cutler D, Murray C, Adams A (2007). *Working paper 12954: Why is the developed world obese?*. National Bureau of Economic Research.

- Borecki IB, Rice T, Pe'russe L, Bouchard C, Rao DC (1995). *Obes Res* **3**: 1.
- Bouchard C, Bray GA, Hubbard VS (1990). Basic and clinical aspects of regional fat distribution. *Am J Clin Nutr* **52**: 946-950.
- Brown CD, Donato KA, Obarzanek E, et al (1998). Body mass index and prevalence of risk factors for cardiovascular disease. *Obes Res.*; submitted for publication.
- Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC (2003) Beta-Cell Deficit and Increased Beta-Cell Apoptosis in Humans With Type 2 Diabetes *Diabetes*.;52(1):102-110
- Caballero B (2007). "The global epidemic of obesity: an overview". *Epidemiol Rev* **29**: 1-5.
- Chakravarthy MV, Booth FW (2004). "Eating, exercise, and "thrifty" genotypes: connecting the dots toward an evolutionary understanding of modern chronic diseases". *J. Appl. Physiol.* **96** (1): 3-10.
- Champe PC, Harvey RA, Ferrier DR (2005). Lippincott's Illustrated Reviews, Biochemistry (3rd edition), , , Lippincott Williams and Wilkins, pp 342
- Chan JM, Rimm EB, Colditz GA, Stampfer MJ, Willett WC (1994). Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. *Diabetes Care* **17**: 961-969
- Chua W, Chediak AD (1994). Obstructive sleep apnea. Treatment improves quality of life-and may prevent death. *Postgrad Med* **95**:123-126, 131, 135-138.
- Cicuttini FM, Baker JR, Spector TD (1996). The association of obesity with osteoarthritis of the hand and knee in women: a twin study. *J Rheumatol* **23**: 1221-1226.
- Cocozza S, Riccardi G, Monticelli A, Capaldo B, Genovese S, Krogh V, et al (1988). Polymorphism at the 5' end flanking region of the insulin gene is associated with reduced insulin secretion in healthy individuals. *Eur J Clin Invest* **18**: 582-586.
- Colditz GA, Willett WC, Stampfer MJ, et al (1990). Weight as a risk factor for clinical diabetes in women. *Am J Epidemiol* **132**: 501-513.
- Cole SA, Mitchell BD, Hsueh WC, et al (2000). The Pro12Ala variant of peroxisome proliferator-activated receptorgamma2 (PPAR-gamma2) is associated with measures of obesity in Mexican Americans. *Int J Obes Relat Metab Disord* **24**: 522-4.
- Comuzzie AG, Blangero J, Mahaney MC, et al. (1995) Major gene with sex-specific effects influences fat mass in Mexican Americans. *Genet Epidemiol.* **12**:475-488.
- Comuzzie AG, Blangero J, Mahaney MC, et el. (1996) Genetic and environmental correlations among hormone levels and measures of body fat accumulation and topography. *J Clin Endocrinol Metab.* **81**:597-600

- Cox NJ, Baker L, Spielman RS (1988). Insulin-gene sharing in sib pairs with insulin-dependent diabetes mellitus: no evidence for linkage. *Am J Hum Genet*.
- Criqui MH, Mebane I, Wallace RB, Heiss G, Holdbrook MJ (1982). Multivariate correlates of adult blood pressures in nine North American populations: The Lipid Research Clinics Prevalence Study. *Prev Med* **11**: 391-402.
- Denke MA, Sempos CT, Grundy SM (1994). Excess body weight. An underrecognized contributor to dyslipidemia in white American women. *Arch Intern Med* **154**: 401-410.
- Dos Santos C, Fallin D, Le Stunff C, LeFur S, Bougneres P (2004). *INS VNTR* is a QTL for the insulin response to oral glucose in obese children. *Physiol Genomics* **16**: 309-313.
- Dunaif A (1992). Polycystic Ovary Syndrome. Boston: Blackwell Scientific Publications;
- Dunger DB, Ong KK, Huxtable SJ, Sherriff A, Woods KA, Ahmed ML, et al (1998). Association of the *INS VNTR* with size at birth. ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. *Nat Genet* **19**: 98-100,
- Dyer AR, Elliott P (1989). The INTERSALT study: relations of body mass index to blood pressure. INTERSALT Co-operative Research Group. *J Hum Hypertens* **3**: 299-308.
- Ek J, Andersen G, Urhammer SA, et al (2001). Homozygosity of the Pro12Ala variant of the peroxisome proliferation-activated receptor-gamma2 (PPAR-gamma2): divergent modulating effects on body mass index in obese and lean Caucasian men. *Diabetologia* **44**: 1170-6.
- Emorine L, Blin N, Strosberg AD (1994). The human b3-adrenergic receptor: the search for a physiological function. *Trends Pharmacol Sci* **15**: 3-7.
- Faith MS, Allison DB (1996). Assessment of psychological status among obese persons. In: Thompson JK, ed. Body Image, Eating Disorders, and Obesity: An Integrative Guide For Assessment and Treatment, Washington, DC, American Psychological Association pp365-387.
- Farooqi S, O'Rahilly S (2006). "Genetics of obesity in humans". *Endocr. Rev.* **27** (7): 710-18.
- Ferrannini E, Natali A, Bell P, Cavallo-Perin P, Lalic N, Mingrone G (1997). Insulin resistance and hypersecretion in obesity. European Group for the Study of Insulin Resistance (EGIR). *J Clin Invest* **100**: 1166-1173,
- Flegal KM, Carrol MD, Kuczmara RJ, Johnson CL (1998). *Int J Obe* **22**: 39.
- Ford ES (2005). Prevalence of the metabolic syndrome defined by the International Diabetes Federation among adults in the U.S. *Diabetes Care* **28**: 2745-9
- Ford ES, Williamson DF, Liu S (1997). Weight change and diabetes incidence: findings from a national cohort of US adults. *Am J Epidemiol* **146**: 214-222.

- Fossati P; Prencipe L (1982) Serum triglyceride determined colourimetrically with an enzyme that produce hydrogen peroxide. *Clin Chem* 28: 2077.
- Frayling TM, Timpson NJ, Weedon MN, et al (2007). "A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity". *Science* **316** : 889–94.
- Friedewald WT, Levy RI, and Fredrickson DS. (1972). Estimation of the concentration of lowdensity lipoprotein cholesterol in plasma, without the use of preparative centrifuge. *Clin Chem*; 18: 499-502.
- Gillespie S, Haddad L (2003). The Double Burden of Malnutrition in Asia: Causes, Consequences and Solutions. New Delhi: Sage Publications, 16–22.
- Giovannucci E, Colditz GA, Stampfer MJ, Willett WC (1996). Physical activity, obesity, and risk of colorectal adenoma in women (United States). *Cancer Causes Control* **7**: 253- 263.
- Gortmaker SL, Must A, Sobol AM, Peterson K, Colditz GA, Dietz WH (1996). "Television viewing as a cause of increasing obesity among children in the United States, 1986-1990". *Arch Pediatr Adolesc Med* **150** (4): 356–62
- Gu D, He J, Duan X, et al (2006). Body weight and mortality among men and women in China. *JAMA* **295**: 776-783.
- Gu D, He J, Duan X, et al. Body weight and mortality among men and women in China. *JAMA* 2006;295:776-783.
- Hansen SK. Gjesing AP. Rasmussen SK. Glümer C. Urhammer SA. Andersen G . et al (2004). Large-scale studies of the HphI insulin gene variable-number-of-tandem-repeats polymorphism in relation to Type 2 diabetes mellitus and insulin release. *Diabetologia* **47**: 1079–1087.
- Hassan Z (2006). Genetic determinant of young-onset diabetes. PhD thesis, University of Dhaka.
- Hasstedt SJ, Ramirez ME, Kuida H., Williams RR (1989). *Am J Hum Genet* **45**: 917.
- Henderson VW, Benke KS, Green RC, Cupples LA, Farrer LA (2005). Postmenopausal hormone therapy and Alzheimer's disease risk: interaction with age. *J Neurol Neurosurg Psychiatry* **76**:103–105.
- Heude B, Dubois S, Charles MA, Deweirder M, Dina C, Borys JM, et al (2004). P VNTR polymorphism of the insulin gene and childhood overweight in a general population. *Obes Res* **12**: 499 –504,
- Hitman GA, Jowett NI, Williams LG, Humphries S, Winter RM, Galton DJ (1984). Polymorphisms in the 50-flanking region of the insulin gene and non-insulin dependent diabetes. *Clin. Sci.* **66**:383–33
- Huang Z, Hankinson SE, Colditz GA, et al (1997). Dual effects of weight and weight gain on breast cancer risk. *JAMA* **278**:1407-1411.

- Huxtable SJ, Saker PJ, Haddad L, Walker M, Frayling TM, Levy JC, et al (2000). Analysis of parent-offspring trios provides evidence for linkage and association between the insulin gene and type 2 diabetes mediated exclusively through paternally transmitted class III variable number tandem repeat alleles. *Diabetes* **49**: 126–130.
- International Diabetes Federation (2003). *Diabetes Atlas*. Second Edition. Brussels, Belgium.
- Julier C, Hyer RN, Davies J, et al (1991). Insulin-IGF2 region on chromosome 11p encodes a gene implicated in HLA- DR4-dependent diabetes susceptibility. *Nature* **354**:155–9.
- Junaid MM (2000). Insulin resistance and insulin secretory dysfunction in obese Bangladeshi type-2 diabetic subjects M. Phill Thesis, BIRDEM Academy, University of Dhaka. 60-64.
- Junien C, van Heyningen V (1991). Report of the committee on the genetic constitution of chromosome 11. *Cytogenet. Cell Genet.* **58**:459–54
- Kahn BB, Flier JS (2000). Obesity and insulin resistance. *J Clin Invest* **106**: 473-481.
- Keith SW, Redden DT, Katzmarzyk PT, et al. (2006). "Putative contributors to the secular increase in obesity: exploring the roads less traveled". *Int J Obes (Lond)* **30** (11): 1585–94.
- Kennedy CG, German MS, Rutter WJ (1995). The minisatellite in the diabetes susceptibility locus IDDM2 regulates insulin transcription. *Nat Genet* **9**: 293–298.
- Khare M, Everhart JE, Maurer KR, Hill MC (1995). Association of ethnicity and body mass index (BMI) with gallstone disease in the United States. *Am J Epidemiol* **141**: S69.
- Kurokawa N, Nakai K, Kameo S, et al (2001). Association of BMI with the b3-adrenergic receptor gene polymorphism in Japanese: meta-analysis. *Obes Res* **9**:741–5.
- Laing,P.(2002).Childhood obesity: A public health threat. *Pediatric Nursing*.14 (10), 14-17.
- Larsson B, Bjorntorp P, Tibblin G (1981). The health consequences of moderate obesity. *Int J Obes* **5**: 97-116.
- Law MR, Wald NJ, Thompson SG (1994). By how much and how quickly does reduction in serum cholesterol concentration lower risk of ischaemic heart disease? *BMJ* **308**: 367-773.
- Le Stunff C, Fallin D, Bougnères P (2001). Paternal transmission of class I INS VNTR alleles predisposes to childhood obesity. *Nat Genet* **29**: 96–99.
- Le Stunff C, Fallin D, Schork NJ, Bougnères P (2000). The insulin gene VNTR is associated with fasting insulin levels and development of juvenile obesity. *Nat Genet* **26**: 444–446.

- Le Stunff C, Fallin D, Schork NJ, Bougneres P (2000). The insulin gene VNTR is associated with fasting insulin levels and development of juvenile obesity. *Nat Genet* **26**: 444–446.
- Lee RE, Cubbin C (2002). Neighborhood context and youth cardiovascular health behaviors). *American Journal of Public Health* **92**: 3.
- Levy et al (1998) Correct Homeostasis Model Assessment (HOMA) Evaluation uses the computer program. *Diabetes Care*; 21: 2191–2.
- Levy JC Matthew DR, Hermans MP (1998) Correct homeostasis model
- Lew EA, Garfinkel L (1979). Variations in mortality by weight among 750,000 men and women. *J Chronic Dis* **32**: 563-576.
- Lindsay RS, Robert L. Hanson, Chris Wiedrich, William C (2003). The Insulin Gene Variable Number Tandem Repeat Class I/III Polymorphism Is in Linkage Disequilibrium With Birth Weight but Not Type 2 Diabetes in the Pima Population. *Diabetes* **52**: 187–193.
- Lingohr MK, Buettner R, and Rhodes CJ. (2002) Pancreatic beta-cell growth and survival—a role in obesity-linked type 2 diabetes? *Trends Mol Med* **8**: 375–384,.
- Loktionov A (2003). Common gene polymorphisms and nutrition: emerging links with pathogenesis of multifactorial chronic diseases (review). *J Nutr Biochem* **14**: 426–51.
- Lorna M. Dickson and Christopher J. Rhodes (2004) Pancreatic B-cell growth and survival in the onset of type 2 diabetes: a role for protein kinase B in the Akt ? *Am J Physiol Endocrinol Metab* 287:192-198.
- Lucassen AM, Julier C, Beressi JP, Boitard C, Froguel P, Lathrop M, et al. (1993). Susceptibility to insulin dependent diabetes mellitus maps to a 4.1 kb segment of DNA spanning the insulin gene and associated VNTR. *Nature Genetics* **4**: 305±310.
- Lucassen AM, Sreaton GR, Julier C, Elliott TJ, Lathrop M, Bell JI (1995). Regulation of insulin gene expression by the IDDM associated, insulin locus haplotype. *Hum Mol Genet* **4**: 501–506.
- Maes HHM, Neale MC, Eaves LJ. (1997) Genetic and environmental factors in relative body weight and human adiposity. *Behav Genet* **27**: 325–51.
- Mainous AG, Diaz VA, Geesey ME (2008). Acculturation and Healthy Lifestyle Among Latinos With Diabetes. *Annals of Family Medicine* **6**: 131-137.
- Manjinder S. Sandhu, Barbara Heude, Elizabeth H. Young, Robert Luben, Jian'an Luan, Kay-Tee Khaw, John Todd, and Nicholas J. Wareham (2005) INS VNTR Class Genotype and Indexes of Body Size and Obesity Population-Based Studies of 7,999 Middle-Aged Men and Women *Diabetes* **54**:2812–2815,
- McGarry JD (2002). Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* **51**:7–18

Medalie JH, Papier C, Herman JB, et al (1974). Diabetes mellitus among 10,000 adult men. I. 5-year incidence and associated variables. *Isr J Med Sci* **10**: 681-697.

Meyer JM (1995). in *Behavior Genetic Approaches in Behavioral Medicine*, J. R. Turner, L. R. Cardon, J. K. Hewitt, Eds. (Plenum Press, New York,

Millman RP, Carlisle CC, McGarvey ST, Eveloff SE, Levinson PD (1995). Body fat distribution and sleep apnea severity in women. *Chest* **107**: 362-366.

Moll PP, Burns TL, and Lauer RM (1991) The genetic and environmental sources of body mass index variability: the Muscatine Ponderosity Family Study.

Myers, S., & Vargas, Z. (2002). Parental perceptions of the preschool obese child. *Pediatric Nursing*, 26 (1), 23-30.

National Institutes Of Health (NIH) National Heart, Lung, and Blood Institute (NHLB) (1998). Clinical guidelines on the Identification, evaluation, and Treatment Of Overweight and Obesity In Adults, The Evidence Report NIH Publication No. 98-4083.

NCEP (2002). Third report of the National Cholesterol Education Program, expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III). Final report. *Circulation* **106**: 3143-3421.

Norberg M, Stenlund H, Lindahl B, Andersson C, Weinehall L, Hallmans G, et al (2007). Components of Metabolic Syndrome Predicting Diabetes: No Role of Inflammation or Dyslipidemia. *Obesity* **15**: 1875-1885.

O'Dell SD, Bujac SR, Miller GJ & Day IN (1999). Associations of IGF2 Apal RFLP and INS VNTR class I allele size with obesity. *European Journal of Human Genetics* **7** 821-827.

OECD data: According to circa 2005. See 3.3, Overweight and obesity, *Health at a Glance 2007: OECD Indicators*, Source OECD (accessed on line January 12, 2008.)

Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, Flegal KM (2006). Prevalence of Overweight and Obesity in the United States, 1999-2004 JAMA. ;295:1549-1555

Ong KK, Petry CJ, Barratt BJ, Ring S, Cordell HJ, Wingate DL, et al (2004). Maternal-fetal interactions and birth order influence insulin variable number of tandem repeats allele class associations with head size at birth and childhood weight gain. *Diabetes* **53**:1128-1133.

Ong KK, Phillips DI, Fall C, Poulton J, Bennett ST, Golding J, et al (1999). The insulin gene VNTR, type 2 diabetes and birth weight. *Nat Genet* **21**: 262-263.

Owerbach D, Bell GI, Rutter WJ, Brown JA, Shows TB (1981). The insulin gene is located on the short arm of chromosome 11 in humans. *Diabetes* **30**:267-70

Owerbach D, Gabbay KH (1993). Localization of a Type 1 diabetes susceptibility locus to the variable tandem repeat region flanking the insulin gene. *Diabetes* **42**:1708-14.

- Owerbach D, Gabbay KH. 1993. Localization of a Type 1 diabetes susceptibility locus to the variable tandem repeat region flanking the insulin gene. *Diabetes* 42:1708–14
- Peiris AN, Sothmann MS, Hoffman RG, et al (1989). Adiposity, fat distribution, and cardiovascular risk. *Ann Intern Med* 110: 867-872.
- Poitout, V. and Robertson, R. P. (2002). Minireview: Secondary beta-cell failure in type 2 diabetes - a convergence of glucotoxicity and lipotoxicity. *Endocrinology* 143, 339-342.
- Pool RM (2001). Fat: fighting the obesity epidemic. Oxford, UK: Oxford University Press. Publication Date: 01-JAN-01
- Popkin BM (2007). "The World Is Fat", Scientific American, pp. 94..
- Powell BL, Haddad L, Bennett A, Gharani N, Sovio U, Groves CJ, et al (2005). Analysis of multiple data sets reveals no association between the insulin gene variable number tandem repeat element and polycystic ovary syndrome or related traits. *J Clin Endocrinol Metab* 90: 2988–2993.
- Price RA (1996). *Int J Obes* 20: 1044.
- Pugliese A, Zeller M, Fernandez A Jr, Zalcberg LJ, Bartlett RJ, Ricordi C et al (1997). The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDD2 susceptibility locus for type 1 diabetes. *Nature Genetics* 1997 15 293±297.
- Qiu FF, Nie QH, Luo CL, Zhang DX, Lin SM, Zhang XQ (2006). Association of Single Nucleotide Polymorphisms of the Insulin Gene with Chicken Early Growth and Fat Deposition. *Poultry Science* 85: 980–985
- Rankinen T, Zuberi A, Chagnon YC, et al (2006). The human obesity gene map: the 2005 update. *Obesity (Silver Spring)* 14: 529–644.
- Reaven GM (1988). Banting lecture: Role of insulin resistance in human disease, *Diabetes* 37: 1595-607
- Rexrode KM, Hennekens CH, Willett WC, et al (1997). A prospective study of body mass index, weight change, and risk of stroke in women. *JAMA* 277: 1539-1545.
- Rice T, Borecki IB, Bouchard C, Rao DC (1993). Segregation analysis of fat mass and other body composition measures derived from underwater weighing. *Am J Hum Genet* 52: 967–973.
- Richmond W. (1973) Quantitative determination of cholesterol in serum or plasma by enzymatic method. *Clin Chem* 19: 1350
- Rosén T, Bosaeus I, Tölli J, Lindstedt G, Bengtsson BA (1993). "Increased body fat mass and decreased extracellular fluid volume in adults with growth hormone deficiency". *Clin. Endocrinol.* 38 (1): 63–71.

Roy MN, Biswas KB, Siddiqua N, Arslan MI, Ali L (2007). Determinants of Insulin Secretion and Sensitivity in Bangladeshi Type 2 Diabetic Subjects. *Metabolic Syndrome and Related Disorders* **5**: 1275-281.

Sanchez-Corona J et al (2004). Polymorphisms in candidate genes for type 2 diabetes mellitus in Mexican population with metabolic syndrome findings. *Diabetes Res Clin Pract* **63**: 47-55.

Sander, A., Ruess, M., Bereswill, S., Schuppler, M. & Steinbrueckner, B. (1998). Comparison of different DNA fingerprinting techniques for molecular typing of *Bartonella henselae* isolates. *J Clin Microbiol* **36**, 2973–2981

Sayeed MA, Mahtab H, Khanam PA, Latif ZA, Ali SAK, Banu A, et al (2003). Diabetes and impaired fasting glycemia in a rural population of Bangladesh. *Diabet care* **26**: 1034-1039.

Shafique S, Akhter S, Stallkamp G, De Pee S, Panagides D, Bloem MW (2007). Trends of under- and overweight among rural and urban poor women indicate the double burden of malnutrition in Bangladesh. *International Journal of Epidemiology* **36**: 449–457.

Stead JD, Buard J, Todd JA and Jeffreys AJ (2000). Influence of allele lineage on the role of the insulin minisatellite in susceptibility to type 1 diabetes. *Hum Mol Genet* **9**: 2929-2935.

Stead JD, Jeffreys AJ (2000). Allele diversity and germline mutation at the insulin minisatellite. *Human Molecular Genetics* **9**: 713± 723.

Stevens J (2003). Ethnic-specific revisions of body mass index cutoffs to define overweight and obesity in Asians are not warranted. *Int J Obes Relat Metab Disord* **27**:1297-1299.

Talukder K, Talukder MSH, Bhadra SK, Talukder MQK (2006). Increasing trend of BMI $\geq$ 23 in Bangladeshi mothers of children aged less than five years. Book of Abstract (089/138). 8th Commonwealth Congress on Diarrhoea and Malnutrition (CAPGAN), Dhaka.

Tjepkema M (2005). "Measured Obesity—Adult obesity in Canada: Measured height and weight", Nutrition: Findings from the Canadian Community Health Survey. Ottawa, Ontario: Statistics Canada.

Trinder P.(1969). *Ann Clin Biochem*; **6**: 24

Ullrich A, Dull TJ, Gray A, Philips JA, Peter S (1982). Variation in the sequence and modification state of the human insulin gene flanking regions. *Nucleic Acids Res.* **10**:2225–40

Ulrich A, Dull TJ, Gray A, Brosius J, Sures I (1980). Genetic variation in the human insulin gene. *Science* **209**: 612–615.

Undlien DE, Bennett ST, Todd JA, Akselsen HE, Ikaheimo I, et al (1995). Insulin gene region-encoded susceptibility to IDDM maps upstream of the insulin gene. *Diabetes* **44**:620–25

- Unger RH (2003). Lipid overload and overflow: metabolic trauma and the metabolic syndrome. *Trends Endocrinol Metab* **14**: 398–403,
- Unger RH, Orci L (2001). Diseases of liporegulation: new perspective on obesity and related disorders. *FASEB J* **15**: 312–21.
- Vafiadis P, Bennett ST, Colle E, et al (1996). Imprinted and genotype specific expression of genes at the *IDDM2* locus in pancreas and leucocytes. *J Autoimmun* **9**: 397–403.
- Vafiadis P, Bennett ST, Colle E, Grabs R, Goodyer CG, Polychronakos C (1996). Imprinted and genotype-specific expression of genes at the *IDDM2* locus in pancreas and leucocytes. *J. Autoimmun.* **9**:397–403
- Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG et al (1997) Insulin expression in human thymus is modulated by *INS VNTR* alleles at the *IDDM2* locus. *Nature Genetics* **15**: 289-292.
- Vioque J, Torres A, Quiles J (2000). "Time spent watching television, sleep duration and obesity in adults living in Valencia, Spain". *Int. J. Obes. Relat. Metab. Disord.* **24** (12): 1683–8.
- Waterworth DM, Bennett ST, Gharani N, McCarthy MI, Hague S, Batty S (1997).. Linkage and association of insulin gene *VNTR* regulatory polymorphism with polycystic ovary syndrome. *Lancet* **349**: 986–990,
- Weaver JU, Kopelman PG, Hitman GA (1992). Central obesity and hyperinsulinaemia in women are associated with polymorphism in the 5' flanking region of the human insulin gene. *European Journal of Clinical Investigation* **22**: 265-270.
- Weiss R, Dziura J, Burgert TS, Tamborlane WV, Taksali SE, Yeckel CW et al (2004). Obesity and the metabolic syndrome in children and adolescents. *N Engl J Med* **350**: 2362–2374.
- WHO (2004). Expert Consultation. Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. *Lancet* **363**:157-163.
- Wild S, Roglic G, Green A, Sicree R, King H (2004). Global Prevalence of Diabetes Estimates for the year 2000 and projections for 2030. *Diabetes Care* **27**: 1047-1053.
- Willett WC, Manson JE, Stampfer MJ, et al (1995). Weight, weight change, and coronary heart disease in women. Risk within the 'normal' weight range. *JAMA* **273**: 461-465.
- World Health Organization (1995). Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. (WHO Technical Report Series no. 854). Geneva, Switzerland,
- World Health Organization (1997). Obesity: preventing and managing the global epidemic. Report of a WHO Consultation on Obesity, 3-5 June, Geneva.

World Health Organization (1998). *Obesity: Preventing and Managing the Global Epidemic* (World Health Organization, Geneva,).

World Health Organization (1999). Report of a WHO consultation: definition of metabolic syndrome in definition, diagnosis, and classification of diabetes mellitus and its complications. I. Diagnosis and classification of diabetes mellitus. Geneva, Switzerland,

World Health Organization (WHO) Consultation (1999). Definition, Diagnosis and Classification of Diabetes Mellitus and its complications, Part 1: Diagnosis and Classification of Diabetes Mellitus, Report of a WHO Consultation. Geneva:.

Yang W, Kelly T, He J (2007). Genetic Epidemiology of Obesity, *Epidemiol Rev*; **29**: 49–61

Zinnat R, Junaid MM, Khan AH, Chowdhury HS, Ali L (2003). Insulin secretory dysfunction and insulin resistance in Bangladeshi obese Type 2 diabetic subjects. *Diabetes Metab* **29**: 4S103.

APPENDICES

Appendix I

Data Collection Sheet

MS Thesis

1. Identification No:

2. Particulars of the Subjects:

i) Name: _____

ii) Father's /Husband's Name: _____

iii) Age: _____ years

iv) Sex: Male / Female

v) Address:

Present: _____

Permanent: _____

vi) Phone/Cell: _____

vii) Date of 1st Examination: _____

3. Past Medical history:

4. Drug history:

Anti-HTN:

Lipid Lowering:

Anti-obesity:

Anti-ischemic:

5. Family history:

- i) Family history of Diabetes
- ii) Family history of HTN

6. Social history:

i) **Marital status:** Married / Unmarried / Widow.

ii) **Scio-economics Status:**

Total members of the family:

Income of the family (Monthly / Yearly):

iii) **Educational Status:**

Class I-VIII / SSC-HSC / Graduate / Others (_____)

iv) **Occupation:**

Current Designation

Duration:

Type of Work

Professional / Housewife / Sales Person / Domestic help /

Laborer / Others (_____)

v) **Habit:**

Exercise:

Present (Type_____, Duration_____)

Past (Type_____, Duration_____)

Not

Smoking:

Present (Duration_____, Sticks/day_____)

Past (Duration_____, Sticks/day_____)

Not

Betel Leaf: Yes / No (_____)

7. Physical Examination:

(b) Anthropometry:

- (ii) Height in meter:
- (iii) Weight in Kg:
- (iv) Waist circumference:
- (v) Hip circumference:
- (vi) Mid-upper arm circumference (MUAC):
- (vii) Skin fold thickness:

Triceps:

Sub-scapular skin folds thickness:

- (viii) Body fat mass:

(b) Blood pressure:

	1 st reading	2 nd reading	Average reading
Systolic			
Diastolic			

Appendix II

ক্রমিক নং

নাম:.....

বয়স:.....

ঠিকানা:.....

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খালী পেটে এবং ৭৫ গ্রাম গ্লুকোজ ড্রিঙ্ক পান করার পর পরিমানমত রক্ত নিয়ে পরীক্ষা করে,
ডায়াবেটিসের আক্রান্তের পূর্বের এবং শরীরে ইনসুলিনের অভাব অথবা কার্যক্ষমতা হ্রাসের
পরিমাপ করা হবে।

স্বাক্ষর:.....

তারিখ:.....