# ASSOCIATION OF VITAMIN D RECEPTOR GENE G>T AND T>C POLYMORPHISMS IN THE PATHOGENESIS OF IMPAIRED GLUCOSE TOLERANT SUBJECTS OF BANGLADESH

A Thesis By

**Md. Sahabuddin**
**Examination Roll No: MS- 090719**
**Session:** 2009 -2010



Submitted To

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# ASSOCIATION OF VITAMIN D RECEPTOR GENE G>T AND T>C POLYMORPHISMS IN THE PATHOGENESIS OF IMPAIRED GLUCOSE TOLERANCE IN SUBJECTS OF BANGLADESHI ORIGIN

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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 G>T and T>C Polymorphisms in the pathogenesis of Impaired Glucose Tolerant Subjects of Bangladesh'** is submitted in partial fulfillment for the degree Masters in Biotechnology and Genetic Engineering under the Faculty of Life Science, Khulna University, Khulna, was carried out in the Laboratory of BMRG, BIRDEM, Dhaka during the period of April 2011 to April 2012.

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 be confirmed that the thesis entitled '**Association of Vitamin D Receptor Gene G>T and T>C Polymorphisms in the pathogenesis of Impaired Glucose Tolerant Subjects of Bangladesh**' a requirement for the degree of Masters in Biotechnology and Genetic Engineering, under the faculty of Life Science School, Khulna University, Khulna, Bangladesh, was carried out in the Laboratory of BMRG, BIRDEM during the period of April 2011 to April 2012.

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*DEDICATED*

*TO*

*MY BELOVED PARENTS*

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## ABSTRACT

Vitamin D receptor (VDR) gene polymorphisms have been found to be associated with pancreatic B cell secretion and glucose intolerance. The present study was undertaken to determine genotype of VDR gene Apa1 (G>T) and Taq1 (T>C) polymorphism of a group of impaired glucose tolerance (IGT) subjects of Bangladeshi origin and investigate its association with its insulin sensitivity and cell secretory capacity. A total number of 54 IGT subjects were recruited in the study and 68 healthy subjects served as control. Oral glucose tolerance test was performed and IGT was diagnosed following WHO Guidelines. Blood glucose was measured by glucose-oxidase and, triglyceride, total cholesterol and HDL-cholesterol by enzymatic colorimetric method. Insulin was estimated by enzyme linked immunosorbent assay (ELISA), and insulin secretory capacity (HOMA%B) and sensitivity (HOMA%S) were determined using HOMA-Sigma Software. DNA was isolated using QIAGEN Blood DNA Kit which utilizes silica gel DNA separation. VDR gene variants [G>T and T>C] were analyzed by PCR-RFLP using restriction enzymes Apa1 and Taq1 respectively. Data were managed using Statistical Program for Social Science (SPSS) for Windows version 10. Unpaired Student's-'t' test and Chi-squared tests were performed where appropriate. Circulatory insulin level was found to be higher ($p<0.001$) and reflected in their lower insulin sensitivity (as judged by HOMA%S) level ($p=0.003$). The G>T genotypes frequencies in the control and IGT were 0.176, 0.618, 0.206 (wild, heterozygous variants and homozygous variants respectively) and 0.185, 0.537, 0.278 respectively. The T>C genotypes frequencies were in the control were 0.500, 0.456, 0.044 (wild, heterozygous variants and homozygous variants respectively) and IGT 0.444, 0.426, 0.130 respectively. Genotypes frequencies of G>T and T>C did not show significant association with IGT ($p=0.606$ and 0.230 respectively). Wild and Variant genotypes of the two marker allele did not show significant difference regarding glucose level (fasting and 2 hour postprandial) and insulinemic status (insulin, HOMA%B and HOMA%S) either in Controls or IGT subjects. It was concluded that i) VDR gene Apa1 (G>T) and Taq1 (T>C) polymorphic alleles are not associated with IGT of Bangladeshi origin; (ii) The polymorphic marker alleles did not have any effect on fasting and two hour blood glucose and insulinemic status of the IGT and Controls; and (iii) The data reconfirmed that lower insulin sensitivity is predominantly present in the IGT subjects of Bangladeshi origin.

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| | |
|---|---|
| ADA | American Diabetes Association |
| BIRDEM | Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders |
| BMI | Body Mass Index |
| BMRG | Biomedical Research Group |
| CI | Confidence Interval |
| CNS | Central Nervous System |
| DBD | DNA-binding Domain |
| DBP | Diastolic blood pressure |
| DM | Diabetes Mellitus |
| DECODA | Diabetes Epidemiology Collaborative of Diagonostic criteria in Asia |
| 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 |
| IFG | Impaired Fasting Glucose |
| IGT | Impaired glucose tolerance |

| | |
|---|---|
| UTR | Untranslated region |
| VDR | Vitamin D receptor gene |
| VLDL | Very Low Density Lipoprotein |
| WGA | Whole-genome Association |
| WHO | World Health Organization |
| WHR | Waist hip ratio |
| µl | Microliter |

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# INTRODUCTION

# 1 Introduction

## 1.1 Diabetes Mellitus (DM)

Diabetes Mellitus (DM) is a disorder of carbohydrate, protein and fat metabolism resulting in persistant hyperglycemia (Wiltshire *et al.*, 2001). The fundamental defect in diabetes is a deficiency of insulin and/ or its action (Reaven, 1998). The typical symptoms of diabetes are polyurea, polyphagia and polydipsia. However, patients often are diagnosed having diabetes on routine examination and/or diabetes related complications.

## 1.2. Classification of Diabetes Mellitus

National Diabetes Data Group in 1979 (NDDG, 1979) first classified diabetes mellitus which later was endorsed by WHO with minor modifications (WHO, 1980). By next two decades there have been major revisions in the diagnostic criteria and classification by both ADA and WHO (WHO, 1985 and 1999; ADA, 1997). The present diabetes classification initially proposed by ADA (1997) and later on adopted by WHO (1999) and mainly considered as etiological classification of diabetes:

### 1.2.1 Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) mellitus is characterized by sudden onset of symptoms, proneness to ketoacidosis 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 seems to occur mainly 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 the incidence of T1DM is similar (10.5/100,000) to many European populations (Ramachandran *et al.*, 1986). T1DM is believed to be less prevalent in Bangladesh, however, population based data are still lacking.

T1DM is subclassified on the basis of type of damage of β-cells; immune mediated type 1 (Type 1A) and non-immune mediated type 1 (idiopathic type 1, T1B) (ADA 1997; WHO

1999). Autoimmune T1DM is characterized by immune mediated damage, targeted against self-antigens, resulting in the destruction of the β cells of the pancreas. A number of autoantibodies have been detected against different islet proteins. A number of T1DM patients have a typical sudden onset of the disease and are ketosis prone, but they lack islet cell related autoantibodies, are called idiopathic T1DM. This form of diabetes is thought to be most common in African and Asian countries (McLarty *et al.*, 1990a and 1990b; Tanaka *et al.*, 2000). Idiopathic T1DM usually occurs in obese teens. Once their blood glucose is controlled by insulin and they can be put on to 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).

**Table 1.1: Major four classes of Diabetes Mellitus**

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

(I) Type 1A- Autoimmune mediated type

(II) 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

(I) Genetic defects of beta- cell function

(II) Genetic defects in insulin action

(III) Diseases of the exocrine pancreas

— Fibrocalculus Pancreatic Diabetes (FCPD)

(IV) Endocrinopathies

(V) Drug- or chemical- induced

(VI) Infections

(VII) Uncommon forms of immune- mediated diabetes

(VIII) Other genetic syndromes sometimes associated with diabetes

D. Gestational diabetes

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### 1.2.2 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 all over the world. 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 40.0% and female 42%) and a very low (0-1.4%; male 0% and female 1.4%) was observed among the Mapuches population in Chile and the prevalence was almost nil in rural and peri-rural population of Papua New Guinea (WHO 1994).

In the non-Europid population the prevalence has been shown to vary from <1% in rural Bantu (Tanzania) and Mainland China to 40-50% in Pima Indians (USA) and Nauru (King and Rewers 1993). Age-standardized prevalence of T2DM among native Asians and non-Indian Asians demonstrated an increased trend from rural to urban and markedly increased trend among the migrants to the west and affluent countries. A similar trend also has been observed among native and migrant Pacific Island populations (Coughlan *et al.*, 1997). In multiethnic US society the prevalence of diabetes has shown to vary in different ethnic groups; 5% in the Caucasian origin, 10% in African-Americans, 16% in Cuban origins and 26% in Puerto Ricans (Harris 1991; Flegal *et al.*, 1991).

Prevalence of diabetes in Bangladesh found to be around 5%. Studies involving rural and suburban population revealed the prevalence of T2DM to be 4.3% and 4.1% respectively (Sayeed *et al.*, 1997 and 2003). Recently the same group has revealed the age adjusted prevalence of T2DM about 5.6% among the rural population, (Sayeed *et al.*, 2008).

## 1.3 Pathogenesis of T2DM

The maintenance of normal glucose homeostasis depends on a precisely balanced and dynamic interaction between tissue sensitivity to insulin (especially in muscle and liver) and insulin secretion (Defronzo, 1988). T2DM is postulated to result from defects in both insulin secretion and/or action; both of these defects can have a genetic as well as an acquired component (Defronzo *et al.*, 1998). The interplay between various factors in the pathogenesis of glucose intolerance and/or diabetes is illustrated in Figure 1.1.

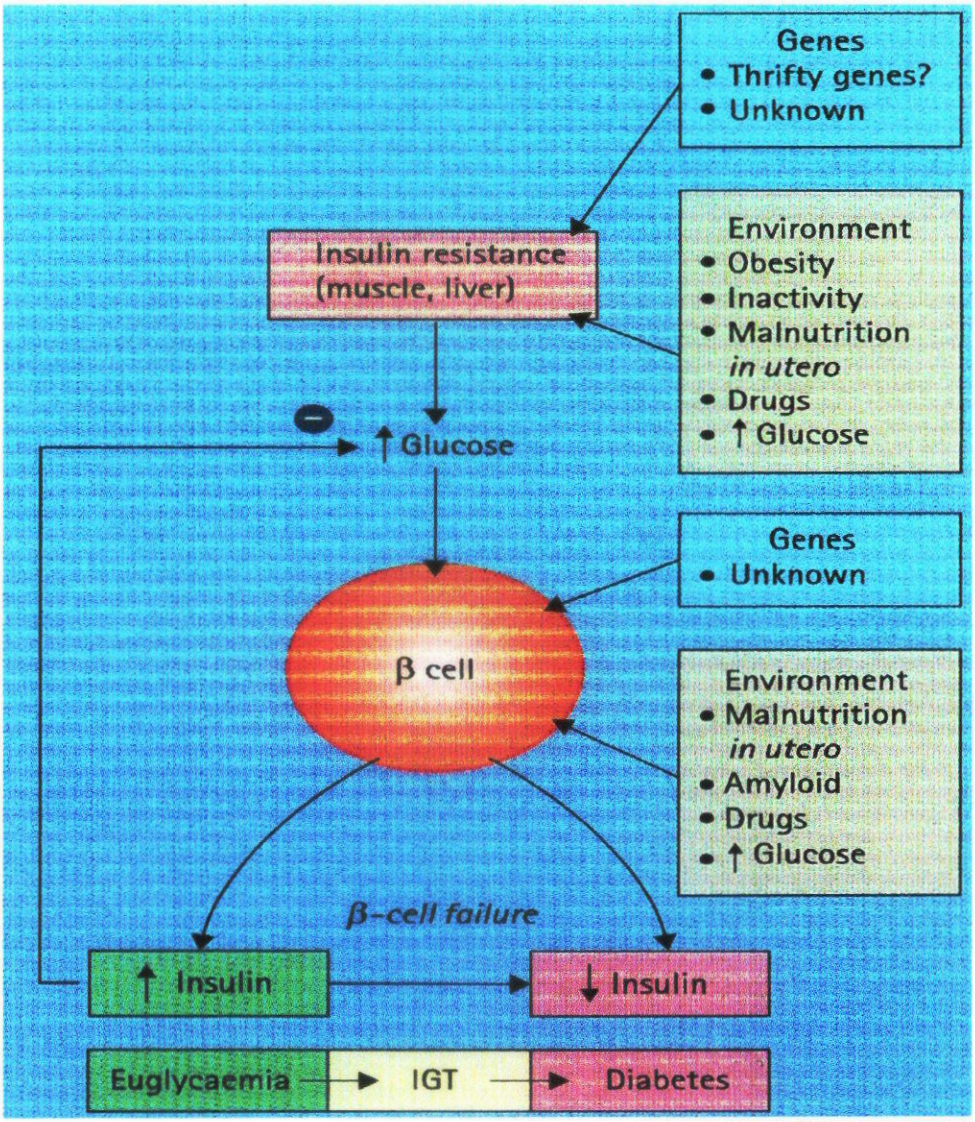


**Figure 1.1:** Pathogenesis of T2DM (Katsilambros and Tentolouris 2003).

## 1.4. Natural history of diabetes mellitus

DM is suggested to pass through an intermediate stage of impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) which together is termed as impaired glucose regulation (IGR) by WHO or pre-diabetes by ADA (Figure1.2). This early stage of diabetes may persist for a variable period of time (Peter *et al.*, 2005) and has recently been adopted as a part of the natural history of diabetes and but not a type of diabetes by ADA (1997) and WHO (1999). The natural history of the patients with IFG/IGT varies, with approximately 25% progress to diabetes, 50% remaining in their abnormal glycemic state and 25% reverting to normal glucose tolerance (NGT) over a period of 3 to 5 years (ADA, 2007)

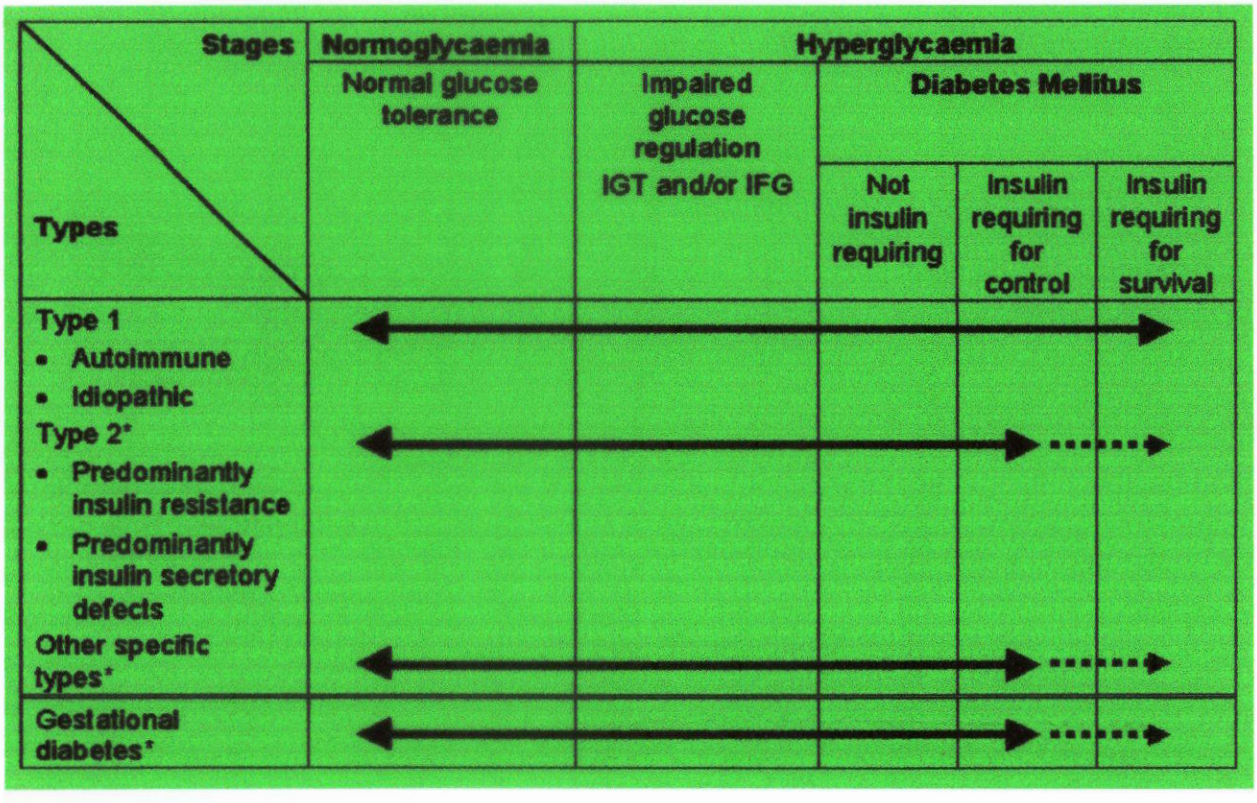


**Figure 1.2:** The stages of glucose regulation in different types of diabetes (Balkau and Eschwege 2003).

## 1.5 Prediabetes

IFG and IGT, a metabolic state intermediate between normal glucose homeostasis and diabetes; are termed as prediabetes by ADA and Impaired glucose regulation (IGR) by WHO. They are not interchangeable and represent different abnormalities of glucose regulation, one in fasting state and the other in postprandial (WHO, 1999). According to fasting and post load glucose concentration, at present patients with IGR or prediabetes may be stratified into three subcategories –(1) Isolated IGT; (2) Isolated IFG; and (3) Combination of the two 'IFG-IGT' (WHO and ADA , 2002).

### IFG

IFG is defined, as fasting plasma glucose between 6.1 and 6.9 mmol/l and 2h plasma glucose <7.8 mmol/l (ADA, 2005). However, ADA has recently lowered the cut off value of fasting plasma glucose in IFG from 6.1 mmol/l to 5.6 mmol/l (ADA, 2005).

### IGT

IGT is defined as fasting plasma glucose <6.1 mmol/l and 2h plasma glucose between 7.8 and 11.0 mmol/l (ADA, 2005). Historically the term IGT was first introduced by the National Diabetes Data Group in 1979 and later, on kept by WHO. In this stage blood glucose values are higher than the defined normal levels but not high enough to meet the diagnostic criteria for diabetes (Rao *et al.*, 2004).

### Combined IFG-IGT

Studies have shown that some subjects may present with of both IFG and IGT and are termed as combined IFG-IGT. They have fasting plasma glucose 6.1- 6.9 mmol/l and 2h plasma glucose 7.8-11.0 mmol/l (ADA 2005). In one particular study in Denmark the progression of IFG-IGT to diabetes found to be 28% per year (Rasmussen *et al.*, 2007). IFG and IGT are asymptomatic and unassociated with any manifested morbidity, but then sole significance lies in the fact that they predict future diabetes or cardiovascular diseases (Stern and Burke 2000).

## 1.6. Factors associated in the pathogenesis of DM

Risk factors found to be associated in the pathogenesis of DM suggested to include (i) environmental factors (obesity, physical inactivity, advancing age diet etc.); and (ii) genetic susceptibility. Environmental factors postulated to play important role in the pathogenesis of diabetes mellitus in genetically susceptible individuals.

### 1.6.1. Environmental factors

***Aging***

Life expectancy has increased from 45 (approx) years in the early 1900s to 75 (approx.) years for men and 80 years for women today (Oeppen *et al.*, 2002). This has suggested contributing to the increasing prevalence of age-related diseases, including T2DM (Davidson *et al.*, 1979; Borkan *et al.*, 1983). In addition, pre-adipocyte capacity to replicate, differentiate and store lipids declines with age (Kirkland *et al.*, 1987) which is also suggested to contribute to increased accumulation of ectopic lipids in muscle and liver, and induction of insulin resistance and ultimately resulting in diabetes mellitus.

***Obesity***

About 80% of T2DM patients found to be obese (Venables and Jeukendrup, 2009). It is postulated that in obesity, there is disparity between uptake of fatty acids into skeletal muscle and oxidation resulting in excessive accumulation of triacylglycerol and fatty acid metabolites in the sarcoplasm of skeletal muscle (Venables and Jeukendrup, 2009). In addition elevation of circulating fatty acids was suggested to play critical role in decreasing insulin signaling and glucose disposal rates (Belfort *et al.*, 2005). An increase in skeletal muscle diacylglycerol (DAC)content in human and animal models of insulin resistance found to be activating specific isoforms of protein kinase C, leading to inhibition of the insulin signal through serine phosphorylation of IRS1 (Itani *et al.*, 2002; Yu et al., 2002). Dysfunction of β-cells found to exists in individuals with high risk of developing T2DM even in normoglycemic state (Kahn *et al.*, 2006).

*Sedentary lifestyle*

Prospective studies have shown strong association between daily physical activity and reduced risk of developing T2DM. Physical activity was found to help to control body weight, by using glucose and restoring cells sensitive to insulin. The effect of physical inactivity on glucose metabolism predominantly includes impairments of peripheral insulin-stimulated glucose uptake (i.e. peripheral insulin resistance) (Balkau *et al.*, 2008; Assah *et al.*, 2008) and increase the risk of T2DM.

*Diet*

In addition to physical inactivity and obesity, diet may play important role in the pathogenesis of T2DM. In particular, intake of total dietary fat and high proportion of saturated fat found to increase the risk of developing impaired glucose regulation and T2DM (Feskens *et al.*, 1995).

*Vitamin D*

Vitamin D deficiency was linked to IGT and T2DM for some years (Chiu *et al.*, 2004; Gedik and Akalin 1986). These observations were confirmed in animal models, which demonstrated that pancreatic insulin secretion is inhibited by vitamin D deficiency (Norman *et al.*, 1980). Several reports have ascribed an active role to vitamin D in the functional regulation of the endocrine pancreas, particularly the β-cells. Not only are receptors for $1,25(OH)_2D3$ found in beta cells (Lee *et al.*,1994), but the effector 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 beta cells from cytokine mediated cell death (Rabinovitch *et al.*, 2001).

### 1.6.2 Genetic factors

Genetic susceptibility has been implicated in the pathogenesis of both T1DM and T2DM. Tendency of aggregation of T1DM in families has been observed (Todd and Farral 1996). The lifelong T1DM risk in the general population is 0.4% but it increases to 6% among the

first degree relatives of T1DM subjects (Risch, 1987). The 15 times higher risk for diabetes among the first degree relatives demonstrates the strong family aggregation of T1DM and sustains the importance of genetic/hereditary factors for this diabetes phenotype. Concordance for T1DM among monozygotic twins is close to 100%, and about 25% of those with the disease foundto have a family history of diabetes (Cotran *et al.*, 1999).

The lifetime risk of a first-degree relative of a patient with T2D has been estimated at about 35% with the relative risk of diabetes compared with the general population of between three- and fourfolds. Furthermore, twin studies have shown much higher concordance among monozygotic compared with dizygotic twins (Newman *et al.*, 1987). However, DM is found to be extremely heterogeneous in nature.

Many genes and its variants have been assigned in the pathogenesis of DM through candidate gene approach. A key example is the gene encoding PPAR$\gamma$ (*PPARG*, MIM 601487) initially identified as a nuclear receptor key for adipogenesis (Rosen *et al.*, 1999). Subsequent studies and meta-analyses have shown that the P12A polymorphism is associated with insulin sensitivity and increase risk for DM (Altshuler *et al.*, 2000; Andrulionyte *et al.*, 2004). Although, the molecular mechanisms responsible for these relationships have not been fully elucidated, *PPARG* P12A substitution found to reduce adipogenesis (Masugi *et al.*, 2000).

Variations in the ATP-sensitive potassium channel Kir 6.2 (*KCNJ1*, MIM 600937) and *ABCC8A* [SUR1 (sulfonylurea receptor 1), MIM 600509] genes which encode components of the β-cell KATP channel, which couples glucose metabolism to membrane depolarization and subsequent insulin release, were also found to be associated with increased T2DM risk (Koo *et al.*, 2007; Florez *et al.*, 2007).

Polymorphisms in VDR (vitamin D receptor, MIM 601769) have shown to be associated with impaired insulin secretion in both T1DM and T2DM (McDermott *et al.*, 1997; Pani *et al.*, 2000).

Positional approaches to candidate gene identification have been largely unsuccessful, with the notable exception of the genes *CAPN10* (calpain10, MIM 605286) (Horikawa *et al.*, 2000) and *TCF7L2* (transcription factor 7-like 2, MIM 602228); (Grant *et al.*, 2006)). Polymorphisms within the *CAPN10* gene en were found to be associated with T2D (Horikawa *et al.*, 2000). Calpains are ubiquitously expressed cysteine proteases that are thought to regulate a variety of normal cellular functions, and recent data indicate a role for calpains in the regulation of both insulin secretion and insulin action (Sreenan *et al.*, 2001)

Although candidate gene studies have been informative, such approaches are inherently limited. Recent improvements in high-throughput identification of genetic variants have made whole-genome association (WGA) studies feasible for the study of complex diseases. Genome-wide association studies have led to the identification of several single nucleotide polymorphisms in genes including *PPARG* and *TCF7L2*. The chromosomal regions that have shown consistent replication of linkage to T2DM in several independent genome scans include 1, 3, 8, 12 and 20 (Salonen *et al.*, 2007).

**Putative loci**

Multiple genetic anomalies at different loci of human genome confer varying degrees of predisposition to DM.

**Table 1.2:** Susceptibility loci linked to pathogenesis of diabetes mellitus

| Loci | Chromosome | Candidate gene | References |
|---|---|---|---|
| IDDM1 (MIM 2221000) | 6p21.3 | HLA DR1, DQ | Donner *et al.*, 2000; Park *et al.*, 1998. |
| IDDM2 (MIM 125852) | 11p15.5 | INS VNTR | Bennette *et al.*, 1996; Huxtable *et al.*, 2000; |
| NIDDM1 (MIM 601283) | 2q37.1 | CAPN10 | Horikawa *et al.*, 2000 |
| NIDDM2 (MIM 601407) | 12q24.1 | 20q12-q13.1 MODY3 | Mahtani *et al.*, 1996; Shaw *et al.*, 1998 |
| NIDDM3 (MIM 603694) | | PKC1 | Zouali *et al.*, 1997; Bowden *et al.*, 1997 |

IDDM1 locus contains human leucocyte antigen (HLA) gene cluster located within the major histocompatibility complex (MHC) on chromosome 6p21.3.The locus has been found to be in linkage disequilibrium in substantial number of T1DDM patients (Wolf *et* al., 1983). Later on variation in HLA class II gene, particularly DQB1 and DR4 allele, found to be strongly associated with type 1 DM (Donnner *et al.*, 2000; Park *et al.*, 1998).

The IDDM2 locus contains the insulin gene (INS, MIM 176730) that is located on chromosome 11. The locus is designated as Ch 11p15.5. Insulin gene is an important candidate gene that is involved in the pathogenesis of DM. INS gene encodes Insulin, a peptide hormone produced by the β-cells of the islets of Langerhans of the pancreas. The effects of insulin on glucose metabolism are most prominent in three tissues: lever or hepatic tissue, muscle tissue and adipose tissue. In each case, the effect of insulin is to enhance the uptake of glucose into the cells. The variable number of tandem repeat (VNTR) that lies immediately adjacent to the 5′ promoter region of INS is believed to have a direct effect on INS expression regulation (Kennedy *et al.*, 1995). INS VNTR found to be associated with both T1DM and T2DM (Bennett *et al.*, 1996; Huxtable *et al.*, 2000).

## 1.7 Vitamin D receptor (VDR) gene polymorphism

Vitamin D receptor (VDR) polymorphism has been reported to be associated with T1DM (McDermott *et al,* 1997; Pani *et al.*, 2000; Chang *et al.*, 2000; Gyapay 1994). VDR gene polymorphisms have been linked to impaired insulin secretion in both 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 associated with T1DM diabetes (Chang *et al.*, 2000). The aa genotype 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 type1 diabetes, 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 for the Taq1 and Apa1 polymorphism in the sample set. Nonetheless, this study indicates that the Bsm1 polymorphism at the VDR gene locus putting on risk for T1DM. Study involving of 152 Caucasian families with T1DM, an excessive transmission of Taq1 and Apa1 alleles alone was observed (Pani *et al.*, 2000). Vitamin D receptor allele combinations found to influence genetic susceptibility to type 1diabetes in Germans. Studies have demonstrated a link between VDR gene polymorphisms and T2DM, although the findings differ from one population to another. A study involving Bangladeshi Asians living in london demonstrated that the ApaI RFLP influences insulin secretion in response to glucose (Hitman *et al.*, 1998). More recently, 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 (Ortlepp *et al.*, 2003).

## 1.8 Rationale of the study

The prevalence of diabetes mellitus in Bangladesh is about 7% (Saeed *et al.*, 2007). There is, however, a difference in prevalence of diabetes in urban and rural areas. Saeed *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. Normal to overweight T2DM subjects found to they have both insulin secretory dysfunction and insulin resistance but former was found to be predominant in the pathophysiology of T2DM (Al-Mahmood, 2000). In another study, it was also observed that the extent of insulin secretory dysfunctional was found to be more pronounced compared to loss of insulin sensitivity in obese T2DM of this population (Junaid, 2000). In contrast most western people with T2DM were relatively obese and impaired insulin action

(Sacks *et al.*, 1999). These studies have demonstrated that insulin secretory dysfunction not the insulin sensitivity is prime features of T2DM of Bangladeshi population.

Some of the established polymorphic genetic markers, implicated in the pathogenesis of T2DM, have been studied at BIRDEM in this population. These include INS-VNTR, CAPN10, NAT2 and VDR gene common polymorphism both in T2DM and young onset diabetes mellitus. NAT2 gene common candidate polymorphic markers 481C>T, 590G>A and 857 G>A are not associated with T2DM in Bangladeshi population. VDR gene Bsm1 (A>G) and Taq1 (T>C) genotype frequencies did not show significant association with Bangladeshi T2DM subjects. VDR gene Apa1 (G>T) genotype frequencies showed significant association with Bangladeshi T2DM subjects. VDR gene G>T and T>C genotype frequencies did not show significant association with Bangladeshi YDM subjects. The defective insulin secretory function, insulin sensitivity and insulin resistance appeared to be prominent in YDM subjects. Therefore, the present study was to evaluate the possible interaction between prediabetes and VDR polymorphism in Bangladeshi population

## Hypothesis

Vitamin D receptor gene Apa1 (G>T) and Taq1 (T>C) variants are not associated with Impaired glucose tolerance in the Bangladeshi population.

## 1.9 Objectives

***General objective***

The general objective of the present study was to determine genotype of VDR gene common variants in a group of Bangladeshi population and find its role in the prediabetes.

***Specific objectives***

The specific objectives of the present study were to:

- Determine the genotype of VDR gene ApaI (G>T) and TaqI (T>C) polymorphisms in IGT subjects of Bangladeshi population.
- Assess the association, if any, ApaI (G>T) and TaqI (T>C) polymorphisms with IGT.
- Estimate circulatory insulin levels of the IGT subjects and determine insulin secretory capacity (as judged by HOMA%B) and insulin sensitivity (HOMA%S) of the study subject
- Explore the effect, if any, of ApaI (G>T) and TaqI (T>C) polymorphisms with in insulin sensitivity and secretory capacity of the IGT subjects.

# LITERATURE REVIEW

# 2 Literature Review

## 2.1 Prevalence of prediabetes

The prevalence of IFG and IGT found to increase with age (Shaw *et al.*, 1999). The prevalence of IFG tends to plateau in the middle age whereas the prevalence of IGT rises the advancement of age (Unwin 2002). IGT is more prevalent than IFG. It was observed that less than or equal to 50% of people with IFG has IGT and 20-30% with IGT also has IFG. In the last decated prevalence of IGT was found to be rising. Between 2003-2005 IGT was found to increase from 8.2% to 9%. At the same, it the change was found to be 7.1 to 7.8 in Bangladesh. Subjects between 40-59 years of age found to have the highest number cases with IGT (Sicree *et al.*, 2003). Crude prevalence of IFG was found to be 12.4% in rural population of Bangladesh (Sayeed *et al.*, 2003).

**Table 2.1: Prevalence of IFG and IGT among different populations (Abdul-ghani *et al.*, 2006)**

| Population | Prevalence of isolated IGT | Prevalence of isolated IFG | Prevalence of IGT with IFG | Prevalence of IFG with IGT |
|---|---|---|---|---|
| European | 8.8 | 6.9 | 26.0 | 31.0 |
| Australian | 8 | 5.7 | 24.5 | 31.3 |
| Mauritius | 13.8 | 4.2 | 19.4 | 44.4 |
| Pima Indian | 10.7 | 1.9 | 18.9 | 56.8 |
| Swedish | 20.3 | 9.7 | 27.2 | 43.9 |
| Chinese | 6.3 | 0.9 | 14.7 | 53 |
| American | 11 | 4.4 | 26.3 | 46.9 |
| Korean | 20.1 | 2.7 | 13.4 | 53 |

The prevalence of IFG found to be similar in men and women, but IGT found to be occurring more frequent in women (Shaw *et al.*, 1999). Study involving Dutch population reported the prevalence of IFG and IGT among men to be 9.7% and 13.8%, respectively and in women is 6.1% and 14.6% (Corpeleijn *et al.*, 2002). AusDiab study, involving of adults aged 25 years and older demonstrated that IGT affected 10.6% of subjects, being more common in women (11.9% v 9.2% in men), and IFG 5.8%, being more prevalent in men (8.1% v 3.4% in women). The overall prevalence of prediabetes was found to be 16.4% in Australian adults (≥25 years) (Dunstan *et al.*, 2002). The prevalence of both IFG and IGT found to vary considerably based on ethnicity, ranging from a low of 6.3% in Chinese (Ko *et al.*, 1998) to a high of 20.3% in a Swedish population (Larsson *et al.*, 1998) (Table1.2). From DECODA (Diabetes epidemiology: Collaborative of Diagonostic criteria in Asia) study , it was found that IGT is more prevalent than IFG in all Asian populations studied for all age- groups (DECODA, 2003).The rising prevalence rate of IGT may be mainly due to diabetogenic lifestyle factors that lead to obesity and increasing life expectancy.

**Features of IFG-IGT**

The main feature of IFG/IGT are: 1) a stage in the natural history of disordered glucose metabolism, 2) can lead to any type of diabetes, 3) increased risk of progression to diabetes, 4) increased risk of cardiovascular diseases 5) little or no risk of micro vascular diseases, and 6) some patient may revert to normoglycemic (Balkau and Eschwege 2003). Both IFG and IGT are similarly associated with an increased risk of diabetes mellitus. Risk is higher where IGT and IFG coexists (Unwin 2002).

## 2.2 Natural history of IGT

Of the two components of prediabtes IGT has shown to follow three distinct courses. About 25% of IGT progress to T2DM within 5 years, while the majority either remain within the category (50%) or revert to normal glucose tolerance (25%) (Jarrett *et al.*, 1984). In the pathogenesis of T2DM it is generally understood that insulin resistance is

compensated by the adaptive capacity of the β-cells by increasing insulin secretion and thus preventing disturbance in glucose homeostasis. Ultimately, whether through worsening insulin resistance or progressive impairment of β-cell function, insulin secretion reaches a plateau, during which blood glucose level rise initially into the subclinical range of IGT. The process usually takes several years. The progression rate IFG to T2DM was found to be 17.6 and for IGT 18.8 cases per 100 persons per year. When analyzing IGT as two separate categories i.e. isolated IGT and combined IFG-IGT, the progression rates found to be 12.0 and 28.1 to diabetes per 100 person years respectively (Rasmussen *et al.*, 2007).

Normal glucose tolerance give rise to a typical bell-shaped curve and has been termed as 'Starling curve of the pancreas' (Katsilambros and Tentolouris 2003) (Figure 2.1) and the changes in glucose metabolisms, insulin secretion and sensitivity during the typical progression through IGT to T2DM are demonstrated in Figure 2.2.

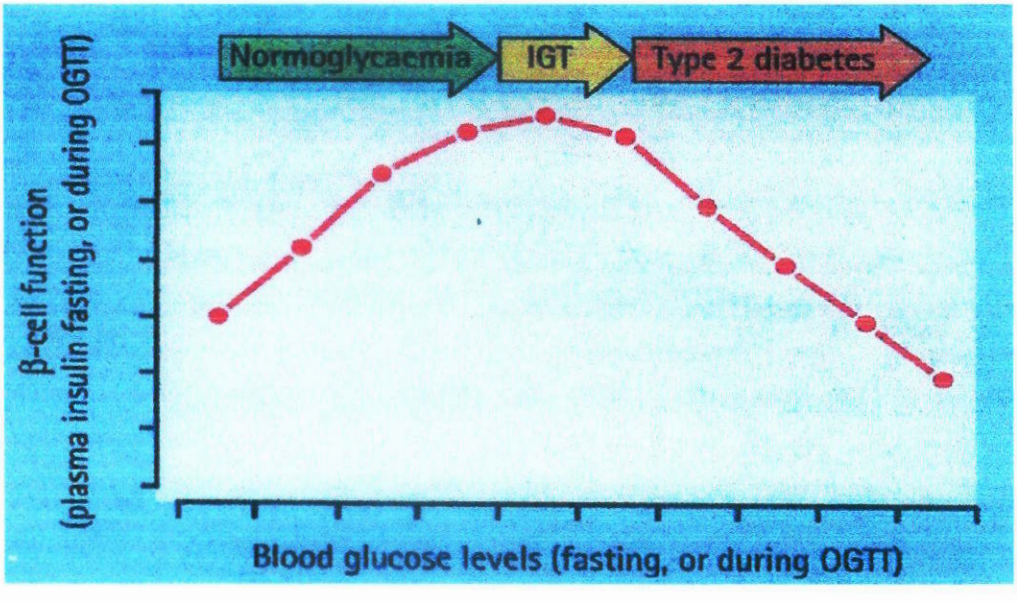


**Figure 2.1:** The 'Starling curve of the pancreas' (Katsilambros and Tentolouris 2003).

Individual destined to develop T2DM pass through five stages. Stage 1: which begins at birth, when glucose homeostasis is normal but individuals are at risk for T2DM because

of non-specific diabetogenic genes; Stage 2: during this stage insulin insensitivity emerges, probably as a result of a genetic predisposition and unhealthy lifestyles which are initially compensated by increased β-cell secretory response so that glucose tolerance remains normal; Stage 3: during this time both β-cell function and insulin sensitivity deteriorate. Stage 4: in this stage as a result of further deterioration in the β-cell function and worsening of insulin sensitivity, fasting plasma glucose concentrations increase due to an increase in basal endogenous glucose production; and Stage 5: finally as a result of further deterioration in β -cell function, both fasting and postprandial glucose levels reach diabetic levels (Gerich and Smith 2003) (FIgure 2.3).

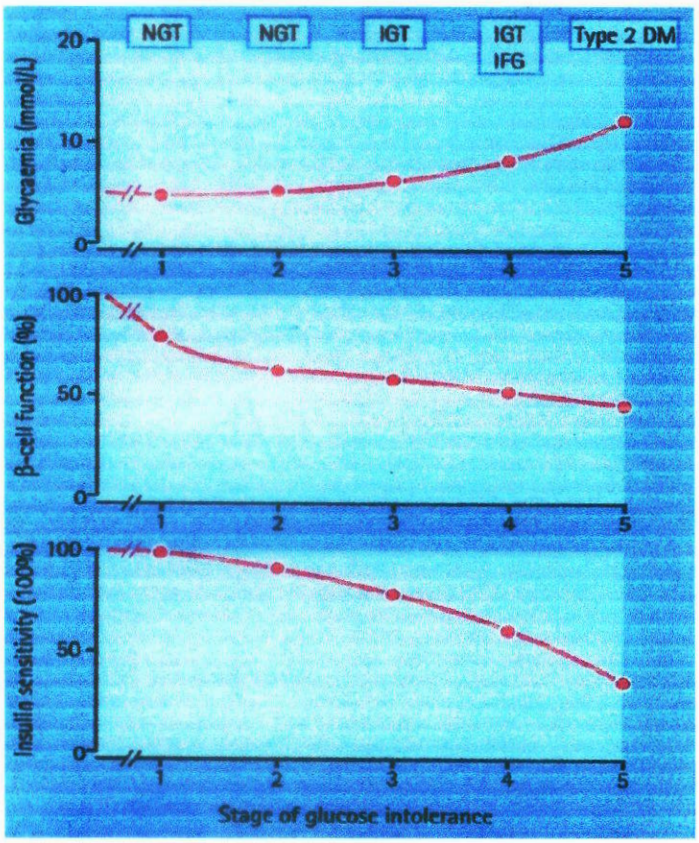


**Figure 2.2:** The stages of glucose tolerance and associated β cell function and insulin sensitivity in natural history of type 2 diabetes mellitus (DM) (Gerich and Smith 2003).

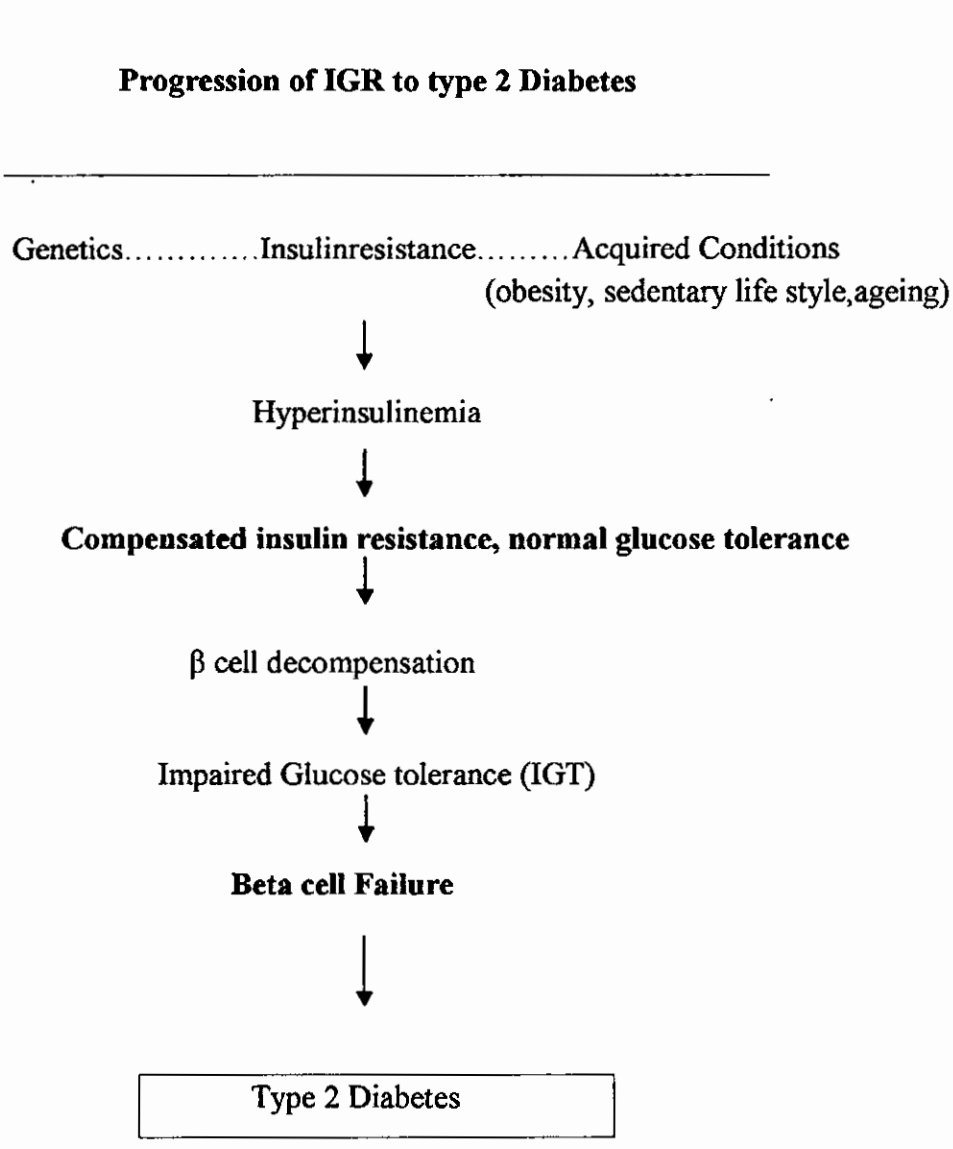


**Figure 2.3:** Progression of type 2 diabetes (Weyer *et al.*, 1999).

## 2.3 Vitamin D Structure and Metabolism

Vitamin D is a group of fat soluble vitamins calcium and phosphorus in the body (Walter *et al.*, 2003). Among the 5 types of vitamin D (vitamin D1, D2, D3, D4, D5); vitamin D2 (ergocalciferol) and D3 (cholecalciferol) are the most important and essential ones.

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 vitamin D, however, foods are after fortified with vitamin D. The "D" represents D2 or D3 (Figure2.2). Vitamin D2 is formed following exposure to the ultraviolet light of ergosterol from yeast, and vitamin D3 through the ultraviolet irradiation of 7-dehydrocholesterol from lanolin (Bouillon *et al.*, 2001; Holick 2004).

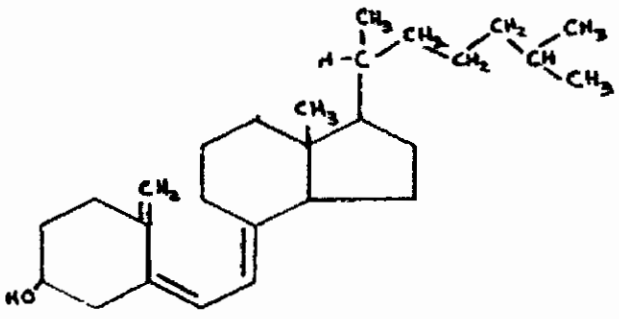

***Figure 2.4:*** *The Chemical Structure of Vitamin D*

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). Renal production of 1,25-dihydroxyvitamin D is tightly regulated by plasma parathyroid hormone and 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. Efficiency of the reabsorption of calcium in renal tubules and of intestinal calcium and phosphorus in the intestine is increased in the absorption of 1,25-dihydroxyvitamin D (Figure 2.2) ( 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).

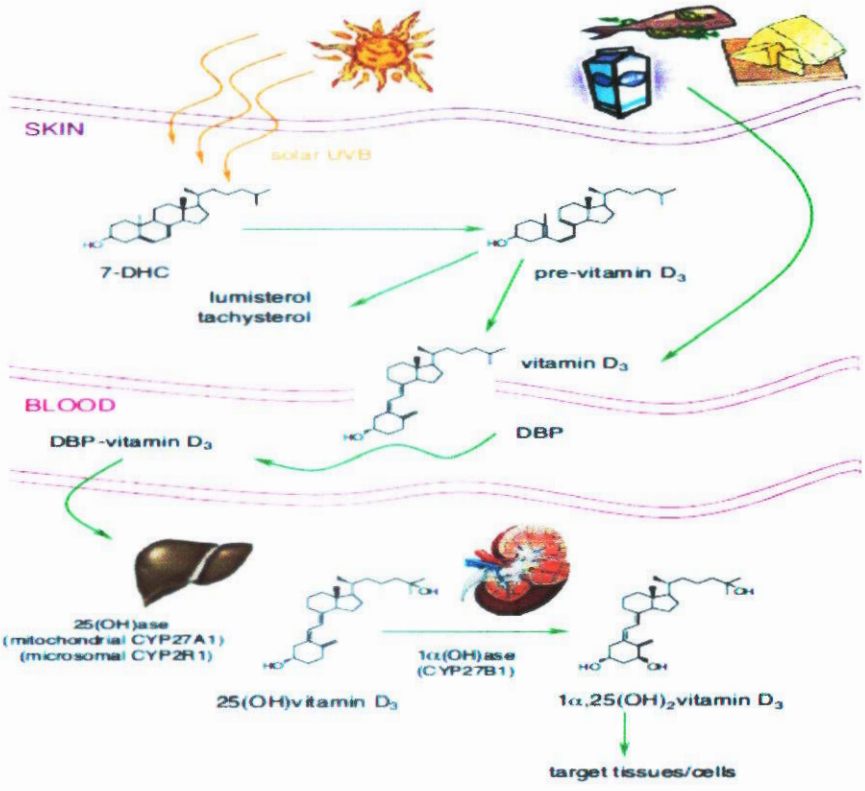


**Figure 2.5:** Synthesis and metabolism of 1α,25-dihydroxyvitamin D3. Vitamin D can be obtained from food (vitamin D2 and D3) or by photobiogenesis in the skin (vitamin D3). In the blood, all vitamin D metabolites are bound to vitamin D-binding protein (DBP). Vitamin D3 is converted by two successive hydroxylations in the liver (25-hydroxylases) and kidney (1α-hydroxylase) into its active hormonal form, 1,25(OH)2D3.

## 2.4 Function of Vitamin D3

Vitamin D3 maintains the blood calcium levels within a narrow range that is vital for normal functioni of the nervous system, as well as for bone growth, and maintenance of bone density. It 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 2) (DeLuca *et al.*, 2004; Heaney *et al.*, 2003).

Bischoff-Ferrari (2006) 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 (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 2) (Holick *et al.*, 2006). Osteoclasts dissolve the mineralized collagen matrix in bone, causing osteopenia, osteoporosis and rickets in children and osteomalacia in adults (Aaron *et al.*, 1974; Holick *et al.*, 2006) also increasing the risk of fracture (Chapuy *et al.*, 1997; Lips *et al.*, 2001). Vitamin D deficiency progresses, the parathyroid glands are maximally stimulated, causing secondary hyperparathyroidism (Malabanan *et al.*, 1998).

Brain, prostate, breast, and colon tissues, among others, as well as immune cells found to have a vitamin D receptor and respond to 1,25-dihydroxyvitamin D. (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 have indicated 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; Feskanich *et al.*, 2004). 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 odds

ratio 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 . 1,25-Dihydroxyvitamin D is also a potent immunomodulator (Penna *et al.*, 2005).

The active form of vitamin D has shown to play important role in insulin secretion under conditions of increased insulin demand (Zeitz *et al.*, 2003). Limited data in humans suggests that insufficient vitamin D levels may have an adverse effect on insulin secretion and glucose tolerance in T2DM (Borissova *et al.*, 2003; Orwoll *et al.*, 1994).

Vitamin D deficiency found to be associated with congestive heart failure (Zittermann *et al.*, 2006) and high 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) (Krause *et al.*, 1998). Vitamin D associated with [$1,25(OH)_2D_3$], has been shown to be inflammatory bowel disease (IBD) (Margherita *et al.*, 2004).

T1DM is an autoimmune disease characterized by the immune-mediated destruction of insulin-producing β-cells of islets of Langerhans in the pancreas. Several cellular effector mechanisms leading to β-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 found to accelerate the onset of T1DM (Giulietti *et al.*, 2004). In fact, when 1,25-$(OH)_2D_3$ was administrated at 3 wk of age before the onset of insulitis, it was found to 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).

Vitamin D3 supplement during pregnancy found to reduce the development of islet autoantibodies in offspring (Chiu *et al.*, 2004). Study involving 10,366 children in Finland were given 2000 IU of vitamin D3 per day during their first year of life and were followed for 31 years, the risk of T1DM was found to be reduced by approximately 80% (relative risk, 0.22; 95% CI, 0.05 to 0.89) (Hypponen *et al.*, 2001). Among children with vitamin D deficiency the risk was shown to be increased by approximately 200% (relative risk, 3.0; 95% CI, 1.0 to 9.0). Chiu et al (2004) have shown that vitamin D deficiency increased insulin resistance, decreased insulin production, and was associated with the metabolic syndrome. It was also observed that a combined daily intake of 1200 mg of calcium and 800 IU of vitamin D lowered the risk for development of T2DM by 33% (relative risk, 0.67; 95% CI, 0.49 to 0.90) as compared to a daily intake of less than 600 mg of calcium and less than 400 IU of 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-γ and stimulated with lipopolysaccharide in a dose-

dependent manner (Mattner *et al.*, 2000). 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 induces arthritis, which was shown 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). 1,25-$(OH)_2D_3$ 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. 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.*, 1999). 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).

In 2006 it was reported that 1 billion people worldwide have vitamin D deficiency or insufficiency. According to Lips et al (2006) 40 to 100% of U.S. and European elderly men and women still living in the community (not in nursing homes) are deficient in vitamin D, which causes osteoporosis in postmenopausal women. Children and young adults are also potentially at high risk for vitamin D deficiency.

Vitamin D deficiency, hypovitaminoses is characterized by inadequate mineralization or demineralization of the skeleton. Inadequate mineralization of the skeleton is the cause of rickets in children, while demineralization of the skeleton results in osteomalcia in

adults. Due to vitamin D deficiency, in children 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 (Lerch *et al.*, 2007), and in those with genetic disorders such as pseudovitamin D deficiency rickets (Zargar *et al.*, 2000). Osteoporosis is similar to ricket. However, this medical condition is seen as vitamin D deficiency symptoms in adults. Osteoporosis occurs due to lack of vitamin D metabolism leading to poor calcification in bones. It is characterized by proximal muscle weakness and bone fragility. The effects of osteomalacia are thought to contribute to chronic musculoskeletal pain (Straube *et al.*, 2009) there is no persuasive evidence of lower vitamin D status in chronic pain sufferers (Stewart *et al.*, 2009).

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; Adams *et al.*, 1997; Koutkia *et al.*, 2001). 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 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 (Adams *et al.*, 2006; Holick *et al.*, 2006).

## 2.5 Vitamin D receptor (VDR) gene

Vitamin D receptor (VDR) bind with cytosolic receptor and exerts rate limiting effort mediates the majority of the effects of vitamin D on gene expression via formation of a heterodimer with the retinoid X receptor which binds to promoter regions of many target genes. Vitamin D is mediated through a nuclear transcription factor known as the vitamin D receptor (VDR) (Sutton *et al.*, 2003). 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 (Figure2.5). 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).

The human VDR is a product of the single chromosomal gene that locates on chromosome 12 at 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).

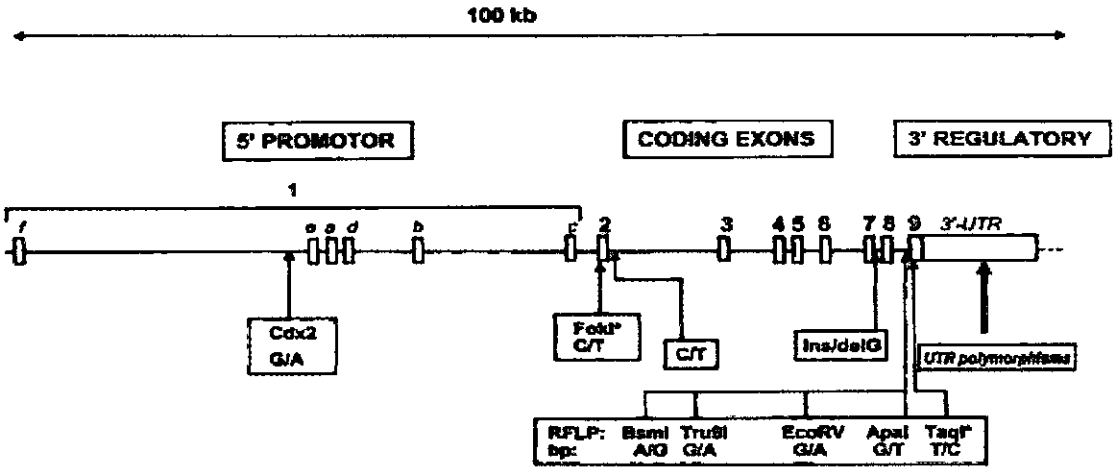


**Figure 2.6:** Exon–intron structure of the VDR gene (on chromosome 12q13.1) and position of known polymorphisms (Uitterlinden *et al.*, 2004).

**Table 2.2: VDR polymorphisms and their proposed disease links**

| **Polymorphism** | **Disease and proposed mechanism if known** |
|---|---|
| Cdx2 (G to A) | Decreased risk of fracture with A allele via increases VDR expression in the intestine increasing calcium transport protein 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 haplotypes and many polymorphisms) | Differences in mRNA expression, levels of serum markers such as osteocalcin<br>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 |

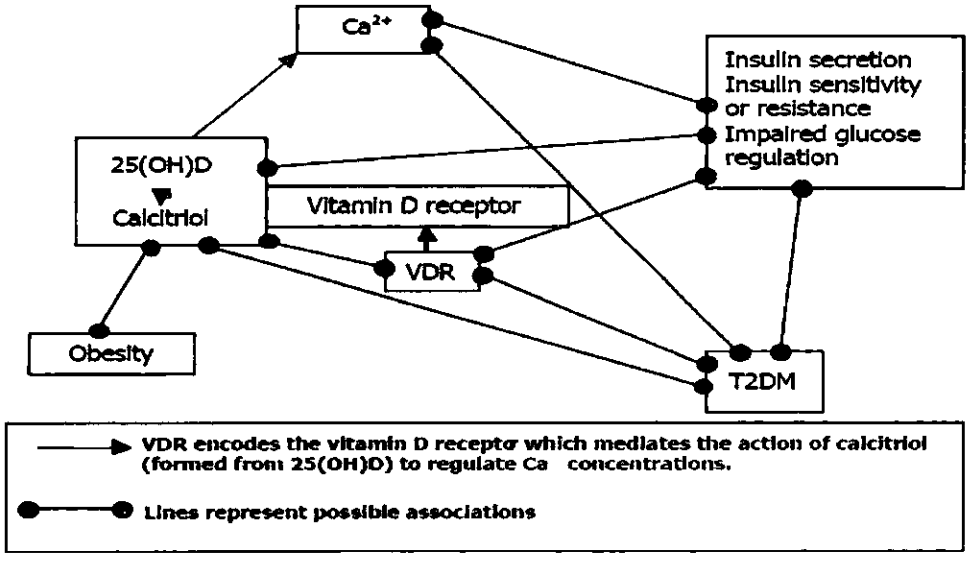


**Figure 2.7:** Possible associations of vitamin D and T2DM. *(DeLuca* et al., *2004).*

## 2.6 Vitamin D Receptor (VDR) gene variants

Six polymorphisms have been identified in the VDR locus (Figure 2.6) with a range of possible effects listed in Table 2.2 (Uitterlinden *et al.,* 2004). Number of observational studies have shown that both associations and lack of it between VDR polymorphisms and T2DM, fasting glucose, glucose intolerance, insulin sensitivity, insulin secretion and calcitriol levels. Possible mechanism postulated regarding the VDR gene polymorphisms and pathogenesis of T2DM still to be clearly under stood. However, schematic diagram (Figure 2.7) summarizes plausible mechanism suggestion in this respect (Uitterlinden *et al.,* 2004).

Four common allelic variants of the VDR gene have so far been identified and found to be exerting risk for T1D. 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.,* 2003). Vitamin D and its receptor complex as a transcription

factor play a regulatory role in β-cell insulin secretion (Lee *et al.*, 1994). The VDR is expressed in pancreatic β-cells (Johnson *et al.*, 1994).

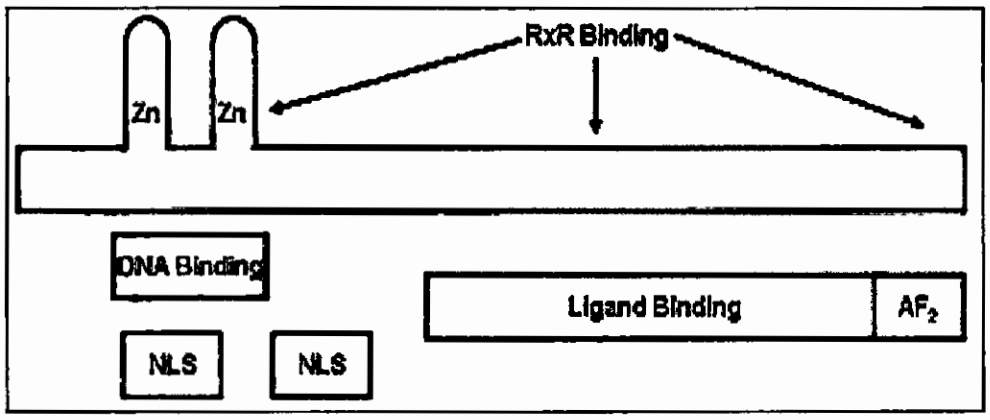


**Figure 2.8:** Model of the vitamin D receptor (VDR).

Variants of the nuclear vitamin D receptor (VDR) gene were found to be associated with T1D and thyroid autoimmunity disease. Pani et al (2002) have shown that the VDR genotype is associated with Addison's disease. They have been demonstrated the 'ff' (13.7% vs 5.5%; p=0.0243; odds ratio = 2.75) and the 'tt' (28.4% vs 14.1%; P=0.0043; odds ratio = 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 but in Caucasian populations. BsmI polymorphisms in the VDR gene were associated with Parkinson's disease in Koreans (Kim *et al.*, 2005).

Vitamin D deficiency has been shown to enhance the prevalence of T2DM and the replacement of vitamin D may increase the secretion of insulin (Lee *et al.*, 1994; Bayan *et al.*, 1997). This impairment of insulin secretion possibly 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, may also play a role. Data from VDR knockout mice were conflicting, with some investigation report of development of IGT (Zeitz *et*

*al.*, 2003) and others reporting no impairment in glucose metabolism (Mathieu *et al.*, 2001)

More convincing support for a beneficial effect of 1,25(OH)2D3 on β cell function has emerged from studies on islets isolated from normal animals in which insulin synthesis and release were provoked by glucose challenge in the presence of high doses of 1,25(OH)2D3 (d'Emden *et al.*, 1989).

Vitamin D receptor (VDR) polymorphism demonstrated to be associated with T1DM (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 T1D among Germans (Pani *et al.*, 2000). In a Taiwanese population, the AA genotype of ApaI polymorphism was fount to be associated with T1DM (Chang *et al.*, 2000). The aa genotype was found to be associated with defective insulin secretion in Bangladeshi Asians, a population at increased risk for T2DM (Gyapay 1994). Study involving 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). This study indicates that the Bsm1 polymorphism at the VDR gene locus is a risk factor of T1DM. In another study involving 152 T1DM Caucasian families, excessive transmission of Taq1 and Apa1 alleles alone were observed (Pani *et al.*, 2000). VDR polymorphism allele combinations found to influence. It was demonstrated in the Taiwanese population (Chang *et al.*, 2000). These two studies in two different populations indicate that VDR Bsm1 polymorphism 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

# SUBJECTS AND METHODS

## 3 SUBJECTS AND METHODS

### Place of the study

The study was conducted in the laboratory of Biomedical Research Group (BMRG), 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 April 2011 to April 2012.

### Study design

It was a cross-sectional study.

### Subjects

A total number of 122 (One hundred and twenty two) subjects were consecutively recruited in the study irrespectively of race, religion and socioeconomic status. Of the total (n=122) study subjects 68 were healthy Controls and 54 Impaired Glucose Tolerance (IGT). Glucose abnormality was diagnosed following WHO criteria (WHO1999).

### Collection of the subjects

Subjects recruited through personal communication from the community. Recruitment of subjects was done in Dhaka and Khulna. Subjects willing to have blood glucose check were invited to volunteer the study. Purpose and method of the study was described to each individual and informed consent was obtained. They were advised to be on their usual diet for 3 days prior to the blood test. For those living in Dhaka city were requested to report BMRG Lab in the morning between 8.00-10.00 am in overnight fasting state (8-10 hours). Those living in Khulna were requested to report Ideal Nursing Home, Gollamari, Khulna, in fasting state (8-10 hrs over night) at the same time. Detailed medical and clinical histories were recorded on pre-design case record form (Appendix I).

### Inclusion criteria

- Healthy adult subjects with age range 30 to 55 yrs.
- Willing to volunteer

**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.
- Pregnancy

**Anthropometric measurements**

***Height (m)***

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

***Weight (kg)***

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

*Calculation of BMI ($kg/m^2$)*

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

*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.

**Measurement of blood pressure**

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

## 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. Subjects were then given glucose to drink (75g Dextrose in 300 ml of water). They were asked not to take any food and be rested for two hours. The second blood sample was obtained after two hours.

An aliquot of blood (5 ml) sample was taken into a EDTA (1 mg/ml) containing tube, mixed thoroughly and preserved in at -30°C for future DNA extraction. Fasting and postprandial blood sample were taken into plain tube allowed to clot for 30 minutes and serum was separated by centrifugation for 10 min at 3000 rpm and preserved at -25°C for further biochemical analyses.

Samples collected in Khulna were preserved at -80°C in the Dept of Fisheries and Marine Technology and transported BIRDEM, Dhaka at cool condition maintained by using icepack.

### *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 reagent were taken in specific cup. They 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***

The 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).

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

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

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

***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. They were arranged serially. Then ID number for each test was entered in the Auto analyzer. 5 µl sample and 500 µl reagent were mixed and incubated at $37^0$C for 5 minutes within the Auto lab. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

***Calculation of result***

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

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

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

***Calculation of result***

Triglyceride concentration was calculated by following formula:

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

**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 10, 20, 40, 50, and 100 µl. The final volume was 200 µl.

***Assay Procedure***

1. 100 ml serum samples 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$^0$C 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 was calculated extrapolating standard four parameter logistic curve 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***

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

**Estimation of insulin**

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

***Principle***

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

*Reagent preparation*

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

*Storage and Stability*

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

*Assay Procedure*

1. The reagents were allowed to come to room temperature. Then each well filled with 300µl of diluted HRP wash buffer and incubated at room temperature for 5 minutes. Then wash buffer was decanted and the residual amount was removed from all wells by inverting the plate and tapping it smartly onto the absorbent towels several times.
2. The microtitre plate was marked. A layout of the plate for blank, standards, QCs and unknown samples were made and recorded on a paper.
3. 20 µl assay buffer was added to the NSB (Non Specific Binding) wells and each of the sample wells.
4. 20 µl matrix solution was added to the NSB, Standard and Control wells.
5. 20 µl human insulin standard in the order of ascending concentration was added to the appropriate wells.
6. 20 µl of QC 1 and QC 2 was added to the appropriate wells.
7. Then 20 µl of unknown samples were added sequentially in wells according to the plate layout.
8. After that 20 µl of detection antibody was added to all wells. All procedures were performed within 30 minutes. Plates were covered using plate sealer and incubated at room temperature for 1 hour on an orbital microtitre plate shaker to rotate at moderate speed approximately 400 rpm.
9. At the end of incubation, plate sealer was removed and solutions were decanted. Residual solutions were removed by tapping as before. Each wells was then washed for 3 times with 300 µl diluted HRP wash buffer. After each wash the plate was tapped on a pad of tissue to dry it off.

10. 100 µl of enzyme solution was added to each well using a multichannel pipette. Plates were covered with sealer and incubated again with moderate shaking at room temperature for 30 minutes on microtitre plate shaker.

11. Then sealer was removed and solutions were decanted from the plate and tapped to remove the residual fluid. Then wells were washed for 5 times using 300 µl HRP wash buffer per well per wash and after each wash the plate was tapped smartly to remove the residual buffer.

12. 100 µl of substrate solution was added to each well and covered with sealer and incubated with moderate shaking at room temperature for 10 minutes on microtitre plate shaker. Blue color was formed in the wells of insulin standards with intensity proportional to increasing concentrations of insulin.

13. Finally sealer was removed and 100µl of stop solution was added. Plate was placed on a microtitre plate shaker for gentle shaking (400 rpm).

14. Reading was taken using a microplate reader at 450 nm.

***Calculation of results for unknown samples***

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

**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 extraction**

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
- Wash Buffer 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 and buffer solution mixed by swirling of the flasks. It was then heated to boiling point in a microwave oven with intermittent mixing until agarose was properly boiled. The gel was cooled nearer to the gelling point and ethidium bromide (0.5µg/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.

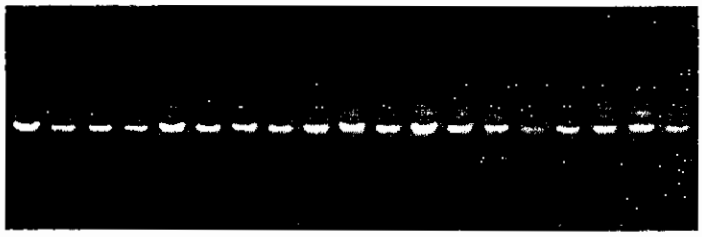

**Figure 3.1:** Gel image of DNA electrophoresis to check the extraction yield

**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 three polymorphic markers were determined by different PCR amplifications and restriction enzyme digestion.

The DNA segment containing A>G (Bsm1 restriction) polymorphic marker was amplified using the following primer set:

Forward primer: 5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′

Reverse primer: 5′-AAC CAG CGG GAA GAG GTC AAG GG -3′

PCR was carried out in 10 µl reaction volume. Product size for the above mentioned primer set is 825 bp. The PCR protocol is as follows:

| Name of the component | Volume (µl) | Concentration |
|---|---|---|
| DNA | 3.50 µl | 10-50 ng/ml |
| Buffer | 1.00 µl | 10x |
| dNTPs | 0.10 µl | 200 µmol |
| Forward Primer | 0.50 µl | 100 µmol |
| Reverse Primer | 0.50 µl | 100 µmol |
| HotStart Taq DNA polymerase | 0.08 µl | 5 U/µl |
| $ddH_2O$ | 4.32 µl | |
| **Total** | **10.0 µl** | |

**PCR conditions**

PCR was carried out using HotStart Taq polymerase. 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 the 35$^{th}$ cycle was followed by a step of final elongation 72°C for 10 minutes.

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 product**

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 3.2:** 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)** | **Concentration** |
|---|---|---|
| PCR product | 4.000 µl | |
| Buffer | 1.500 µl | 10 x |
| Restriction enzyme | 0.160 µl | 50 U/µl |
| BSA | 0.200 µl | 10µg/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.

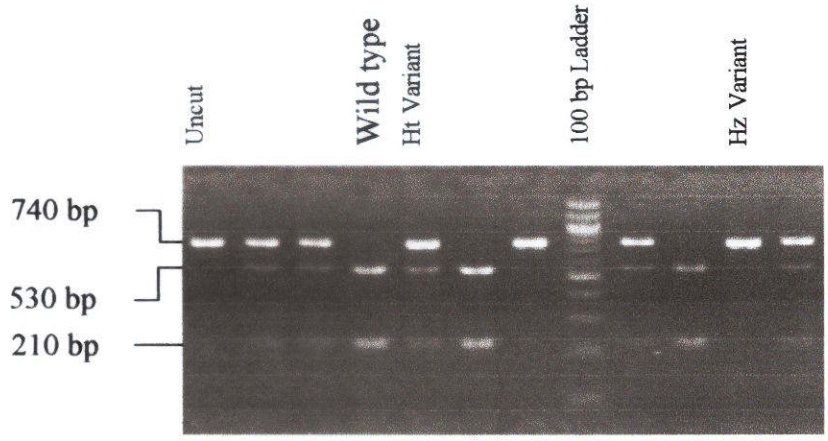


**Figure 3.3:** Agarose gel image of VDR gene Apa1 G>T candidate marker analysis by Apa1 restriction enzyme digestion

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)** | **Concentration** |
|---|---|---|
| PCR product | 4.000 µl | |
| Buffer | 1.500 µl | 10 x |
| Restriction enzyme | 0.250 µl | 20 U/µl |
| BSA | 0.200µl | 10µg/µl |
| 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.

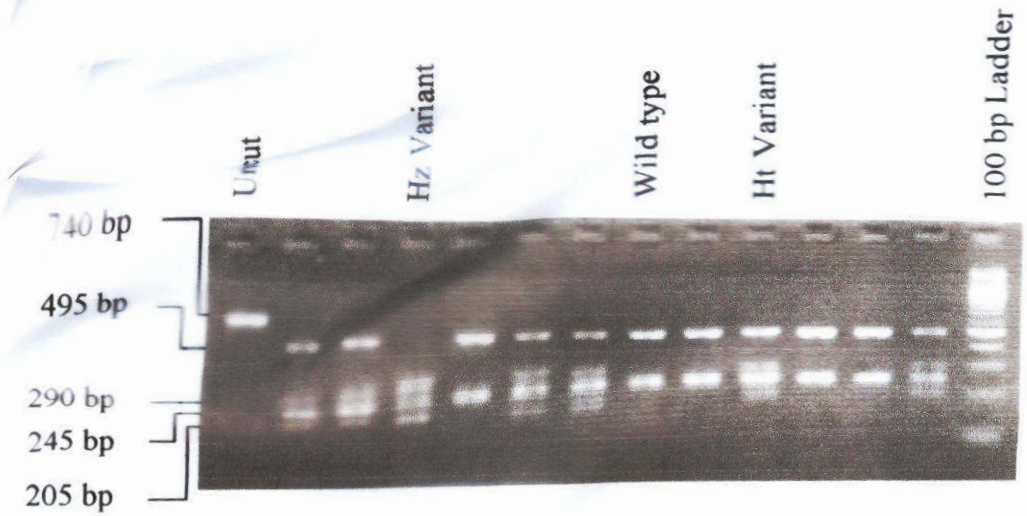


**Figure 3.4:** Agarose gel image of VDR gene Taq1 T>C candidate marker analysis by Taq1 restriction enzyme digestion.

**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.

# RESULTS

## 4 RESULTS

A total number of 122 unrelated subjects were screened for glycemic status. Of them 68 were healthy controls and 54 were Impaired Glucose Tolerance (IGT). In control group male and female distribution was 40 (58.8%) and 28 (41.2%) respectively. In IGT group the distribution was 27 (50.0%) and 27 (50.0%) respectively.

### 4.1 Age, BMI and blood pressure of the study subjects

Mean (±SD) age (yrs) of the control and IGT subjects was 41.8± 5.4 and 42.3± 6.2 respectively which did not show statistical significant difference (p=0.62) (Table 4.1).

Mean (±SD) body mass index (BMI) of the control and IGT subjects was 24.7± 4.1 and 24.9± 4.4 respectively which did not show statistical significant difference (p=0.73) (Table 4.1).

Mean (±SD) systolic blood pressure (SBP, mmHg) of the control and IGT subjects was 113±13 and 115±15 respectively which did not show statistical significant difference (p=0.40) (Table 4.1).

Mean (±SD) diastolic blood pressure (DBP, mmHg) of the control and IGT subjects was 75±9 and 75±11 respectively which did not show statistical significant difference (p=0.88) (Table 4.1).

Mean (±SD) waist to hip ratio (WHR) of the control and IGT subjects was 0.91±6.02and 0.93±0.07 respectively. which did not show statistical significant difference (p=0.20(Table 4.1).

### 4.2 Serum glucose levels and insulinemic status of the study subjects

Mean (±SD) fasting serum glucose (FSG, mmol/l) of the control and IGT subjects was 5.0± 0.4 and 5.4 ± 0.65 respectively. Mean (±SD) 2 hour glucose (PPG, mmol/l) of control and IGT subjects was 6.0 ± 1.1 and 9.3 ± 0.87 respectively (Table 4.2).

Mean (±SD) fasting serum insulin (μU/ml) in the control and IGT subjects was 9.8± 3.1 and 12.7± 5.12 respectively. Mean serum insulin in the IGT group was significantly higher compared to the Controls ($p<0.001$) (Table 4.2).

Mean (±SD) of HOMA%S in the Control and IGT subjects was 88.3± 43 and 68.2± 26 respectively. Mean HOMA%S values in IGT group was significantly much higher compared to the Control ($p<0.003$) group (Table 4.2).

Mean (±SD) HOMA% B values in the control and IGT subjects was 109 ± 26 and 118± 44 respectively. Mean HOMA%B value in IGT group did not show significant difference compared to the controls (p=0.58) (Table 4.2).

### 4.3 Lipid levels of study subjects

Mean (±SD) serum triglycerides (TG, mg/dl) of the control and IGT subjects was 125 ± 54 and 172 ± 69 respectively, TG value in IGT group was statistical significant difference higher compared to the controls (p<0.001) (Table 4.3).

Mean (±SD) serum total cholesterol (mg/dl) of the control and IGT subjects was 192 ± 32 and 195 ± 43 respectively which did not show significant difference (p=0.69) (Table 4.3).

Mean (±SD) high density lipoprotein cholesterol (HDL-c, mg/dl) of the control and IGT subjects was 34.5± 10 and 30 ± 10 respectively HDL-c level was significantly lower compared to the controls (p=0.02) (Table 4.3).

Mean (±SD) low density lipoprotein cholesterol (LDL-c, mg/dl) of the control and IGT subjects were 132 ± 34 and 130± 41 respectively which did not show statistical significant difference (p=0.76) (Table 4.3).

**Table 4.1: Age, BMI and blood pressure of the study subjects**

| **Variables** | **Control** (n=68) | **IGT** (n=54) | *t/p values* **Control vs IGT** |
|---|---|---|---|
| **Age** (yrs) | 41.8± 5.4 | 42.3± 6.2 | 0.49/0.62 |
| **BMI** ($kg/m^2$) | 24.7± 4.1 | 24.9± 4.4 | 0.33/0 .73 |
| **SBP** (mmHg) | 113± 13 | 115± 15 | 0.83/0.40 |
| **DBP** (mmHg) | 75± 9 | 75±11 | 0.16/0.88 |
| **WHR** | 0.91±6.02 | 0.93±0.07 | 1.26/ 0.20 |

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, WHR , Waist Hip Ratio; IGT, Impaired Glucose Tolerance.

**Table 4.2: Glycemic and insulinemic status of the study subjects**

| Variables | Control (n=68) | IGT (n=54) | *t/p values* Control vs IGT |
|---|---|---|---|
| **FSG** (mmol/l) | 5.0± 0.4 | 5.4 ± 0.65 | |
| **PPG** (mmol/l) | 6.0 ± 1.1 | 9.3 ± 0.87 | |
| **Insulin** (μU/ml)* | 9.8± 3.1 | 12.7± 5.12 | 3.80 /<0.001 |
| **HOMA % S*** | 88.3± 43 | 68.2± 26 | 3.03 /<0.003 |
| **HOMA % B*** | 109 ± 26 | 118± 44 | 1.42 /0.58 |

Results were expressed as mean±SD.

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

FSG, Fasting Serum Glucose; PPG, Postprandial Glucose; HOMA%S, Insulin sensitivity assessed by Homeostatic Model Assessment; HOMA%B, B-cell function assessed by Homeostatic Model Assessment; IGT, Impaired Glucose Tolerance.

**Table 4.3: Lipid levels of the study subjects**

| Variables | Control (n=68) | IGT (n=54) | *t/p values* Control vs IGT |
|---|---|---|---|
| **TG** (mg/dl) | 125 ± 54 | 172 ± 69 | 4.26 /<0.001 |
| **T Chol** (mg/dl) | 192 ± 32 | 195 ± 43 | 0.39 /0 .69 |
| **HDL-c** (mg/dl) | 34.5± 10 | 30 ± 10 | 2.31 /0 .02 |
| **LDL-c** (mg/dl) | 132 ± 34 | 130± 41 | 0.30 / 0.76 |

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, IGT, Impaired Glucose Tolerance.

## 4.4 CANDIDATE GENE MARKER ANALYSES

### 4.4.1 VDR gene G>T (Apa1 restriction) variant

Genotype frequencies of the VDR gene G>T variants were 0.185, 0.537 and 0.278 for wild type, heterozygous (Ht) variant and homozygous (Hz) variant respectively in the IGT group. In the controls, the frequencies were 0.176, 0.618 and 0.206respectively. This frequency distribution did not show statistical significant association ($\chi^2$= *1.003*; p= *p=0.606*) (Table 4.4i). Hardy-Weinberg distribution in the IGT group ($\chi$2=0.375, p=0.540) as well as Control ($\chi$2=0.3.799; p=0.51) found to be in equilibrium.

When heterozygous and homozygous variant genotypes were grouped together, the distribution was as follows: IGT 0.185 and 0.815 for wild and variant genotype respectively; Control 0.176 and 0.824 respectively. This distribution showed statistical significant association ($\chi^2$= 0.015 p= 0.901) for the genotype frequencies (Table 4.4ii).

Allele frequency of VDR gene Apa1 the distribution was as follows: Control G and T allele frequency 0.485 and 0.515 respectively; IGT 0.471 and 0.529 respectively. (Table 4.4iii)

**Table 4.4i: VDR gene Apa1 G>T genotype of the study subjects**

| **VDR Apa1 G>T genotype** | **Control** (n= 68) | **IGT**(n= 54) | **Total** (n= 122) |
|---|---|---|---|
| **Wild (GG)** | 0.176 (12) | 0.185 (10) | 0.180 (22) |
| **Ht variant (GT)** | 0.618 (42) | 0.537(29) | 0.582(71) |
| **Hz variant (TT)** | 0.206 (14) | 0.278 (15) | 0.238 (29) |
| | $\chi^2$= *1.003* | *p=0.606* | |

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; IGT, Impaired Glucose Tolerance.

**Table 4.4ii: VDR gene Apa1 G>T genotype (Ht and Hz variant together) of the study subjects**

| VDR Apa1 G>T genotype | Control (n=68) | IGT (n= 54) | Total (n= 122) |
|---|---|---|---|
| **Wild GG)** | 0.176 (12) | 0.185 (10) | 0.18 (22) |
| **Variant (GT and TT)** | 0.824 (56) | 0.815 (44) | 0.82 (100) |
| | $\chi^2$= 0.015 | p= 0.901 | |

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical association.

**Table 4.4iii: VDR gene Apa1 Allele Frequency of the study subjects**

| VDR Apa1 Allele | Control (n=68) | IGT (n= 54) | Total (n= 122) |
|---|---|---|---|
| **Allele G** | 0.485 | 0.471 | 0.471 |
| **Allele T** | 0.515 | 0.529 | 0.529 |

### 4.4.2VDR gene T>C (Taq1 restriction) variant

Genotype frequencies of the VDR gene T>C variants were 0.444, 0.426 and 0.130 for wild type, heterozygous (Ht) variant and homozygous (Hz) variant respectively in the IGT group. In the controls, the frequencies were 0.500, 0.456 and 0.044 respectively. This frequency distribution did not show statistical significant association ($\chi^2$= *2.942*; *p=0.230*) (Table 4.5i). Hardy-Weinberg distribution IGT group ($\chi$2=0.160, p=0.689) as well as in the Control ($\chi$2=1.550; p=0.213) found to be in equilibrium.

When heterozygous and homozygous variant genotypes were grouped together, the distribution was as follows: IGT 0.444and 0.556 for wild and variant genotype respectively; Control 0.500 and 0.500 respectively. This distribution also did not show significant association ($\chi^2$= 0.372; p= p=0.542) for the genotype frequencies (Table 4.5ii).

Allele frequency of VDR gene Taq1 the distribution was as follows: Control T and C allele frequency 0.728 and 0.272 respectively; IGT 0.657 and 0.343 respectively. (Table 4.5iii)

**Table 4.5i: VDR gene Taq1 T>C genotype of the study subjects**

| **VDR Taq1 T>C genotype** | **Control** (n= 68) | **IGT** (n= 54) | **Total** (n= 122) |
|---|---|---|---|
| **Wild (TT)** | 0.500 (34) | 0.444(24) | 0.475 (58) |
| **Ht variant (TC)** | 0.456 (31) | 0.426 (23) | 0.443 (54) |
| **Hz variant (CC)** | 0.044 (3) | 0.130 (7) | 0.082 (10) |
| | *$\chi^2$= 2.942* | *p=0.230* | |

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; IGT, Impaired Glucose Tolerance

**Table 4.5ii: VDR gene Taq1 T>C genotype (Ht and Hz variant together) of the study subjects**

| **VDR Taq1 T>C genotype** | **Control** (n= 68) | **IGT** (n= 54) | **Total** (n= 122) |
|---|---|---|---|
| **Wild (TT)** | 0.500 (34) | 0.444 (24) | 0.475 (58) |
| **Variant (TC and CC)** | 0.500 (34) | 0.556 (30) | 0.525(64) |
| | $\chi^2$= 0.372 | p=0.542 | |

Results were expressed as frequency (number).

Chi-square test was performed to calculate statistical association.

**Table 4.5iii: VDR gene Taq1 Allele Frequency of the study subjects**

| **VDR Apa1 Allele** | **Control** (n=68) | **IGT** (n= 54) | **Total** (n= 122) |
|---|---|---|---|
| **Allele T** | 0.728 | 0.657 | 0.697 |
| **Allele C** | 0.272 | 0.343 | 0.303 |

### 4.5 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 was found to be almost similar in the control (5.22±0.4 and 5.03±0.4 respectively; p=0.13) as well as in IGT subjects (5.33±0.54 and 5.43±0.68 respectively; p=0.680) (Table 4.6).

Mean (±SD) postprandial glucose levels in subjects with wild and variant (heterozygous and homozygous together) genotype was similar in the control (6.33±1.9 and 5.91±1.1 respectively; p=0.24). The IGT subjects with variant genotype (heterozygous and homozygous together) had significantly lower glucose (9.4±0.86 and 9.18±0.85 respectively; p=0.042) (Table 4.6).

Mean (±SD) insulin levels in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (10.13±3.2 and 9.8±1.1 respectively; p=0.72) as well as in IGT subjects (13.1±4.5 and 12.6±5.3 respectively; p=0.772) (Table 4.6).

Mean (±SD) HOMA%B in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (106±27.9 and 110±25 respectively; p=0.66) as well as in IGT subjects (128±51 and 116±43 respectively; p=0.459) (Table 4.6).

Mean (±SD) HOMA%S in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (81.3±21.2 and 89.7±46 respectively; p=0.54) as well as in IGT subjects (63.2±21 and 69.26±26.8 respectively; p=0.507) (Table 4.6).

The Odd Ratio was calculated for Apa1 (G>T) polymorphism in case of wild and variant genotypes which was 0.58 with CI of 95% from 0.25 to 1.37 (p=0.22).It was not significant.

**Table 4.6: Glycemic and insulinemic status of the study subjects on the basis of Apa1 G>T genotype**

| | FSG (mmol/l) | PPG (mmol/l) | F Insulin (pmol/l)* | HOMA%B* | HOMA%S* |
|---|---|---|---|---|---|
| ***Control subjects*** | | | | | |
| **Wild** (n=12) | 5.22±0.4 | 6.33±1.9 | 10.13±3.2 | 106±28 | 81.3±21 |
| **Variant** (n=56) | 5.03±0.4 | 5.91± 1.1 | 9.8±1.1 | 110±26 | 89.7± 46 |
| *t/p values* | *1.53/0.13* | *1.16 /0.24* | *0.34/0.72* | *0.43/0.66* | *0.61/0.54* |
| ***IGT subjects*** | | | | | |
| **Wild** (n=10) | 5.33± 0.54 | 9.40± 0.86 | 13.12±4.5 | 128±51 | 63.23±21 |
| **Variant** (n=44) | 5.43± 0.68 | 9.18± 0.85 | 12.59±5.3 | 116± 43 | 69.26± 27 |
| *t/p values* | *0.42/0.680* | *2.08/0.042* | *0.29/0.772* | *0.74/0.459* | *0.668/0.507* |

Results were expressed as mean±SD.

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

### 4.6 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 was found to be almost similar in the control (5.0±0.43) and 5.1±0.38 respectively; p=0.427) as well as in IGT subjects (5.5±0.54 and 5.4±0.74) respectively; p=0.63) (Table 4.7).

Mean (±SD) postprandial glucose levels in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (5.9±1.7 and 6.01±1.1) respectively; p=0.825) as well as in IGT subjects (9.44± 0.86 and 9.18±0.83 respectively; p=0.276) (Table 4.7).

Mean (±SD) insulin levels in subjects with wild and variant (heterozygous and homozygous together) genotype was found in the control (10.2±2.9 and 9.5±3.2 respectively; p=0.388) as well as in IGT subjects (12.9±5.7 and 12.6±4.7 respectively; p=0.81) (Table 4.7).

Mean (±SD) HOMA%B in subjects with wild and variant (heterozygous and homozygous together) genotype was found in the control (113±24.02 and 106±27.1 respectively; p=0.266) as well as in IGT subjects (12.5±52.0 and 119±37 respectively; p=0.93) (Table 4.7).

Mean (±SD) HOMA%S in subjects with wild and variant (heterozygous and homozygous together) genotype was found to be almost similar in the control (83.2±23.7 and 93.3±56.1 respectively; p=0.337) as well as in IGT subjects (67.2±24.6 and 68.9±26.9) respectively; p=0.80) (Table 4.7).

The Odd Ratio was calculated for Taq1 (T>C) polymorphism in case of wild and variant genotypes which was 1.25 with CI of 95% from 0.61 to 2.56 (p=0.54).It was not significant.

**Table 4.7: Glycemic and insulinemic status of the study subjects on the basis of Taq1 T>C genotype**

| | FSG (mmol/l) | PPG (mmol/l) | F Insulin (pmol/l)* | HOMA%B* | HOMA%S* | IR* |
|---|---|---|---|---|---|---|
| ***Control subjects*** | | | | | | |
| **Wild** (n=34) | 5.0±0.43 | 5.95±1.7 | 10.2±2.9 | 113±24 | 83.20±24 | 1.3±0.3 |
| **Variant** (n=34) | 5.1±0.38 | 6.01±1.1 | 9.5±3.2 | 106±27 | 93.31±56 | 1.6±1.3 |
| *t/p values* | *0.800/0.427* | *0.222/0.825* | *0.870/0.388* | *1.121/0.266* | *0.968/0.337* | *0.855/0.398* |
| ***IGT subjects*** | | | | | | |
| **Wild** (n=24) | 5.5± 0.54 | 9.44± 0.86 | 12.9±5.7 | 12.5±52 | 67.2±25 | |
| **Variant** (n=30) | 5.4± 0.74 | 9.18± 0.83 | 12.6±4.7 | 119±37.27 | 68.9±27 | |
| *t/p values* | *0.48/0.63* | *1.10/0.276* | *0.247/0.81* | *0.088/0.93* | *0.253/0.80* | |

Results were expressed as mean±SD.

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

# DISCUSSION

## 5 DISCUSSION

Diabetes mellitus has become a major health problem all over the world. About 90-95% of the total diabetic patients are of type 2 variety (WHO 1999). Both insulin resistance and insulin secretory defect have been implicated in its pathogenesis. Vitamin D has been suggested to play important role in the pathogenesis of T2DM (Chiu *et al*, 2004; Scagg et al, 2004). Study involving Bangladeshi Asians living in East London, UK revealed that those at risk for T2DM had lower vitamin D level compared to subjects without risk (Boucher *et al.*, 1995). Polymorphisms in VDR gene, however, found to be associated with insulin secretion (Hitman *et al.*, 1998; Ongunkolade *et al.*, 2002), insulin sensitivity (Chiu *et al.*, 2001), fasting glucose of normal and T2DM (Oh and Barrett-Cornor 2002; Ortlepp *et al.*, 2003).

In the natural history of diabetes, in particular T2DM variety, prediabetes (IFG and IGT) has been recognized as the intermediate state (WHO and ADA, 2002). Like that of T2DM the prediabetes is found to be increasing fast all over the world. The prevalence of T2DM in Bangladesh has been increased from 4% to 8% to over the decades (Sayeed *et al.* 2007). IGT is also shown to increase fast in this population. Recently, Sayeed and his group have shown that in a rural population prevalence of IGT was 11% (Sayeed *et al* 2007). This trend also has been reflected among normal to overweight adult subjects living in the Dhaka city. A study has revealed that 30% of subjects with normal to over weight found to have IGT on screening (unpublished data). T2DM in the Bangladeshi population found to show both insulin resistance and secretory defect (Al- Mahmood, 2000; Junaid MM 2000). However, the exact pathophisiological mechanism in the development of IGT is still to be clearly understood. Since VDR gene polymorphisms have been linked to the pancreatic B insulin secretory status of normal and abnormal glucose tolerance state, in the present study VDR gene Apa1 (G>T) and Taq1 (T>C) polymorphic markers were studied in the IGT subjects of Bangladeshi origin to explore its association with the phenotype and explore its effect in their glycemic and insulinemic status.

A Total number of 54 IGT and 68 Control subjects were included in the study. The male-female proportion in Control group (58.8% and 41.2%) and in IGT group (50.0% and 50%). The study subjects in the present study were age and BMI matched (Table 4.1). The WHR of the IGT group also did not show any significant difference compared to the controls (p=0.20) (Table 4.1). IGT subjects had higher (mean±SD) circulatory level of insulin and determination of HOMA%S revealed significantly lower sensitively of these subjects (Table 4.2). The finding, however, is consistent with previous report (Rahman *et al.*, 2010; Shefin *et al.*, 2008).

Dyslipidemia is more common in IGT subjects than glycemic control group. In the present study, higher level of serum total cholesterol and triglyceride ($p<0.001$ both) in IGT (Table 4.3) also supported the issue.

The present study investigates the association of VDR gene Apa1 (G>T) and Taq1 (T>C) polymorphism in the pathogenesis of IGT of Bangladeshi origin. The genotypes frequencies of Apa1 (G>T) polymorphism, in the controls were 0.176, 0.618, 0.206 (wild, heterozygous and homozygous variant) and in the IGT were 0.185, 0.537, 0.278 respectively which did not show any significant association ($\chi^2= 1.003$, p=0.606) (Table 4.4.1i). The genotype frequencies of VDR Apa1 (G>T) polymorphic allele in the control subjects found to be consistent the healthy subjects with previous report [0.183, 0.516 and 0.301] (Paul 2010). It is appeared that genotype frequency of Apa 1 (G>T) polymorphic alleles are more frequent in this populations. A study involving 500 women of Europoid origin showed frequencies to be 0.258 and 0.742 (wild and heterozygous respectively) (McCullough *et al.*, 2007). Among the Japa.nese Apa1 genotype frequencies were found to be 0.475, 0.413 and 0.113 respectively (Naito *et al.*, 2007). It suggests that Bangladeshi population might have relatively different VDR Apa1 (G>T) genotype than that of Caucasoid and Japanese. The differences in the Apa1 (G>T) frequency might be attributed in the smaller number of subjects recruited in the present study. The facts may to be substantiated by increasing the number of subjects and complete the study.

The genotypes frequencies of Taq1 (T>C) polymorphism, in the controls were 0.500, 0.456, 0.044 (wild, heterozygous and homozygous variant respectively) and in the IGT group were 0.444, 0.426, 0.130 respectively which did not show statistical association ($\chi^2$=2.942, p=0.230) (Table 4.4.2i). The genotype frequencies of Taq1 (T>C) polymorphic marker in the controls in this study was found to be consistent with healthy subjects of a previous report (0.370, 0.522 and 0.109) (Paul, 2010). Taq1 (T>C) polymorphic allele frequencies were 0.491, 0.399 and 0.110 among the North Indians (Mittal *et al.*, 2007). Among the French population the frequencies were 0.424, 0.444 and 0.132 respectively (Taverna *et al.*, 2002). It is appeared that VDR Taq1 (T>C) polymorphic allele frequencies found among people of Bangladeshi origin are consistent with that of the other populations.

Fasting and 2hour glucose and insulinemic status were evaluated in the study subjects (both Controls and IGT) on the basis of their genotypes of VDR Apa1 (G>T) and Taq1 (T>C) genotypes. When Apa1 (G>T) allele was considered in the control subjects no difference was observed between those with will 'GG' allele compared to the counterpart with 'GT and TT' alleles for fasting and 2hour glucose and insulinemic status. The IGT subjects with variant 'GT and TT' alleles had significantly lower 2hour glucose compared to those with 'GG' allele (p=0.042). However, their mean (±SD) insulin level did not show significant difference compared to those with wild genotype. It may be argued that mean difference of 2hour glucose value between those with wild and 'GG' and variant 'GT and TT' genotype was very small. To conclusively comment on this issue the observations need to be reconfirmed by increasing the number of study subjects ie, the power of the study. When Taq1 (T>C) Polymorphic marker was considered subjects with wild 'TT' and variant 'TC and CC' alleles did not show any statistical difference regarding fasting and 2h glucose and insulinemic status in both Controls and IGT groups.

From the above discussion, it can be concluded that

i) VDR gene Ap1 (G>T) and Taq1 (T>C) polymorphic alleles are not associated with IGT of Bangladeshi origin.

ii) The polymorphic marker alleles did not have effect on fasting and two hour blood glucose and insulinemic status of the IGT and Controls.

iii) The Study reconfirmed that lower insulin sensitivity is prominently present in the IGT subjects of Bangladeshi origin.

# LIMITATIONS AND RECOMMENDATIONS

## LIMITATIONS AND RECOMMENDATIONS

- Vitamin D was not measured in this study and the number of subjects was small.
- Based on these preliminary data a study involving statistically appropriate number of subjects and determination of their vitamin D and insulin level suggested to be producing interesting data to understand effect of VDR gene polymorphism in the pathogenesis prediabetes.

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## 7 REFERENCES


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# APPENDIX

# Appendix I
# Data Collection Sheet

MS Thesis

**1. Code No:**

**2. Particulars of the volunteer:**

i) Name: _____

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

iii) Mother's Name: _____

iv) (a) Age: _____ years (b) DOB:

v) Sex: Male / Female

vi) Address:

Present: _____

_____

_____

Permanent: _____

_____

_____

vii) Phone/Cell: _____

viii) Date of recruitment: _____

ix) Address of two contact persons:

1:

2:

**3. Present medical Status**

**4. Present medication:**

**5. Past medical history:**

**6. Past medications :**

**7. Family medical history:**

I. Diabetes
II. HTN
III. Abdominal Pain

**8. Social history:**

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

ii) **Scio-economics Status:**

(a) Number of family-members:

(b) Monthly family income: Low / Middle / Upper

iii) **Educational Status:**

iv) **Occupational history:**

(a) Present occupation: Duration:

(b) Past history of work:

v) **Obstetrical history:**

vi) **Physical activity**

(a) Daily activity:

(b) Exercise: