ASSOCIATION OF VITAMIN D RECEPTOR GENE POLYMORPHISM WITH YOUNG ONSET DIABETES MELLITUS 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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DECLARATION OF ORIGINALITY OF THE WORK

This is to be confirmed that the Thesis entitled **'Association of vitamin D receptor gene polymorphism with young onset diabetes mellitus in a Bangladeshi population'** is submitted in partial fulfillment for the degree of Masters in Biotechnology, Department of Mathematics and Natural Sciences, BRAC University, Dhaka, was carried out in the Laboratory of BMRG, BIRDEM during the period of June 2009 to February 2010.

No part of the work has been submitted for another degree or qualification in any other institutes at home or in abroad.

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CERTIFICATION OF ORGINALITY OF THE WORK

This is to certify that the Thesis entitled **'Association of vitamin D receptor gene polymorphism with young onset diabetes mellitus in a Bangladeshi population',** a requirement for the degree of Masters of Science (MS) in Biotechnology under the Dept of Mathematics and Natural Science, BRAC University was carried out in the Laboratory of Biomedical Research Group, BIRDEM, Dhaka during the period of June 2009 to February 2010, under our joint supervision.

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MY BELOVED PARENTS

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ABSTRACT

Vitamin D has an important immunomodulatory property and also has been suggested to play an important role in cellular metabolism. Vitamin D receptor (VDR) gene polymorphism has also been found to be associated with insulin secretory capacity and glucose intolerance. The present study was undertaken to determine genotype of VDR gene common variants in young onset diabetic subjects of Bangladesh to explore its association with diabetes and B cell secretory capacity. A total number of 94 young diabetic subjects and 92 healthy controls were recruited. Detailed clinical and anthropometric measurements were recorded. Blood glucose was measured by glucose-oxidase, lipids by standard methods. C-peptide was estimated by enzyme linked immunosorbent assay (ELISA), and insulin secretory capacity (HOMA%B), sensitivity (HOMA%S), resistance (HOMA-IR) were calculated using HOMA Sigma software. DNA was extracted using QIAGEN Blood DNA Kit. VDR gene variants [G>T and T>C] were determined by PCR-RFLP using restriction endonuclease Apa1 and Taq1 respectively. Data were managed using Statistical Program for Social Science (SPSS). Unpaired Student's -'t' test and Chi-squared tests, as appropriate, were performed. C-peptide level was found to be significantly lower 3.297 ($p<0.001$) and HOMA%B found lower 3.102 ($p=0.003$). The G>T and T>C genome frequencies (wild, heterozygous and homozygous variants) were in the control (0.183, 0.516, 0.316 and 0.370, 0.522, 0.109 respectively) and (0.198, 0.484, 0.319 and 0.484, 0.418, 0.090 respectively) in YDM which did not show significant association with YDM ($p=0.906$ and 0.288 respectively). Genotype frequency of the marker allele did not show significant association with glucose, C-peptide, HOMA%S and IR either in Controls or YDM subjects except the HOMA%B shown to have relatively lower.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders
BMI	Body Mass Index
DBP	Diastolic blood pressure
DM	Diabetes Mellitus
EDTA	Ethylene diamine tetracetic acid
ELISA	Enzyme Linked Immunosorbent Assay
FSG	Fasting serum glucose
GDM	Gestational Diabetes Mellitus
HDL	High Density Lipoprotein
HDL-c	High density lipoprotein cholesterol
HLA	Human Leukocyte Antigen
HOMA %B	Homeostasis model assessment B-cell Function
HOMA %S	Homeostasis model assessment insulin sensitivity
Ht	Heterozygous
Hz	Homozygous
IDF	International Diabetes Federation
IGT	Impaired glucose tolerance
Kg	Kilogram
LBD	Ligand-binding domain;
LDL	Low Density Lipoprotein
MAC	Mid arm circumference
MHC	Major Histocompatibility complex
MODY	Maturity onset Diabetes in Young

mRNA	Messenger Ribonucleic Acid
MRDM	Malnutrition-related Diabetes Mellitus
NIDDM	Non-insulin dependent Diabetes Mellitus
PPDM	Protein-deficient Diabetes Mellitus
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SBP	Systolic Blood Pressure
SD	Standard Deviation
SGPT	Serum Glutamic Pyruvic Transaminase
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for Social Sciences
SSF	Sub-scapular skin fold thickness
STR	Sub scapular Triceps Ratio
T Chol	Total Cholesterol
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TSF	Triceps Skin Fold
UTR	Untranslated region
VDR	Vitamin D Receptor
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization
WHR	Waist Hip Ratio
YDM	Young Onset Diabetes Mellitus
µl	Microliter

CHAPTER 1

INTRODUCTION

INTRODUCTION

Diabetes mellitus is a complex heterogeneous group of metabolic condition characterized by elevated levels of blood glucose, caused mainly by impairment of insulin action and or insulin secretion (ADA 2005). Diabetes is one of the most prevalent and devastating chronic non-communicable diseases having serious health, economic and social consequences (IDF 2006). Diabetes mellitus, long been considered as a disease of minor significance to world health, however has become one of the main threats to human health in the 21st century (Zimmet *et al.,* 2000). In the past two decades there has been an explosive increase in the number of people diagnosed with diabetes all over the world (Amos *et al.,* 1997; King *et al.,* 1998).

The World Health Organization (WHO) sponsored estimate in 1995 showed total world diabetic subjects to be 135 million and which however projected that the number would increase to around 300 million in the year 2025 (King *et al.,* 1998). Subsequently global prevalence of diabetes for all age-groups was estimated to rise from 2.8% in 2000 to 4.4% in 2030 and also demonstrated that total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild *et al.,* 2004). In Europe, an over all prevalence of diabetes is about 7.8% in the adult population (20-79 years) (IDF 2003). Without effective prevention program of diabetes prevalence in Europe is expected to increase to 9.1% in 2025 as estimated by the International Diabetes Federation (IDF 2003). The number of Americans with diabetes is expected to be almost double from 18.2 million in 2003 to 30.3 million in 2030 (Wild *et al.,* 2004).

Classification of Diabetes of Mellitus

Diabetes mellitus has long been classified based on its clinical presentation; age of onset and special feature, and need for insulin of the individual to control blood glucose. Lawrence (1951) was first to introduce the nomenclature type 1 and type 2 to describe two distinct forms of diabetes mellitus. In late seventies the National Institute of Health (NIH), United States of America (USA) set up the National Diabetes Data Group (NDDG) that formulates the classification and diagnostic criteria. The NDDG introduced the term insulin dependent diabetes mellitus (IDDM, Type 1) and noninsulin dependent diabetes mellitus (NIDDM, Type 2) to describe two main classes of diabetes (NDDG 1979). In the following year a WHO Expert Committee on Diabetes endorsed the NDDG proposed classification of diabetes with some modifications especially

regarding diagnostic criteria (WHO 1980). According to the present classification of diabetes four main classes of the disease are type 1, type 2, Other Specific types and Gestational Diabetes Mellitus (Table 1) (WHO 1999 and ADA 1997).The other subclass was protein deficient diabetes mellitus (PDDM). Among the young diabetes FCPD was found to be 13% and PDDM 42% (Azad Khan and Ali 1997). The others with BMI>19 and diabetes diagnosed at younger age were termed as non insulin dependent diabetes mellitus in young (NIDDY).

Table 1: Etiological Classification of Diabetes Mellitus (WHO, 1999)

A. Type 1 (beta-cell destruction, usually leading to absolute insulin deficiency)

Type 1A- Autoimmune mediated type

Type 1B- Non-immune mediated idiopathic type

B. Type 2 (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance)

C. Other Specific Types of Diabetes

- Genetic defects of beta-cell function
- Genetic defects in insulin action
- Diseases of the exocrine pancreas
- Endocrinopathies
- Drug- or chemical-induced
- Infections (Congenital rubella, Cytomegalovirus, Others)
- Uncommon forms of immune-mediated diabetes
- Other Genetic Syndromes Sometimes Associated with Diabetes

(Down's syndrome, Friedreich's ataxia, Turner's syndrome, etc)

D. Gestational diabetes

Type 1 Diabetes Mellitus

T1DM is characterized by sudden onset of symptoms, proneness to kitoacidosis and need of insulin for survival. The hallmark of the T1DM is pancreatic B cell damage resulting in very low to absolute loss of insulin secretion. T1DM mainly occur in children and young adults and accounts for about 10% of all diabetic patients (ADA 1997). The incidence of T1DM was found to be lowest in Asia (0.1 per 1000,000 in China) and highest in Scandinavia (35.5 per 1000,000 in Finland). However, there are notable exceptions, for instance the incidence is low in Iceland but in Sardinia, Italy the incidence is similar to that of Finland (Karvonen *et al.,* 2000). A report from Southern India has shown that these the incidence of T1DM is similar (10.5/1000,000) to many European populations (Ramachandran *et al.,* 1986). T1DM is believed to be less prevalent in Bangladesh, however, population based studies are still lacking.

T1DM is subclassified on the basis of type of damage of B cells; immune mediated type1 and non-immune mediated type1 (idiopathic type 1, T1DM) (ADA 1997; WHO 1999). Autoimmune T1DM is characterized by immune mediated damage, targeted against self-antigens, resulting in the destruction of the B cells of the pancreas. A number of autoantibodies have been detected against different islet proteins. The rate of B cell destruction is found to vary widely. It has been seen to be usually faster in children and slower in adults. A number of T1DM patients have a typical sudden onset of the disease and are ketosis prone. But they lack classical autoantibodies directed to islets and/or islet cell proteins and are called idiopathic T1DM. This form of diabetes is thought to be most common in African and Asian countries (McLarty *et al.,* 1990 and; Tanaka *et al.,* 2000). Idiopathic T1DM usually occurs to obese teens. Once their blood glucose is controlled by insulin oral hypoglycemic agents can be subsequently used before the onset of complete insulin dependence as in T1DM. While the exact etiological factor(s) are still unknown, multiple genetic and environmental factors are thought to be involved (ADA 1997; WHO 1999).

In another in depth study, autoantibody and candidate gene markers were investigated in young onset diabetic subjects. T1DM diabetes related antibodies; anti Glutamic acid decarboxylase (GAD) antibody and anti IA2 antibodies were found to be positive around 25% of the study subjects (Hassan *et al.,* 2005). INS-VNTR polymorphism did not show any association with diabetes. Although FCPD is suggested to be the secondary type of

diabetes according to the present classification. T1DM related auto antibodies were found to be positive in 20% of study subjects (Hassan 2006). This suggested diabetes in the young subjects of Bangladesh may represent a group of atypical T1DM.

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is characterized by the presence of disorders of insulin action and/or insulin secretion (Reaven 1988). Both defects are usually present in a T2DM patient; however, the primacy of the two factors for the development of diabetes still remains to be clearly understood.

T2DM constitutes more than 90% of all diabetics and in most populations. However, in American Indians and South Pacific Islanders, T2DM is found to be the only form of the diabetes. Overall prevalence of diabetes varies between 15-20%. The highest prevalence of T2DM (50%) was found among Pima Indians (49.4% in male and 51.1% in female) in USA and Nauru (41%; male 40% and female 42%) and a very low (0-1.4%; male 0% and female 1.4%) was observed among the Mapuches population in Chile. The prevalence was almost nil in rural and peri-rural population of Papua New Guinea (WHO 1994).

Based on recent community based studies the prevalence of diabetes in Bangladesh is found to be around 5%. Studies involving rural and suburban population revealed the prevalence of T2D to be 4.3% and 4.1% respectively (Sayeed *et al.*, 1997 and 2003).

Pathogenesis of DM

Diabetes is probably caused by a complex interaction of environmental factors and genetic predisposition. Environmental risk factors are thought to act as either damage or dysfunction among the genetically predisposed subjects.

Environmental factors

Despite the strong evidence for environmental trigger in the pathogenesis of T1DM the precise pathology still remains elusive. Among the environmental factors implicated in the pathogenesis of T1DM most notable are diet, stress and viral infections (Knip and Akerblom 1999; Dahlquist 1998). Early exposure to cow's milk, duration of breast-feeding and food preservatives, particularly nitroso compound are claimed to be associated with the development of T1DM (Karjalainen *et al.*, 1992).

Coxsackie virus B (CVB) and rubella have been implicated in the pathogenesis of T1DM (Lukie *et al.,* 1992). However, the pathological basis in its development remains to be clearly understood.

Among the environmental factors in the pathogenesis of T2DM age, physical activity, dietary habits, lifestyle and obesity play a crucial role in the modulation of development of the disease (Hu *et al.,* 2003). Glucose tolerance has been shown to decrease with age (DeFronzo 1981; Shimokata *et al.,* 1991). However, whether the deterioration is caused by increased age or secondary to age related factors, such as decreased physical activity, is still to be clearly understood.

Difference has been observed in the prevalence of T2DM in Europoid populations between men and women. Some studies had shown a higher age-adjusted prevalence in women (Damsgaard *et al.,* 1987; Bruno *et al.,* 1992), whereas other investigators demonstrated male preponderance (Lintott *et al.,* 1992). Variation in T2DM prevalence between men and women also has been shown to vary in different age group in the same community.

The major environmental risk factors for T2DM are found to be obesity (>120% ideal body weight or a body mass index >30Kg/m) and a sedentary lifestyle (Bleich *et al.,* 2007). Thus, the tremendous increase in the rates of T2DM in recent years has been attributed, primarily, to the dramatic rise in obesity worldwide (Zimmet *et al.,* 2001). It has been shown that approximately 80% of all new T2DM cases are due to obesity. In the Pima Indians, 85% of the T2DM children were either overweight or obese (Fagot *et al.,* 2000). Although excess weight and obesity (BMI>25 kg/m^2) account for 64% cases of diabetes in men and 74% in women, many cases of diabetes occur in lean subjects as well (Chan *et al.,* 1994; Colditz *et al.,* 1995).

The risk for T2DM also found to be associated with physical activity and a gradient was observed between exposure and outcome (Knowler *et al.,* 2002). Eriksson et al (1993) has shown that high physical activity reduces the progression from IGT to T2DM in the Sweden population. A low prevalence of T2DM was found in physically active japaness-Hawaiian men (Benoist *et al.,* 1997). In Chinese (Pani *et al.,* 1997) and Indian population the progression from IGT to T2DM was also shown to be reduced (Eriksson *et al.*, 1993).

Role of vitamin D in diabetes

Vitamin D has been suggested to play important role in the immunomodulation (DeLuca *et al.,* 2004). It has been implicated in the pathogenesis of T1DM although the mechanism is yet to be clearly understood. Several studies in rats and humans (Norman *et al.*, 1980 and Boucher *et al.*, 1995) have demonstrated vitamin D deficiency to reduce insulin secretion, and $1\alpha25(OH)_2D_3$ supplementation improve in B cell secretory function and consequently in glucose tolerance (Kumar *et al.*, 1994). Glucose tolerance and insulin secretion were found to improve in vitamin D deficient rat treated with $1\alpha25(OH)_2D_3$ treatment (Cade *et al.*, 1997). Rudnicki and Molsted-Petersen (1997) reported fall of glucose to 4.8 from 5.6 mmol/l after intravenous treatment with $1\alpha,25(OH)_2D_3$ in gestational diabetes mellitus patients. In a study by Giulietti et al (2004),it was demonstrated that vitamin D deficiency in early life might increase risk for development of T1DM. They showed that at 250 days, 35% male and 66% female vitamin D deficient mice were diabetic compared to 15 and 45% of the control mice. In the vitamin D deficient mice, higher IL-1 expression was detected in islets. Thymus and lymph nodes also contained less CD4CD62L+ cells; a defect in this cytokine profile might trigger the diabetes. The higher rate of development of diabetes in vitamin D deficient mice was substantiated by the observation of Chiu et al (2004). They have demonstrated inhibitor of insulin secretion in vitamin D deficient state.

Molecular action of $1\alpha,25$-dihydroxyvitamin D_3

Not only vitamin D but also its metabolites play important role in regulation of the endocrine pancreatic function. The possible mechanism is not only via the plasma calcium levels but also directly on the B cells. $1\alpha,25(OH)_2D_3$ may influence both endocrine and exocrine pancreatic function (Johnson *et al.,* 1994). The effects of $1\alpha,25(OH)_2D_3$, a biologically active metabolite of vitamin D, and its analogs have been examined regarding binding to nuclear VDR (nVDR) and membrane VDR (mVDR) receptors, through which they might induce genomic and nongenomic responses respectively.

Kajikawa et al (1999) studied the effect of $1\alpha,25$-dihydroxyluminesterol3 $1,25(OH)_2$lumisterol3 – an analog of $1,25(OH)_2D_3$ that is preferred for its nongenomic action through putative signal transduction by binding to mVDR (Dormanen *et al.,* 1994) on insulin release from rat pancreatic B cells. They found an insulinotropic effect of this

vitamin analog with increasing intracellular Ca^{2+} concentration in pancreatic B cells through nongenomic signal transduction. There is also evidence that $1\alpha,25(OH)_2D_3$ directly influences insulin secretion in the B cell through a rise in intracellular-free calcium concentration via the nonselective calcium channel, rather than the calcium dependent inositol 1,4,5-triphosphate receptor-mediated pathway (Sergeev *et al.,* 1995). $1\alpha,25(OH)_2D_3$ also exerted a stimulating effect on insulin release via protein kinase A activation, but reduced the supranormal cyclic adenosine monophosphate (AMP) synthesis (Bourlon *et al.,* 1997). $1\alpha,25(OH)_2D_3$ may provide supplementary calcium to the B cell by regulating the intracellular signaling processes involving phospholipids metabolism, protein kinase C induction, Ca^{2+} mobilization, and Ca^{2+} entry by Ca^{2+} channels (Billaudel *et al.,* 1995).

Norman et al (1993) reported the presence of a vitamin D–dependent calcium-binding protein in pancreas and cytosol receptor for the hormonal form of vitamin D, 1α,25-dihydroxyvitamin D_3, suggesting an important role of vitamin D in the endocrine function of the pancreas. Vitamin D deficient rats were unable to respond to a glucose challenge by secreting appropriate amounts of insulin (Norman *et al.*, 1989) since insulin release in vivo is dependent on acute change in plasma calcium (Curry *et al.,* 1968). Glucagon secretion is also calcium-dependent (Lundquist *et al.,* 1996), but secretion of this hormone was unaffected by $1\alpha,25(OH)_2D_3$ treatment. The genomic actions of $1\alpha,25(OH)_2D_3$ on B cells of the endocrine pancreas have been reported.

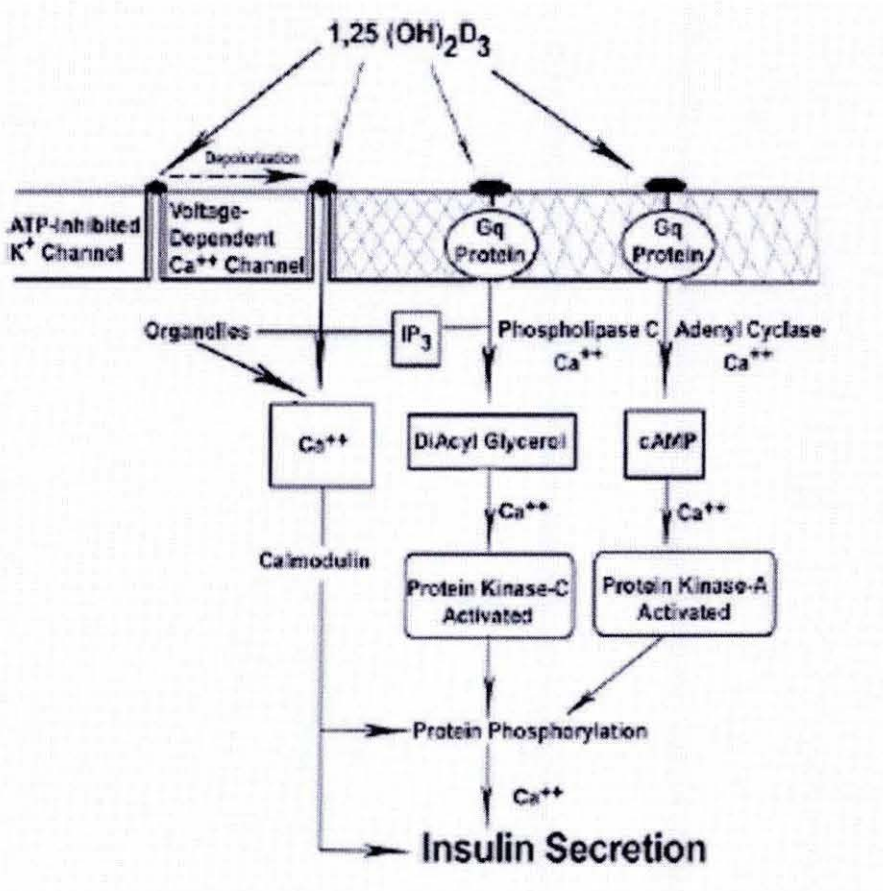

Figure 1: Schematic nongenomic model for $1\alpha,25(OH)_2D_3$ effects on insulin secretion in B cells

Calbindin-D28K, a calcium-binding protein that is thought to act as a facilitator of calcium diffusion in intestine and kidney is known to be regulated by vitamin D in these tissues (Christakos *et al.,* 1989). In cells transfected with Calbindin D28K, there was a marked increase in the expression of insulin mRNA. In addition, Calbindin D28K overexpression was also associated with an increase in insulin content and release. In chicken, pancreatic cells Calbindin D28K is altered with variations in vitamin D and mineral status (Kadowaki *et al.,* 1984). $1,25(OH)_2D_3$ activates the B cell insulin response to glucose in vivo after a delay of 3 to 20 h (Kadowaki *et al.,* 1985), or after 6-h in vitro (Billaudel *et al.,* 1990*)*, via an improvement of calcium handling occurring after a 4-h delay (Billaudel *et al.,* 1990), increasing both Ca^{2+} entry by voltage-dependent channels and Ca^{2+} mobilization from Ca^{2+} stores (Billaudel *et al.,* 1993). Bourlon et al (1999) reported that synthesis of numerous proteins is decreased during vitamin D3 deficiency

and is gradually restored by $1\alpha,25(OH)_2D_3$ repletion in the islets of Langerhans of rats. The existence of variable delays for the actions of $1\alpha,25(OH)_2D_3$ supports the hypothesis

of a genomic action of the steroid on the biosynthesis of several B cell protein implicated in the different steps of the insulin excitation-secretion coupling. $1\alpha,25(OH)_2D_3$ causes transcriptional activation of the human insulin receptor gene in U-937 human promonocytic cells (Maestro *et al.*, 2002).

Genetic factors

Genetic susceptibility has been implicated in the pathogenesis of both T1DM and T2DM diabetes. Tendency of aggregation of T1DM in families has been observed by different researchers (Risch *et al.*, 1993; Todd and Farrall 1996). The concordance rate for T1DM among monozygotic twins found to be higher between 20-71% compared to that of among dizygotic twins (Kyvik *et al.*, 1995; Redondo *et al.*, 1999) suggesting strong genetic role in its pathogenesis. The human histocompatibility antigen locus has been found to be in linkage disequilibrium in substatial number of T1DM patients (Wolf *et al.*, 1983). Later on variation in HLA Class II gene, particularly DQB1 and DR4 allele was found to be strongly associated with T1DM (Donner *et al.*, 2000; Park *et al.*, 1998). Among other gene locuses found to be implicated with T1DM are in chromosome 4p15 (IDDM2 and IDDM4) (Sawicki *et al.*, 1997; Hayes *et al.*, 1998) chromosome 2q13 (IDDM12), chromosome 2q3-2q34 (IDDM13) (Morahan *et al.*, 1996; Larsen *et al.*, 1999), chromosome 6q21 (IDDM 15) (Temple *et al.*, 2000).

INS gene is located in the IGF2-INS-TH region on short arm of human chromosome 11. The locus is designated as 11p15.1 (Powell *et al.*, 2005). The INS gene has a variable number of tendem repeats (VNTR) immediate adjacent to the 5' promoter.

The VNTR polymorphism can be classified into two main groups: small class I alleles (28-44 repeats) and large class III allele (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 *et al.*, 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 T1DM (Julier *et al.,* 1991; Lucassen *et al.,* 1993).

CTLA-4 (cytotoxic T lymphocyte-associated 4) gene is located on chromosome 2q31-35, where multiple T1DM genes may be located. CTLA-4 gene variants have been associated with T1DM, as well as other autoimmune disease. CTLA-4 is a protein that plays a key role in T cell activation (Barbara Angel *et al.,* 2009) by recognizing its specific antigen which is presented by an antigen presenting cell in a peripheral lymphoid organ, the T cell receives co-stimulatory signals to differentiate and proliferate into armed effectors T cells but the principal co-stimulatory molecule for activation and clonal expansion expressed by T cells are the CD^{28} receptors. CD^{28} proteins bind to B7 molecules, which are expressed by antigen presenting cells (Janeway *et al.*, 2005).

Also, subsequent to T cell activation, the lymphocyte secretes the cytokine interleukin-2 (IL-2), which drives proliferation and differentiation of the cell. Activated T cells express a high affinity for IL-2 receptors, which binds to the cytokine IL-2. However, it was discovered that when CTLA-4 is induced, it decreases IL-2 production (Alegre *et al.*, 1998). The extra cellular domain of CTLA-4 can be fused with the antibody IgG (Freeman *et al.*, 1998). CTLA-4 Ig is a protein that is induced to block the production IL-2 and CD28/B7 co-stimulation (Alegre *et al.*, 1998).

A meta-analysis was done by Fotini (2005) of 33 studies examining the association of T1DM mellitus with polymorphisms in the cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) gene, including the *A49G* (29 comparisons), *C* (*–318*) *T* (three comparisons) and (*AT*) *n* microsatellite (six comparisons) polymorphisms.

T2DM is also found to be concentrated in families than in general population (Klein *et al.,* 1996; Weijnen *et al.,* 2002). Reduced concordance of T2DM in both mono-and dizygoric twins suggested that nongenetic factors, possibly environmental trigger the development of this type of diabetes (Newman *et al.,* 1987). Family studies have revealed that first degree relatives of individuals with T2DM are about 3 times more likely to develop the disease than individuals without a positive family history of the disease (Hansen 2003; Gloyn 2003). Moreover, T2DM mainly results from interaction of many genes. Genome wide scan identified several chromosomal regions strongly linked with T2DM. One approach that is used to identify disease susceptibility genes is based

on the identification of candidate genes (Barroso *et al.,* 2003; Stumvoll, 2004). Most predisposing candidate genes are PPARγ (peroxisome proliferator-activated receptor-γ), KCNJ11, ABCC8 and CALPN 10 (Calpain 10) involved in T2DM.

PPARγ gene is an important regulator of adiposite development and lipid metabolism (Tontonoz *et al.*, 1994) has become a potential therapeutic target for the treatment of a diverse array of disorders, including T2DM, dyslipidaemia, inflammation and malignancy. One form of the *PPARγ* gene (Pro) decreases insulin sensitivity and increases T2DM risk several fold.

CAPN 10 encodes an intracellular calcium-dependent cysteine protease that is ubiquitously expressed (Cox *et al.,* 2001). A haplotype that was initially allowed to T2DM included an intronic A to G mutation at position 43, which appears to be involved in *CAPN10* transcription.

Vitamin D receptor (VDR) gene

The vitamin D receptor mediates the majority of the effects of vitamin D (as the active form 1α,25-dihydroxyvitamin D (calcitriol)) on gene expression via formation of a heterodimer with the retinoid X receptor which binds to promoter regions of many target genes. There are 6 known polymorphisms in the VDR locus with a range of possible effects (Table 1) (Uitterlinden *et al.,* 2004).

A number of observational studies have reported on associations (or lack of) between VDR polymorphisms and T2DM; fasting glucose, glucose intolerance, insulin sensitivity, insulin secretion and calcitriol levels.

Studies have demonstrated a link between VDR polymorphism and T2DM, although the findings differ from one population to another. A study involving Bangladeshi population demonstrated that the Apa1 polymorphism influences insulin secretion in response to glucose (Hitman *et al.,* 1998) while association between the VDR Apa1 and higher fasting plasma glucose levels and glucose intolerance were observed in a community based study of older adults without known diabetes (Oh *et al.,* 2002). Subsequently common VDR polymorphism was investigated in another population. In this study rather Bsm1 polymorphism was found to be associated with higher fasting glucose levels in young males with low physical activity (Ortlepp *et al.,* 2003).

Table 2: VDR polymorphisms and its association (Uitterlinden *et al.*, 2004)

Polymorphism	Disease and proposed mechanism
Cdx2(G to A)	Decreased risk of fracture with A allele increases VDR expression in the intestine increasing calcium transport protien transcription leading to increased calcium absorption and increased bone mineral density.
FokI (424 and 427 amino acid variants)	Appears to be functional and the 424aa variant is more active in terms of transactivation capacity
3'UTR polymorphisms (2 frequent haplotype and many polymorphism)	Difference in mRNA expression, level of serum markers such as osteocalcin With FokI one haplotype has higher VDR transcription efficiency
BsmI,ApaI and TaqI	Are in linkage disequilibrium with the 3' UTR and functionality currently assumed to be due to that

Diabetes in Bangladesh

At present, prevalence of diabetes mellitus in Bangladesh is about 7% (Sayeed *et al.*, 2007). This rise in prevalence is found to be consistent with the previous WHO estimate which has suggested a rise of prevalence of diabetes in Bangladesh like other developing Asian countries.

There is, however, a difference in prevalence of diabetes in urban and rural areas. Sayeed et al (2007) have shown that the prevalence of diabetes to be 4% and 11% in urban and rural areas respectively. Another study has shown the prevalence to be around 8% which also showed male and female the prevalence to be 9.8 and 8.0% respectively (Rahman *et al.*, 2007).

Studies were undertaken also to explore etiopathogenesis of T2DM in the Bangladeshi population as well. Study on newly diagnosed normal to overweight T2DM subjects reporting that they have both insulin secretory dysfunction and insulin resistance but

former was found to be predominant in the pathophysiology of T2DM (Al-Mahmood 2000). It was also observed that the extent of insulin secretory dysfunction was found to be more pronounced compared to loss of insulin sensitivity in obese type 2 subjects of our population (Junaid 2000). In contrast most western people with T2DM were relatively obese and had impaired insulin action (Sacks 1999). These studies have demonstrated that insulin secretory dysfunction not the insulin sensitivity is prime features of T2DM of Bangladeshi population.

Young Diabetes Mellitus (YDM) in Bangladesh

A substantial number of patients diagnosed and registered at BIRDEM are of younger age i.e. less than 30 years. They account for about 7% of patients of all ages who are registered a BIRDEM termed as young onset diabetes mellitus (YDM). They are lean, normal to moderate over weight, low BMI (BMI<19) and usually present with moderate to sever hyperglycemia. A subset of these diabetic patients has pancreatic calcification and/or fibrosis and is termed fibrocalculous pancreatic diabetes (FCPD). In the present diabetes classification they are classified as secondary diabetes of pancreatic origin under other specific types of diabetes. However, in the previous WHO classification these case have been classified as one the two subclass of malnutrition related diabetes mellitus (MRDM).

In the very first of these kinds of studies, Islam et al., (1998) investigated a series of young onset diabetic patients by glucagon stimulating test. They demonstrated that among the lean young subjects 33% had intermediate secretory capacity and 67% had low capacity compared to 100% with intermediate secretory capacity of the normal to moderately over weight young diabetic patients. The findings clearly showed that large number of lean-young diabetic patients had low B cell secretory capacity. However, they were nonketotic at the time of recruitment even with very high serum glucose level (Islam *et al.*, 1998). In later a study involving a series of lean under-30 diabetic patients insulin sensitivity was assessed by short insulin tolerance test and insulin secretory capacity was measured to explore the primary of defect in the pathogenesis of diabetes. The study revealed substantial reduction of insulin secretory capacity, both in absolute and relative terms, in these lean young patients (Ali *et al.*, 2003). They were not insulin resistant compared to age and BMI matched healthy controls (Biswas *et al.*, 2003).

Vitamin D itself and VDR gene polymorphism have links to the pathogenesis of both type 1 and type 2 diabetes since YDM seems to be representing unique groups of diabetic patients. An investigation of VDR gene common polymorphism would help in further understanding the development of diabetes in the young onset diabetes in Bangladesh.

Hypothesis

Vitamin D receptor gene common polymorphisms are not associated with diabetes mellitus in the young of Bangladeshi population.

Objectives:

General objectives:

The general objectives of the study were to determine the VDR gene common polymorphisms in the young diabetic subjects of Bangladesh.

Specific objectives:

The specific objectives of the present study were to:

- Determine VDR gene G>T and T>C polymorphisms in a group of young onset diabetic patients of Bangladeshi origin.
- Estimate C-peptide level of the study subjects to evaluate residual B cell secretory status
- Explore the relationship, if any, with B cell secretory status and VDR gene G>T and T>C polymorphism of the study subjects.

CHAPTER 2

REVIEW OF LITERATURE

Review of Literature

Vitamin D is a group of fat-soluble molecules that are essential for calcium and phosphorus absorption in our body. Among the 5 types of vitamin D (vitamin D_1, D_2, D_3, D_4, D_5); vitamin D_2 (ergosterol) and D_3 (cholecalciferol) are the most important and essential ones (Walter *et al.*, 2003). Vitamin D is a generic term and indicates a molecule of the general structure shown for rings A, B, C, and D with differing side chain structures. The A, B, C, and D ring structure is derived from the cyclopentanoperhydro-phenanthrene ring structure for steroids. Vitamin D is classified as a seco-steroid in which one of the rings has been broken; in vitamin D, the 9, 10 carbon-carbon bond of ring B is broken.

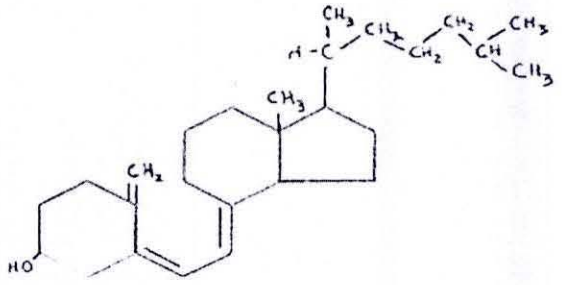

Figure 2: The Chemical Structure of Vitamin D (Crowfoot-Hodgkin *et al.*, 1957).

Source and Metabolism of vitamin D

Good sources of vitamin D are butter, cheese, cream, yogurt, milk eggs and sunlight. The richest sources of vitamin D are fish-liver oils, particularly those of the halibut and the cod (NIH 2010).

Few foods naturally contain or may be fortified with vitamin D. Vitamin D_2 is formed through the ultraviolet irradiation of ergosterol from yeast, and vitamin D_3 through the ultraviolet irradiation of 7-dehydrocholesterol from lanolin which is called cholecalciferol (Figure 2).

7-Dehydrocholesterol is the precursor of vitamin D_3 and forms cholecalciferol only after being exposed to solar UV radiation (Bouillon *et al.*, 2001; Holick; 2004). Cholecalciferol is then hydroxylated in the liver to become calcifediol (25-hydroxyvitamin D_3). 25-hydroxyvitamin D is metabolized in the kidneys by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) to its active form, 1,25-

dihydroxyvitamin D (Holick; 2004). The renal production of 1,25-dihydroxyvitamin D is tightly regulated by plasma parathyroid hormone, serum calcium and phosphorus levels (Holick; 2004). Fibroblast growth factor 23, secreted from the bone, causes the sodium–phosphate cotransporter to be internalized by the cells of the kidney and small intestine and also suppresses 1,25-dihydroxyvitamin D synthesis. The efficiency of the absorption of renal calcium and of intestinal calcium and phosphorus is increased in the presence of 1,25-dihydroxyvitamin D (Figure 2) (Dusso *et al.*, 2005). It also induces the expression of the enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24), which catabolizes both 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D into biologically inactive, water-soluble calcitroic acid (Holick *et al.*, 2005).

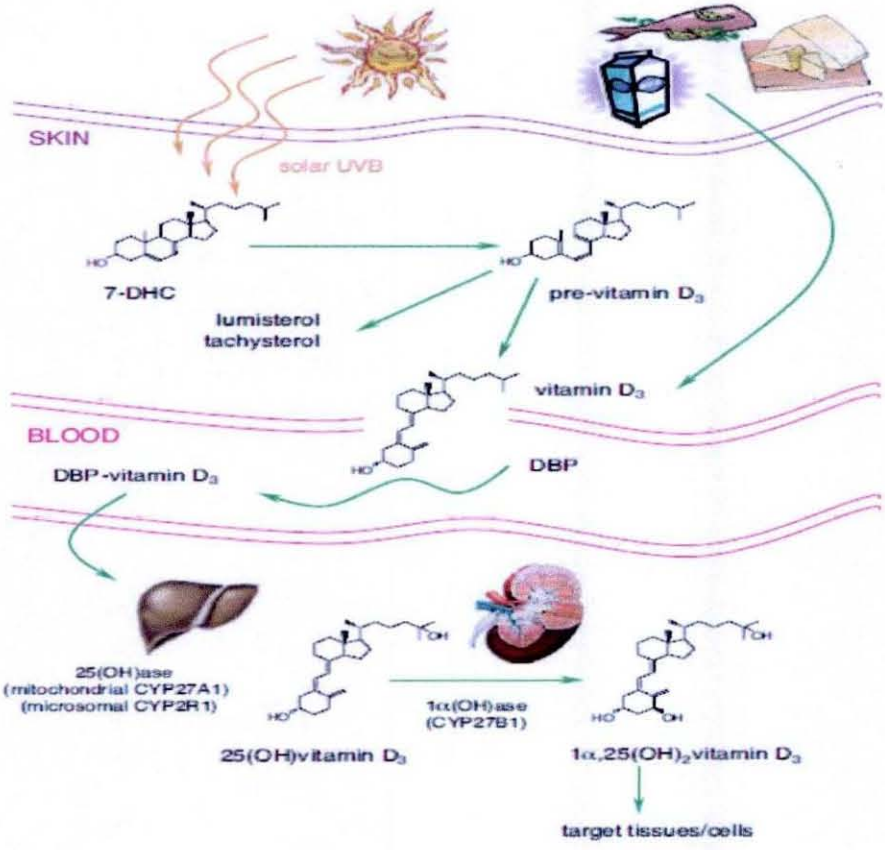

Figure 3: Synthesis and metabolism of 1α,25-dihydroxyvitamin D_3. Vitamin D can be obtained from food (vitamin D_2 and D_3) or by photobiogenesis in the skin (vitamin D_3). In the blood, all vitamin D metabolites are bound to vitamin D-binding protein (DBP). Vitamin D_3 is converted by two successive hydroxylations in the liver (25-hydroxylases) and kidney (1α-hydroxylase) into its active hormonal form, $1,25(OH)_2D_3$ (Mathieu *et al.*,2006).

Function of Vitamin D_3

Vitamin D_3 maintains the blood calcium level within a narrow range is vital for normal functioning of the nervous system, as well as for bone growth, and maintenance of bone density. Vitamin D is essential for the efficient utilization of calcium by the body (Holick; 2004). Without vitamin D, only 10 to 15% of dietary calcium and about 60% of phosphorus is absorbed (DeLuca *et al.,* 2004). The interaction of 1,25-dihydroxyvitamin D with the vitamin D receptor increases the efficiency of intestinal calcium absorption to 30 to 40% and phosphorus absorption to approximately 80% (Figure 3) (DeLuca *et al.,* 2004; Heaney *et al.,* 2003).

Bischoff-Ferrari (2006) has shown that serum levels of 25-hydroxyvitamin D were directly related to bone mineral density in white, black, and Mexican-American men and women, with a maximum density achieved when the 25-hydroxyvitamin D level reached 40 ng/ml or more (Boonen *et al.,* 2006; Lips *et al.,* 2001). When the level was found to be 30 ng/ml or less, there was a significant decrease in intestinal calcium absorption (Heaney *et al.,* 2003) that was associated with increased parathyroid hormone secretion (Thomas *et al.,* 1998). Parathyroid hormone enhances the tubular reabsorption of calcium and stimulates the kidneys to produce 1,25-dihydroxyvitamin D (Holick *et al.,* 2006; Dusso *et al.,* 2005). Parathyroid hormone also activates osteoblasts, which stimulate the transformation of preosteoclasts into mature osteoclasts (Figure 3) (Holick *et al.,* 2006). Osteoclasts dissolve the mineralized collagen matrix in bone, causing osteopenia, osteoporosis and rickets in children and osteomalacia in adults (Holick *et al.,* 2006) also increasing the risk of fracture (Chapuy *et al.,* 1997; Lips *et al.,* 2001). If vitamin D deficiency progresses the parathyroid glands are maximally stimulated and may cause secondary hyperparathyroidism (Malabanan *et al.,* 1998).

Vitamin D deficiency results in muscle weakness (Holick *et al.,* 2006; Bischoff-Ferrari *et al.,* 2006; Pettifor *et al.,* 2005). Skeletal muscles have a vitamin D receptor and may require vitamin D for maximum function (Holick *et al.,* 2006; Bischoff-Ferrari *et al.,* 2006). Performance speed and proximal muscle strength were markedly improved when 25- hydroxyvitamin D levels increased from 4 to 16 ng/ml (10 to 40 nmol/l) and continued to improve as the levels increased to more than 40 ng per milliliter (100 nmol/l) (Malabanan *et al.,* 1998).

Nonskeletal organ such as brain, prostate, breast, and colon tissues, among others, as well as immune cells have a vitamin D receptor and respond to 1,25-dihydroxyvitamin D_3 (Holick *et al.,* 2006; Dusso *et al.,* 2005). In addition, some of these tissues and cells express the enzyme 25-hydroxyvitamin D-1α-hydroxylase (Holick *et al.,* 2006; DeLuca *et al.,* 2004). Both prospective and retrospective epidemiologic studies indicate that the levels of 25-hydroxyvitamin D below 20 ng/ml are associated with a 30 to 50% increased risk of incident colon, prostate, and breast cancer, along with higher mortality from these cancers (Gorham *et al.,* 2005; Giovannucci *et al.,* 2006). An analysis from the Nurses' Health Study cohort (32,826 subjects) showed that the odds ratios for colorectal cancer were inversely associated with median serum levels of 25-hydroxyvitamin D (the OR at 16.2 ng/ml [40.4 nmol/l] was 1.0, and the odds ratio at 39.9 ng/ml [99.6 nmol/l] was 0.53; $p \leq 0.01$). Serum 1,25-dihydroxyvitamin D levels were not associated with colorectal cancer (Giovannucci *et al.,* 2006). 1,25-Dihydroxyvitamin D is also a potent immunomodulator (Penna *et al.,* 2005).

Vitamin D deficiency found to be associated with congestive heart failure (Zittermann *et al.,* 2006) and blood levels of inflammatory factors, including C-reactive protein and interleukin-10 (Zittermann *et al.,* 2003). Patients with hypertension who were exposed to ultraviolet B radiation three times a week for 3 months, 25-hydroxyvitamin D levels found to be increased by approximately 180%, and blood pressure became normal (both systolic and diastolic blood pressure reduced by 6 mm Hg) (Gorham *et al.,* 2005). The 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], has been shown to inhibit the development of autoimmune diseases, including inflammatory bowel disease (IBD) (Margherita *et al.,* 2004).

Low dietary vitamin D intake or vitamin D levels found to inversely related to glucose intolerance, insulin resistance, decreased insulin secretion and found the associations between VDR gene polymorphisms and T2DM have been sparse and yielded inconsistent (Yiqing *et al.,* 2010).

Vitamin D deficiency was linked to IGT and T2DM in humans for sometimes (Chiu *et al.,* 2004). These observations were confirmed in animal model, which demonstrated that pancreatic insulin is inhibited by vitamin D deficiency (Norman *et al.,* 1980). Several reports have ascribed an active role of vitamin D in the functional regulation of the endocrine pancreas, particularly the B cells. Not only are receptors for 1,25-

dihydroxyvitaminD_3 found in B cells (Lee *et al.,* 1994), but the effectors part of the vitamin D pathway is also present in the form of vitamin D dependent calcium-binding protein, also known as calbindin-D28K (Sooy *et al.,* 1999). The expression of calbindin D28K has been shown to protect B cells from cytokine mediated cell death (Rabinovitch *et al.,* 2001).

Vitamin D_3 has been suggested to play important role in modulation of immune process. Destruction of B cells in pancreatic islets in T1DM is highly selective. Presence of T1DM related autoantibodies strongly linked an involvement of auto immune mechanism. Several cellular effector mechanisms possibly leading to B cell destruction have been identified, including CD4+ and CD8+ T cells and macrophages (Benoist *et al.,* 1997). Epidemiological studies have shown an increase in the disease incidence when vitamin D deficiency was present in the first month of life in children. Moreover, in a recent study in NOD mice, vitamin D deficiency accelerated the onset of T1DM (Giulietti *et al.,* 2004). In fact, when 1,25-$(OH)_2D_3$ was administrated at 3 weeks of age before the onset of insulitis, it effectively prevented the progression of diabetes in NOD mice. However, treatment was ineffective if administrated at 8 wk of age when insulitis was well established (Mathieu *et al.,* 1994).

Supplementation of vitamin D during pregnancy reduces the development of islet autoantibodies in offspring (Chiu *et al.,* 2004). A cohort of children in Finland were given 2000 IU of vitamin D_3 per day during their first year of life and followed for 31 years. In this children found to reduce by approximately 80% (RR, 0.22; 95% CI, 0.05 to 0.89) (Hypponen *et al.,* 2001). Among children with vitamin D deficiency the risk was increased by approximately 200% (RR, 3.0; 95% CI, 1.0 to 9.0). Chiu et al (2004) has shown that vitamin D deficiency increased insulin resistance, decreased insulin production, and was associated with the metabolic syndrome. Another study showed that a combined daily intake of 1200 mg calcium and 800 IU of vitamin D lowered the risk of T2DM by 33% (relative risk, 0.67; 95% CI, 0.49 to 0.90) as compared with a daily intake of less than 600 mg calcium and less than 400 IU vitamin D (Pittas *et al.,* 2006). Vitamin D deficiency has been shown to impair insulin synthesis and secretion in humans and in animal models of diabetes, suggesting a role in the development of T2DM (Mathieu *et al.,* 2006).

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS) in which self-epitopes on myelinated nerve fibers is in appropriately recognized by adaptive immune cells of the host (Yoshiko *et al.,* 2002). The ensuing immune response recruits T cells and macrophages into the CNS, resulting in localized areas of inflammation and demyelination known as MS lesions. Self-antigen reactive T helper I (Th1) cells have been demonstrated to play an essential role in both induction and effective phase of disease. Th1 cell differentiation is controlled by both antigen stimulation and cytokines, particularly IL-12 and IL-23, which subsequently induce synthesis of Th1-specific transcription factor, T-bet, and drives Th0 cells toward Th1 differentiation (Szabo *et al.,* 2000). Therefore, controlling Th1 development by inhibiting IL-12 production will benefit the Th1-mediated disease, such as MS. VDR ligands have been shown to inhibit IL-12 p70 production in freshly isolated human monocytes that are primed with IFN-? and stimulated with lipopolysaccharide in a dose-dependent manner. Women who ingested more than 400 IU of vitamin D per day had a 42% reduced risk of developing multiple sclerosis (Munger *et al.,* 2004).

Rheumatoid arthritis one of the most common chronic inflammatory diseases, affects about 1% of the population and is characterized by articular infiltration of neutrophils, macrophages, T and B cells, and Dendritic Cells, resulting in subsequent tissue damage (Feldman *et al.,* 1996). Immunization of mice with type II collagen found to induces arthritis, which was shown to be prevented by dietary supplementation or oral administration of 1,25-$(OH)_2D_3$ in both mouse and rat (Tetlow *et al.,* 1999). Epidemiological studies have reported low serum levels of vitamin D and its metabolites in RA patients (Kroger *et al.,* 1993). Vitamin D3 has been detected in synovial fluid of arthritic joints, and the expression of VDR has also been reported in rheumatoid synovial tissue and at the site of cartilage erosion. Matrix metalloproteinases (MMPs) play an important role in the chondrolytic process of rheumatoid lesion (Gerstenfeld *et al.,* 1990). Animal studies have shown that the production of some MMPs may be up-regulated in rat chondrocytes by administration of 1,25-$(OH)_2D_3$ (Gerstenfeld *et al.,* 1990). Therefore, VDR ligand can suppress both the IL-1ß-stimulated production of MMP and PGE2 in rheumatoid synovial fibroblasts, suggesting that VDR-mediated biological processes are important in controlling RA (Tetlow *et al.,* 1999).

Vitamin D deficiency is characterized by inadequate mineralization or demineralization of the skeleton. It is also called hypovitaminosis D. Inadequate mineralization of the

skeleton causes of rickets in children while demineralization of the skeleton results in osteomalcia in adults. Due to vitamin D deficiency, the bones become weak and the legs begin to bow down due to the body's pressure exerted by the weight. As the vitamin D is in deficit, the bone tissues do not mineralize, which makes them soft and deformed (Hollis *et al.,* 2007). Today it is largely found in low-income counties in Africa, Asia or the Middle East and in those with genetic disorders such as pseudovitamin D deficiency rickets (Suda *et al.,* 1992). Osteoporosis is similar to rickets. However, this medical condition is seen as vitamin D deficiency symptoms in adults. It is a disease of the bone, wherein the bones become prone to fractures. Osteoporosis occurs due to lack of vitamin D metabolism leading to poor calcification in bones. Due to low vitamin D in the body, the bones become soft and brittle. Osteomalacia is a bone-thinning disorder that occurs exclusively in adults and is characterized by proximal muscle weakness and bone fragility. The effects of osteomalacia are thought to contribute to chronic musculoskeletal pain there is no persuasive evidence of lower vitamin D status in chronic pain sufferers (Fukumoto *et al.,* 2007).

Vitamin D deficiency has been linked to an increased incidence of schizophrenia and depression (McGrath *et al.,* 2002; Gloth *et al.,* 1999). Maintaining vitamin D sufficiency in utero and during early life, to satisfy the vitamin D receptor transcriptional activity in the brain, may be important for brain development as well as for maintenance of mental function later in life (Eyles *et al.,* 2005). Vitamin D deficiencies during pregnancy are at increased risk for wheezing illnesses (Camargo *et al.,* 2007).

Vitamin D intoxication is extremely rare but can be caused by inadvertent or intentional ingestion of excessively high doses. Doses of more than 50,000 IU per day raise levels of 25-hydroxyvitamin D to more than 150 ng/ml (374 nmol/l) and are associated with hypercalcemia and hyperphosphatemia (Holick *et al.,* 2006; Vieth *et al.,* 2004). Doses of 10,000 IU of vitamin D3 per day for up to 5 months, however, do not cause toxicity (Vieth *et al.,* 2004). Vitamin D toxicity (hypervitaminosis D) induces abnormally high serum calcium levels (hypercalcemia), which could result in bone loss, kidney stones, and calcification of organs like the heart and kidneys. The hypercalcemia associated with hypervitaminosis D may cause multiple debilitating effects. Anorexia, nausea and vomiting have been observed in hypercalcemic individuals treated with 1,250 to 5,000 micrograms (50,000 to 200,000 IU)/day of vitamin D. Patients with chronic granulomatous disorders are more sensitive to serum 25-hydroxyvitamin D levels above

30 ng/ml because of macrophage production of 1,25-dihydroxyvitamin D, which causes hypercalciuria and hypercalcemia (Holick *et al.*, 2006).

Vitamin D Receptor (VDR) gene variants

Vitamin D is mediated through a nuclear transcription factor known as the vitamin D receptor (VDR) (Schuster *et al.*, 2001). Upon entering the nucleus of a cell, 1,25-dihydroxyvitamin D associates with the VDR and promotes its association with the retinoic acid X receptor (RXR). In the presence of 1,25-dihydroxyvitamin D the VDR/RXR complex binds small sequences of DNA known as vitamin D response elements (VDREs) and initiates a cascade of molecular interactions that modulate the transcription of specific genes.

VDR protein consists of 427 amino acids, with a molecular mass of ~48 kDa. VDR can be divided by function into several domains (Figure 4). At the amino terminus there is an A/B domain 20 amino acids long. DNA-binding domain (DBD), termed also C domain, locates between amino acids 21 and 92. D or flexible linker region locates approximately between amino acids 93 and 123, followed by the E- or legend-binding domain between amino acids 124 and 427 (Jones *et al.*, 1998).

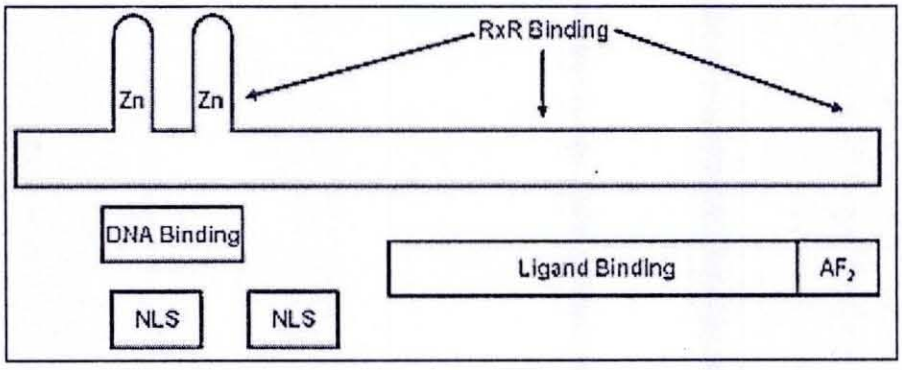

Figure 4: Model of the vitamin D receptor (VDR) (Daniel *et al.*, 2009).

The human VDR is a product of the single chromosomal gene that locates on chromosome 12; locus 12q13-14 (Labuda *et al.*, 1992). The gene is comprised of 11 exons that, together with intervening introns, an approximately 75 kb. The noncoding 5"-end of the gene includes 3 exons 1A, 1B, and 1C. Eight additional exons (exons 2-9) encode the structural portion of the VDR gene product. Three VDR mRNA transcripts are synthesized depending on how exons 1A, 1B, and 1C are spliced during transcription. The promoter sequence lying upstream of exon 1A is GC rich and does not

contain an apparent TATA box. A unique feature for the VDR gene is an additional exon (V) that is not found in other nuclear hormone receptor genes. It codes an insertion peptide about 40 amino acids long that locates in the LBD of receptor (Miyamoto *et al.*, 1997). Four common allelic variants of the VDR gene have been identified and they are genetic risk for T1DM. FokI, BsmI, ApaI and TaqI restriction site polymorphisms occur in exon 2, the intron between exons 8 and 9 (BsmI and ApaI) and exon 9 respectively (Deluca *et al.*, 2004). Vitamin D and its receptor complex as a transcription factor play a regulatory role in B cell insulin secretion (Lee *et al.*, 1994). The VDR was found to express in pancreatic B cells (Johnson *et al.*, 1994).

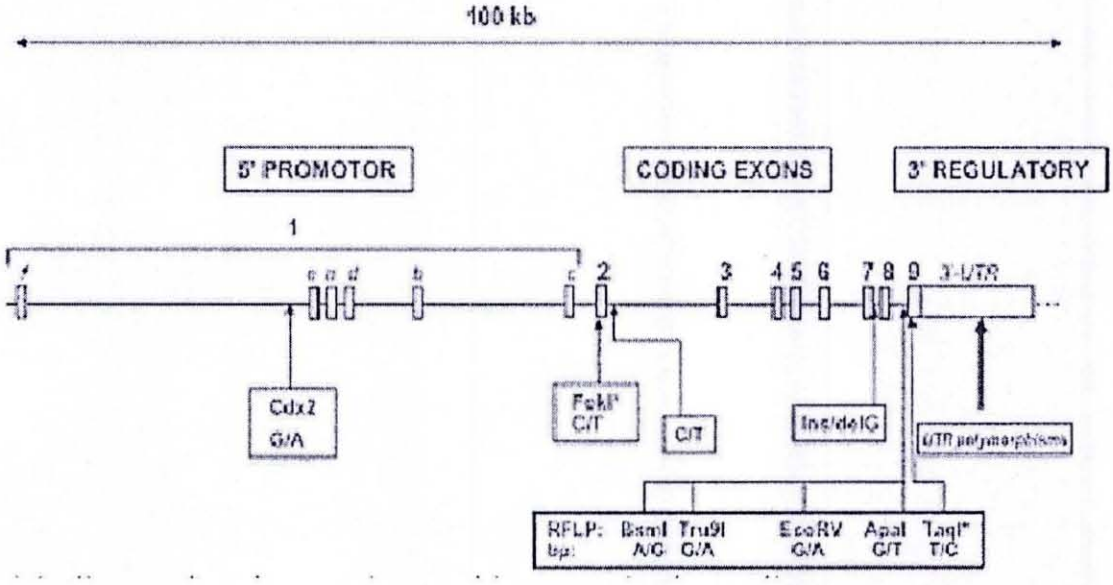

Figure 5: Exon–intron structure of the VDR gene (on chromosome 12q13.1) and position of known polymorphisms (Uitterlinden *et al.*, 2004).

Diseases associated with VDR gene variants

Variants of the nuclear vitamin D receptor (VDR) gene were found to be associated with T1DM and thyroid autoimmunity disease. Pani et al (2002) have shown that the VDR genotype is associated with Addison's disease. They have found that the 'ff' (13.7% vs 5.5%; p=0.0243; OR = 2.75) and the 'tt' (28.4% vs 14.1%; p=0.0043; OR = 2.42) genotypes were significantly more frequent in patients (n=95) than in controls (n=220). Haiyang et al (2009) have shown that ApaI, BsmI and FokI polymorphisms in the VDR gene were associated with susceptibility to Graves ' disease in Asian populations, while ApaI, BsmI, TaqI and FokI polymorphisms were not associated with the condition in Caucasian populations. BsmI polymorphism in the VDR gene was found to be associated

with Parkinson's disease in Koreans (Kim *et al.,* 2005). Two VDR polymorphisms (TaqI and FokI RFLPs) in determining Prostate cancer (PRCa) risk in patients with benign prostatic hypertrophy (BPH) in Scottish population.

Polymorphism of the vitamin D receptor (VDR) gene, which was considered to regulate bone metabolism, aroused considerable interest as a risk factor for osteoporosis (Tobin *et al.,* 2004). Bennett et al (1996) have reported that the VDR gene polymorphism of related to the occurrence of tuberculosis and infection of chronic hepatitis B virus. This may be interpreted to indicate a close relationship between VDR gene polymorphism and the immunological action, because vitamin D activates monocytes, stimulates cell-mediated immunity, and suppresses lymphocyte proliferation. VDR gene polymorphism (TT genotype) found to be a risk factor for chronic periodontitis (CP), independently of smoking and diabetes (Yoichi *et al.,* 2003). A case control study was performed on a group of 168 unrelated Japanese subjects ages ranged from 35 to 65 years. The Taq I polymorphism in the VDR gene was found to be significantly associated with CP (X^2 = 4.48, p = 0.034). They performed multiple logistic regression analyses on the TT genotype, which was found to be associated with CP, and on well-recognized risk factors, smoking and diabetes.

Vitamin D deficiency suggested enhancing the prevalence of T2DM and the replacement of vitamin D may increase the secretion of insulin (Lee *et al.,* 1994). This impairment is primarily caused by the direct effect of vitamin D deficiency on the beta cell, but other effects of vitamin D deficiency, such as impaired food intake and hypocalcaemia, might also play a role. Data from VDR knockout mice were however, conflicting, with some groups reporting IGT (Zeitz *et al.,* 2003) and others reporting no impairment in glucose metabolism (Mathieu *et al.,* 2001). The findings, however, suggested that genetic background of VDR knockout strain might be of critical importance.

Vitamin D receptor (VDR) polymorphism found to be related to T1DM (Pani *et al.,* 2000; Chang *et al.,* 2000). In the nonobese diabetic mouse model for insulin-dependent diabetes mellitus, vitamin D is necessary for normal insulin release and maintenance of glucose tolerance (Mathieu *et al.,* 1994). Recently, described the association between allelic variants of the VDR gene and impaired insulin secretion in T1DM and T2DM (McDermott *et al.,* 1997; Pani *et al.,* 2000; Chang *et al.,* 2000). BsmI polymorphism was shown to be associated with T1DM in South Indians (McDermott *et al.*, 1997) and

combinations of BsmI/ApaI/TaqI found to influence susceptibility to T1DM among Germans (Pani *et al.,* 2000). In a Taiwanese population, the AA genotype of ApaI polymorphism was found to be associated with T1DM (Chang *et al.,* 2000). The aa genotype, however, was found to be associated with defective insulin secretion in Bangladeshi Asians, a population at increased risk for T2DM (Gyapay 1994). In 93 Southern Indian families with T1DM, transmission disequilibrium testing analysis demonstrated the preferential transmission of the "b" allele of the Bsm1 polymorphism to affected subjects (Pani *et al.,* 1997). However, it was less certain that for the Taq1 and Apa1 polymorphism in the sample set. This study indicates that the Bsm1 polymorphism at the VDR gene locus is a risk factor of T1DM. In a study of 152 Caucasian families with T1DM, there was an excessive transmission of Taq1 and Apa1 alleles alone (Pani *et al.,* 2000). Vitamin D receptor allele combinations influence genetic susceptibility to T1D in Germans. Recently it was confirmed the association of this marker of Taiwanese population (Chang *et al.*, 2000). These two studies in two different populations indicate that Bsm1 polymorphism at the VDR gene locus confers susceptibility to T1DM. Studies have demonstrated a link between VDR gene polymorphisms and T2DM, although the findings differ from one population to another. A study in Bangladeshi Asians demonstrated that the ApaI RFLP influences insulin secretory capacity of the B cells in response to glucose (Hitman *et al.,* 1998) while associations between the VDR ApaI RFLP and higher fasting plasma glucose levels and glucose intolerance were observed in a community-based study of older adults without known diabetes (Oh *et al.,* 2002). Ortlepp *et al.,* (2003) shown that the genotyping for TaqI, ApaI, BsmI and FokI RFLPs revealed that the BsmI RFLP is associated with high fasting glucose levels in young males with low physical activity.

CHAPTER 3

SUBJECTS AND METHODS

SUBJECTS AND METHODS

Place of the study

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

Study period

This study was done during the period of June 2009 to February 2010.

Study design

This was a case control study. Variant allele was taken as exposure factor.

Subjects

Ninety four (94) healthy subjects under 30 years of age search as control and 92 (ninety two) subjects consecutively attending the out-patient department of BIRDEM, the central institute of Bangladesh Diabetic Association (BADAS) were recruited. The subjects included different races, religions and socioeconomic status. Diabetes was diagnosed following WHO criteria (WHO1999).

Detail medical and clinical history was recorded in a predesigned case record form (Appendix I).

Inclusion Criteria

- Age of the subject's ≤30 years.
- Duration of diabetes < 3 months.

Exclusion Criteria

- Subjects with co-morbid diseases (infection, stroke, myocardial infarction, major surgery, essential hypertension, malabsorption etc.) were excluded.
- Subjects with history of medication which may significantly affect glucose metabolism (glucocorticoids, oral contraceptives containing; levonorgestral or high-dose estrogen, phenytoin, high-dose thiazide diuretics etc.) were excluded.
- Pregnant women were excluded.

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 0.1 kg.

Calculation of BMI (kg/m^2).

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

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

Waist circumference (cm)

Waist circumference was measured to the nearest 1m 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.

MAC (mm)

Mid Upper Arm Circumference (MAC) 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 bellow the inferior angle of right scapula with skin fold lying at 45 degree. Values were recorded at the nearest millimeter.

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 (WHO-IHS).

METHODS

Collection of blood samples

Overnight fasting (8-10 hours) blood was collected between 8.00-9.00 am. Venous blood (10 ml) was obtained by venepuncture following standard procedure. A portion of blood (5 ml) sample was taken into a tube containing EDTA (1 mg/ml), mixed thoroughly and preserved at -30°C for future DNA extraction. The other portion of blood sample was taken into plain tube and allowed to clot for 30 minutes and serum was separated by centrifugation for 10 min at 3000 rpm using refrigerated centrifuge and preserved at -30°C for further biochemical analyses.

Biochemical methods

Estimation of Glucose

Serum glucose was estimated by enzymatic colorimetric (GOD-PAP) method in the Hitachi 704 Automatic Analyzer, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

Principle

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

$$\text{Glucose} + H_2O \xrightarrow[\text{Oxidase}]{\text{Glucose}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

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%

Procedure

The method determines glucose without deproteinization. The instrument was calibrated before estimation. Serum and reagents were taken in specific cup. These were arranged serially into the Auto analyzer. 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.

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

Estimation of 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

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

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholestene- 3-one} + H_2O_2$$

$$2H_2O_2 + \text{phenol} + \text{4-aminoantiphyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + H_2O$$

Reagents

Contents	Initial Concentration of Solution
4-Aminoantipyrine	0.30 mmol/l
Phenol	6 mmol/l
Peroxidase	$\geq$ 0.5 U/ml
Cholesterol esterase	$\geq$ 0.15 U/ml
Cholesterol oxides	$\geq$ 0.1 U/ml
Pipes Buffer	80 mmol/l; pH 6.8
Standard	5.17 mmol/l (200 mg/dl)

Procedure

Serum and reagents were taken in specific cup or cell. These were arranged serially. Then ID number for each test was entered in the Auto analyzer. Five microlitter sample and 500 µl reagent were mixed and incubated at 37^0C 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.

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

Estimation of Triglyceride

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in the Hitachi 704 Automatic Analyzer, 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).

$$\text{Triglyceride} + H_2O \xrightarrow{Lipase} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[Kinase]{Glycerol} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow[Oxidase]{Glycerol\ Phosphate} \text{Dihydroxy acetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4- chlorophenol} \xrightarrow{Peroxidase} \text{Quinoneimine} + HCl + 4\,H_2O$$

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	0.5 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)

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 37^0C for 5 minutes within the cell. Reading was taken at 500 nm.

Calculation of result

Triglyceride concentration was calculated by following formula:

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

Estimation of high density lipoprotein (HDL) cholesterol

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

Principle

HDL (High Density Lipoproteins) is separated from chylomicrons, VLDL (very low density lipoproteins) and LDL (Low density lipoproteins) by precipitating reagent (phosphotungstic acid-magnesium chloride). 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).

Materials and reagents

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

Reagents composition:

Phosphotungstic Acid: 0.55 mmol/l

Magnesium Chloride: 25 mmol/l

Standard Preparation

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

Assay Procedure

1. 100 ml serum sample taken in microcentrifuge tube
2. Add 250 µl 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 37^0C on orbital microtiter plate shaker.
9. Absorbance was read at 490 nm.

Calculation

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

Estimation of LDL-cholesterol

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

Formula

$$\text{LDL cholesterol} = \{\text{Total cholesterol} - (\text{HDL Cholesterol} + \frac{1}{5} x \quad \text{Triglyceride})\}$$

Estimation of C-peptide

Serum C-peptide was estimated by an enzyme linked immunosorbent assay (ELISA) method using kit from DRG International Inc., Germany.

Principle

The DRG C-peptide ELISA kit is based on the competition principle and microplate separation. An unknown amount of C-peptide present in the sample and fixed amount of C-peptide conjugate compete for the binding sites of a polyclonal C-peptide antiserum coated onto the wells. In a second step an enzyme complex binds to C-peptide conjugate. The unbound Enzyme complex is washed off. Having added the substrate solution, the concentration of C-peptide in the samples is inversely proportional to the optical density measured.

Reagents

1. Microtiter wells coated with C-peptide antiserum
2. C-peptide conjugates in stabilizing buffer solution
3. Enzymes complex containing Horseradish peroxidase
4. References standard set, lyophilized for 1.0 ml, 5 vials (0.2-16 ng/ml)
5. Zero standard or Specimen diluent (3 ml)
6. Substrate solution – TMB (22 ml)
7. Stop solution (0.5 M H_2SO_4)
8. Wash solution (10X concentrated)

Material Required

- A microtitre plate reader (450nm)
- Micropipettes with disposable tips for 100, 200 and 1000 µl
- Absorbent paper
- Deionized water

Procedure

1. Desired numbers of coated microtitre wells were placed in the holder.
2. 100 µl of C-peptide standards were dispensed into wells.
3. 100 µl of samples was dispensed into selected wells.
4. The wells were incubated for 5 minutes at room temperature.

5. 200 µl of C-peptide conjugate was dispensed into each well.
6. The plate was incubated for 3 hours at room temperature.
7. The contents of the well were shacked out briskly.
8. The wells were rinsed 3 times with diluted wash solution (400 µl per well). The wells were striked sharply on absorbance paper to removed residual droplets.
9. 200 µl of enzyme complex was dispensed into each well.
10. The plate was incubated for 60 minutes at room temperature without agitation.
11. The contents of the wells were shacked out briskly. The wells were rinsed 5 times with dilute wash solution (400 µl per well). Then the wells were stroked sharply on absorbance paper to remove residual droplets.
12. 200 µl of substrate solution was added to each well.
13. The plate was incubated for 30 minutes.
14. 100 µl of stop solution was added to each well and
15. Absorbance of each well was determined at 450 nm by using a microwell plate reader (Bio-Tek, EL-340, USA).

Determination of insulin secretory capacity and insulin sensitivity

Homeostasis Model Assessment (HOMA%S) 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.

DNA Method

Extraction of DNA was performed using GenElute DNA extraction kit (QIAGEN, USA). The kit uses the principal of silica gel DNA isolation from whole blood adapted in spin column.

Equipment, reagent and accessories

- Water bath
- Vortex
- Centrifuge
- Microcentrifuge tubes (1.5 ml)
- Pipette tips at different capacities (10 µl, 20 µl, 100 µl, 200 µl and 1000 µl)
- Micropipettes (4-50 µl, 100-1000 µl)
- Absolute ethanol (95-100 %)

Content of DNA kit

- Extraction column
- Collection tubes
- Proteinase K
- RNase-A solution
- Lyses Buffer AL
- Wash Buffer AW1 and AW2
- Elution Buffer AE

Preparation of reagents

Wash buffer AW1: 125 ml ethanol (96-100%) was added to obtain 125 ml Buffer AW1 (95 ml concentrated).

Wash buffer AW2: 160 ml ethanol (96-100%) was added to obtain 226 ml Buffer AW2 (66 ml concentrated).

Extraction procedure

1. Frozen blood was brought to room temperature and made homogenous by brief vortexing. Aliquot of blood (200 µl) was transferred into 1.5 ml microcentrifuge tube.
2. 20 µl QIAGEN proteinase K was added inside the cap of the microcentrifuge tube.
3. 4 µl of an RNase-A stock solution (100mg/ml) was added to the sample before adding of Buffer AL.
4. 200 µl Buffer AL was added to the sample. In order to ensure efficient lyses, pulse-vortexed for 15 sec to make a homogenous solution.
5. Incubated at 56°C for 10 min.
6. To remove drops from the inside of the lid at the tube briefly centrifuged.
7. 200 µl ethanol (96-100%) was added to the sample, and mixed by pulse-vortexing for 15 sec and briefly spanned.
8. The mixture from the step 7 was transferred to the QIAamp Mini Spin Column (mounted on 2 ml collection tube) without wetting the rim and after closing the cap, centrifuged at 6000 x g for 1 min. The QIAamp Mini Spin column was placed on a fresh 2 ml collection tube.
9. The QIAamp Mini Spin column was opened carefully and 500 µl Buffer AW1 was added without wetting the rim. Centrifuged at 6000 x g for 1 min. the collection tube was discarded and the spin column was placed on fresh collection tube.
10. The QIAamp Mini Spin column was opened carefully and 500 µl Buffer AW2 was added without wetting the rim. Cap was screwed carefully and the centrifuged at 20000 x g for 3 minutes. At this stage the column appeared to be clean.
11. The QIAamp Mini spin column placed again on a 2 ml collection tube. 200 µl Buffer AE was added to the column. Incubated at room temperature for 5 minutes. Centrifuged at 6000 x g for 1 minute at 25°C. This elute supposed to contain DNA.

Check for DNA extraction

DNA yield for each sample was checked by agarose gel (1%) electrophoresis. To prepare agarose gel, appropriate amount of agarose was taken into polypropylene conical flasks containing required volume of working tris-borate EDTA (TBE) buffer. Agarose in working TBE buffer was mixed by swirling of the flasks. It was then boiled using a microwave oven at medium temperature. The gel was cooled nearer to the gelling point and ethidium bromide (0.5μg/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.

To resolve DNA extract 3 μl of DNA elute mixed with appropriate amount of loading buffer and then the mixer was loaded in agarose gel prepared earlier. The gel was run for at medium voltage for required time. DNA presence was visualized under UV light and gel image was captured.

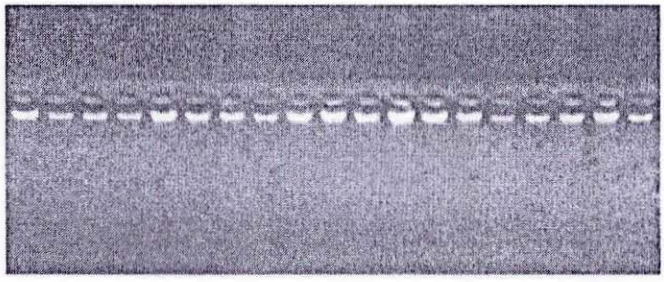

Figure 6: Gel image of DNA electrophoresis to check the extraction yield

Vitamin D receptor (VDR) gene polymorphic markers analyses

Vitamin D receptor (VDR) gene polymorphic markers (G>T and T>C) were analyzed by PCR and RFLP.

The DNA segment containing G>T (Apa1 restriction) and T>C (Taq1 restriction) polymorphic marker was amplified using the following primer set:

Forward primer: 5′-CAG AGC ATG GAC AGG GAG CAA G-3′

Reverse primer: 5′-GCA ACT CCT CAT GGC TGA GGT CTC A-3′

PCR was carried out in 15 µl reaction volume. Product size for the above mentioned primer set is 740 bp. The PCR protocol is as follows:

Name of the component	Volume (µl)	Concentration
DNA	4.00 µl	10-50 ng/ml
Buffer	1.50 µl	10x
dNTPs	0.12 µl	200 µmol
Forward Primer	0.70 µl	10 µmol
Reverse Primer	0.70 µl	10 µmol
HotStart Taq DNA polymerase	0.105 µl	5 U/µl
ddH_2O	7.875 µl	
Total	**15.0 µl**	

PCR condition

Conditions for the amplification of the above mention product include initial step of denaturation 94°C for 15 min followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 45 seconds and elongation at 72°C for 45 seconds and a 35^{th} cycle was followed by a step of final elongation 72°C for 10 minutes.

Evaluation of PCR

3 µl of PCR product was checked for amplification in a 1.5 % agarose gel. The optimum size of the product was ascertained comparing it with 100 bp DNA ladder. The amplified DNA was visualized using under UV light and gel image captured and documented.

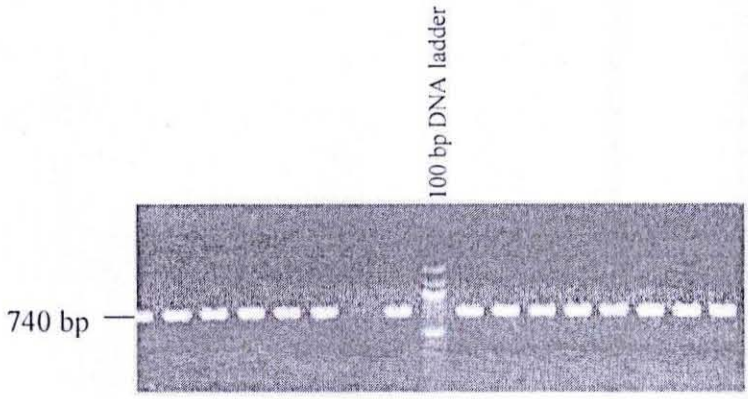

Figure 7: Gel image of VDR PCR for G>T and T>C containing DNA fragment in agarose gel

RFLP analysis of VDR gene candidate markers

G>T polymorphism restricts Apa1 site. Hence the polymorphism was determined by Apa1 restriction endonuclease digestion. The digestion was carried out in a reaction volume of 15µl. The enzyme digestion protocol is as follows:

Apa1 restriction enzyme digestion protocol:

Name of the component	**Volume (µl)**
PCR product	4.000 µl
Buffer	1.500 µl
Restriction enzyme	0.160 µl
BSA	0.200 µl
Spermidine	0.187 µl
H_2O	8.952 µl
Total reaction volume	**15.00 µl**

Apa1 restriction enzyme digestion was carried at 25°C for 2 hours in water bath. Enzyme digestion product was resolved in 3% agarose gel and digested product was visualized using gel documentation system following ethidium bromide straining.

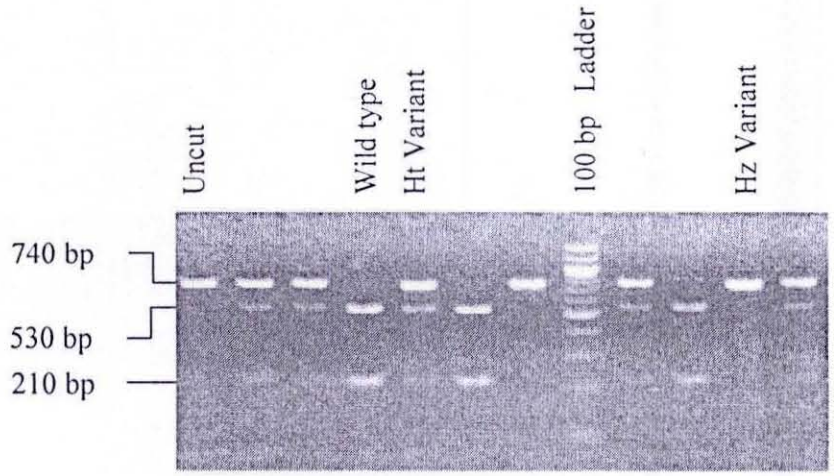

Figure 8: Agarose gel image of VDR gene Apa1 G>T candidate marker analysis by Apa1 restriction enzyme digestion

T>C polymorphism creates a restriction site for Taq1 endonuclease. Hence the polymorphism was determined by Taq1 restriction enzyme digestion. Restriction enzyme digestion was performed using standard digestion protocol. The digestion was carried out in a reaction volume of 15µl. The enzyme digestion protocol is as follows:

Taq1 restriction enzyme digestion protocol:

Name of the component	**Volume (µl)**
PCR product	4.000 µl
Buffer	1.500 µl
Restriction enzyme	0.250 µl
BSA	0.200µl
Spermidine	0.187 µl
H_2O	8.862 µl
Total reaction volume	**15.00 µl**

Taq1 restriction enzyme digestion was carried at 65°C for 2 hours in water bath. Enzyme digestion product was resolved in 3% agarose gel and digested product was visualized using gel documentation system following ethidium bromide straining.

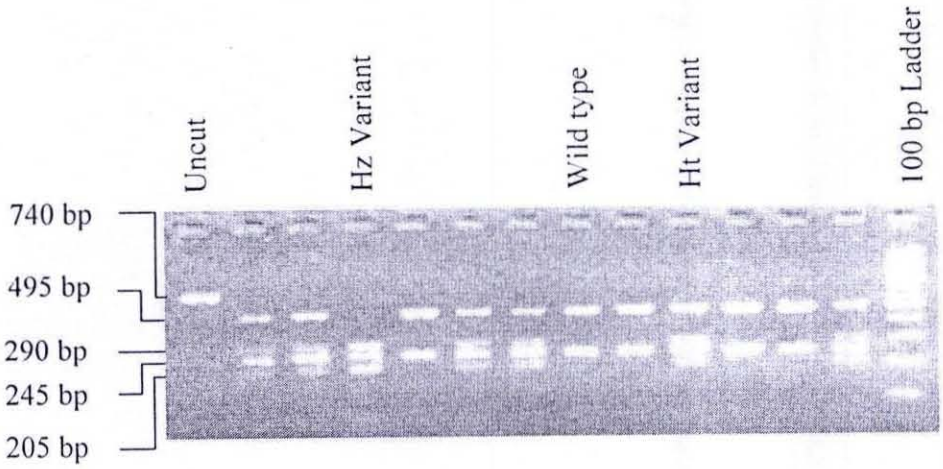

Figure 9: Agarose gel image of VDR gene Taq1 T>C candidate marker analysis by Taq1 restriction enzyme digestion.

Statistical Methods

Data were expressed as mean (±SD) and number (percentage) as appropriate. Difference between two groups was determined by unpaired Student's 't' test, Chi-square test where applicable. Data were managed using statistical package for social science (SPSS) for Windows Version 10.

CHAPTER 4

RESULTS

RESULTS

A total number of 186 unrelated subjects were included in the study. Of them 94 were healthy controls and 92 were young diabetes mellitus (YDM). In control group male and female distribution was 55 (58.5%) and 39 (41.5%) respectively. In YDM group the distribution was 37 (40.2%) and 55 (59.8%) respectively.

Age, BMI and blood pressure of the study subjects

Mean (±SD) age (yrs) of the control and YDM subjects was 23±4 and 19±6 respectively. Mean of YDM subjects was statistically significant lower ($p<0.001$) compared to the control (Table 3).

Mean (±SD) body mass index (BMI) of the control and YDM subjects was 20.4±3.7 and 18.2±5.1 respectively. Mean BMI was statistically significantly lower ($p=0.001$) in the YDM group compared to the controls (Table 3).

Mean (±SD) systolic blood pressure (SBP, mmHg) of the control and YDM subjects was 112.4±7.4 and 103.7±12.4 respectively. Mean SBP was statistically significantly lower ($p<0.001$) in the YDM compared to the controls (Table 3).

Mean (±SD) diastolic blood pressure (DBP, mmHg) of the control and YDM subjects was 71.6±5.9 and 68.8±9.1 respectively. Mean DBP in this two groups did not show statistical significant difference ($p=0.16$) (Table 3).

Anthropometric measurements of the study subjects

Mean (±SD) mid arm circumference (MAC, cm) of the control and YDM subjects was 24.4±3.3 and 21.3±5.7 respectively. Mean MAC was statistically significantly lower ($p<0.001$) in the YDM group compared to the controls (Table 4).

Mean (±SD) triceps skin fold (TSF, mm) of the control and YDM subjects was 11.1±5.9 and 11.5±8.2 respectively. Mean TSF in the two groups did not show significant difference ($p<0.716$) (Table 4).

Mean (±SD) subscapular skin fold thickness (SSF, mm) of the control and YDM subjects was 17.2±7.9 and 14.3±10.5 respectively. Mean SSF was statistically significantly lower ($p=0.036$) in the YDM group compared to the controls (Table 4).

Serum glucose levels and insulinemic status of the study subjects

Mean (±SD) fasting serum glucose (FSG, mmol/l) of the control and YDM subjects was 4.9±0.5 and 15.5±5.2 respectively. (Table 5).

Mean (±SD) C-peptide values (ng/ml) in the control and YDM subjects was 1.6±0.6 and 1.2±0.9 respectively. Mean C-peptide value was statistically significantly lower ($p<0.001$) in the YDM group compared to the controls (Table 5).

Mean (±SD) of HOMA%S in the control and YDM subjects was 94.1±39.8 and 102.6±83.3 respectively. Mean HOMA%S values was statistically significantly lower ($p<0.001$) in the YDM group compared to the controls (Table 5).

Mean (±SD) HOMA% B values in the control and YDM subjects was 112.6±38.5 and 19.4±21.3 respectively. Mean HOMA%B value was statistically significantly lower ($p=0.003$) in the YDM group compared to the controls (Table 5).

Mean (±SD) HOMAIR values in the control and YDM subjects was 0.36±0.15 and 0.74±0.54 respectively. Mean HOMAIR value was statistically significantly higher ($p<0.001$) in the YDM group compared to the controls (Table 5).

Lipid levels of study subjects

Mean (±SD) serum triglycerides (TG, mg/dl) of the control and YDM subjects was 109±48 and 128±57 respectively. Mean TG was statistically significantly higher ($p<0.019$) in the YDM group compared to the controls (Table 6).

Mean (±SD) serum total cholesterol (mg/dl) of the control and YDM subjects was 147±32 and 148±39 respectively. Mean total cholesterol in the two genotypes did not show statistical significant difference ($p<0.962$) (Table 6).

Mean (±SD) high density lipoprotein cholesterol (HDL-c, mg/dl) of the control and YDM subjects was 37±8 and 32±10 respectively. Mean HDL-c was statistically significantly lower ($p<0.001$) in the YDM group compared to the controls (Table 6).

Mean (±SD) low density lipoprotein cholesterol (LDL-c, mg/dl) of the control and YDM subjects were 89±32 and 91±36 respectively. Mean LDL-c in the groups did not show statistical significant difference ($p=0.725$) (Table 6).

Table 3: Age, BMI and blood pressure of the study subjects

Variables	Control (n=94)	YDM (n=92)	*t/p values*
Age (yrs)	23±4	19±6	4.780/<0.001
BMI (kg/m^2)	20.4±3.7	18.2±5.1	3.355/0.001
SBP (mmHg)	112.4±7.4	103.7±12.4	5.775/<0.001
DBP (mmHg)	71.6±5.9	68.8±9.1	2.433/0.16

Results were expressed as mean±SD.

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

BMI, Body Mass Index; SBP, Systolic Blood Pressure DBP, Diastolic Blood Pressure; YDM, Young Diabetes Mellitus.

Table 4: Anthropometric measurements of the study subjects

Variables	Control (n=94)	YDM (n=92)	*t/p values*
MAC (cm)	24.4±3.3	21.3±5.7	4.518/<0.001
TSF (mm)	11.1±5.9	11.5±8.2	0.364/0.716
SSF (mm)	17.2±7.9	14.3±10.5	2.117/0.036

Results were expressed as mean±SD.

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

MAC, Mid Arm Circumferences; TSF, Triceps Skin Fold; SSF, Sub Scapular Skin fold; YDM, Young Diabetes Mellitus

Table 5: Glycemic and Insulinemic status of the study subjects

Variables	Control (n=94)	YDM (n=92)	*t/p values*
FSG (mmol/l)	4.9±0.5	15.4±5.2	19.040/<0.001
C-peptide (ng/ml)	1.6 ±0.6	1.2±0.9	3.297/<0.001
HOMA % S	94.1±39.8	102.6±83.3	6.541/<0.001
HOMA % B	112.6 ±38.5	19.4±21.3	3.102/0.003
HOMA IR	0.36±0.15	0.74±0.54	6.364<0.001

Results were expressed as mean±SD.

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

FSG, Fasting Serum Glucose; HOMA%S, Insulin sensitivity assessed by Homeostatic Model Assessment; HOMA%B, B-cell function assessed by Homeostatic Model Assessment; YDM, Young Diabetes Mellitus.

Table 6: Lipid levels of the study subjects

Variables	Control (n=94)	YDM (n=92)	*t/p values*
TG (mg/dl)	109±48	128±57	2.365/0.019
T Chol (mg/dl)	147±32	148±39	0.048/0.962
HDL-c (mg/dl)	37±8	32±10	3.810/<0.001
LDL-c (mg/dl)	89±32	91±36	0.353/0.725

Results were expressed as mean±SD.

Statistical 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, YDM, Young Diabetes Mellitus.

CANDIDATE GENE MARKER ANALYSES

VDR gene G>T (Apa1 restriction) variant

Genotype frequencies of the VDR gene G>T variants in the control group were 0.183, 0.516 and 0.301 for wild type, heterozygous (Ht) variant and homozygous (Hz) variant respectively. In the YDM group, the frequencies were 0.198, 0.484 and 0.319 respectively. The genotype frequency in the two groups did not show statistical significant association (χ^2=0.198; p=0.906) (Table 8i). Hardy-Weinberg distribution of the genotype frequency in the Control (χ2=0.508; p=0.476) and in the YDM group (χ2=0.032, p=0.859). For all the study subjects the distribution was (χ2=0.387; p=0.844). Hardy-Weinberg distributor found to be in compliance for the marker.

When heterozygous and homozygous variant genotypes were grouped together, the distribution was as follows: Control - 0.183 and 0.817 for wild and variant genotype respectively; YDM - 0.198 and 0.802 respectively which did not show significant association (χ^2=0.067; p=0.795) between the two groups (Table 8ii). Odd-Ratio for the variant genotype was to the condition 0.907 (range 0.434-1.895), which did not show any significant risk association (p=0.852).

Table 7i: VDR gene Apa1 G>T genotype of the study subjects

VDR Apa1 G>T genotype	Control (n= 94)	YDM (n= 92)	Total (n= 186)
Wild (GG)	0.183 (17)	0.198 (18)	0.190 (35)
Ht variant (GT)	0.516 (48)	0.484 (44)	0.500 (92)
Hz variant (TT)	0.301 (25)	0.319 (29)	0.310 (57)
	χ^2= 0.198	*p=0.906*	

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical association.

Hz Wild, homozygous wild; Ht variant, Heterozygous variant; Hz variant, Homozygous variant; VDR, Vitamin D receptor; YDM, Young Diabetes Mellitus.

Table 7ii: VDR gene Apa1 G>T genotype (Ht and Hz variant together) of the study subjects

VDR Apa1 G>T genotype	Control (n=94)	YDM (n= 92)	Total (n= 186)
Wild GG)	0.183 (17)	0.198 (18)	0.190 (35)
Variant (GT and TT)	0.817 (76)	0.802 (73)	0.810 (149)
	$\chi^2 = 0.067$	$p = 0.795$	

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical association.

VDR gene T>C (Taq1 restriction) variant

Genotype frequencies of the VDR gene T>C variants were 0.370, 0.522 and 0.109 for wild type, heterozygous (Ht) variant and homozygous (Hz) variant respectively. In the YDM group, the frequencies were 0.484, 0.418 and 0.09 respectively. The genotype frequency distribution did not show statistical significant association (χ^2=2.492; p=0.288) (Table 9i). Hardy-Weinberg distribution of the genotype frequency in the Control was (χ2=1.318; p=0.0.251) and in the YDM group (χ2=0.034, p=0.850). For all the study subjects the Hardy-Weinberg distribution was the found to be in equilibrium (χ2=0.661; p=0.416). The T>C genotype frequency found to be in compliance with Hardy-Weinberg equilibrium.

When heterozygous and homozygous variant genotypes were grouped together, the distribution was as follows: Control - 0.37 and 0.63 for wild and variant genotype respectively; YDM - 0.484 and 0.516 respectively which also did not show significant association (χ^2=2.429; p=0.119) between the two groups (Table 10). Odd-Ratio for the variant genotype to the condition was 0.626 (range 0.347-1.230), which did not show any significant risk association (p=0.136).

Table 8i: VDR gene Taq1 T>C genotype of the study subjects

VDR Taq1 T>C genotype	Control (n= 94)	YDM (n= 92)	Total (n= 186)
Wild (TT)	0.370 (34)	0.484 (44)	0.426 (78)
Ht variant (TC)	0.522 (48)	0.418 (38)	0.470 (86)
Hz variant (CC)	0.109 (10)	0.090 (9)	0.104 (19)
	$\chi^2 = 2.492$	$p=0.288$	

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical association.

Hz Wild, Homozygous wild; Ht variant, Heterozygous variant; Hz variant, Homozygous variant; VDR, Vitamin D receptor; YDM, Young Diabetes Mellitus.

Table 8ii: VDR gene Taq1 T>C genotype (Ht and Hz variant together) of the study subjects

VDR Taq1 T>C genotype	Control (n= 94)	YDM (n= 92)	Total (n= 186)
Wild (TT)	0.370 (34)	0.484 (44)	0.426 (78)
Variant (TC and CC)	0.630 (58)	0.516 (47)	0.574 (105)
	$\chi^2 = 2.429$	$p=0.119$	

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical association.

Glycemic and insulinemic status of study subjects on the basis of Apa1 G>T genotype

Mean (±SD) fasting glucose levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control (5.1±0.6 and 4.9±0.5 respectively; p=0.227) and YDM group (16.2±4.5 and 15.3±5.5 respectively; p=0.463) (Table 9i).

Mean (±SD) C-peptide levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (1.6±0.7 and 1.6±0.6 respectively; p=0.829) and YDM group (0.99±0.83 and 1.28±0.95 respectively; p=0.243) (Table 9i).

Mean (±SD) HOMA%B in subjects with wild and variant (heterozygous and homozygous together) genotype was in the control was (101.0±31.6 and 115.0±39.8 respectively; p=0.146) and in YDM group (11.6±11.6 and 20.9±22.7 respectively) (Table 9i). HOMA%B in the subjects with variant allele was significantly higher (p=0.019) compared to these with wild allele.

Mean (±SD) HOMA%S in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (95.0±30.9 and 94.1±41.8 respectively; p=0.929) as well as in YDM group (106.5±46.2 and 102.4±90.7 respectively; p=0.855) (Table 9i).

Mean (±SD) insulin resistance in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (0.3±0.1 and 0.3±0.1 respectively; p=0.910) and YDM group (0.6±0.6 and 0.7±0.4 respectively; p=0.720) (Table 9i).

Mean (±SD) BMI levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (20.5±2.4 and 20.4±4.1 respectively; p=0.087) and in YDM group (16.7±4.1 and 18.6±5.3 respectively; p=0.108) (Table 9ii).

Mean (±SD) SBP levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (112.9±6.1 and 112.4±7.7 respectively; p=0.788) and YDM group (98.1±11.0 and 104.1±12.4 respectively; p=0.035) (Table 9ii).

Mean (±SD) DBP levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (71.7±5.2 and 71.5±6.1 respectively; p=0.886) and YDM group (64.7±7.7 and 69.7±9.1 respectively; p=0.035) (Table 9ii).

Mean (±SD) triglyceride levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (107.1±36.8 and 110.1±51.1

respectively; p=0.840) and YDM group (106.9±40.2 and 132.1±60.1 respectively; p=0.097) (Table 9ii).

Mean (±SD) cholesterol levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (162.1±39.2 and 144.1±30.5 respectively; p=0.039) and YDM group (134.1±28.7 and 150.7±40.7 respectively; p=0.107) (Table 9ii).

Mean (±SD) LDL levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (102.7±37.7 and 85.6±30.3 respectively; p=0.048) and YDM group (81.2±21.7 and 92.7±38.8 respectively; p=0.102) (Table 9ii).

Table 9i: Glycemic and insulinemic status of the study subjects on the basis of Apa1 G>T genotype

	FSG (mmol/l)	C-peptide (ng/ml)	HOMA%B	HOMA%S	HOMA-IR
Control subjects					
Wild (n=17)	5.1±0.6	1.6±0.7	101.0±31.6	95.0±30.9	0.3± 0.1
Variant (n=76)	4.9±0.5	1.6± 0.6	115.0±39.8	94.1±41.8	0.3±0.1
t/p values	*1.243/0.227*	*0.219/0.829*	*1.496/0.146*	*0.090/0.929*	*0.114/0.910*
YDM subjects					
Wild (n=30)	16.2± 4.5	0.9± 0.8	11.6±11.6	106.5±46.2	0.6±0.6
Variant (n=71)	15.3± 5.5	1.2±.9	20.9±22.7	102.4±90.7	0.7±0.4
t/p values	*0.637/0.463*	*1.177/0.243*	*2.424/0.019*	*0.183/0.855*	*0.359/0.720*

Results were expressed as mean±SD.

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

Variant, heterozygous and homozygous variant together; FSG, fasting serum glucose; PPG, postprandial glucose; F insulin, Fasting Insulin; HOMA %B, Homeostasis model assessment B-cell Function; HOMA %S, Homeostasis model assessment insulin sensitivity; YDM Young Diabetes Mellitus.

Table 9ii: Glycemic and insulinemic status of the study subjects on the basis of Apa1 G>T genotype

	BMI	SBP	DBP	TG	Cholesterol	LDL
Control subjects						
Wild (n=17)	20.5±2.4	112.9±6.1	71.7±5.2	107.1±36.8	162.1±39.2	102.7±37.7
Variant (n=76)	20.4±4.1	112.4±7.7	71.5±6.1	110.1±51.1	144.1±30.5	85.6±30.3
t/p values	*0.158/0.875*	*0.270/0.788*	*0.143/0.886*	*0.202/0.840*	*2.094/0.039*	*2.003/0.048*
YDM subjects						
Wild (n=30)	16.7±4.1	98.1±11.0	64.7±7.7	106.9±40.2	134.1±28.7	81.2±21.7
Variant (n=71)	18.6±5.3	104.1±12.4	69.7±9.1	132.1±60.1	150.7±40.7	92.7±38.8
t/p values	*1.650/0.108*	*2.143/0.035*	*2.135/0.035*	*1.676/0.097*	*1.631/0.107*	*1.668/0.102*

Results were expressed as mean±SD.

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

Variant, heterozygous and homozygous variant together; BMI, Body Mass Index; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; TG, triglyceride; LDL, Low density Lipoprotein.

Glycemic and insulinemic status of study subjects on the basis of Taq1 T>C genotype

Mean (±SD) fasting glucose levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (5.0±0.5 and 4.9±0.5 respectively; p=0.117) and YDM group (15.4±5.4 and 15.6±5.1) respectively; p=0.815) (Table 10i).

Mean (±SD) C-peptide levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (1.6±0.6 and 1.6±0.6 respectively; p=0.776) and YDM group (1.2±0.9 and 1.2±0.9 respectively; p=0.922) (Table 10i).

Mean (±SD) HOMA%B in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (107.6±40.2 and 114.7±37.8 respectively; p=0.393) and YDM group (20.6±24.5 and 17.6±17.9 respectively; p=0.512) (Table 10i).

Mean (±SD) HOMA%S in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (90.8±31.2 and 96.8±44.5 respectively; p=0.491) and in YDM group (96.4±60.8 and 109.4±99.7 respectively; p=0.0.467) (Table 10i).

Mean (±SD) insulin resistance in subjects with wild and variant (heterozygous and homozygous together) in the control was (0.3±0.1 and 0.4±0.1 respectively; p=0.670) and in YDM group (0.7±0.5 and 0.8±0.5 respectively; p=0.674) (Table 10i).

Mean (±SD) BMI levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (19.4±2.8 and 21.1±4.1 respectively; p=0.046) and YDM group (18.1±5.4 and 18.2±4.9) respectively; p=0.910) (Table 10ii).

Mean (±SD) SBP levels in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (110.1±7.9 and 113.8±6.8 respectively; p=0.021) as well as in YDM group subjects (100.1±10.3 and 106.1±13.4 respectively; p=0.009) (Table 10ii).

Mean (±SD) DBP levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (69.7±5.9 and 72.7±5.8 respectively; p=0.023) and YDM group (67.8±9.3 and 69.5±8.8 respectively; p=0.366) (Table 10ii).

Mean (±SD) TG levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (108.1±41.8 and 110.5±52.9 respectively; p=0.821) and YDM group (120.1±46.2 and 133.7±66.3 respectively; p=0.263) (Table 10ii).

Mean (±SD) cholesterol levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (149.2±34.4 and 145.7±34.9 respectively; p=0.627) and YDM group (145.1±36.2 and 149.5±41.7 respectively; p=0.603) (Table 10ii).

Mean (±SD) LDL levels in subjects with wild and variant (heterozygous and homozygous together) genotype in the control was (90.6±32.6 and 87.2±32.2 respectively; p=0.626) YDM group (89.1±30.8 and 91.6±41.1 respectively; p=0.746) (Table 10ii).

Table 10i: Glycemic and insulinemic status of the study subjects on the basis of Taq1 T>C genotype

	FSG (mmol/l)	C-peptide (ng/ml)	HOMA%B	HOMA%S	IR
Control subjects					
Wild (n=34)	5.0±0.5	1.6±0.6	107.6±40.2	90.8±31.2	0.3±0.1
Variant (n=58)	4.9±0.5	1.6±0.6	114.7±37.8	96.8±44.5	0.4±0.1
t/p values	*1.581/0.117*	*0.286/0.776*	*0.858/0.393*	*0.691/0.491*	*0.428/0.670*
YDM subjects					
Wild (n=44)	15.4± 5.4	1.2± 0.9	20.6±24.5	96.4±60.8	0.7±0.5
Variant (n=47)	15.6± 5.1	1.2± 0.9	17.6±17.9	109.4±99.7	0.8±0.5
t/p values	*0.235/0.815*	*0.098/0.922*	*0.658/0.512*	*0.731/0.467*	*0.422/0.674*

Results were expressed as mean±SD.

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

Variant, heterozygous and homozygous variant together; FSG, Fasting Serum Glucose; HOMA %B, Homeostasis model assessment B-cell Function; HOMA %S, Homeostasis model assessment insulin sensitivity.

Table 10ii: Glycemic and insulinemic status of the study subjects on the basis of Taq1 T>C genotype

	BMI	SBP	DBP	TG	Cholesterol	LDL
Control subjects						
Wild (n=34)	19.4±2.8	110.1±7.9	69.7±5.9	108.1±41.8	149.2±34.4	90.6±32.6
Variant (n=58)	21.1±4.1	113.8±6.8	72.7±5.8	110.5±52.9	145.7±34.9	87.2±32.2
t/p values	*2.024/0.046*	*2.351/0.021*	*2.306/0.023*	*0.227/0.821*	*0.487/0.627*	*0.488/0.626*
YDM subjects						
Wild (n=44)	18.1±5.4	100.1±10.3	67.8±9.3	120.1±46.1	145.1±36.2	89.1±30.8
Variant (n=47)	18.2±4.9	106.1±13.4	69.5±8.8	133.7±66.3	149.5±41.7	91.6±41.1
t/p values	*0.113/0.910*	*2.651/0.009*	*0.909/0.366*	*1.127/0.263*	*0.522/0.603*	*0.325/0.746*

Results were expressed as mean±SD.

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

Variant, heterozygous and homozygous variant together; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; LDL, low density lipoprotein.

CHAPTER 5

DISCUSSION

DISCUSSION

Diabetes Mellitus has emerged as a major health problem all over the world and is posing additional burden to the already exhausted health care budget like many other developing countries including Bangladesh.

Over all the prevalence of diabetes in Bangladesh is 7% (Sayeed *et al.*, 2007). Majority of diabetic patient are of type 2 variety. However, a substantial number of patients do not fit to either to type 1 or type 2 groups. They are termed as young onset diabetes mellitus (YDM). This group of patients mainly encompasses previously termed FCPD and PDDM patients, who are classified as MRDM.

To characterize the nature of diabetes in this group and explore its etiopathogenesis a number of studies have been under taken so far. One study involving lean young diabetic patients has revealed secretory defects in these subjects (Zinnat *et. al.*, 2003). Subsequently (Hassan *et. al.*, 2005) in thesis study showed that YDM subjects may be representing atypical T1DM in Bangladesh. These subjects also have been found to belong to low insulin secretory group. The hallmark of T1DM is destruction of pancreatic B cell leading to severely compromised insulin secretion resulting in need for insulin for metabolic control.

Vitamin D has been suggested to play an important role in the pathogenesis of diabetes mellitus, both in type 1 and type 2 varieties (Norman *et al.*, 1980). Moreover, VDR gene polymorphisms have been found to be associated with T1DM in different population (Yoshiko *et al.*, 2009). However, studies also revealed lack of association of VDR gene polymorphism with either T1DM or T2DM (Ortlepp *et al.*, 2003).

The lack or presence of association of VDR gene polymorphism with diabetes mellitus points to the importance of gene environment and gene to gene interaction in its pathogenesis. In this respect ethnic variation appeared to be an important factor.

The novelty of present study is the inclusion of a group of unique young onset diabetic patients for the determination of VDR gene G>T and T>C polymorphism. The YDM subjects (n=94) were of low to near normal BMI (18.2±5.16) and matched with control (20.4±3.74) (Table: 3). Male-female distribution in the control and YDM group did not

show significant association (p= 0.009). The leanness of the YDM subjects in the present study has been further confirmed by their significantly lower mid-arm circumference and sub scapular skin fold thickness (p=0.001 and 0.036 respectively) compared to the controls.

The YDM subjects in the present study were found to be having low insulin secretory capacity as revealed by HOMA%B (19.4±21.3) and HOMAIR (0.74±0.54) values compared to the control group. The present finding is in agreement with the observation of Ali et al., (2003).

VDR gene polymorphic (G>T and T>C) markers genotype in the present study determined were in compliance with the Hardy-Weinberg equilibrium. VDR gene G>T polymorphism did not show any association with YDM (χ^2= 0.198; p=0.906). This is in agreement with Ichiro et al (2002) who did not finding any association between VDR gene G>T variation and juvenile onset diabetes in the Japanese population. The finding is also substantiated by the lack of association in the study involving in Finnish type 1 diabetic patients. They have concluded that single nucleotide polymorphism is unlikely to be associated with type 1 diabetes in Finnish population (Turpeinen *et al.,* 2003). The lack of association of the VDR gene G>T polymorphism in the present study is, however, in contrast to that observed in the type 2 diabetic patients (Samiun 2009). Presence of wild or variant allele in the study subjects did not show any difference regarding their blood glucose, insulin secretion as evaluated by measurement of C-peptide, in absolute term or HOMA-IR. However, YDM subjects with variant allele had significantly higher HOMA%B compared to those with wild allele. The findings suggest, important interaction between insulin gene expression and VDR gene G>T polymorphism considering the effect of VDR polymorphism in its expression.

In the present study VDR gene T>C (Taq1 restriction) polymorphism also did not show any association with YDM (χ^2= 2.49; p=0.288). This is again in agreement with findings of Ichiro et al., (2003) in the Japanese population. VDR gene T>C polymorphism also lacked any association with type 1 diabetes in Caucasoid population (Oh and Berrot *et al.,* 2002).Presence of VDR gene T>C variant allele also did not show any relationship regarding insulin secretory status of pancreatic B cell in YDM subjects.

The lack of any relationship of VDR gene G>T and T>C polymorphism with YDM as a group and there insulinemic status does not exclude possible role of other polymorphic marker in the pathogenesis of diabetes in the young which needs to be evaluated.

It may be concluded that; (i) VDR gene G>T and T>C polymorphic marker are not associated with diabetes mellitus in the young Bangladeshi diabetic patients; (ii) Insulin secretory dysfunction is main feature of pathogenesis of diabetes in the young; (iii) VDR gene G>T genotype is associated with higher secretory capacity however, need to be further substantiated.

Limitations of the study

- Small number of young onset diabetic subjects is the major weakness of this study.
- Measurement of Vitamin D level was not done this would have strengthened the data substantially.

Recommendations

- The study needs to be completed involving adequate number of patients to provide proper statistical power.
- Further study should design to explore the relationship of VDR gene common variants with YDM found to be related in other populations.

CHAPTER 6

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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 (MUPC):

(vii) Skin fold thickness:

Triceps:

Sub-scapular skin folds thickness:

(viii) Body fat mass:

(b) Blood pressure:

	1st reading	2nd reading	Average reading
Systolic			
Diastolic			

Appendix II

ক্রমিক নং .....

নাম:.....

বয়স:.....

ঠিকানা:.....

.....

.....

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:.....