GENETIC DETERMINANTS OF YOUNG ONSET DIABETES

GIFT

PhD Thesis

Submitted By

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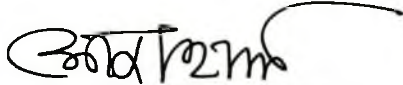
2006

Declaration

This thesis entitled **'Genetic Determinants of Young Onset Diabetes'** is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD), under the Faculty of Postgraduate Medical Science and Research, University of Dhaka. Laboratory analyses for this thesis were carried out in the Biomedical Research Group (BMRG) Laboratory, BIRDEM, Dhaka; Molecular Genetics Laboratory, Centre for Diabetes and Metabolic Medicine, Barts and the London, Queen Mary's School of Medicine and Dentistry, University of London, UK; and Dept of Pathology, Exeter University, Exeter, UK.

Any part of result of this work has not been submitted for any degree from any University at home or in abroad.



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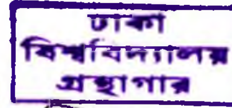

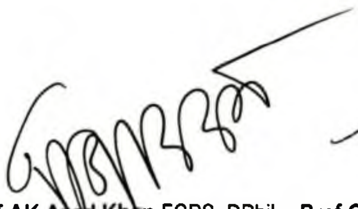

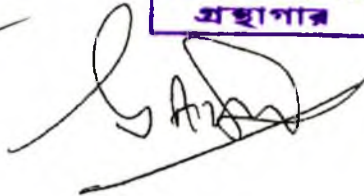

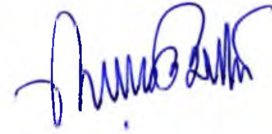

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Dedicated to
The memory of my mother
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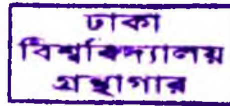

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SUMMARY

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A remarkable heterogeneity in clinical and biochemical presentation creates a special problem in characterization and classification of young diabetic patients in Bangladesh. A small subset of patients can be grouped as fibrocalculus pancreatic diabetes (FCPD) by relatively simple radiological or other imaging techniques, but the nature of diabetes of these subjects still remains unclear. Other subjects with young onset diabetes mellitus (YDM) present features of both T1 and T2 diabetes and some atypical findings further complicate the picture. In the above context it has now become necessary to analyze the clinical and biochemical observations with immunological and genetic tools for a more meaningful insight on the etiopathogenesis of the disorders constituting FCPD and YDM in Bangladesh. The present study was undertaken to investigate two major autoantibodies as well as some of the genetic markers of pancreatitis and diabetes in a young Bangladeshi population. Some of the candidate gene markers were also investigated in additional subjects from South India and UK origin in an attempt to substantiate the result further.

A total number of 423 young diabetic patients, with their disease diagnosed under- 30 years of age, were consecutively recruited from the under-30 diabetic clinic, Out-patient department and Dept of Pediatrics of BIRDEM Hospital. Of the 423 subjects 51 individuals were diagnosed to have FCPD and rest (n=372) were termed as young diabetes mellitus (YDM). Healthy subjects (n=337) of almost comparable age and having no family history of diabetes up to second generation served as controls. A collection of 70 families, consisted of FCPD probands and parents, were investigated mainly for genes related to pancreatitis. Additional subjects consisted of 65 unrelated FCPD patients, 7 FCPD families, 77 T2D, 56 IGT, and 312 controls from South India; 142 T2D and 156 controls from UK immigrants of Bangladesh; and 235 young T2D and 152 controls of British Caucasoid origin.

Basal insulin secretion of the study subjects was evaluated by measurement of fasting C-peptide level (by ELISA). GAD and IA2-ic autoantibodies were determined by radioimmunoprecipitation. Candidate gene markers [*SPINK1* (N34S), trypsinogen (R122H, A16V and N29I), INS VNTR (–23bp A/T), *EIF2AK3* (Indel15 AT^+/AT^-), *TCF1*

(A98V), *NEUROD1* (A45T) and *NEUROG3* (G167R and S199F)] were investigated by PCR-RFLP and/ or direct DNA sequencing.

Both FCPD and YDM subjects had significantly compromised basal insulin secretion compared to the controls [C-peptide value, nmol/l, median (range); 0.12 (0.023-0.84) in FCPD and 0.20 (0.02-1.33) in YDM vs 0.39 (0.16-1.44) in controls, $p<0.0001$ for both the groups]. About 20% of FCPD subjects were positive for GAD as well as IA2-ic antibodies. In YDM subjects about one-quarter were positive for GAD Ab and about 12% for IA2-ic Ab. Among FCPD subjects the GAD Ab positive and negative groups did not differ significantly in fasting insulin levels; however, in YDM subjects the GAD Ab positive group had significantly lower values compared to the negative subjects.

SPINK1 gene N34S mutation was found to be present in about 32% of FCPD subjects (unrelated and probands together) against 5.7% in the background population ($p<0.0001$). N34S mutation was also significantly associated with unrelated FCPD subjects from South Indian origin [35% in FCPD and 3.5% in controls, ($p<0.0001$)]. The *SPINK1* gene 'G' allele variant resulting in N34S mutation was found to be preferentially significantly transmitted to the FCPD probands from heterozygous parents [20 transmission against 2 non-transmission, $p<0.0001$]. Among total subjects (Bangladeshi and southern Indian origin) the N34S variant was present in 33% of 188 subjects with FCPD, 4.3% of 857 (including 56 IGT cases) nondiabetic subjects (odds ratio 10.91; $p<0.0001$ compared with FCPD), 3.7% 219 subjects with T2D, and 12.1% of 372 subjects with YDM. Trypsinogen gene mutations (R122H, A16V and N29I) were not present in the FCPD subjects (unrelated cases and probands) and these mutations were also absent in South Indian unrelated FCPD patients. Direct DNA sequencing was done in selected number of FCPD subjects and revealed absence of R122H and N29I mutations.

INS VNTR A/T polymorphism at –23bp was not found to be associated either with FCPD or YDM subjects. However, polymorphic 'A' allele was found to be significantly associated with GAD Ab negativity of the YDM subjects ($p<0.018$). YDM subjects with variant 'A' allele was found to have significantly higher fasting C-peptide levels [0.23 (0.02-1.32)] compared to those with wild 'A' allele [0.19 (0.03-1.13) ($p<0.05$)].

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Common variants of transcription factor genes responsible for neonatal and early childhood T1D [*EIF2AK3* gene Indel15 AT^{+}/AT^{-}], and maturity onset diabetes of the young (MODY) [*TCF1* (A98V), *NEUROD1* (A45T) and *NEUROG3* (G167R, S199F)] was found not to be associated with diabetes in FCPD and YDM. Subjects with variant allele of these genes did not show significant difference regarding the age of onset of diabetes and fasting insulin secretion of the FCPD and YDM subjects.

The data indicate that: (i) Autoimmunity is involved in a substantial number of young Bangladeshi patients of both FCPD and YDM varieties, however, in contrast to classical T1D there is some preservation of B cell function in these patients. Based on autoantibodies alone T1D, as defined by the latest WHO and ADA Expert Committee criteria, may be more common in Bangladeshi patients than generally thought; (ii) *SPINK1* gene N34S mutation is strongly associated with pancreatitis of FCPD in Bangladesh (indeed in South Asia) and *SPINK1* gene (N34S variant) 'G' allele is preferentially transmitted to the FCPD probands from heterozygous parents. However, it is unclear whether this mutation has any direct association with diabetes in FCPD patients or simply it is the etiological factor for chronic pancreatitis in this condition. Interestingly, an association has been found between the *SPINK1* N34S mutation and young onset diabetes (YDM) in the Bangladeshi population. It is yet to be established whether they are subclinical cases of FCPD; (iii) Trypsinogen gene R122H, A16V and N29I mutations are not commonly associated with pancreatitis either in Bangladeshi or Southern Indian FCPD subjects; (iv) INS VNTR A/T polymorphic 'T' allele is not associated either with FCPD or the YDM patients in Bangladeshi population. However, within the YDM patients those carrying the T1D associated wild AA genotype had a lower insulin secretory capacity than those carrying the variant allele; and (v) Common variants of transcription factor genes, linked to neonatal and early childhood T1D and MODY, are not associated with diabetes (either FCPD or YDM) in Bangladeshi population.

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Abbreviations

ADA	American Diabetes Association
APM1	Adeponectin gene 1
BB	Bio-breeding
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders
bp	base pairs
CAPN10	Calpain-10
CBV	Coxsackie B virus
CD	Cluster differentiation
CMV	Cytomegalovirus
CODIAB Study	Stands for a French study of diabetes complications
CF	Cystic fibrosis
cpm	count per minute
CRS	Congenital rubella syndrome
CTLA-4	Cytotoxic T-lymphocyte associated molecule 4
CV	Coefficient of variation
DASP	Diabetes antibody standardization program
DNA	Deoxyribonucleic acid
DZ	Dizygotic
EBV	Epstein-Barr virus
ECIGS	European Consortium for IDDM Genome Studies
EDTA	Ethylenediaminetetraacetic acid
EIF2AK3	Eukaryotic initiation factor 2 alpha kinase3
ENSA	Endosulfine alpha
ER	Endoplasmic reticulum
ETDT	Extended transmission disequilibrium test
FADD	Fas-activated death domain
FCPD	Fibrocalculus pancreatic diabetes
GAD	Glutamic acid decarboxylase
GBP28	Gelatin-binding protein 28
GCK	Glucokinase
GK	Goto-Kakizaki
GLK	Glucokinase
HLA	Human leukocyte antigen
HNF	Hepatocyte nuclear factor
HP	Hereditary pancreatitis
Ht	Heterozygous
Hz	Homozygous
IA-2	Islet antigen-2
IA-2ic	Islet antigen-2 intra-cellular fragment
IAA	Insulin autoantibody
ICA	Islet cell antibody
ICOS	Inducible costimulator
ICSA	Islet cell surface antibody
IDDM	Insulin dependant diabetes mellitus
IDX-1	Islet/duodenum homeobox-1
IGF2	Insulin like growth factor 2
IGFBP	IGF binding protein
IGRP	Islet specific glucose-6-phosphatase catalytic-related protein
IL-12B	Interleukin-12B
INS	Insulin gene

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IPF-1	Insulin promoter factor-1
IVS	Intervening sequence
LADA	latent autoimmune diabetes in adult
LOD	Logarithm of the odds
LPR5	Liproprotein receptor related protein
LWB	Low birth weight
MC3R	Melanocortin3 receptor
MHC	Major histocompatibility complex
MIM	Mendelian Inheritance in Man
MnSOD	Manganese superoxide dismutase
MODY	Maturity onset diabetes mellitus of the young
MRDM	Malnutrition related diabetes mellitus
MZ	Monozygotic
NEUROD1	Neurogenic differentiation factor 1
NEUROG3	Neurogenin differentiation factor 3
NIDDM	Noninsulin dependant diabetes mellitus
NOD	Non-obese diabetic
NRAMP1	Natural-resistance–associated macrophage protein 1
NZO mouse	New Zealand obese mouse
PCK1	phosphoenolpyruvare carboxykinase gene
PCOS	Polycystic ovary syndrome
PDX-1	Pancreatic and duodenal homeobox-1
PPARG	Peroxisome proliferator-activated receptors gama
PPARs	Peroxisome proliferator-activated receptors
PRSS1	Protease serine 1
PSP	Pancreatic stone protein
REG	Regeneration
RR	Relative risk
RT6	Rat protein 6
SDS	Sodium dodesyle sulfate
SEL1L	Suppressor of lin-12-like protein
SNP	Single nucleotide polymorphism
SPINK1	Serine protease inhibitor kazal type 1
SUR	Sulphonylurea
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
TATI	Tumor associated trypsin inhibitor
TCF	Transcription factor
TCP	Tropical calcific pancreatitis
TCRB	T cell receptor B
TNF-LT	Tumor necrosis factor-lymphotoxin
TPD	Tropical pancreatic diabetes
TRYP	Trypsinogen gene
UTR	Untranslated region
VDR	Vitamin D receptor
VNTR	Variable number tandem repeat
WHO	World health organization
WRS	Wolcott-Rallison syndrome
YDM	Young onset diabetes mellitus
ZFM1	Zinc finger gene in Men1

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CHAPTER 1

INTRODUCTION

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1. INTRODUCTION

1.1 Historical Perspectives

Diabetes mellitus has been known to affect human individuals for thousands of years. The oldest documentation of diabetes was traced back to Egyptian Papyrus around 1550 years BC (Major 1939). Diabetes mellitus was also known to the ancient Indian genius Acharya Charak and Sushruta. Graphic pictures, presumably depicting diabetes like symptoms, had been mentioned in the compiled teachings of Acharya Charak in 'The Charak Samihta' around 600 BC (Tripathy *et al.*, 2002). Sushruta, an astute physician, around 400 BC described a comprehensive account of diabetes mellitus in his famous writing 'The Sushruta Samihta' (Tripathy *et al.*, 2002; Frank 1957). Sushruta termed the disease as Madhumeha 'rain of honey' observing the feature of ants attracted to the urine, a suggestion of sweetness of urine. He clearly mentioned two distinct forms of diabetes - one associated with emaciation, dehydration, polyuria and lassitude, a hint of present day type 1 diabetes mellitus and the 'other group' associated with stout built, gluttony, obesity and sleeplessness, which refers to present day type 2 (noninsulin dependant diabetes mellitus; NIDDM).

The Greek word Diabetes, 'means flow through siphon', was coined by 'Aretaeus' of Cappedocia in Asia Minor in the 2nd century AD because of profuse amount of urine passed by a diabetic patient (Papaspyros 1964) and the adjectives 'Mellitus' (both in Latin and Greek it means honey) used by Thomas Wills in 1675 after rediscovering the sweetness of urine and blood of patients 'first noticed by the ancient Indians'. It was only in 1776 that Dobson firstly confirmed the presence of excess sugar in urine and blood as a cause of their sweetness (Dobson 1776). The role of the pancreas in pathogenesis of diabetes was discovered by von Mering and Minkowski (von Mering and Minkowski 1889). However, this finding was preceded by discovery of small islands 'heaps' of cells within the pancreas by Paul Langerhans in 1869, later termed as Islets of Langerhans (Langerhans 1869). Laguesse (1894) hypothesised that 'islets of langerhans' might be the site of internal secretion related to diabetes mellitus. Subsequently Jean de Meyer (1909) coined the name 'insulin', (Latin 'insula' means an island), for glucose lowering hormone secreted from islets. The final breakthrough in

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the understanding of diabetes and its treatment was unravelled when pancreatic extract was found to be reducing blood sugar in experimental animal (Banting *et al.*, 1922; cited in Rosenfeld 2002)

Important discoveries and advancement in the understanding of diabetes have been achieved within the last hundred years, but the most fundamental observation seemed to be made by the ancient Indian genius Sushruta. More than almost 2500 years ago he made the astonishing observations; 'Modhumeha' or diabetes sometimes associated with family history of diabetes, the genetic basis 'seed' and some associated with injudicious diet, the environmental cause. Importantly this was the first recorded reference to the etiology of diabetes being of multifactorial origin. With the tremendous progress in the experimental technique it is now possible to examine the gene and its role in the etiopathogenesis of diabetes.

1.2 Classification of Diabetes Meilitus

Diabetes mellitus has long been classified based on its clinical presentation; age of onset and special features, and need for insulin of the individuals to control blood glucose (Appendix 5.1). Lawrence (1951) was first to introduce the nomenclature Type 1 and Type 2 to describe two distinct forms of diabetes mellitus. It was in late seventies National Institute of Health (NIH), United States of America (USA), took the first collective efforts on formulation of classification and diagnostic criteria of diabetes by setting up the National Diabetes Data Group (NDDG), who introduced the term insulin dependent diabetes mellitus (IDDM, Type 1) and noninsulin dependent diabetes mellitus (NIDDM, Type 2) to describe two main classes of diabetes (NDDG 1979). In the following year a WHO Expert Committee on Diabetes endorsed the ADA classification with some modifications especially regarding diagnostic criteria (WHO 1980). Subsequently another WHO Study Group on Diabetes Mellitus brought out a revised classification in 1985, which introduced Malnutrition Related Diabetes Mellitus (MRDM) as the third major type of diabetes mellitus (WHO 1985). MRDM comprised of two subclasses i) fibrocalculus pancreatic diabetes (FCPD) and ii) protein deficient pancreatic diabetes (PDPD) (WHO 1985). Although the WHO (1985) Study Group classification of DM was widely accepted internationally many issues remained to be addressed. All the classifications mentioned above, including the one by WHO Study Group in 1985, were based on a combination of clinical and etiological backgrounds

designed to be useful for the purpose of management and disease epidemiology. With the expansion of knowledge the need for a revised and more useful classification of diabetes mellitus was keenly felt. Therefore, the ADA set up a Committee, which, in 1997 suggested major changes in the classification. In this approach etiological factors were used as the basis for the formulation of the classification (ADA Expert Committee 1997) and this was subsequently endorsed by the WHO Consultation Group on Diabetes (WHO 1999). In the latest classification the two major classes have been renamed as type 1 and type 2 instead of IDDM and NIDDM respectively. MRDM has been removed and FCPD, one of the two subclasses of MRDM, was assigned under 'disease of exocrine pancreas' under major head of 'Other Specific Types'.

A shortened version of the current WHO classification is as follows:

Table 1.1: Etiological classification of diabetes mellitus (WHO 1999)

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
   - 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

1.3 Type 1 Diabetes Mellitus

Type 1 diabetes (T1D) mellitus is characterised by sudden onset of symptoms, proneness to ketoacidosis and need of insulin for survival. The hallmark of the T1D is the destruction of pancreatic B cells resulting in very low to absolute loss of insulin secretion.

T1D occurs mainly in children and young adults and accounts for about 10% of all diabetic patients (ADA Expert Committee 1997). The incidence of T1D shows a wide range of variation worldwide (Figure 1.1). Karvonen et al (2000) have analyzed data from 100 populations across the world and demonstrated a clear difference in incidence between northern and southern hemisphere.

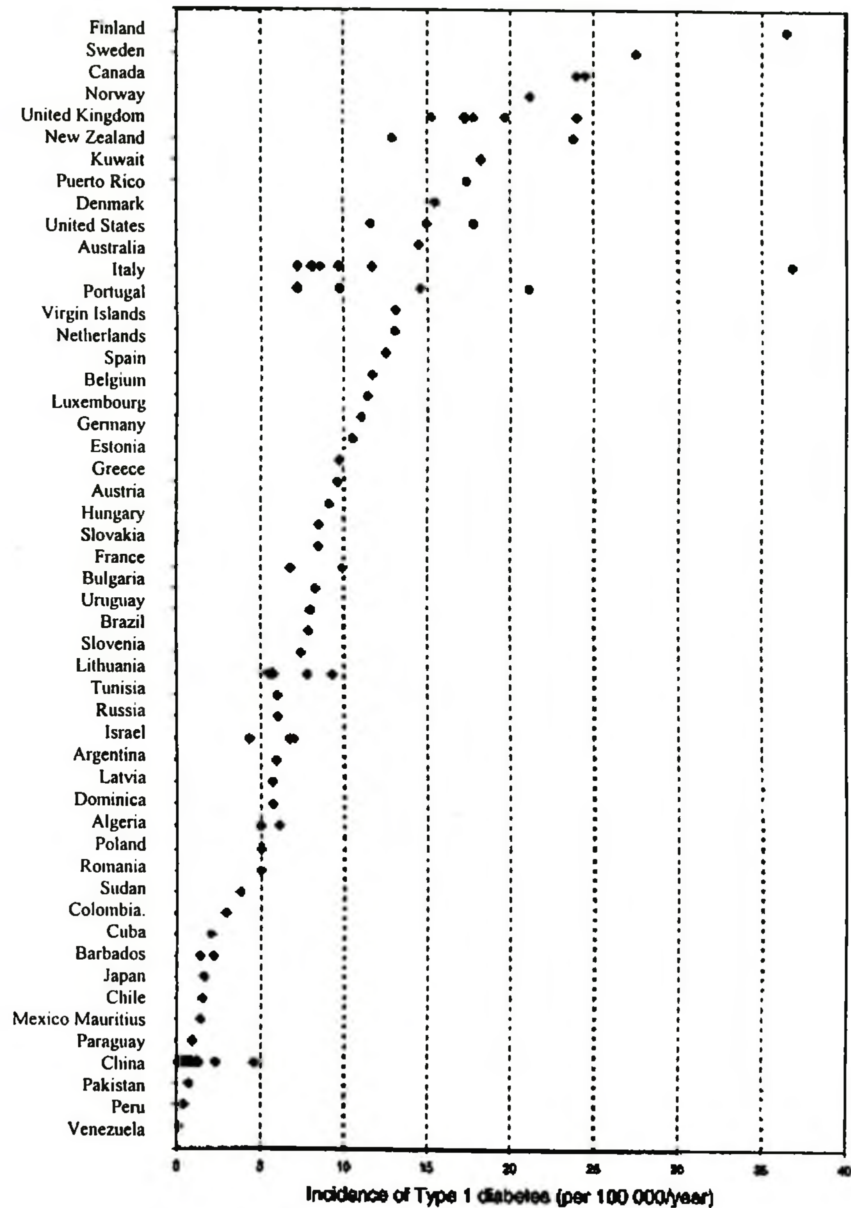

Figure 1.1: Age adjusted incidence (per 100,000 per year) of T1D in children ≤14 years of age in 100 populations (Boys and girls were pooled) (Karvonen *et al.*, 2000).

In their earlier report they demonstrated that above the equator the incidence of T1D is common and countries lies below the line had lower incidence (Karvonen *et al.*, 1993). The incidence 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 Finland (Karvonen *et al.*, 2000). A report from Southern India has shown that the incidence of T1D is similar (10.5/1000,000) to many European populations (Ramachandran *et al.*, 1986). T1D is believed to be less prevalent in Bangladesh, however, population based studies are still lacking.

T1D is divided on the basis of mode of damage of B cells; immune mediated type 1 (Type 1A) and non-immune mediated type 1 (idiopathic type 1, T1B) (ADA Expert Committee 1997; WHO 1999). Autoimmune T1D is characterized by immune mediated damage, targeted against self-antigens, resulting in the destruction of the B cells of the pancreas. A number of autoantibodies have been detected against different islet proteins. The rate of B cell destruction varies and is usually faster in children and slower in adults. A number of T1D patients have a typical sudden onset of the disease and are ketosis prone, but they lack classical autoantibodies directed to islets and/ or islet cell proteins, are called idiopathic T1D. 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; Imagawa *et al.*, 2000). Idiopathic T1D usually occurs to obese teens. Their blood glucose is controlled by insulin initially and oral hypoglycemic agents can be subsequently used before the onset of complete insulin dependence as in T1D. While the exact etiological factor(s) are still unknown, multiple genetic and environmental factors are thought to be involved (ADA Expert Committee 1997; WHO 1999).

1.3.1 Genetics of T1D

1.3.1.1 Familial aggregation

Although an increased clustering of T1D is observed in the family, more than 80% of newly diagnosed T1D cases occur in individuals with no family history of diabetes (WHO 1999). The average prevalence of T1D was calculated to be about 6% in siblings of a parent with T1D compared to 0.4% in the US population. Familial clustering (λs) is calculated as the ratio of risk to the siblings over the disease

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prevalence in the general population. It has been estimated that for T1D, familial clustering has a λs value of 15 (λs=6/0.4=15), i.e the disease is 15 times more common in siblings of the diabetic patients than in the general population (Risch *et al.*, 1993; Todd and Farrall, 1996). This simple epidemiological observation imply that genetic factors play a role in the multifactorial origin of this complex disease.

A number of studies have demonstrated increased familial clustering of T1D within families. Kobberling and Tillil (1984) have suggested an empirical risk to children of parents with T1D as 1.8% up to the age of 25 years and 3.5% up to the age 80 years. In their report Tilli and Kobberling (1987) demonstrated overall risk for lifetime recurrence of T1D (risk up to age 80 years) in three consecutive generations. The overall risk for siblings was 6.6±1.1 and for children 4.9±1.7. In addition to the familial aggregation they have also examined parent-of-origin of diabetes. Regardless of age of onset, the offspring of male probands always had a higher risk than the offspring of female probands. Among all probands, fathers were significantly more often affected than mothers (4.1±0.9% vs 1.7±0.6%). In other words the risk to children of T1D mothers for diabetes is less compared to that of T1D fathers (Tilli and kobberling 1987). Several other studies have showed that, by 20 years of age, the risk for diabetes in the children is lower if the diabetic parent is a mother than if the diabetic parent is a father; 1% compared to 6% (Warram *et al.*, 1984), 3% compared with 9% (Bleich *et al.*, 1993) and 4% compared with 8% (Tuomilehto *et al.*, 1995). The difference in parent-of-origin in transmission of diabetes was observed also for expression of anti-islet autoantibodies. The frequency and levels of expression of GAD, IAA and ICA were increased in offspring of fathers with diabetes compared to those offspring of mothers with diabetes (Yu *et al.*, 1995).

The above reports clearly demonstrate the clustering of T1D in families and in addition unmask the gender bias in transmission of diabetes to offspring from diabetic parents. However, the factors related to gender-bias in high rate of transmission of diabetes have not yet been clearly understood. Environmental factors, including maternal sex hormones modulating susceptibility *in utero*, immunogenic tolerance to autoantigens or selective fetal loss of potential diabetic offsprings have been suggested (Warram *et al.*, 1988). But, evidence in support of these environmental factors is still to be substantiated.

Genetic imprinting has been hypothesized to explain the difference in parent-to-offspring transmissions. Polychronakos et al (1995) in their review hypothesized about presence of an imprinting gene close to INS-IGF2 locus region and its role in the pathogenesis of T1D. However, the exact nature of imprinting is yet to be ascertained.

1.3.1.2 Twin studies

Twin studies demonstrated consistently higher T1D concordance rate in monozygotic (MZ) twins than dizygotic (DZ) twins. The concordance rate for T1D among monozygotic twins has been reported between 21%-70% (Barnett *et al.*, 1981a; Verge *et al.*, 1995; Kyvik *et al.*, 1995; Redondo *et al.*, 1999) and in dizygotic twins to be quite low 13% (Hawa *et al.*, 1997; Kumar *et al.*, 1993; Redondo *et al.*, 1999). This is a clear indication for genetic susceptibility to disease. The MZ twin concordance rate also provides a rough idea on the degree of genetic involvement compared with nongenetic determinants, in particular environmental factors. However, the concordance rate has been variously estimated as 34% by the age of 30 (Olmos *et al.*, 1988), 43% within 12 years of diagnosis of index cases (Hawa *et al.*, 1997), and 50% within 40 years of index diagnosis (Redondo *et al.*, 2001). This shows that rates of developing T1D in the co-twins declines sharply in the years after diagnosis of the index twin, which suggests that the initiation of the process leading to T1D occurs within a finite, not a prolonged, period. It also indicates the importance of nongenetic factors, reflected as disconcordance, in the etiology of T1D. In this regard Eisenbarth (1986) in his review suggested that a somatic cell rearrangements of the immunoglobulins and T cell receptor genes is responsible to certain stimuli. Another explanation could be the genetic susceptibility to T1D is not an all or none phenomenon, and that there is a graded susceptibility depending on the genetic susceptibility of the individual. Thus discordant twins would have gene combinations leading to a lower penetrance of the disease whilst in the concordant twins different gene combinations would lead to higher penetrance of disease (Hitman 1994).

1.3.1.3 Animal model

The spontaneous nature of T1D was demonstrated in animal models especially in rodents. Most widely studied models are bio breeding (BB) rats and non-obese

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diabetic (NOD) mouse. The two complementary pathogenic factors, genetic predisposition and autoimmune phenomenon, both are seen in those two models.

BB rats show phenotypic variability of diabetes like that in human. Cellular infiltration of inflammatory cells in the pancreas was also seen similar to that in human T1D. These include both helper and cytotoxic/suppressor subsets of T lymphocytes, with macrophages, natural killer (NK) cells and B lymphocytes (Seemayer *et al.*, 1982; Walker *et al.*, 1988). Experiments designed to interfere with immune response have been reported to prevent development of diabetes in BB rats further strengthening the hypothesis of immune involvement in the pathogenesis (Rossini *et al.*, 1984; Laupacis *et al.*, 1983; Like *et al.*, 1986). Autoantibodies have been detected in the preclinical BB rats. GAD and islet cell surface antibodies (ICSA) but ICA were not detected in diabetes prone and newly detected BB rats (Baekkeskov *et al.*, 1984; Dyrberg *et al.*, 1982; Dyrberg *et al.*, 1984).

NOD mice have typical hypoinsulinemia, hyperglycemia and glycosuria and dependency on insulin replacement for survival. Insulitis with both helper and cytotoxic T lymphocyte and NK cell infiltration has been observed in all prediabetic mice (Kanazawa *et al.*, 1984). It has been clearly shown that insulitis and overt diabetes in the NOD mice can be prevented by various immunomodulatory agents (Tochino 1987). Humoral antibodies, ICA, ICSA, IAA and GAD, have been found in the sera of prediabetic mouse (Reddy *et al.*, 1988; Kaufman *et al.*, 1993). Diabetes developed in 80% of female compared to less than 20% male mice whereas mice bear the same genetic background and were influenced by same environment (Makino *et al.*, 1980). However, the high incidence of events in female mice is critically dependent on environment for instance role of male sex hormone and factors(s) yet to be found out which triggers destruction of B cell.

1.3.1.4 HLA genes in T1D

HLA molecules associated with diabetes susceptibility

The best evidence for a genetic component in the susceptibility to T1D come from studies of HLA genes (IDDM1, MIM 222100) in both populations and families as well as in animal models. It has been estimated that HLA provides up to 40-50% of familial clustering of T1D (Noble *et al.*, 1996). The HLA region is a cluster of genes located

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within major histocompatibility complex (MHC) on chromosome 6p21. The HLA complex is classically divided into four regions, known as class I, class II, class III and class IV (Gruen and Weissman, 2001). The class II MHC region encode the class II HLA molecules which are involved in immune process and turn out to be important candidate in the pathogenesis of T1D.

Serological typing was first demonstrated to have positive association between HLA-B8 and HLA-B15 class I molecules and T1D mellitus. Subsequently a strong association between T1D and DR4 and DR3 class II molecules was observed. Over 95% of Caucasian T1D patients were found to posses HLA-DR3 and/ or DR4 antigen emphasizing its strong association with the disease phenotype (Wolf *et al.*, 1983). Studies of segregation analysis of HLA haplotypes in families with T1D implicated disease-associated gene(s) closely linked to class II region. Since, DR3 and DR4 were also found in background population, other gene(s) possibly in the class II region were suggested to be associated with T1D (Spielman *et al.*, 1989). Linkage disequilibrium between HLA-DR and DQ region was too strong and familial recombination was unlikely between DQA1 and DRB1.

Subsequently polymorphism in the DQB1 gene was identified and suggested that DQB1 conferred greater susceptibility to T1D than the DRB1 locus (Owerbach *et al.*, 1983). The HLA-DQ8 molecule was later identified to be encoded by DQB1*0302 allele and was found to be very frequent (90%) in DR4 positive Caucasian T1D patients than HLA-DQ7 (DQB1*0301) (Nepom *et al.*, 1986; Kim *et al.*, 1985) but both are still prevalent in the background population.

Several studies have confirmed the earlier findings and concluded that rather than individual gene(s), HLA-DRB and DQB haplotypes confer disease associated risk and protection in individuals. This DRB1 and DQB1 gene association with T1D was later confirmed as the major contribution to the T1D by chromosomal peak (Herr *et al.*, 2000).

The strongest genetic association was demonstrated with T1D and HLA-DQB alleles by She (1996). In white Caucasians T1D is positively associated to two combinations DQA1 and DQB1 alleles: DQA1*0501-DQB1*0201 (DQ2 molecule) and DQA1*0301-DQB1*0302 (DQ8). Heterozygous individuals with DQ2 and DQ8 are associated with the highest risk of disease, which suggest that these two DQ molecules may act

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synergistically. HLA-DRB1*03 is in linkage disequilibrium with DQA1*0501-DQB1*0201 and DRB1*04 with DQA1*0301-DQB1*0302. HLA-DRB1*04 (*0401, DRB1*0402 and DRB1*0405) alleles with DQA1*0301-DQB1*0302 form high-risk haplotypes associated with diabetes. HLA-DRB1*0301-DQA1*0501-DQB1*0201 is another high-risk haplotype.

DRB1*0401 and DRB1*0405 has been reported to increase the risk independent of DQB8 locus in different ethnic groups whereas DRB1*0403 DRB1*0406 confer protection from diabetes (She 1996; Donner *et al.*, 2000; Undlien *et al.*, 1997; Park *et al.*, 1998). However, DQ2/DQ8 genotype has been shown to be disease susceptible even in the presence of HLA-DRB1*0403 (Roep *et al.*, 1999; van der Auwera *et al.*, 1995).

The predominance of DQB1*0302 over DR4 was also demonstrated in northern Indian Asian, Afro-Caribbean and Southern Chinese diabetic populations but not in Japanese (Fletcher *et al.*, 1988, 1998; Mijovic *et al.*, 1991; Penny *et al.*, 1992; Jacobs *et al.*, 1992). The findings suggest that disease susceptibility of DQB1*0302 may be modified by other factors. The DQA1 allele associated with DR4 is DQA1*0301. It was demonstrated that DQA1*0301 allele occurs on predisposing DR7 haplotypes in Negroid (Todd *et al.*, 1989). This suggested that DQA1*0301 is disease susceptible. However, it also occurs on predisposing DR9 in Negroid and Japanese (Mijovic *et al.*, 1991; Jacobs *et al.*, 1992). DR7-DQB1*0201 is, however, positively associated with disease in Negroid. In Negroid the haplotype possesses HLADQA1*0301 allele where as the Europid had DQA1*0201 allele (Todd *et al.*, 1989).

HLA has also been found to confer protection from diabetes. The best known protective allele is DQB1*0602, usually found on DR2 (DRB1*1501-DQA1*0102-DQB1*0602) haplotypes. Approximately 20% of American and European children without diabetes have DRB1*1501-DQA1*0102-DQB1*0602 where as less than 1% of children with diabetes carry (DRB1*1501-DQA1*0102-DQB1*0602). Protection conferred by DRB1*1501-DQA1*0102-DQB1*0602 haplotypes have been found to be dominant. A similar frequency of DQB1*0602 was demonstrated in two groups of subjects who had first-degree relatives with T1D: ICA positive and autoantibody negative. Antibody positive and DQB1*0602 positive patients who did not develop diabetes had restricted pattern of ICA staining but it was associated with high titers of

GAD antibody (Verge *et al.*, 1996). The first-degree relatives who were both ICA and DQB1*0602 positive had more limited response to islet antigen compared to ICA positive relatives without the DQB1*0602 allele (Gianani *et al.*, 1992 and 1996). DRB1*0403 and DRB1*0406 alleles have been shown to confer protection from diabetes. Another study by Pugliese et al (1995) demonstrated that first-degree relatives with DQB1*0302/DQB1*0201 genotype had tendency to develop diabetes at an earlier age than those with negative genotype. They observed that relatives with DQB1*0602 genotype did not develop diabetes, but those with negative genotype developed diabetes. The protective allele DQB1*0602 is found in ICA positive relatives who have minimal risk of progression to T1D.

Mechanisms of diabetogenicity of HLA molecules

Class II molecules are critical to present antigens to CD4+ T cell. Thus different disease associations of class II molecules suggest that particular HLA molecules may be more effective either in binding antigen, or in inactivating T cell receptor and thus influencing disease susceptibility. It has been postulated that the predisposing effect of DR3 and/or DR4 associated alleles arises as a result of more efficient binding of a particular 'diabetogenic peptide'.

The model presented by Nepom (1990) describes that peptide bound with HLA molecule present with the highest affinity. Diabetes susceptibility or protection associated with HLA molecules may be related to their ability to present peptides of relevance to diabetogenic T cells.

Diabetes susceptibility or protection associated with HLA molecules may be related to their ability to present peptides of relevance to diabetogenic T cells. Individuals with HLA molecules that are not able to effectively present specific peptide to naive T cells in thymus might fail to engender tolerance. Alternatively, specific HLA alleles may selectively present an islet peptide to mature T lymphocytes that have escaped negative selection. These proposed mechanisms 'lack of central tolerance affecting the T cell receptor in the thymus or abnormal tolerance in the periphery' — are not mutually exclusive and could coexist. The ability to present antigens may depend on conformational properties of the HLA molecule with some alleles binding to peptides in such a fashion that antigens would never be effectively presented to T cells.

1.3.1.5 Non-HLA genes in T1D

1.3.1.5.1 IDDM2

Insulin gene was sequenced and mapped to 11p15.5 (Bell *et al.*, 1980; Harper *et al.*, 1981). Subsequently association was observed between polymorphism at 5' flanking region of the *INS* gene and IDDM (Bell *et al.*, 1984). The polymorphism was demonstrated related to a unique variable tandem (VNTR) repeats of 14 base pairs (ACAGGGGTGTGGGG), in the 5'-regulatory region of the *INS* gene. The number of repeats varies from 26 to over 200 and VNTR has been seen in three classes: class 1 (26-44 repeats), class II (54-138, mean 80 repeats) and class III (140-209 repeats) (Bell *et al.*, 1982; Rotwein *et al.*, 1981; Bennett *et al.*, 1995). Class II alleles are extremely rare in Caucasoid population, in whom the frequency of class 1 and class III are 70% and 30% respectively (Bennett and Todd 1996). The class I VNTR allele has been shown to predispose to T1D, while class III allele has dominant protective effect (Julier *et al.*, 1991; Hitman *et al.*, 1985; Bennett *et al.*, 1995; Rotwein *et al.*, 1981). The association between T1D and *INS* gene polymorphism was further confirmed by genome wide linkage analysis by Davies et al (1994) who demonstrated significant linkage of T1D on chromosome 11p15 and designated it as IDDM2 (OMIM 125852). Cross-matched haplotype analysis and linkage disequilibrium mapping also mapped NIDDM2 to a site within the *INS* VNTR locus (Bennett *et al.*, 1995).

INS gene (gene access number; gi: 307071) -23bp A/T polymorphism is strongly associated with the 5'-VNTR of the gene with the A allele being in linkage disequilibrium with class I allele and the T allele with the class III allele (Ullrich *et al.*, 1980; Julier *et al.*, 1991). The *INS* VNTR has been suggested to regulate expression of insulin and insulin-like growth factor 2 (IGF2) genes and is thought to be relevant to the pathogenesis of T1D. In adult and fetal pancreas, class I VNTR allele was found to be associated with higher levels of insulin gene transcription compared with class III allele (Bennett *et al.*, 1996; Vafladis *et al.*, 1997). The reverse pattern was observed in thymus with low insulin messenger RNA (mRNA) levels occurring in the presence of class III alleles (Vafidias *et al.*, 1997; Pugliese *et al.*, 1997). Implication of *INS* gene in the pathogenesis of T1D was also strengthened by the observation of Kennedy et al (1995). Since the VNTR is located 365bp upstream of the transcription start site of the *INS* gene they hypothesized that the VNTR might be an important transcriptional regulatory region. They demonstrated greater transcriptional activity in pancreatic beta

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cells of long VNTR compared to the short VNTR. The VNTR contains numerous high-affinity binding sites for the transcription factor Pur-1 (PUR1) and transcriptional activation of Pur-1 is modulated by naturally occurring sequences in the VNTR. Thus, the authors speculated that this unique minisatellite might have important implications for T1D.

The increased expression of insulin in thymus might lead to the deletion of autoreactive T cells or selection of a suppressor T cell population which may explain why the class III allele confer dominant protection against T1D. In subjects homologous for class I alleles, the lower thymic insulin expression and higher pancreatic expression may result in an increased risk of pancreatic autoimmunity. However, another study showed no correlation between VNTR class and induction of tolerance to insulin (Sarugeri *et al.*, 1998). In a recent publication Nielsen et al (2006) have shown a direct connection *in vivo* between INS VNTR class III alleles, a decreased humoral immune response to insulin, and preservation of beta cell function in recent-onset T1D.

The *IGF2* gene, located in close proximity to the *INS* gene, has also been implicated in IDDM2 associated susceptibility. Although INS VNTR class I and class III alleles were found to be associated with similar levels of *IGF2* gene transcription in pancreas, thymus and leukocytes (Vafiadis *et al.*, 1998a; Vafiadis *et al.*, 1998b) increased IGF2 expression was found to be associated with the class I allele in placenta (Paquette *et al.*, 1998). This increased placental IGF2 expression has been suggested to influence intra-uterine growth and birth size, which have both been reported to affect the risk of T1D (Dahlquist *et al.*, 1996).

Insulin gene VNTR was found to be associated with different kind of clinical conditions such as polycystic ovary syndrome (PCOS), predominantly associated with insulin resistance (Vankova *et al.*, 2002; Xita *et al.*, 2002).

Various studies were conducted to unmask the effect of INS VNTR genotype on insulin sensitivity and secretory capacity. However, available data are conflicting. The study by Ahmed *et al.*, (1999) following IVGTT found no difference regarding the amount or rate of insulin secretion, but in class I genotype group there was a difference in pattern of pulsatile insulin secretion with more 9-minute oscillations. One recent study demonstrated significantly higher fasting insulin secretory level in children with homozygous (HZ) class III genotype group compared to HZ class I group (Bazaes *et al.*, 2003). This was supported by earlier study by Weaver et al (1992) who have

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demonstrated association of INS VNTR class III allele with fasting hyperinsulinemia, stimulated insulin secretion and insulin resistance. In a more recent study Bennett et al (2005) observed no difference between VNTR genotype with measures of insulin secretion, insulin sensitivity, glycemia and, adiposity and suggested that the effects of INS VNTR variation on insulin transcription observed *in vitro* does not appear to translate into detectable differences in basal insulin secretion in humans.

1.3.1.5.2 Putative T1D loci

T1D loci, IDDM1 and IDDM2, susceptibility have been identified by association studies and linkage analysis using various analytical methods. However, these two loci only contribute to a portion of disease familial clustering, suggesting the existence of additional loci. The increasing availability of polymorphic markers (microsatellites, single nucleotide polymorphism (SNPs)) and development of fluorescence-based automated typing technology has greatly facilitated studies in large collection of families (simplex and large multigenerational families, and affected sib-pairs).

In an effort to map T1D loci a number of genome-wide scans have been performed and approximately 20 chromosomal regions have been linked with the disease and it also has emerged that T1D is a polygenic disease. Among these the loci IDDM12 has been well characterized. Putative loci for T1D diabetes so far identified are shown in the table 1.2. Of the linked loci, nine lie on just three (6, 2 and 11) chromosomes and rests are scattered in other chromosomes (Table 1.2).

IDDM12 (MIM 6013388) locus maps to chromosome 2q33 and the locus is very close to cytotoxic T-lymphocyte associated molecule 4 (CTLA-4) and CD28 genes which encode CTLA4 and T-cell costimulatory receptor, CD28, also called CD152. Maximum evidence for linkage was obtained in D2s72-CTLA4-D2s116 region (Nistico *et al.*, 1996). A multi-ethnic study (involving Spanish, French, Mexican-American, Chinese Korean) revealed a highly significant deviation of transmission of alleles at (AT)n microsatellite marker in 3' untranslated region and a polymorphism (49A>G) in exon1 of the CTLA-4 gene. However, the 49G>A polymorphism has not been replicated in British, Sardinian and Chinese data, while the Caucasian American had week transmission deviation. The deviation was strong in French, Italian and Spanish populations (Marron *et al.*, 1997; Marron *et al.*, 2000). The CTLA-4 gene polymorphism has also been suggested to influence gene expression (Kouki *et al.*, 2000; Ligers *et al.*, 2001). There were evidences for general role of CTLA-4 in other autoimmune

Table 1.2: Putative loci for T1D

Locus	Chromosome	Candidate genes	References
IDDM3 (MIM 600318)	15q26	Unknown	Field *et al.*, 1994; Zamani *et al.*, 1996; [a]Concannon *et al.*, 1998; [a]Mein *et al.*, 1998; [a]Cox *et al.*, 1994)
IDDM4 (MIM 600319)	11q13	ZFM1, FADD, LPR5, CD3, RT6	Davies *et al.*, 1994; Hashimoto *et al.*, 1994; Sawicki *et al.*, 1997; Kim *et al.*, 1996; Hay *et al.*, 1998
IDDM5 (MIM 600320)	6q25	MnSOD	Concannon *et al.*, 1998; Fox *et al.*, 2000
IDDM6 (MIM 601941)	18q21	Unknown	Merriman *et al.*, 1997
IDDM7 (MIM 600321)	2q31	IGRP	Luo *et al.*, 1995; Hutton and Eisenbarth 2003; Martin *et al.*, 2001
IDDM8 (MIM 600883)	6q25-q27	Unknown	Luo *et al.*, 1995 and 1996
IDDM9	3q21-q25	Unknown	Davies *et al.*, 1994; Mein *et al.*, 1998
IDDM10 MIM 601942	10p10-q11	Unknown	Davies et 1994; Reed *et al.*, 1997; Cox *et al.*, 2001; Mein *et al.*, 1998;
IDDM11 (MIM 601208)	14q24.3-q31	ENSA, SEL1L,	Field *et al.*, 1996; Corder *et al.*, 2001; Heron *et al.*, 1999; Harada *et al.*, 1999; Bataille *et al.*, 1999; [a]Pociot *et al.*, 2001
IDDM13 (MIM 601318)	2q3, 2q34	IGFBP2, IGFBP5, IA-2, NRAMP1	Morahan *et al.*, 1996; Larsen *et al.*, 1999; Owerbach *et al.*, 1997
IDDM15 (MIM 601666)	6q21	Unknown	Delepine *et al.*, 1997; European Commission Study 2001; Temple *et al.*, 2000; Cave *et al.*, 2000
IDDM16	14q32.3	Unknown	Field *et al.*, 2002;
IDDM17 (MIM 603266)	10q25	Unknown	Verge *et al.*, 1998; [a]Davies *et al.*, 1994; [a]Concannon *et al.*, 1998; [a]Mein *et al.*, 1998
IDDM18 (MIM 605598)	5q3.1-q331	IL-12B	Morahan *et al.*, 2001; Trembleau *et al.*, 1995; Huang *et al.*, 2000; Hall *et al.*, 2000

[a] Failed to replicate/find linkage.

disease (Karandikar *et al.*, 1996; Kristiansen *et al.*, 2000) and suggested that involvement is related to down regulation of T cell function (Greene *et al.*, 1996). Further functional studies revealed another member of this family of molecules 'inducible costimulator', termed ICOS, has structural and functional similarity to CD28 and CTLA-4 (Hutloff *et al.*, 1999). Studies using ICOS knockouts demonstrated that ICOS is essential for development of normal T cells and thus play protective roles against development of autoimmune disease (Dong *et al.*, 2001).

1.3.1.5.3 Susceptible T1D loci

It has been suggested that *EIF2AK3* gene, assigned to 2p12 (Hayes *et al.*, 1999), may be a candidate gene for T1D based on linkage data, a monogenic model of neonatal diabetes and a recent case control study in South Indian subjects. Since the gene has been studied for this study, it will be discussed in detail.

Eukaryotic initiation factor 2 (eIF2) plays an important role in environmental stress and in translation controls of number of genes (Wek *et al.*, 2006). Mutations were identified in the *EIF2AK3* gene in patients with Wolcott-Rallison syndrome (WRS) (Delepine *et al.*, 2000). WRS is a rare syndrome, with less than 20 cases described so far. It is associated with permanent neonatal or early childhood insulin dependent diabetes mellitus and epiphyseal dysplasia. Other clinical features that show variability between WRS cases include mental retardation, hepatic and kidney dysfunction, cardiac abnormalities, exocrine pancreatic dysfunction and neutropenia (Senee *et al.*, 2004). Biason-Lauber et al (2002) demonstrated a missense T/C mutation in exon 13 resulting S877P in the peptide of a WRS patients. The mutated protein although retained autophosphorylation activity was unable to phosphorylate its natural substrate. So far a limited number of different mutations have been demonstrated in families with WRS (Delepine *et al.*, 2000; Senee *et al.*, 2004; Brickwood *et al.*, 2005). However, in a recent publication Durocher et al (2006) demonstrated a novel mutation in the EIF2AK3 gene with variable expressivity in two patients with Wolcott-Rallison syndrome.

The EIF2AK3 gene has been studied in South Indian type 1 diabetic patient by Allotey et al (2004). They observed excess transmission of the D2S1786 located 3' to the gene and 15INDEL (within the gene either) singly or in combination suggesting an

association between the region around *EIF2AK3* and T1D susceptibility. The *EIF2AK3* gene has been studied in French early onset type 2 diabetic patients by Vaxillaire et al (2001) but no mutations were identified.

An *EIF2AK3* knock out mouse model has been developed. The phenotype is remarkably similar to human WRS, with neonatal diabetes, skeletal defects and small size with delayed growth (Harding *et al.*, 2001; Zhang *et al.*, 2002); additionally it was characterized by malabsorption. The heterozygous had a milder phenotype characterized by glucose intolerance. *EIF2AK3* has been demonstrated to be involved in the coordination of gene expression in response to endoplasmic reticulum (ER) stress and therefore is a key enzyme in pancreatic B cell function (Jiang *et al.*, 200).

Recent studies have demonstrated linkage of T1D with additional chromosomal regions (LOD score >10.5) but the loci are not designated yet. Susceptible loci are listed in the table 1.3.

Table 1.3: Chromosomal regions had linkage (LOD score >1.5) to T1D (ECIGS 2001[a]; Cox *et al.*, 2001[b])

Chromosome	Marker/position	LOD score	*p* values
1	1q42	2.2 [a]	$7.4x10^{-4}$
2	D2S113	1.82 [b]	0.002
4	D4S403	1.84 [b*]	-
5	D5S507	2.16 [b*]	-
12	D12S99	1.97 [b*]	-
16	D16S405-D16S287	2.80 [b]	$2x10^{-4}$
16	16p11-p13	1.74 [a*]	-
16	D16S3098	3.93 [a]	$2.2x10^{-5}$
17	17q25	1.81 [a*]	-
19	19q11	1.80 [a*]	-

[*]Corresponding p value not mentioned.

1.3.2 Environmental factors

Despite the strong evidence for importance of environmental triggers for development of T1D the precise pathology remain(s) elusive. T1D is increasing world-wide and this

strongly suggests the importance of environmental factors in the prediabetic process. Among the environmental factors diet, stress and viral infection have been proposed (Knip and Akerblom, 1999, Dahlquist 1998; Atkinson *et al.*, 1994).

Among the dietary factors exposure to cow's milk, duration of breast feeding and N-nitroso compounds are claimed to be associated with the development of T1D. Epidemiological studies have revealed that giving cow's milk to the new-born increases the risk of development of T1D in later life. It was hypothesized that limited amino acid homologies between bovine albumin and ICA69, an islet antigen, may contribute to the development of T1D (Karjalainen *et al.*, 1992). Increased concentration of IgG and IgA antibodies to cow's milk formula have been found to be associated with risk of developing T1D (Dahlquist *et al.*, 1992; Virtanen *et al.*, 1994). Both infant feeding patterns and current milk consumption have also been suggested affecting cow milk antibody titers. A follow up of the initially nondiabetic siblings of T1D children showed that higher milk consumption is associated with greater risk of developing T1D in 10 years time (Virtanen *et al.*, 1998; Virtanen *et al.*, 2000). When amount of milk consumption and child's genotype was considered together there was 5-fold increase of risk of developing diabetes, substantiating an interaction between milk consumption and genetic risk (Virtanen *et al.*, 2000). Even the time of exposure to cow's milk also has been found to be important. Hypponen et al (1999) has observed that an early exposure to cow's milk-formula and rapid growth in infancy confer independent risk factors for childhood diabetes. However, case control studies have revealed an inconsistency in the association between milk consumption during childhood and risk of T1D (Dahlquist *et al.*, 1990; Verge *et al.*, 1994; Dahlquist *et al.*, 1994).

In the recent years vitamin D has been suggested to be associated with increased risk of T1D. Low vitamin D level was demonstrated in T1D patients' sera compared to controls and has been claimed to play a key role in T1D susceptibility (Baumgart *et al.*, 1991; Hypponen *et al.*, 2001). In addition use of cod liver oil during pregnancy was found to be with low incidence of T1D in the offspring (Stener *et al.*, 2000) Allelic variations in the vitamin D receptor (VDR) gene of T1D patients were studied. McDermott et al (1997) found association of polymorphism in the *VDR* gene with T1D and suggested a possible link in its pathogenesis. It has also been observed that VDR

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polymorphism had significant determinant effect on VDR mRNA and VDR protein (Ogunkolade *et al.*, 2002). Although a number of studies supported these initial observations (Pani *et al.*, 2000; Chang *et al.*, 2000; Skrabic *et al.*, 2003) a recent comparative study has cast doubt on those findings (Nejentsev *et al.*, 2004).

A possible role of vitamin D in the glucose metabolism has also been supported by animal studies. Vitamin D supplementation has been shown to prevent insulitis in NOD mice (Mathieu *et al.*, 1992) and later on short-term treatment with high dose of vit-D3 has been shown to protect the development of diabetes in NOD mice (Zella *et al.*, 2003). The abnormality in glucose metabolism has been suggested as a result of an alteration of mitochondrial metabolism (Billaudel *et al.*, 1998). Vitamin D was suggested to play important role in the modulation of immune system (Mathieu and Adorini, 2002). However, the role of vitamin D and the mechanism involved in the etiopathogenesis of diabetes are yet to be clearly understood.

Food preservatives have emerged as a great health concern. Case control studies have demonstrated that dietary *N-nitroso* compounds, nitrate and nitrite or a combination thereof, may play a role in the pathogenesis of T1D (Helgason and Jonasson, 1981; Dahlquist *et al.*, 1990; Virtanen *et al.*, 1994). The most important exogenous source of *N-nitroso* compound is food and in addition they may also come from cigarettes, car interiors, and cosmetics. Nitrates and nitrites are used as food additives in the processing of meat for their antimicrobial property and also the ability to improve color and taste. In food and in gastrointestinal tract, nitrate can be reduced to nitrite which may react with certain amines and amides leading to formation of toxic *N-nitroso* compounds. Studies in Canada and Australia assessed consumption of *N-nitroso* compounds but did not find any difference between controls and diabetes (Verge *et al.*, 1994; Siemiatycki *et al.*, 1989). In contrast in a Swedish study the risk of a 2.5 fold rise of diabetes was found with the use of foods containing nitrosamines (Dahlquist *et al.*, 1990). In another study, nitrite but not nitrate intake in the highest quartile was found to be associated with 2.4 fold increased risk for T1D compared to lower quartile and among the children of less than 7 years of age the risk was 4.5 fold (Virtanen *et al.*, 1994). Although different groups have conflicting results the importance of those compounds cannot be ignored. Toniolo et al (1980) had shown

that nitrosamines increase the diabetogenic effect of certain viruses which further suggest importance of environmental factors.

Many viruses have been implicated in the pathogenesis of the disease. Among those, most important viruses are coxsackie B, rubella, cytomegalovirus, and Epstein-Barr viruses. The viral hypothesis in the development of T1D is mainly derived form epidemiological studies, although some direct evidences do exist (Szopa *et al.*, 1993). Among the viruses Coxsackie B virus (CBV) has been extensively studied in the pathogenesis of T1D. The association of CBV with T1D was based on isolation of CBV from pancreas of T1D patients and increased prevalence of antibodies to CBV in T1D patients at diagnosis (Yoon *et al.*, 1979; Frisk *et al.*, 1985; D'Alessio 1992). The mechanism of CBV induced B cell death leading to T1D has not been fully understood. However, one possible mechanism is 'molecular mimicry'. This is based on a sequence homology between foreign antigen, ie viral protein and a host protein. It is postulated that immune reactivity against the virus can lead to a cross reactivity to the homologous sequence of the host protein (Vreugdenhil *et al.*, 1998; Atkinson *et al.*, 1994; Jaeckel *et al.*, 2002). Viral protein PC2 (non-capsid protein of CBVB4) has been demonstrated to have a sequence homology with human GAD65, an enzyme that converts glutamic acid to gamma-amino butyric acid. Human GAD65 amino acid residue 250-273 and PC2 protein residue of 28-50 carry PEVKEK amino acid sequence. The PEVKEK motif found in both CBV and GAD65 bind to T1D associated DR3, but not to DR1 or DR4 (Tian *et al.*, 1994; Vreugdenhil *et al.*, 1998). Antibodies isolated from T1D patient's serum reacted with both PC2 and GAD which further substantiated the molecular mimicry theory (Hou *et al.*, 1994). It has also been reported that B cell critically depend on antiviral interferons (INFs) to lower their permissiveness to CBV4 infection and that in mice with pancreatic B cells that had defective INF responses, CBV4 manifest an acute diabetes which resembled T1D that develops in humans after severe enteroviral infection (Flodstrom *et al.*, 2002).

A high incidence of T1D is observed among patients with congenital rubella syndrome (CRS). Approximately 12-20% of subjects with congenital rubella infection develop T1D within 5-20 years of age. Islet cell surface antibodies were found in 50-80% patients of CRS with glucose abnormality and in 20% of the total CRS patients (Ginsberg-Fellner *et al.*, 1985). Cytomegalovirus (CMV) and Epstein-Barr virus (EBV)

has also been shown to be associated with newly diagnosed T1D. Twenty percent of T1D patients had CMV's DNA in their islets and 80% of this subgroup showed ICA antibody in the serum. EBV has been implicated in the etiology of several autoimmune diseases and a few cases showed temporal link to onset of diabetes. A capsid assembly protein, encoded by *BOLF1* gene, of EVB has an 11-residue sequence homology with DQ8 HLA class II molecule. Thus, EBV may be capable of triggering autoimmune T1D through molecular mimicry. All the viruses implicated in the etiology of T1D have been associated with disease involving autoimmune process either by directly triggering autoimmune response or as a result of molecular mimicry with viral and host protein.

1.3.3 Autoimmunity in T1D

Features of inflammatory response was first observed in and around pancreatic islets, 'termed insulitis', in T1D at post-mortem examination suggesting immune mediated damage of pancreatic islets. Subsequently T1D was found to be associated with other autoimmune diseases, again implicating autoimmune process involved in its pathogenesis. A number of islet related antigens have been characterized and autoantibodies against some of these islet antigens have been identified in the serum of T1D patients (Bottazzo *et al.*, 1974; Palmer *et al.*, 1983; Baekkeskov *et al.*, 1990; Payton *et al.*, 1995).

Autoantibodies are found in T1D patients either in singly or in combination. In general a single autoantibody is usually not associated with progression to T1D while autoantibodies against more than one antigen is a strong predictor of diabetes.

1.3.3.1 Islet cell autoantibody

The first autoantibody to be identified in serum of patients with T1D was the islet cell antibody (ICA) directed against islet tissue. Bottazzo et al (1974) observed the presence of ICA in T1D patients with polyendocrine disease. Subsequently different other groups demonstrated the association of ICA with T1D. Lendrum et al (1975) reported that about 50% of their study subjects were ICA positive independent of other autoimmune markers. In another study 80% of newly diagnosed T1D patients were found to be positive for ICA (Bruining *et al.*, 1984). However, several subsequent studies have found the frequency of ICA positivity of 35-80% among new and recently

diagnosed T1D patients in different populations (Shinjyo *et al.*, 1983; Bruining *et al.*, 1984; Merchant *et al.*, 1996; Savola *et al.*, 1998). In the background population ICAs are reported to be around 1-4% in different studies (Samuelsson *et al.*, 1994; Levy-Marchal *et al.*, 1992; Knip *et al.*, 1998). Among the nondiabetic sibs ICA is reported to be positive between 4 to 13% (Eskola *et al.*, 2003, Knip *et al.*, 1998; Deschamps *et al.*, 1984).

Different prospective studies have revealed that ICA titre is relatively transient; the titre falls with progression of the disease. However, a small subset of patients have been shown to have persistently high ICA levels and this is associated with a more rapid loss of residual endogenous insulin secretion (Marner *et al.*, 1985; Wallensteen *et al.*, 1988; Schiffrin *et al.*, 1994; Bruining *et al.*, 1984).

Prospective family based studies have shown that, in nondiabetic first degree relatives of T1D patients, the presence of ICA is associated with an increased risk of subsequent clinical T1D (Tarn *et al.*, 1988). Bonifacio et al (1990) followed up first-degree relatives of T1D patients for 10 years and demonstrated an increased risk of T1D. ICA has also been shown to have high predictive value for T1D among unrelated children with persistently high titer of the antibody (Bruining *et al.*, 1989; Karjalainen 1990).

The highest predictive value of 75% for development of T1D among the first-degree relatives was obtained in individuals with high titer of ICA and positive for HLA-DR-3/4 (Robert *et al.*, 1991). However, it was also evident that some apparently high-risk first-degree relatives remain without diabetes despite persistent ICA positivity, reduced B cell function and at risk HLA status (McCulloch *et al.*, 1990; Robert *et al.*, 1991).

1.3.3.2 Insulin autoantibody

Insulin autoantibodies (IAA) was first reported in 34% of a group of subjects with T1D as compared to 4% in control population in early sixties (Pav *et al.*, 1963). In 1983 Palmer et al however, found IAA in 18% of untreated and newly diagnosed T1D subjects. IAA was subsequently reported positive in around 40-50% of newly diagnosed diabetic subjects in different studies (Wilkin *et al.*, 1985; Karjalainen *et al.*, 1988; Sochett and Daneman, 1989). IAAs was found to be as high as 100% in T1D patients of 5 years old and younger at onset of diabetes, but positivity for IAA

progressively declined as age progressed (Vardi *et al.*, 1988). However, a correlation between IAA titre and age of onset of T1D has been demonstrated; IAA titre remaining within a limited range for many years. IAA has also been found to have strong correlated with ICAs. It was observed that IAA was positive in 53% of ICA positive subjects, compared to only 2.7% of ICA negative individuals (Vardi *et al.*, 1988; Ziegler *et al.*, 1988). Detection of IAAs and ICAs in combination among first-degree relatives of T1D conferred highest risk of developing T1D. More than 70% of individuals with both antibodies develop diabetes compared to 42% of those positive for ICA alone, and 17% for IAA alone (Ziegler *et al.*, 1989).

In the background population IAAs are found positive in around 4% or less (Pav *et al.*, 1963; Hegewald *et al.*, 1992; Samuelsson *et al.*, 1994; Strebelow *et al.*, 1999) and among the first relatives of T1D is 10% or lower (Kuglin *et al.*, 1989; Hegewald *et al.*, 1992).

IAA positivity in T1D patients has been found to be associated with HLA-DR4 (Ziegler *et al.*, 1991; Gorus *et al.*, 1994; Pugliese *et al.*, 1994). Subsequently, in a population based study, it was observed that IAA were associated with HLA DR-4 DQ8 haplotype (DQB1*0302-DQA1*0301), but not with DQ2 haplotype (DQB1*0201-DQA1*0501) (Vandewalle *et al.*, 1993; Hagopian *et al.*, 1995).

1.3.3.3 Antibody against glutamic acid decarboxylase (GAD)

An antibody was demonstrated against a protein of molecular size 64,000 *Mr* in the newly diagnosed T1D patients' sera (Baekkeskov *et al.*, 1982). It was observed that the antibody against the 64000 *Mr,* possibly a human islet protein, is an early marker of B cell autoimmunity and it is useful to predict development of T1D in later life and it persists after the onset of diabetes (Baekkeskov *et al.*, 1987; Christie *et al.*, 1990b). True identification of this newly evolved 64000 *Mr* protein and antibody against it remained elusive for some time. Later Christie et al (1990a) demonstrated that the 64000*Mr* protein was restricted to B cell plasma membrane which further strengthened the hypothesis that this protein may be a target antigen of B cell specific autoimmunity of T1D. Subsequently this 64000 *Mr* protein was revealed to be an enzyme protein, namely glutamic acid decarboxylase (GAD) which catalyses the formation of inhibitory neuropeptide γ-amino butyric acid (GABA) from glutamic acid. Pancreatic beta cells and a subpopulation of central nervous system neurons express high levels of this

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enzyme (Baekkeskov *et al.*, 1990). GAD was found to exist as multiple isoforms with different tissue specific pattern of expression, but two isoforms GAD65 and GAD67 (having molecular weight 65,000 and 67,000 *Mr* respectively), were found in nerves and islet cells as doublet of proteins (Kaufman *et al.*, 1991; Christgau *et al.*, 1991). Both GAD65 and GAD67 have been cloned and sequenced (Michelsen *et al.*, 1991; Karlsen *et al.*, 1991). Gene encoding GAD65 lies on Chromosome 3 and GAD67 on Chromosome 10. Molecular sequences of GAD65 and GAD67 have been determined in different mammalian species and have shown a remarkable degree of evolutionary conservation. The two isoforms had approximately 65% identity at the amino acid level and most of the variation was observed in primary structure, located within the 100 amino acids (Karlsen *et al.*, 1991; Bu *et al.*, 1992). Antibodies in T1D found to be primarily recognizing the 65,000 *Mr* isoform of GAD (Hagopian et al 1993)

Numerous studies have determined the frequency of GAD Ab in newly diagnosed T1D subjects from a wide range of populations. Between 50-80% of newly diagnosed subjects are positive for GAD against <2% in the background population. The frequency of GAD positivity is found much higher in Caucasoid compared to other populations. In Caucasian T1D patients GAD antibody positivity was shown to be 76% and 77% (Velloso *et al.*, 1993; Hawa *et al.*, 1997) compared to 54% in Mexican, 41% in Asians in Singapore, 35% in Japanese and 39.6% in Chinese T1D patients (Mendoza-Morfin *et al.*, 2001; Lee *et al.*, 2001; Tsuruoka *et al.*, 1995; Thai *et al.*, 1997). The low positivity of GAD Ab in Asian population was also supported by the study of Tuomi et al (1995) who found a frequency of GAD Ab positivity as that of 30% in Koreans, 51% in Thais and 84% in Europid Australians. In India, between north and south, frequency of GAD positivity showed some variations. Mohan et al (1998a) have shown frequency of GAD Ab positivity in 47.5% in T1D patients. In the study by Goswami et al (2001) involving north Indian population GAD Ab was demonstrated in 14.2% of typical T1D patients, but in ketosis resistant T1D it was 38%. However, Singh et al (2000) in their series observed 40% positivity of GAD Ab in T1D patients of North India.

The frequency of GAD antibodies in the background population was found to be 2.2% (Tsuruoka *et al.*, 1995) in Japanese and 0.4% in southwestern Netherlands population (Batstra *et al.*, 2001). Among the background Indian population the frequency was 2% for GAD Ab. In one study none was found to be positive for GAD Ab among 20 controls (Singh *et al.*, 2000). In another study involving the same north Indian

population revealed zero positivity for GAD Ab (Goswami *et al.*, 2001). First-degree relatives of T1D patients had comparatively higher frequency of GAD Ab positivity than the background population. Among French school children 3.6% of first-degree relatives had GAD Ab positive (Thivolet *et al.*, 2002) and the value was 9.4% in Germans (Hatziagelaki *et al.*, 1999).

Autoantibody to GAD has been reported to increase with age and the frequency of this autoantibody was found highest in the patients with HLA-DR3 genotype type (Hawa *et al.*, 1997; Lohmann *et al.*, 1997).

1.3.3.4 Antibody against tyrosine phosphatase like protein

In 1998 Christie et al (1988) demonstrated a novel antibody against an islet cell protein in the sera of a group of newly diagnosed T1D patients. Subsequent study suggested that this newly found antibody might be different from ICAs (Christie *et al.*, 1993). This led to the search for the identification of the newly found antibody against a 64000 *Mr* protein. Partial proteolysis of the 64000 *Mr* protein generates three major fragments: 50,000 *Mr*, 40,000 *Mr* and 37,000 *Mr* (Christie *et al.*, 1990c). The 40,000 *Mr* protein fragment was found to precipitate in high percentage of sera from patients with T1D, but it did not react with GAD antibody, confirming that the antibody against the 40,000 *Mr* fragment was unique (Christie *et al.*, 1993). Subsequently the 40,000 *Mr* fragment was identified as the intracellular domain of a B cell transmembrane protein (islet antigen; IA-2) (Lampasona *et al.*, 1996; Payton *et al.*, 1995).

In the meantime Rabin et al (1994), based on the screening of a human islet cell cDNA expression library, isolated a islet cell protein, termed as ICA512, that reacted with sera from patients with T1D. They found that this autoantigen was reactive with 48% of sera from 80 T1D patients tested. Another group independently demonstrated that the ICA512 is expressed in neuroendocrine cells and was restricted to neurosectretory granules (Solimena *et al.*, 1996). Lan et al (1994), by molecular cloning, independently identified a protein tyrosine phosphatase like protein. They termed it as islet antigen 2 (IA2) which differentially expressed in human beta islet cells of the pancreas. Subsequently Pietropaolo et al (1997) demonstrated that ICA512 and IA2 had little dissimilarity in amino acid sequences and confirmed they were the same molecules.

The gene encoding protein tyrosine phosphatase-2 or islet antigen 2 (IA2) has been assigned to the chromosome 2q35-q36.1 (van den Maagdenberg *et al.*, 1996; Morahan *et al.*, 1998). IA2 gene encodes a 979-amino acid protein with an

approximate mass of 105 kD. In contrast to most other receptor PTPases, which have 2 tandem PTPase domains, IA2 has a single transmembrane domain and only 1 PTPase domain (Lan *et al.*, 1996). The extracellular domain (576 amino acid) is thought to reside within the secretory granules and intracellular domain (378 amino acid) protrudes in the cytoplasm. On stimulation of cells, the secretory granules fuse with the plasma membrane and extracellular domain of IA-2 becomes part of the plasma membrane (Lan *et al.*, 1994; Hawa *et al.*, 1997).

Attempts have been made to clarify the major antigenic regions of the ICA512/IA2 membrane protein. Extracellular domain itself does not react with diabetic sera, but all the sera reacted with intracellular domain; 95% of sera reacted with carboxyl-terminus (amino acid 771-979) and 40% with amino-terminus (amino acid 604-776) of IA-2. It was confirmed that 40, 000 *Mr* is the intracytoplasmic domain of the IA-2 (IA-2ic) (Christie *et al.*, 1993).

The frequency of IA-2 was found to be positive in 55-80% of newly diagnosed T1D patients compared to 0-2.5% of controls. Positivity for IA-2 in type 1 diabetic patients is higher in Caucasoid compared to Indian population. For example frequency of IA-2 antibody was 58%, 66%, 67% and 81% involving Caucasoid serum samples (Gorus *et al.*, 1997; Lan *et al.*, 1996; Hawa *et al.*, 1997; Ongagna and Levy-Marchal, 1997). Whereas one study involving Indian population showed frequency of IA-2 antibody about 23% (Fida *et al.*, 2001).

Batstra et al (2001) found the frequency of IA-2 positivity 0.1% in the control subjects of south western Netherlands. Lan et al (1996) in their study observed that none of the healthy controls was IA-2 antibody positive. First-degree relatives of T1D patients had much higher frequency of IA-2 positivity. In the Europid population this frequency was observed between 3-6% in number of studies; 4.4% by Seissler et al (1996), 4.9% by Thivolet et al (2002) and 3.6% by Hatziagelaki et al (1999).

The frequency of IA-2 antibodies varies with age and HLA genotype. The frequency has been observed highest in younger age group and patients with HLA DR4 (HLA DQA1*0301-DQB1*0302) genotype (Hawa *et al.*, 1997; Gorus *et al.*, 1997; Genovese *et al.*, 1996; Lohmann *et al.*, 1997). A number of studies have demonstrated that the estimation of GAD alone or GAD and IA-2 antibodies in combination better identifies the cases of T1D compared to ICA and IAA.

1.4 Latent autoimmune diabetes in adult

A subset of diabetic patients diagnosed as adult onset T2D has little difference with T1D in clinical presentation and they also very difficult to be distinguished from true T1D. The development of clinical symptoms is often insidious, without pathognomonic features for T1D such as polydypsia, polyurea, weight loss or ketoacidosis. This group of patients are usually younger, over 25 years but some has suggested the age to be above 35 yrs, and non-obese compared to the typical T2D patients. They have been found to have low residual insulin secretory capacity compared to their T2D counterpart (Turner *et al.*, 1997; Abiru *et al.*, 1996). However, they have pronounced hyperglycemia and after being put on to oral hypoglycemic agents (OHA) for months to years, become insulin dependent for their glycemic control.

In a number of studies circulating autoantibody ICA (Groop *et al.*, 1986; Gottsater *et al.*, 1994) and more frequently anti-GAD (Tuomi *et al.*, 1993) were demonstrated to occur in clinically diagnosed T2D patients. Later on autoantibody IA2 also have been shown to occur in this group of T2D patients (Gambelunghe *et al.*, 2000). Positivity for ICA or GADAb shows a strong correlation with insulin deficiency or relative insulin requirement in white Caucasians and Asian patients (Gottsater *et al.*, 1993; Toumi *et al.*, 1993; Zimmet *et al.*, 1994; Niskanen *et al.*, 1995).

This group of patients is described by the term latent autoimmune diabetes in adult (LADA) (Groop *et al.*, 1986; Tuomi *et al.*, 1993; Zimmet *et al.*, 1994) although in the latest classification of diabetes by the WHO they would be termed as autoimmune T1D. Some have suggested that T1D and LADA are two distinct forms of disease. They thought that both are autoimmune in nature and they coined the term 1.5 diabetes (Juneja and Palmer, 1999).

Based on the presence of either GAD or IA2 antibody the prevalence of LADA found to show wide range worldwide. The prevalence was found to be as low as 3.7% (Soriguer-Escofet *et al.*, 2002) in Spain where as in the UK it was 34% (Turner *et al.*, 1997).

1.4.1 Genetics of LADA

GAD Ab positve, ie LADA, patients had shown increased frequency (13%) of HLA-DQ B1* 0201/0302 compared to non-LADA patients and control subjects (4%) and other genotypes containing the *0302 allele 22 vs 12%. However, the high-risk genotypes

were significantly lower in the LADA, GAD Ab positive T2D patients, than the T1D of young and adult onset (0201/0302 or 0302/X: 36 vs 66 vs 64%) (Tuomi *et al.*, 1999).

Frequency of HLA-DQB1*0201/0302 was lower in GAD antibody positive diabetic patients (12%) compared to both young onset (34%) and late onset (41%) T1D, although it did not differ from the frequency of DR3/DR4 in adult onset patients in another Finish study (12.5%) (Karjalainen *et al.*, 1989). Also the proportion of GAD positive insulin requiring T2D patients possessing genotypes including HLA-DQB1*0302 or 0602 was different from the proportion of T1D patients possessing the same genotypes, whereas no difference was observed in the genotype frequencies between the two T1D group (Caillat-Zucman *et al.*, 1992).

T2D patients from families in which T1D is also found are more frequently GADab (18% vs 8%) and DQB1*0302/X genotype (25% vs 12%) positive than patients from families with only T2D; however, they have a lower frequency of DQB1*02/0302 genotype compared with adult-onset type I (LADA) patients (4% vs 27%). In the mixed families, the insulin response to oral glucose load was impaired in patients who had HLA class II risk haplotypes, either DR3-DQA1*0501-DQB1*02 or DR4*0401/4-DQA1*0301-DQB1*0302, compared with patients without such haplotypes independent of the presence of GAD ab (Li *et al.*, 2001). This led to a conclusion that T1D and T2D cluster in families and share a common genetic background defined by HLA irrespective of autoantibody positivity and thus in turn leads to impaired insulin secretion.

1.5 Type 2 Diabetes Mellitus

Type 2 diabetes (T2D) mellitus is characterised by the presence of disorders of insulin action and/ or insulin secretion (Reaven 1988). Both defects are usually present in a diabetic patient; however, the primacy of the two factors for the development of diabetes still remains to be clearly understood.

T2D constitutes more than 90% of all diabetics and in most populations. However, in American Indians and South Pacific islanders, T2D is the only form of the disease. Overall prevalence of diabetes varies between 15-20%. The highest prevalence of T2D (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).

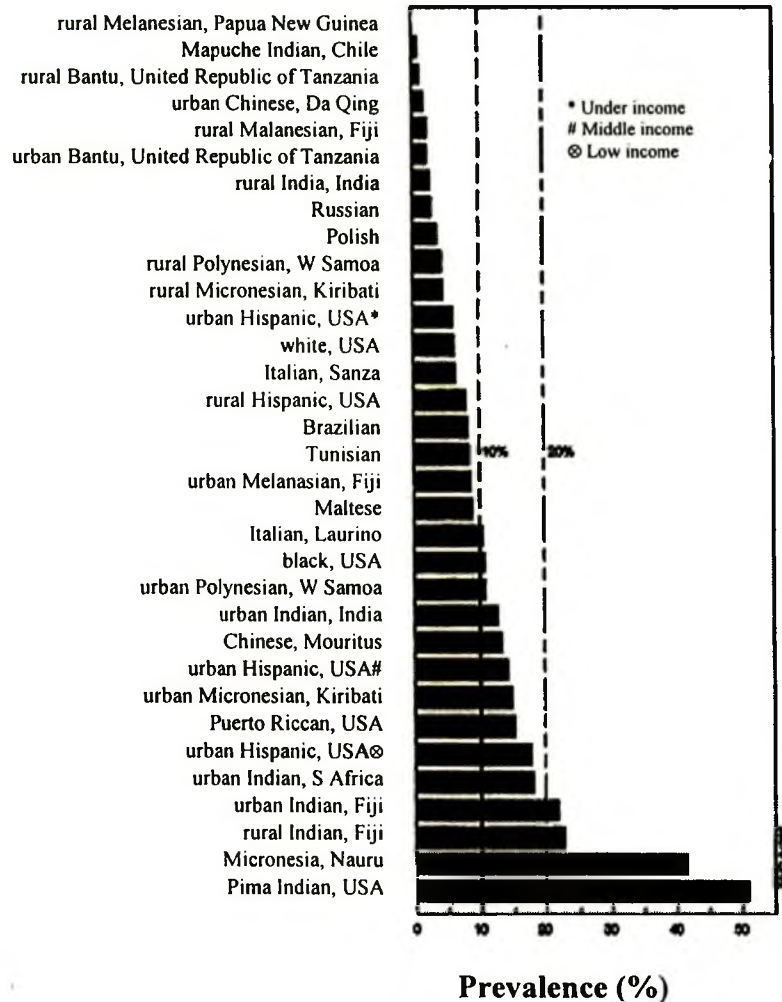

Figure 1.2: Prevalence of T2D in the age range 30-64 years in the selected populations (gender combined) (WHO 1994).

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 T2D 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 USA the prevalence of diabetes has shown to vary among different ethnic groups; 5% in the Caucasian origin, 10% in

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African-Americans, 16% in Cuban origins and 26% in Puerto Ricans (Harris 1990; Flegal *et al.*, 1991).

Part of this variability in the prevalence of diabetes can be attributed to environmental and cultural factors; however, the observation that disease prevalence varies substantially between ethnic groups that all share similar environments support the fact that genetic factors might be contributing to disease predisposition. This is reflected in the prevalence of diabetes in different ethnic groups living in the US and UK, where minority ethnic groups have very different prevalence of diabetes compared with mixed subjects of European descents. There are around 1.4 million people with diabetes in UK; the prevalence of disease is 2.4% (approx) in Caucasians, but in some minority groups, such as African-Caribbean and South Asian descents, the estimated risk of diabetes is 3-6 fold higher (Greenhalgh 1997; Mather and Keen, 1985; Chaturvedi *et al.*, 1993; Simmons *et al.*, 1991). Although part of this variability may be accounted for cultural and socio-economic status, it is unlikely that these factors alone explain the observed difference.

1.5.1 Genetics of T2D

1.5.1.1 Familial aggregation

Familial aggregation of the disease is another line of evidence for a genetic contribution. A higher prevalence within family members of a proband than expected within the general population was observed in different studies. This is thought to be due to an increased number of shared genes between family members, including genes that play a role in disease predisposition.

Several studies showed increased prevalence of T2D in the off springs of parental diabetes. Klein et al (1996) reported that if neither parent has the disease the prevalence of T2D is 10.4% in the siblings, but if one parent is affected the prevalence in the siblings was 17.8% and 25.2% when both parents are diabetic. In another study the risk for diabetes was demonstrated to be strongly associated with family history: 14.8% for those with no family history of diabetes, 22% for those with one parent having diabetes, and 41% in those with both parents having history of diabetes (Weijnen *et al.*, 2002). The CODIAB Study in France showed that 66% of T2D patients had at least one relative with diabetes. The life-time risk of developing T2D was about

40% in offsprings with one diabetic parent, but the risk further increases up to 70% if both parents have the disease (Groop and Tuomi, 1997). Although T2D is 10 time more common than T1D, it has λs = 3.5 compared to T1D of 15 (Rich 1990). This phenomenon was observed across different races. In a study involving South Indian population Ramachandran et al (1988) observed that 36% offsprings of one parent compared to that of 54% with both parents diabetic had the disease. Clustering of T2D in the families was also reported in the Chinese population of Hong Kong. Approximately 36% of T2D patients had at least one affected parent or siblings (Lee *et al.*, 2000).

Family studies, however, showed an increased rate of transmission of T2D from diabetic mothers than fathers. The French CODIAB study showed that maternal history (34.2% and 31.5%, diabetic men and women) of diabetes was more than twice of the paternal diabetic history (16.7% and 17.6%) (Thomas *et al.*, 1994).

A cross-sectional study through a diabetic registry involving Caucasoid, Afro-origins and Asian patients showed very high maternal diabetosity than that of the father. Over 60% had history of mother with diabetes. Similar trend was reflected in the Caucasoid and Afro-origin patients who had incidence of maternal diabetes 68.1% and 67.1% respectively and paternal diabetes 27.3 vs 22%. Among the Asian patients the incidence of maternal diabetes was observed rather less than that of father ie 34% vs 56% respectively. However, under reporting of maternal diabetes have been explained by the author as the reason of high paternal incidence of diabetes in the Asian study (Young *et al.*, 1995).

Studies involving African Americans, Asians/Pacific Islander, Hispanic and Native Americans revealed that for all races 20% and 16.7% of the offsprings had type 2 diabetic mothers and fathers respectively. Race-wise mother was affected in 20.3%, 18.7%, 23.3% and 26.1% in African Americans, Asian/Pacific Islanders, Hispanic and Native Americans compared to 20.1%, 16.2%, 18.7% and 24.1% fathers respectively (Karter *et al.*, 1999). A 22 year follow up study revealed that family history of diabetes was reported in 15.3% maternal compared to 10.8% parental history and 6.4% with no history (Bjornholt *et al.*, 2000). They have showed that maternal history conferred a relative risk (RR) of 2.5, combined maternal and paternal RR of 3.96 and paternal history RR of 1.4 for development of diabetes. However, the Framingham Study

showed no different level of incidence of T2D with maternal (10.5%) and paternal (11.55) history of diabetes (Meigs *et al.*, 200).

1.5.1.2 Twin studies

One of the earliest twin studies by Barnett and co-workers showed that 91% (48 out of 53) monozygotic twin pairs were concordant for diabetes. Thirty five (35) out of 48 pairs (73%) became diabetic within one year and of the remaining 13 pairs 11 became diabetic between 6 and 10 years (Barnett *et al.*, 1981a). Following a glucose challenge the remaining discordant twin members had severely impaired insulin response. These findings, taken with the high concordance rate for non-insulin-dependent diabetic twins, suggest that T2D has a strong genetic component (Barnett *et al.*, 1981b). In contrast in a study conducted by Newman et al (1987) on a cohort of MZ and DZ twins, who were examined at 10 years apart, at the age of 47 and 57 years, demonstrated lower concordance rate. Initially 28% of both MZ and DZ twins were concordant for diabetes and, after 10 years, at the mean age of 57, 58% MZ and 17% DZ twins had diabetes against 10% expected prevalence of diabetes at that age. Even after 10 years 1 of 15 discordant for the disease remained the same. In the same study it was demonstrated that concordance for diabetes was less than 100% even by the age 52-65 years and twins varied in age at the onset of the disease. They suggested non-genetic factors, possibly environmental, to trigger development of the diabetes. This was supported by the fact that among the discordant MZ twin pairs for diabetes, diabetic twins did not differ from their nondiabtic co-twins regarding obesity, diet, alcohol consumption or education. However, compared with unrelated nondiabetic twins of the same ages, nondiabetic co-twins of diabetic twins gained more weight as adults and had higher glucose levels (Newman *et al.*, 1987). A population based twin study by Kaprio et al (1992) investigated the cumulative incidence, concordance rate and heritability of the disease. Cumulative incidence of the disease was 1.4% in males and 1.3% in females between 28-50 years of age, and 9.3% and 7% respectively by age of 60 years and above. The probands-wise and pair-wise concordance rates for T2D were 34% and 20% among monozygotic twins and 16% and 9% in dizygotic twins, respectively. In another study by Medici and colleagues demonstrated that after a 15- year follow-up 76% of MZ twins that were initially selected as being discordant for disease became concordant (Medici *et al.*, 1999). Ghosh and Schork (1996)

suggested that age-adjusted concordance rate in MZ twins may be as high as 70-80% for T2D. In spite of the differences in different twin studies, the evidence is compelling that T2D has a substantial genetic component.

1.5.1.3 Animal model

Numerous syndromes of glucose intolerance including moderate and severe T2D have been found to occur spontaneously or can be induced experimentally in various animal species. These models can be found to support all possible proposed etiologies of diabetes from environmental to wholly genetic and multifactorial in origin. Spontaneous models can result from single gene mutations, such as autosomal recessive traits in obese (ob/ob), diabetic (db/db) mice and fat (fa/fa) rats or from polygenic defects as in the New Zealand obese (NZO) mouse, Goto-Kakizaki (GK) rat and Chinese hamster. However, the manifestation of different phenotypes between strains of the same species and between species with the same gene defects emphasizes the complexity of physiological processes involved.

The study of monogenic models of diabetes potentially enables the uncovering of novel metabolic pathways in the disease pathogenesis. A novel hormone 'leptin' was found to have critical role in the pathogenesis of diabetes in ob/ob mouse and led the way of its role in the pathogenesis of T2D in human (Zhang *et al.*, 1994). The knowledge further suggested a number of different possible therapeutic approaches to the prevention of obesity and subsequent diabetes. Among the polygenic models GK rat and NZO mouse have been mostly studied. The NZO mouse polygenic model exhibited typical symptoms of T2D - hyperphagia, mild hyperglycemia, and hyperinsulinemia with marked insulin resistance (Holemans *et al.*, 1991). The GK model demonstrated typical features of T2D like that in human. At least seven loci controlling diabetes related sub-phenotypes have been identified in GK rat (Gauguier *et al.*, 1996; Galli *et al.*, 1996). Moreover, one of the loci, GK2, was found in the syntenic region on human chromosome 1 in which linkage with T2D has been found in several genome scans (Vionnet *et al.*, 2000; Hanson *et al.*, 1998; Elbein *et al.*, 1999).

1.5.1.4 Gene discovery for T2D

T2D is an example of a multifactorial disease and is a component of the metabolic syndrome consisting of fasting plasma glucose ≥6.0 mmol/l or insulin resistance and at

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least two of the three factors of raised arterial pressure, dyslipidemia (raised plasma triglyceride an/ or low HDLc) and central obesity (WHO 1999). About 75% of deaths in diabetes is due to cardiovascular disease.

An early study showed heterogeneity of body weight in probands with T2D and in the affected siblings; 38% of affected siblings were nonobese compared to 10% in moderate to over-weight proband group (Kobberling 1971). Fajans (1986) in a review article described the variability in the pathology of patients with T2D including structural abnormalities in pancreatic A and B cells. The heterogeneity in the pathology of T2D includes decreased B cell insulin secretion, delayed insulin response, decreased insulin sensitivity and peripheral insulin resistance. This wide range of variability in individuals strongly suggests involvement of multiple genes 'polygenes' in the etiology of T2D rather than a single locus.

In search of genetic involvement in T2D both positional approach 'phenotype down' and 'candidate gene' approach 'phenotype up' have been applied. In the first approach pedigrees are analyzed by linkage analysis using parametric or non-parametric linkage methods in affected large pedigrees or sibling pairs respectively. Alternatively families and population samples were used for genetic linkage disequilibrium analyses. There has been remarkable progress in identifying gene variants causing monogenic (maturity onset diabetes mellitus (MODY)) forms of T2D (section 1.5) principally by analysis of large pedigrees in whom a clear mode of inheritance can be identified. In common T2D in whom the inheritance is complex the study methods have been nonparametric linkage analysis using sib pairs or association studies.

Since the nature of presentation of T2D is complex, alternative strategies have been applied, mostly focusing on analysis of 'small nuclear families' and 'sibships' containing multiple affected siblings'. However, a large number of affected sib pairs are required to achieve sufficient power to detect and assert susceptibility loci. A large number of genome scans for T2D have been performed. Most studies have been carried out in Caucasian populations of European descent.

1.5.1.4.1 Linkage study

No T2D susceptibility gene has been identified of the magnitude of HLA in T1D. However, several chromosomal regions have been found in strong linkage with T2D.

Some of the loci have showed linkage in more than one population. Important hotspots for T2D have been identified on chromosome 2q37 (Hanis *et al.*, 1996), chromosome 12q24 (Mahtani *et al.*, 1996; Shaw *et al.*, 1998), chromosome 20q12-q13 (Bowden *et al.*, 1997), chromosome 1q21-q23 (Vionnet *et al.*, 2000; Wiltshire *et al.*, 2001), chromosome 3q27 (Vionnet *et al.*, 2000; Ehm *et al.*, 2002) and chromosome 8p21.3-22 (Wiltshire *et al.*, 2001). Among these loci those important ones are discussed below.

Locus 2q37.1

A genome wide search in Mexican American families and revealed a susceptibility locus on chromosome 2q37.1 and inferred the locus as NIDDM1 (MIM 601283) (Hanis *et al.*, 1996). However, majority of the linkage studies failed to replicate those findings (Ghosh *et al.*, 1998). Nonetheless using positional cloning Horikawa et al (2000) identified a T2D susceptibility gene located in NIDDM1 region in Mexican Americans and northern European population in Botnia region in Finland. The gene encodes a member of calpain like cystein protease family, calpain-10 (CAPN10). They found a single nucleotide polymorphism (SNP, UCSNP 43) and high-risk haplotypes (consisting of 3 SNPs; UCSNP43, -19 and -63) associated with T2D. Subsequently a large number of association studies were performed worldwide. Many confirmed disease associations with either T2D, insulin action, insulin secretion or an aspect of adipocyte function. However, these findings have been tempered by a number of negative studies. Furthermore, even in the positive studies it has not always been with same SNP or haplotypes. Studies in different populations- Scandinavian (Rasmussen *et al.*, 2002), northern Chinese (Sun *et al.*, 2002), American Samoa (Tsai *et al.*, 2001), and Oji-Cree population of isolated northern Ontario (Hegele *et al.*, 2001) failed to replicate earlier findings. In Japanese population SNP-19 variant genotype was found to be associated with obesity and glucose intolerance, but SNP-43 and SNP-63 variant did not show any association with the disease (Horikawa *et al.*, 2003) and suggested that *CAPN10* is not a major gene in Japanese T2D patients. The UK group, however, postulated other alleles, in particular SNP 44 may increase at this locus may increase the risk for T2D (Evans *et a.l* 2001) and other observers subsequently confirmed by metaanalysis with an odds ratio of 1.17 (Weedon *et al.*, 2003). Another study by Elbein et al (2002) involving Caucasoid population also failed to confirm the *CAPN10*

as a major diabetes susceptibility gene; but they observed that the gene variant influences insulin sensitivity and glucose homeostasis in nondiabetic members of the family and thus confer high risk for T2D.

Among few studies showing *CAPN10* gene associated with T2D were those done in Mexican American population by del Bosque-Plata et al (2004) and in South Indian population by Cassell et al (2002). Cassell et al (2002) observed that *CAPN10* gene variant haplotypes to increase the risk for IFG/IGT and T2D in that population.

Locus 12q24.1

A study in the Botnian population of isolated Western Finland demonstrated a T2D susceptibility locus and mapped it to chromosome 12q24.1. The locus was termed 'NIDDM2' (MIM 601407) (Mahtani *et al.*, 1996). The locus is very close to MODY3 genes and is suggested to be representing the same gene. Shaw et al (1998) studied whether this T2D gene is segregated in an autosomal-dominant pattern and linked to 12q24.2. These families were negative for HNF-1α mutations and the data suggested the presence of a novel gene in that region. To confirm the susceptibility locus the region was scanned in both Caucasoid and non-Caucasoid US families. MODY3 was excluded as a candidate gene in the studied population but linkage between markers D12S375 and D12S1052 was observed (Bektas *et al.*, 1999). More recently a number of association studies have implicated *TCF1* as a candidate gene for T2D but the data is insufficient to explain the linkage peak. However, an extended genome wide scan by Lindgren et al (2000) confirmed the susceptible locus at 12q24.

Locus 20q12-q13.1

In different populations T2D susceptibility locus has been identified on chromosome 20q12-q13.1 and designated as NIDDM3 (MIM 603694) (Bowden *et al.*, 1997; Ji *et al.*, 1997; Zouali *et al.*, 1997; Price *et al.*, 1999). Several genes are located in the region including two important genes, phosphoenolpyruvare carboxykinase gene (PCK1) and HNF-4α. Zouali et al (1997) have found NIDDM3 susceptibility in the vicinity of the PCK1 gene and they excluded MODY1 gene. However, association of PCK1 gene with T2D has not yet been clearly understood. In another study NIDDM3 susceptible locus at 20q13 has also been confirmed in French Caucasoid population and suggested melanocortin3 receptor gene (*MC3R*) as a candidate gene. They identified

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two missense mutations in the *MC3R* gene: V81I and K6T. These two variants, which are in complete linkage disequilibrium, are also found in the controls. Although MC3R gene-coding variants were observed not to be associated with diabetes and obesity, however, it was marginally associated with insulin and glucose levels during oral glucose tolerance test in normoglycemic subjects (Hani *et al.*, 2001).

Genomewide linkage study was performed in T2D of Icelandic population (Reynisdottir *et al.*, 2003). Multipoint linkage analysis linked these diabetic patients to 5q34-q35.2 (lod score of 2.9) and designated the locus NIDDM4 (MIM 608036). The obese subset of diabetic patients did not show any linkage to same region, but the nonobese group had lod score 3.64. Reynisdottir et al (2003) also observed a potential interactions between the 5q locus and T2D susceptibility loci on chromosome 10 (Duggirala *et al.*, 1999) and T2D locus on chromosome 12q24.2. Duggirala *et al.*, (1999) had earlier suggested a susceptibility gene for type 2 on chromosome 10q by linkage analysis in Mexican-American diabetic families.

Other susceptible locus

In addition to above loci more chromosomal regions have been linked to T2D. Genomewide scan in Utah Caucasians families of North European ancestry revealed a susceptibility locus to chromosome 1q21-q23 (Elbein *et al.*, 1999) and the finding was replicated in a French family by Vionnet et al (2000). A region on chromosome 3q27 has been linked toT2D (Kissebah *et al.*, 2000; Vionnet et al 2000). In Japanese T2D patients an adipocyte derived peptide, adiponectin (also known as GBP28) was found to be decreased. Another genomewide scan has mapped diabetes susceptibility locus to chromosome 3q37 where the adiponectin gene (APM1) is located. APM1 gene variant was found to be associated with T2D Japanese patients (Hara *et al.*, 2002). Another study in indigenous Australians (Busfield *et al.*, 2002) and Utah Caucasians (Elbein *et al.*, 1999) revealed a susceptible locus for T2D on chromosome 8.

1.5.1.4.2 Candidate genes of T2D

A large number of candidate genes have been studied in T2D. Many of them have not been replicated and very few have survived meta-analyses. In subsequent sections those confirmed by metaanalysis and those genes directly related to the thesis will be discussed.

Genes in B cell function

Pancreatic B cell function is central to glucose homeostasis. There is substantial evidence to support an inherited defect in B cell function in T2D (Hitman and McCarthy, 1991). Glucose stimulated insulin secretion involves many steps - glucose transport and metabolism, oxidative phosphorylation, membrane depolarization and fluxes of intracellular calcium. Genes connected to any of these steps are candidates for B cell dysfunction.

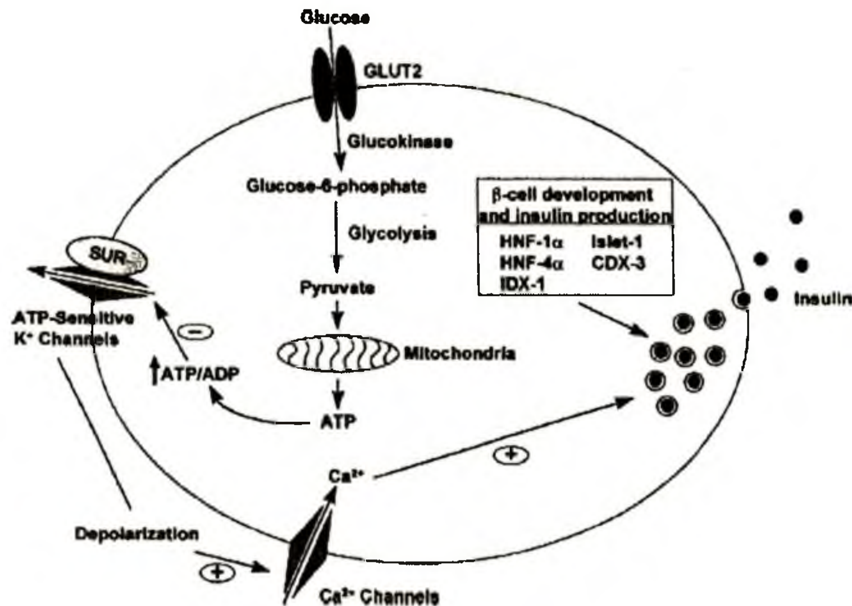

Figure 1.3: Schematic representation of glucose-stimulated insulin secretion by the pancreatic B cell. It involves glucose entry into the cell, metabolism, production of ATP leading to cascade of cellular events leading to build up of Ca^{2+} and finally release of insulin secretion.

Insulin gene: Case-control studies have suggested association between T2D and polymorphism at the regulatory minisatellite, upstream of insulin gene (Owerbach *et al.*, 1982; Bell *et al.*, 1984) which later found to be class III alleles (-23 bp AA genotype) of the 5' VNTR to the gene. However, T2D in Pima Indians did not show association with the INS VNTR (Knowler *et al.*, 1984). Subsequent study demonstrated the relative risk of 1.86 (p=0.03) for developing T2D with *INS* gene class III allele (Bennett and Todd, 1996). A recent study involving parent-offspring trios showed association between T2D and INS VNTR class III allele, only when the class III allele was preferentially inherited (Huxtable *et al.*, 2000). In a large scale study INS gene

class III allele did not demonstrate any impact on insulin secretion following oral GTT and the allele also did not confer susceptibility to T2D (Hansen *et al.*, 2004). However, this study did not account for the maternal or paternal origin that can only be determined by family studies. INS VNTR has been shown to influence insulin expression *in vitro* (Lucassen *et al.*, 1995; Kennedy *et al.*, 1995) and, *in vivo*, class III allele showed association with decreased level of insulin mRNA in the pancreas (Vafiadis *et al.*, 1996; Bennett *et al.*, 1995). The allelic variation also has been shown to affect IGF-2 transcription in human placenta (Paquette *et al.*, 1998). Since, allelic variation of the 5'VNTR has been shown to be a determinant of insulin gene transcription and fetal growth, the association with T2D seems unlikely to be just by chance (Dunger *et al.*, 1998). As INS gene class I allele has been found to be a major determinant of T1D its association with T2D indicates that some candidate genes may contribute to both the major type of diabetes. In a recent study it has been demonstrated that heterogeneity of class I INS VNTR allele is also associated with insulin gene secretion in obese children (Le Fur *et al.*, 2006)

Birth weight is an important determinant of T2D. Since 5' VNTR of the INS gene influences its transcription and insulin is a primary growth factor the association between the INS VNTR and birth weight was investigated (Bennett and Todd, 1996). The study revealed significant genetic association with birth weight (LBW); HZ class III allele was associated with a larger baby in terms of head circumference, length and weight. They further postulated that the class III allele bestowed a perinatal survival during human history by conferring a large size at birth (Bennett and Todd, 1996). This would therefore be consistent with the thrifty genotype hypothesis first postulated by Neel (1962). Subsequently Hales et al (1991) have already described a link between low birth weight and impaired glucose tolerance (IGT)/ T2D and termed this 'the thrifty phenotype' hypothesis. Ong et al (1999) showed that LBW was strongly related to IGT/T2D in those gaining weight soon after birth compared to those who did not 'catch up' the growth. This indicated important role of restraint of fetal growth according to the intrauterine environment and nutrition. Fetal metabolic and endocrine adaptations to maternal nutritional restraint *in utero* may persist throughout life and lead to insulin resistance and increased susceptibility to type II diabetes.

B cell K_{ATP} channel- SUR1 and KIr6.2 genes: The B cell K_{ATP} channel is composed of subunits - a specific K-channel (Kir6.2) surrounded by regulatory sulphonylurea (SUR) binding subunits and is encoded by the SUR1 of Kir6.2 genes respectively.

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Theses two genes are postulated to be clustered at locus 11p15.1 (Thomas *et al.*, 1995; Inagaki *et al.*, 1995). Although associations between T2D the SUR1 have been described, but the association with the E23K variant of Kir6.2 is more consistent and supported by several meta-analyses.

The 11p15.1 locus has been linked to T2D in Caucasoid population and a gene variant was found to be associated with disease phenotype (Inoue *et al.*, 1996; Hart *et al.*, 1999). However, the variant of Kir6.2 gene was also found to be associated with T2D. The association is suggested to be related to the missense E23K mutation in Kir6.2 gene in European Caucasoid (Hani *et al.*, 1998; Gloyn *et al.*, 2001). However, E23K was not in linkage disequilibrium with SUR1 gene and evidence in support of its functional effect is lacking (Gloyn *et al.*, 2001; Sakura *et al.*, 1996).

Other genes: Adipocyte has a complex endocrine function modulating metabolism and playing a key role in the pathogenesis of the dysmetabolic syndrome. On the basis of physiological functions several adipocyte-expressed genes have been associated to T2D. The strongest evidence of a candidate gene is one that is encoding peroxisome proliferator-activated receptors (PPARs) (MIM: 601487). PPARs form the members of the nuclear hormone receptor subfamily of transcription factors and peroxisome proliferator-activated receptors gama (PPARG) is believed to be involved in adepocyte differentiation. The *PPARG* gene is mapped to chromosome 3p25 (Greene *et al.*, 1995; Beamer *et al.*, 1997). Elbrecht et al (1996) cloned and characterized two genes encoding PAPR-gama 1 (*PPARG1*) and PPAR-gama-2 (*PPARG2*) and the third form of the gene *PPARG* was identified by Martin et al (1998). A mutation Pro115Gln was identified in *PPARG2* and suggested to accelerate the differentiation of adipocytes and also to cause obesity (Ristow *et al.*, 1998). A mutation resulting P12A change in *PAPRG2* gene was identified and has been postulated to modify susceptibility to T2D and obesity (Yen *et al.*, 1997; Deeb *et al.*, 1998). This has been confirmed by Lohmueller et al (2003) who have done a meta-analysis and found that that disease association was related to P12A polymorphism and resistance was conferred by A12A genotype. In familial syndromes of insulin resistance inherited susceptibility to T2D and insulin resistance insulin resistance may involve the interplay of several genetic loci, until recently no clear examples of interactions among genes had been reported. Savage et al (2002) described a family in which five individuals with severe insulin resistance who were doubly heterozygous with respect to frameshift/premature stop

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mutations in two unlinked genes, peroxisome proliferator-activated receptors gama (*PPARG*) and Protein phosphatase-1 regulatory subunit 3A (*PPP1R3A*). PPP1R3A is the muscle-specific regulatory subunit of protein phosphatase 1, which is centrally involved in the regulation of carbohydrate metabolism. Mutation in these molecules primarily involved in either carbohydrate (PPP1R3A) or lipid (PPARG) metabolism when combined can produce a phenotype of extreme insulin resistance and provides a theoretical model for the pathogenesis of T2D.

1 .5.2 Environmental factors

Major environmental factors so far suggested in the etiopathogenesis of T2D include age and sex, obesity, weight at birth and childhood development. T2D mainly occurs in middle age and elderly people. In the Europe and USA about 70% of patients are over 55 years and average age at diagnosis is 60 years. The prevalence of T2D increases markedly with age in all populations. However, it is also prevalent among highly susceptible young population in Pima Indians. Specific subtypes of T2D, maturity onset-diabetes of the young (MODY), occurs in younger age, usually around 25 years of age (Hattersley 1998).

Age

Glucose tolerance has been shown to decrease with age (DeFronzo 1981; Shimokata *et al.*, 1991). However, whether the deterioration is caused by increased chronological age or secondary to age related factors, such as decreased physical activity, is still to be clearly understood.

Sex

Differences have been observed in the prevalence of T2D in Europoid populations between men and women. Some studies had showed a higher age-adjusted prevalence in women (Damsgaard *et al.*, 1987; Eriksson *et al.*, 1992; Bruno *et al.*, 1992), where as some investigators demonstrated male preponderance (Glatthaar *et al.*, 1985; Verrillo *et al.*, 1985; Lintott *et al.*, 1992). Variation in T2D prevalence between men and women also has been shown to vary in different age group in the same community. A study in Finland revealed that age specific prevalence of known diabetes is higher in men up to the age of 60 years and in older age group prevalence is higher in women (Eriksson *et al.*, 1992). A male preponderance in the prevalence was also noticed among the people with higher socioeconomic status (Stern *et al.*, 1984).

Obesity

T2D is strongly associated with obesity. There is a close relationship between diabetes and obesity as defined by body mass index (BMI) with 50-90% of patients with T2D patients being obese. The risk for developing T2D has been seen to rise steadily with increasing BMI (used to define obesity). With BMI >30 kg/m^2 being associated with a life-time risk of 50% of T2D (Chan *et al.*, 1994). Although excess weight and obesity (BMI >25 kg/m^2) account for 64% cases of diabetes in men and 74% in women, many cases of diabetes occur in lean subjects (Chan *et al.*, 1994; Colditz *et al.*, 1995). However, there is some evidence that this may partly be related to ethnic and genetic factors that determine susceptibility to diabetes related fatness (Ruderman *et al.*, 1998).

Physical activity

Decreased physical activity is linked to an increased risk of developing T2D. Data regarding the relationship between physical activity and diabetes are mainly derived from European populations. The risk for T2D is associated with physical activity and a gradient was observed between exposure and outcome (Frisch *et al.*, 1986; Helmrich *et al.*, 1991; Manson *et al.*, 1991 and 1992; Knowler *et al.*, 2002). Increased physical activity has been shown to reduce the progression from IGT to T2D in several different populations: in Sweden (Eriksson and Lindgarde, 1991), Finland (Tuomilehto *et al.*, 2001). Data, involving non-European populations also showed some relevance in physical activity and progression to T2D. A low prevalence of T2D was found in physically active Japanese-Hawaiian men (Burchfiel *et al.*, 1995), in a Chinese (Pan *et al.*, 1994) and in Indian population (Ramachandran *et al.*, 2006) increased exercise was shown to reduce the progression from IGT to T2D.

Diet

Dietary components have also been believed to play a major role in the etiopathogenesis of T2D. Diets with a high-fat, low-carbohydrate content have differential effects on insulin action. High-fat, low-carbohydrate diets impair the action of insulin on endogenous glucose production, glucose oxidation, and probably lipolysis, whereas high-fat, low-carbohydrate diets do not unequivocally affect the action of insulin on total glucose disposal and tend to enhance the action of insulin on nonoxidative glucose disposal (Bisschop *et al.*, 2001). Dietary fatty acid composition

significantly influenced fat oxidation but did not impact insulin sensitivity or secretion in lean individuals. Overweight individuals were more susceptible to developing insulin resistance on high-saturated fat diets (Lovejoy *et al.*, 2002). The true composition of carbohydrate in the diet has also drawn attention. In one study complex carbohydrates have been shown to exert low glycemic and high insulinemic indices and hence believed to reduce the risk of glucose intolerance (Noriega *et al.*, 2001). Among other nutritional factors vitamin D has been implicated to be another candidate. Low vitamin D was reported in a group of at risk population (Boucher *et al.*, 1995). In another recent study Borissova *et al.*, (2003) have suggested vitamin D3 deficiency may at least partly contribute to the impairment of insulin secretion and probably of insulin action. Improvement in glucose tolerance and B cell function has al been reported (Kumar *et al.*, 1994; Baynes *et al.*, 1997). However, study in mice revealed that hypovitaminosis D was associated with insulin resistance and beta cell dysfunction (Chiu *et al.*, 2004).

Birth weight

As previously described nutrition *in utero* may be an important component of the susceptibility to T2D. Hales et al (1991) have hypothesised that 'fetal and childhood malnutrition, by programming metabolism, predispose to certain diseases in adult life' including T2D.

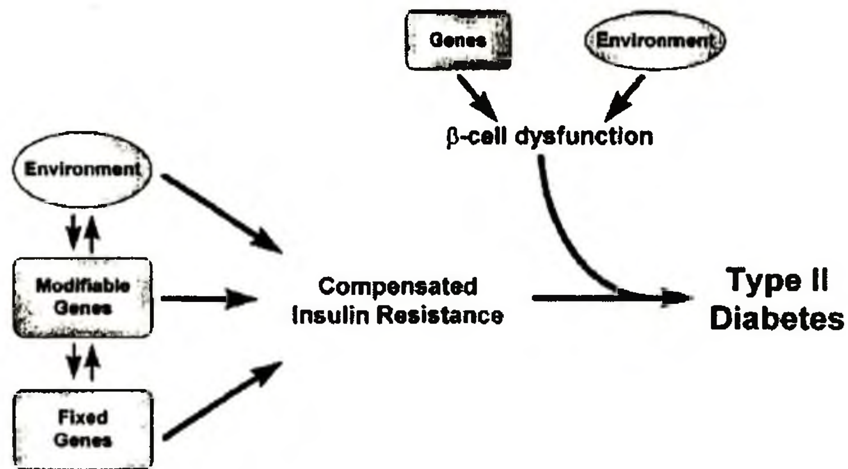

Figure 1.4: Schematic model of the origin of T2D. Compensated insulin resistance and B cell dysfunction are influenced by multiple genetic and environmental factors. Penetrence of T2D is secondary to the expression of several different genes, some of which are likely fixed and act independently of environmental factors. Predisposing genes might be modifiable in that their expression or action is influenced by environmental factors. Interactions between genes are also likely to contribute to insulin resistance and b-cell dysfunction (Hales and Barker 1992).

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In the original observation 40% of subjects had IGT or diabetes who had lower birth weight, where as only 10% had IGT or diabetes who had normal birth weight. Subsequently their observations have been well replicated including in the Southern Indian sub-continent although not in Pima Indians. These associations have been suggested to be mediated through impairment of B cell function but more recent findings suggested that diabetogenesis was mediated through insulin resistance and depend on interaction with obesity in adult life (Phillips *et al.*, 1994; Lithell *et al.*, 1996). A thrifty phenotype has been proposed, in which inadequate nutrition programs the fetus to develop insulin resistance in adult life (Hales and Barker, 1992).

2.6 Maturity Onset Diabetes of the Young

A subset of T2D patients have been found to occur at younger age, usually before 25 years and is inferred as an autosomal disorder. Tattersall (1974) described three families in which diabetes was inherited as an autosomal dominant trait and they had distinct clinical features: early onset of the disease, treated without insulin for long time and not prone to ketoacidosis. Tattersall and Fajans, (1975) termed this unique diabetic group as maturity onset diabetes of the young (MODY). However, there is considerable clinical heterogeneity among MODY and this is known to be due to genetic heterogeneity.

Table 1.4: Comparison between T2D and MODY

Characteristics	T2D	MODY
Inheritance	Polygenic	Monogenic, autosomal dominat
Age of onset	Usually >40 years of age	Usually <25 years of age
Pedigree	Rarely seen across the generation	Usually seen across the generation
Obesity	Usually obese	Non-obese
Metabolic syndrome*	Usually present	Absent

*Metabolic syndrome; diabetes, insulin resistance, hypertension and hypertriglyceridemia

1.6.1 Prevalence of MODY

MODY is believed to be an uncommon form of diabetes. In the European population prevalence of MODY has been found to be 1-2% of all the T2D cases. In UK the prevalence of MODY was suggested to be 1% (Shepherd *et al.*, 2001). Velho and Froguel (1997a) in their review pointed out that 10% of families with more than one family member with T2D in France are MODY. Winter et al (1999) have showed that less than 5% of cases of childhood diabetes in Caucasians are caused by MODY. In Southern India 4.8% of T2D patients were found to have MODY on clinical grounds but that is limited in that a molecular diagnosis by mutation screening was not performed (Mohan *et al.*, 1985).

1.6.2 Pathogenesis

MODY is usually of insidious origin and typically diagnosed on routine examinations. MODY patients show reduced secretion of insulin in response to glucose load (O'Rahilly *et al.*, 1988). However, hyperinsulinemia has been described in few families, although there was very little pathophysiological evidence of insulin resistance. The longitudinal studies suggested that, in general, insulin response declines with increasing duration of the disease (Fajans 1989).

Genetic studies have defined a number of subtypes of MODY. Mutations in the genes encoding hepatocyte nuclear factor 4 (HNF4), glucokinase (GCK), hepatocyte nuclear factor 1 alpha (*HNF1A*) and HNF I beta (*HNF1B*) (official symbols *TCF1* and *TCF2* respectively), insulin promoter factor 1 (*IPF1*) and *NEUROD1* genes are the causes of six known forms of MODY. MODY2 is caused by mutant glucokinase enzyme that fails to accurately sense the circulating glucose. All the other MODY genes encode transcription factors- namely *HNF4A, TCF1, TCF2, IPF-1* and *NEUROD 1*, which form crucial links and control appropriate expression of B cell genes such as insulin, glucose transporter 2 (GLUT2) and glycolytic genes essential for normal B cell function (Matschinsky 1990; Parrizas *et al.*, 2001). Several studies, performed in the extrapancreatic tissue such as liver, gut and visceral endoderm, have shown that HNFs form a hierarchical transcriptional network that regulates the differentiation and metabolism in these cells (Duncan *et al.*, 1998; Li *et al.*, 2000; Shih *et al.*, 2001; Boj *et al.*, 2001). Mutations in these genes may disrupt the development of B cell in the

embryo and result in dysfunctional B cells in adult life. However, the precise role of these proteins in the adult pancreatic islets is only beginning to be unraveled.

HNF4A transcription factors regulate wide range of genes expressed in the liver. HNF4A has a key role in the transcription hierarchy since it controls the *TCF1* which, in turn, regulates the expression of several important genes in the liver. However, the relationship between *TCF1* and *HNF4A* is not unidirectional, rather *TCF1* itself is an important activator of *HNF4A* gene (Boj *et al.*, 2001). Two independent studies demonstrated functional binding site in an alternative *HNF4A* gene promoter, termed P2 (Thomas *et al.*, 2001; Boj *et al.*, 2001). Experiment in mice model demonstrated that P2 promoter is the major regulatory site for islet specific HNF-4α transcription factor. Tcf1 (hnf-1α) deficient mice has been found to contain more than 10 fold reduction in pancreatic mass compared to wild type and it occupies the HNF-4αP2 promoter *in vivo* (Tian and Schibler, 1991). This suggests that *HNF-4α* and *TCF1* genes autoregulate each other and defect in this regulatory feed-forward loop is responsible for insulin secretory defect.

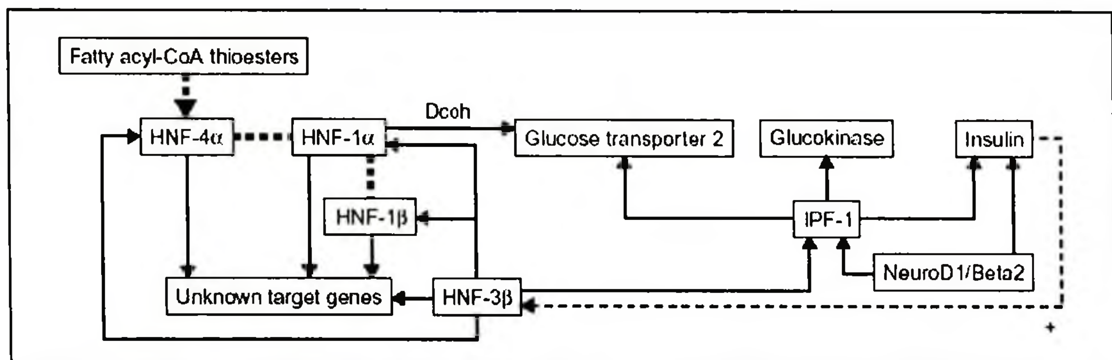

Figure 1.5: Network of DNA-binding factors involved in transcription in the pancreas. Dcoh is a cofactor of HNF1Aand NEUROD*1* is a β-cell transcription factor (So *et al.*, 2000).

Another important gene in differentiation of B cells is *IPF1* (also known as *PDX-1/IDX-1*), initially expressed both in exocrine and endocrine pancreatic precursors, but later becomes restricted to B cells. Loss of *IPF1* expression has been shown to cause agenesis of pancreas and haploinsufficiency of the IPF1 gene resulting in defects of glucose stimulated insulin secretion in human and mice (Stoffers *et al.*, 1997a; Ahlgren *et al.*, 1998). Expression of *IPF1* in the adult islet is regulated in feed-forward loop by *TCF1* and *HNF4A* as well as by *HNF-3β*. HNF1A molecule has been shown to bind on

conserved site of IPF1 promoter and activate its transcription (Ben-Shushan *et al.*, 2001; Marshak *et al.*, 2001). Shih et al (2001) demonstrated reduced expression of Pdx-1 in hnf-1$\alpha^{-/-}$ mice indicating that HNF-1α is required for normal *IPF1* gene expression in pancreatic B cells. HNF-3β molecule has also been shown to bind to the conserved area of the regulatory elements in mouse and human *IPF1* gene promoter and to activate their transcription (Ben-Shushan *et al.*, 2001; Wu *et al.*, 1997; Gerrish *et al.*, 2000).

Thomas et al (2001) has identified a link between *HNF4A* and IPF1. They found a mutation (-146T-C) in the HNF-4αP2 promoter. The mutation has been suggested to decrease binding activity to this site of other factors resulting in reduced transcriptional activity and affecting disruption in transcription hierarchy.

1.6.3 MODY classes and candidate gene

There are six different well characterized MODY classes. Frequencies of MODYs show great deal of variation. Genes responsible, the frequency of the each MODY type and their penitrance are shown in the tabulated form (Table 1.5).

Table 1.5: Types of MODY and candidate genes

Types	Genes	Frequency, (%) MODY families	Penetrance
MODY1	*HNF-4α*	2-4	High
MODY2	*GCK*	11-63	100%
MODY3	*TCF1*	21-73	High
MODY4	*IPF-1/PDX-1*	1-4	High
MODY5	*HNF-1β*	1-5	High
MODY6	*NEUROD1*	-	-

1.6.3.1 MODY1

Tight genetic linkage was demonstrated for MODY1 (MIM 125850) on to long arm of chromosome 20 and mapped the locus to 20q11.2-13.1 (Bell *et al.*, 1991; Bowden *et al.*, 1992) in a single family (R-W family). The locus was found to contain a gene which encodes HNF4A transcription factor and the mutation (Q268X) leading to the disease

was identified (Yamagata *et al.*, 1996). They also demonstrated that the mutation cosegregated with the at risk haplotype in MODY and but not in T2D patients and healthy individuals of the R-W MODY family.

Moller et al (1999) screened 10 Danish MODY probands for mutation in the promoter and the 12 exons of the *HNF4A* gene. They found 1 frameshift (phe75fsdelT) mutation in 1 proband and concluded that defects in the HNF4α gene is a rare cause of MODY in Denmark. Barrio et al (2002) have screened Mexican MODY patients and mutations in the *HNF4A* gene were frequently found.

Mutation in the *HNF4A* gene has been suggested as an uncommon cause of MODY. So far 13 families have been identified having MODY1 (Fajans *et al.*, 2001). MODY1 causes a mild form of diabetes but ultimately progress to insulin dependency. They usually have full spectrum of complications, particularly those involving the retina or kidneys. However, in a recent report common variations of the *HNF4A* gene P2 promoter have been described in T2D (Weedon *et al.*, 2004).

1.6.3.2 MODY2

MODY has been found to be linked to chromosome 7 in two different populations and the locus was mapped to 7q15-q13 locus (Froguel *et al.*, 1992; Hattersley *et al.*, 1992). It was identified that MODY2 (MIM 1125851) results from mutations in the glucokinase (GCK) gene located on chromosome 7q15-q13.

MODY2 has been found to be a common form of MODY. More than 130 mutations have been found in the GCK gene and have been found in many ethnic groups (Fajans *et al.*, 2001; Froguel *et al.*, 1993; Sakura *et al.*, 1992) and some of those are shown in the table 1.5 and 1.6. Heterozygous mutations in GCK gene are associated with a mild form of nonprogressive hyperglycemia that is usually asymptomatic at diagnosis and is treated with diet alone. The mild fasting hyperglycemia with blood glucose concentrations of 6.1-8.1 mmol/l and impaired glucose tolerance in most affected carriers may be recognized by biochemical testing at a young age, possibly as early as birth. About 50% of the women who are carriers may have gestational diabetes. Less than 50% of the carriers have overt diabetes; many of those who don't are obese or elderly. About 2% of MODY2 patients require insulin therapy. Diabetes-associated complications are rare in this form of disease (Fajans *et al.*, 2001).

Table 1.6: Novel GCK Mutations Identified in UK and European populations (Thomson *et al.*, 2003)

Patient ID	EXON	Nucleotide	Protein Effect	Age at diagnosis /testing
DUK 465	2	c.131de1G	G44fsdelG	5
DUK 795	3	c.232G>C	D78H	27
DUK 118	3	c.234C>G	D78E	5
DUK 363	3	c.322T>C	Y1O8H	12
DUK 200	3	c.322T>C	YI08H	5
DUK 808	3	c.324C>A	YI08X	16
DUK 828	3	c.332C>T	PIIIL	33
DUK 746	4	c.389T>C	1130T	3
DUK 475	4	c.466C>T	H156Y	Birth
DUK 361	7	c.856C>T	Q286X	14
DUK 157	8	c.884de1G	G295fsdeiG	18
DUK 293	8	c.952G>A	G318R	16
DUK 404	8	c.928_929delGT	V31OfsdelGT	27
DUK 703	8	c.I000_I018delTTCGTGTCGCAGGTGF334fs del19 GAGA		25
DUK 291	9	c.I024A>C	T342P	43
DUK 511	9	c.1160C>T	A387V	30
DUK 481	9	c.1159G>A	A387T	28
DUK 649	10	c.1335_1345delTGGCCGGGGC G	S445fsdelll	33
DUK 552	10	c.I336G>C	G446R	14
DUK 790	10	c.1340G>A	R447Q	9
DUK 397	10	c.1358C>T	S453L	30
DUK 589	10	c.1361C>T	A454V	31

Demonstration of linkage for MODY2 to 7q15-q13 locus and identification of mutations in the GCK gene preceded the suggestion of the role of enzyme in the pathogenesis of diabetes (Matschinsky 1990 and 1993). It was hypothesized earlier that the rate of glucose metabolism and insulin secretion is closely linked, with both being determined by the plasma glucose concentration. The rate of glucose metabolism in B cells and hepatocytes is determined by the rate of glucose phosphorylation catalyzed by glucokinase. B cells and hapatocytes also contains glucose transporter 2 (GLUT2), an insulin dependent cellular protein that mediates the transport of glucose into cells. It facilitates rapid equilibrium between intracellular and extracellular glucose. Thus, in effect, the glucokinase senses extracellular glucose concentrations and is therefore known as the glucose sensor of the B cell (Matschinsky *et al.*, 1990 and 1993).

Table 1.7: Characterization of the mutations and polymorphisms in the GCK gene in families with MODY (Froguel *et al.*, 1993)

FAMILY	Location	Codon	Nucleotide change	Amino acid change
Mutations associated with NIDDM				
F392	Exon 2	70	GAA-AAA	Glu-Lys
F393	Exon 3	98	CAG-TAG	Gin to Stop
F423*	Exon4/intron 4	161	'15-bp deletion at splice donor site	
F386	Exon 5	175	GGA-AGA	Gly-Arg
F114, F331ŧ	Exon 5	182	GTG-ATG	Val-Met
F446ŧ	Exon 5	186	CGA-TGA	Arg-stop codon
F422	Exon 6	203	GTG-GCG	Val-Ala
F85Ŧ	Intron 6/exon 7	227	Mutation of splice acceptor site	
F39lŧ	Exon 7	228	ACG-ATG	Thr-Met
F388. F390ŧ	Exon 7	261	GGG-AGG	Gly-Arg
F8t	Exon 7	279	GAG-TAG	Glu-stop codon
F28ŧ	Exon 8	300	GAG-AAG	Glu-Lys
F51ŧ	Exon8	300	GAG-CAG	Glu-Gln
Fl60	Exon 8	309	CTC-CCC	Leu-Pro
F385	Exon 9	414	AAG-GAG	Lys-Glu
F397§	Intron 9/exon 10	418	Mutation of splice acceptor site	
Polymorphisms not associated with NIDDM				
F8t	Exon la	4	GAC-AAC	Asp-Asn
F370ŧ	Exon la	10	GCC-GCT	Ala-Ala
Fl5	Intron lc	C-T substitution 12 bp upstream from splice acceptor site		
F51ŧ	Exon 3	116	ACC-ACT	Thr-Thr
F85, F388, F390, F391	Intron 9	C-T substitution 8 bp downstream from splice donor site		

*Deletion of 15 bp including the messenger RNA splice donor site (Lys at codon 1611intron 4): AAG/G(TGGGCCG*GGTGGAGG)GGC→AAG/GGGC.*

ŧ Mutations occurring in the context of a cytosine-phosphate-guanosine dinucleotide,

Ŧ introt 6 splice acceptor site/Gly at codon 227: AG/GC→AT/GC.

§ Intron 9 splice acceptor site/Ser at codon 418: *AG/C→AC/C*

Studies in animal models further substantiated the role of the GCK gene in the pathogenesis of MODY2. Heterozygous mice with disrupted GCK gene were observed to have mildly elevated blood glucose, phenotypically similar to MODY (Bali *et al.*, 1995). Aizawa et al (1996) studied pancreatic beta-cell function in the isolated pancreatic islets of heterozygous GCK knockout mice. These GCK knockout mice displayed impaired glucose sensitivity, poor discrimination of alpha- and beta-glucose anomers, and normal activity of the ATP-sensitive K+ channel. Sensing the change of

glucose concentration by pancreatic B cells is critical to maintain the glucose homeostasis. Glucose sensing has been suggested to be mediated through increased rate of intracellular catabolism of glucose and the enzyme glucokinase plays a key role in maintaining the homeostasis and thought to be the rate limiting step for glucose catabolism in B cells. Grupe et al (1995) generated mice completely deficient in GLK and transgenic mice in which GLK was expressed only in B cells. In mice with only 1 GLK allele, blood glucose levels were elevated and insulin secretion was reduced. GLK-deficient mice died perinatally with severe hyperglycemia. Expression of GLK in beta cells in the absence of expression in the liver is sufficient for survival.

1.6.3.3 MODY3

Genome-wide analysis in 12 French MODY families localized a gene for MODY susceptible to locus (MODY3) to 12q in 6 families (Vaxillaire *et al.*, 1995). These families did not show linkage to previously identified MODY loci. They were later called MODY3 (MIM 600496). These patients exhibited marked hyperglycemia with severe insulin secretory defect, suggesting a causal gene implicated in pancreatic B cell function. Linkage to chromosome 12 was also revealed in another study involving families from Denmark, Germany, and US (Menzel *et al.*, 1995) and the MODY3 loci to be located in the 12q24.1-12q24.32 region (Bach et al 1990, 1991). Yamagata et al (1996) refined the localization of MODY3 gene to 12q24.1 and identified it as *TCF1* (Genecard Access No: M57732). Analysis of 4 large Finish kindred further confirmed the linkage of MODY to 12q and functional analysis showed severe impairment of insulin secretion in the diabetic phenotype and also in the normoglycemic subjects who inherited the MODY3 gene mutations (Lehto *et al.*, 1997).

Gonzalez et al (1990) demonstrated that the new-born mice homologous for chromosomal deletion, containing hnf1 gene, had no increased activity of CYP2E, which is regulated by the hnf1 gene (the rodent homologue of *TCF1*). It was found that mice with inactive hnf1 gene failed to thrive and died around weaning and transcription rate of albumin and □-1 antitrypsin genes was reduced, while the gene coding phenylalanine hydroxylase was totally absent. The mice also suffered from renal tubular functional abnormality, like human Fanconi syndrome (Pontoglio *et al.*, 1996). Vaxillaire et al (1997) examined 10 unrelated Caucasian families in whom MODY/NIDDM cosegregated with polymorphic markers for MODY3 and found 10 different mutations in the *TCF1* gene, all of which cosegregated with diabetes.

Lindner et al (1999a) demonstrated combination of different mutations in the *TCF1* gene in German MODY families. Mutations identified in the *TCF1* gene include P291fsinsC, R131Q, R229Q and R272fsdelGC. It was observed that 55% of families had different MODY mutations, of which 36% were accounted by mutations in T*CF1* gene.

Urhammer et al (1997a and 1997b) studied a cohort of MODY3/T2D patients and identified a number of nucleotide substitutions- out of which 4 resulted in amino acid substitution in the TCF1 peptide (I27L, A98V, S487N, R583Q) and 5 silent (L17, G228, L459 and T515) and 5 intronic substitutions. Frequency of amino acid substitution of codon 27, 98 and 487 were similar among the Controls. However, first-degree relatives of T2D with A98V variant had significantly decreased insulin secretion compared to those with wild genotype (Urhammer *et al.*, 1998). In a study involving unrelated Danish Caucasoid MODY3 patients Hansen et al (1997) identified 2 novel and 1 previously reported mutation and 2 new frameshift mutations, but not in any of the T2D and controls subjects. They opined that mutation in TCF1 gene is common in Danish MODY patients.

Frayling et al (1997) studied 15 UK MODY families and identified 8 different mutations (3 frameshift (P291fsinsC, P379fsdelCT, and A443fsdelCA) and 5 missense mutations (P129T, R131W, R159W, P519L, and T620I)) in 11 families (73%) in the *TCF1* gene. The previously reported mutation P291fsinsC was found in four pedigrees. They also screened a further 32 probands with early onset (<40 years of age) T2D who showed the mutation in two additional families. They suggested that HNF1A mutations are a common cause of MODY in UK families.

TCF1 gene was studied for mutations in a total of 130 Norwegian families; 42 with clinical MODY, 75 with suspected MODY and 13 pedigrees with multiplex type 1 diabetes. Twenty-two clinical MODY, 15 suspected MODY, and 1 family with type 1 diabetes multiplex had TCF1 mutations. Eight of the 18 different mutations identified were novel (G47E, T196fsdelCCAA, IVS3-1G>A, S256T, A276D, S445fsdelAG, M522V, and S531T). Functional study revealed that beta-cell dysfunction in MODY3 is caused by loss-of-function mechanisms like reduced DNA binding, impaired transcriptional activation, and defects in subcellular localization (Bjorkhaug *et al.*, 2003).

TCF1 screening in Japanese MODY3 patients identified 3 new mutations- L518P519fsTCC→A mutation in 3 unrelated families, other two mutations (T521I and V617I) in one family. The known promoter site mutation +102G→C was also found. They demonstrated relatively higher (10%, 5/50) prevalence of *TCF1* gene mutations among Japanese patients with MODY (Ikema *et al.*, 2002). *TCF1* gene was also studied in Chinese MODY families. Xu et al (2002) reported 10% (5 of 50) families with mutations in the coding region of HNF1A gene: 1 novel nonsense mutation (Q176X) and 4 already reported mutations (frameshift mutation P379fsdelCT; nonsense mutation R171X; missense mutations G20R and P112L). Recently they have reported MODY3 mutations in 13 families and 4 of the 12 mutations detected were new (Q243E, A311D, P379R and P488fsdelC). They also observed that MODY3 accounted 9% of total MODY families (Xu *et al.*, 2005).

TCF1 gene mutations have been identified in all racial and ethnic backgrounds that have been studied, including European, Chinese, Japanese, African, and American Indian. Surprisingly there have been no studies from the Indian Sub-continent. Mutations in the TCF1 gene appear to be the most common cause of MODY among adults seen in diabetic clinics (Fajans *et al.*, 2001). Ellard (2000) stated that 65 different mutations in the TCF1 gene had been found to cause MODY3 in a total of 116 families worldwide. Approximately 15% of MODY3 families have the same mutation - a C insertion (P291fsinsC) in the exon4. They noted that diagnostic and predictive genetic testing is possible for the majority of patients with MODY, opening new avenues for the classification, prediction,. and perhaps ultimate attempt in the prevention of diabetes in the MODY families.

1.6.3.4 MODY4

Insulin promoter factor 1 (IPF1) is a transcription factor that regulates insulin transcription in pancreatic B cells. This is also known as somatostatin transcription factor-1 (STF1). Stoffel et al (1995) determined that the *IPF1*gene is located on chromosome 13. Later on the assignment was refined and assigned to 13q12.1. A mouse homologue of the human gene was named ipf1 and mapped it to the distal end of chromosome 5 (Fiedorek and Kay 1995). Stoffers et al (1997b) has further confirmed the linkage for MODY (MODY4, MIM 606392).

IPF1 has been suggested to serve as the master control switch for expression of both exocrine and endocrine pancreatic development program. IPF1 is initially expressed in both exocrine and endocrine cells, but eventually becomes restricted to beta and delta cells of the islets, where it appears to regulate expression of the insulin and somatostatin genes respectively. Sharma et al (1997) characterized a composite enhancer that directs *IPF1* expression to pancreatic islet cells via 2 functional elements that recognize the nuclear factors HNF 3-beta and 2-beta. Watada et al (1996) showed that IPF1, can activate the promoter of human islet amyloid polypeptide (IAPP) gene and this activation is mediated by at least 2 element-like regions in the 5-prime flanking sequence. IPF1 has been shown to be required by fibroblast growth factor receptor (FGFR1) signaling component in beta cells, indicating that IPF1 acts on upstream of FGFR1 signaling in B cells to maintain proper glucose sensing, insulin processing, and glucose homeostasis (Hart *et al.*, 2000).

Macfarlane et al (1999) identified 3 novel missense mutations (C18R, D17N and R197H) *IPF1* gene in diabetic and nondiabetic subjects from UK. The mutations, however, were not highly penetrant, as the carrier had the mutation for 25-53 years of age and remained non-diabetic. Hani et al (1999) investigated 192 French type 2 diabetic families and identified 3 novel IPF1 mutations, including 2 substitutions (59L) and D76N) and in-frame proline insertion (insCCG243). Functional analysis revealed that the CCG243 mutation was linked in two families in autosomal dominant- like late onset form of type 2 DM. Most prevalent D76N and Q59L mutations were associated with increased relative risk for diabetes and decreased glucose stimulated insulin secretion in nondiabetic subjects. In another study 200 Danish late-onset type 2 DM and, 44 Danish and Italian MODY patients were screened for *IPF1* mutations. In the Danish T2 patients noncoding –108G In/del, silent G45C substitution, and rare variant D75N was observed. Moreover, one Danish MODY patient carried an A140T variant. Neither D75N nor A140T variant segregated with diabetes (Hansen *et al.*, 2000). It was suggested that mutations in *IPF1* gene is a rare cause for MODY.

1.6.3.5 MODY5

Hepatocyte nuclear factor 1B (HNF1B) gene encodes transcription factor 2 (*TCF2*) which is homodomain-containing transcription factor structurally related to *TCF*1. With

TCF1 it forms a homodimer or heterodimer (Bach *et al.*, 1991). The gene is assigned to human chromosome 17cen-q23.1 (MIM 604282).

Horikawa et al (1997) had screened unrelated Japanese subjects with MODY. They found 4 nucleotide substitutions, including a nonsense mutation (R177X), which were absent in nondiabetic subjects. Lindner et al (1999a) did not find mutation in *TCF2* gene of the German MODY patients. However, in a Norwegian family with mild diabetes, progressive nondiabetic renal disease and severe genital malformation Linder et al (1999b) identified a 75bp deletion in exon2 of *TCF2* gene, which was producing a truncated protein. The deletion has been found to be cosegregated with diabetes and renal disease in the pedigree. Bingham et al (2000) found that MODY5 patient had B cell functional defect. Although TCF2 is a good candidate gene, it became clear that mutations in the gene are not common cause of MODY (Horikawa *et al.*, 1997).

1.6.3.6 MODY6

Mutations in the *NEUROD1* gene also lead to the MODY (MODY6 (MIM 606394)) (Malecki *et al.*, 1999). The *NEUROD1* gene had already been mapped to chromosome 2q32 (*Tamimi et al.*, 1996). The gene encodes a basic helix-loop-helix (bHLH) protein, termed NeuroD1, which functions as regulatory switch for endocrine pancreatic development. In man NeuroD1 regulates insulin gene expression by binding to a critical E-box motif of the INS gene promoter (Naya *et al.*, 1995). A mouse model with Neurod$^{-/-}$ 'knock out' leads to abnormal pancreatic islet morphogenesis and later develops diabetes (Naya *et al.*, 1997).

MODY6 has been mapped to long arm of chromosome 2 (2q33), which is in close vicinity of three IDDM loci (IDDM7, IDDM12 and IDD13). The importance of *NEUROD1* gene in the abnormal transcription/regulation of B cell development and function suggested that it might be involved in the development of more common form of diabetes. Iwata et al (1999) have studied the NeuroD/BETA2 gene in Japanese T1D. They observed G>A substitution in NeuroD1 gene (Gene Access No; AF045152.1 in the exon2 leading to A45T was associated with adult onset T1D in Japanese patients. In this study 25% of T1D had A45T variant compared to 9.8% in Controls and 9.5% in T2D. In another study involving Japanese population NEUROD1 gene A45T polymorphism was found to be contributing risk for early deterioration of B cell function

and development of diabetes (Mochizuki *et al.*, 2002). However, in a larger study from the Todd group the finding has not been repeated (Vella *et al.*, 2004).

Malecki et al (1999) has studied the *NEUROD1* gene in families and unrelated cases with T2D. They found 4 variants of the *NEUROD1* gene. The frequency of a G>A (A45T) variant was found to be similar (32.9% and 35.9% in index and unrelated cases respectively). This polymorphism was not associated with T2D unlike other studies (Furuta *et al.*, 1998; Dupont *et al.*, 1999). The C>A (P196H) was found in the controls and diabetic patients in equal frequency 3.6% vs 3.1% respectively). The G>T (R111L) and cytosine residue insertion (206+C) were present in two index cases and were not present in the controls. The mutation leading to R111L has been found to be located in the proximal basic portion of the HLH domain, which is responsible for DNA binding. A functional study by Malecki et al (1999) demonstrated that development of T2D in carriers of R111L and 206+C mutations was due to disruption of *NEUROD1* control by impairment of NeurodD1 binding to INS gene promoter or elimination of function of the C-terminal transactivation domain of NEUROD1.

Neuroginin3 (*NEUROG3*) gene is involved in triggering *NEUROD1* expression in the endocrine precursor cells. The *NEUROG3* gene is on 10q21.3 (Gene Bank No: AJ133776 and AF234829), has only one exon and is another member of the subfamily of basic-helix-loop helix (bHLP) class B transcription factors. *NEUROG3* has been suggested to be functioning as a 'pro-endocrine' switch involved in controlling of pancreatic endocrine and exocrine cells (Gradwohl *et al.*, 2000). *NEUROG3* knockout mice seemed normal at birth, but die from diabetes within a few days due to lack of pancreatic cells. These mice also lack Pax4, Pax6 and NeuroD, providing further evidence that *NEUROG3* is the 'upstream' pro-encrine switch controlling the fate of endocrine cells (Gradwohl *et al.*, 2000). Genetic polymorphisms were identified in the *NEUROG3* gene in two distinct populations. Jensen et al (2001) demonstrated G to A (g.499G/A) substitution leading to G167R substitution and common C to T (g.596T/C) substitution causing S199F in the protein. They also found two non-coding variations in the 5'-UTR, a c→t nucleotide variation at position –10 in one MODY patient and a 2bp(CA) deletion polymorphism at –44/45 upstream of the start codon. In another study a nucleotide variant in 5' UTR and CA deletion at -44/45 was identified in one individual. Jensen et al (2001) observed Ser199Phe and -45/-45delCA in linkage disequilibrium with the disease.

However, the genetic variability was not associated with T1D, MODY, T2D or changes in the insulin secretion in the Danish Caucasians. *NEUROG3* gene was also investigated in the Japanese MODY patients. del Bosque-Plata et al (2001) revealed 2bp deletion in the 5'-untranslated region at -44/-45delCA and the two coding region variations S199F and G167R in the Japanese MODY. However, none of the coding and/ or non-coding region variation was associated with diabetes. They concluded that *NEUROG3* gene variations are not common in Japanese MODY patients. However, as MODY/T2D is a heterogeneous disorders mutations in another member(s) of the bHLH family of transcription factors, involved in the development and maturation of pancreatic B cells, may be responsible for MODY.

1.7 Fibrocalculus Pancreatic Diabetes

Fibrocalculus Pancreatic Diabetes (FCPD) is characterized by a form of diabetes in the young associated with chronic calcific pancreatitis in the absence of any known causes of pancreatitis ie alcoholism, cholecystitis and billiary obstruction. FCPD patients sometimes have history of recurrent abdominal pain since childhood but remain unnoticed until the diagnosis on routine examinations. They either have pancreatic stones revealed on abdominal plain X-ray or pancreatic fibrosis by ultrasound examination. FCPD patients are usually lean and require insulin for the control of blood glucose, but they rarely develop ketoacidosis even if insulin is withdrawn for a prolonged period. FCPD is generally regarded as secondary to tropical calcific pancreatitis (TCP) (Mohan *et al.*, 1998b). However, a study conducted by Rahman et al (2002), although on a relatively small number of subjects involved, showed that FCPD is distinct from TCP, suggesting that the relationship between the two may not be straightforward, which was earlier been indicated in a report by Azad Khan and Ali (1997).

1.7.1 Prevalence of FCPD

Patients with tropical pancreatitis and diabetes was first reported by Zuidema (1959) from Indonesia. Following this first report Shaper in 1966 demonstrated presence of the similar condition in Uganda. It was also reported from Southern India (Geevarghese 1967). Since the unique condition was mostly found in the tropical

countries the condition was termed as tropical pancreatic diabetes (TPD) (Geevarghese 1967). However, it attracted the attention of diabetologists and physicians when a WHO Study Group Report on Diabetes Mellitus in 1985 recognized the condition and termed it as fibrocalculus pancreatic diabetes (FCPD) (WHO 1985). FCPD has subsequently been reported from several tropical developing countries including Brazil, Congo, Nigeria, Madagasker, Kenya, Zambia, Zimbabwe, Sri Lanka, India, Bangladesh, Singapore, Thailand and New Guinea (Abu-Bakare *et al.*, 1986).

Almost all reported prevalence of FCPD is derived from clinic-based studies. Balaji et al (1988) carrier out a systematic survey in a region of Kerala and observed that the prevalence of FCPD is about 0.1% (28 out of 28507 people surveyed). In another door-to-door survey by the prevalence was found to be 0.2% (8 out of 4000 persons) (Augustine 1996). FCPD constituted about 1% of all the diabetic patients and 4% of those below 30 years of age registered in MV Diabetes Specialties Centre, Chenni, India. The condition, however, is not common throughout India. It is more prevalent in the Southern and Eastern States and rare in the northern areas (Mohan *et al.*, 1998b). The clinic-based prevalence of FCPD was reported to be about 8.6% in Nigeria in 1971, but in a later study involving MRDM patients, the rate was demonstrated to be 6% (Osuntokun *et al.*, 1971; Akanji 1990). The condition was found to be rare in South Africa (Omar and Asmal 1984). It has been observed that among all diabetic patients registered in BIRDEM, the central diabetic centre of Diabetic Association of Bangladesh (DAB), about 7% belonged to the group whose onset of diabetes under 30 years of age. In a cohort of these young (<30 yrs) diabetic patients 13% were found to be constituted by the FCPD patients (Azad Khan and Ali 1997).

1.7.2 Etiopathogenesis of FCPD

1.7.2.1 Etiology of FCPD

In western industrialized countries the most common etiological factors in chronic pancreatitis is long-term alcohol abuse. However, in 10-30% of chronic pancreatitis patients there is no apparent underlying cause and these are classified as idiopathic chronic pancreatitis (ICP). Small percentage, less than 5%, of chronic pancreatitisis is cause by hereditary pancreatitis (HP) and cystic fibrosis (CF), and others (Andren-Sandberg 2003). In the tropical developing countries both HP and cystic fibrosis are

rare and the main bulk of patients with chronic pancreatitis is contributed by TCP. About 90% of the patients with TCP, of all age, ultimately develop diabetes. However, the proportion of TCP patients among the young becoming FCPD is still to be ascertained.

HP is a multigenerational disease and mostly found among the American and European Caucasoid. The occurrence of diabetes in HP patients is low although it clearly depends on how assiduously diabetes is sought. Sibert (1978) has reported that 12.5% of HP patients in 7 families had diabetes. Cystic fibrosis related diabetes increases with age and an age-dependent rate of diabetes was demonstrated about 5% per year; and at 30 years 50% of patients have diabetes (Lanng *et al.*, 1991, Lanng 2001 Solomon et al 2003).

Little is known about the etiology of pancreatitis in FCPD. Chronic pancreatitis due to alcoholic abuse occurs at 4th and 5th decades of life; on the other hand FCPD occur at much earlier age. Since the condition is mostly restricted in tropical developing countries and the earlier reports mainly involved poorer section of people, environmental factors have been implicated in the etiopathogenesis of FCPD, so was the name chosen malnutrition related diabetes mellitus (MRDM) (WHO 1985). Chronic undernutrition has been suggested to be important determinant of diabetes in an individual, either by progressively impairing beta cell function or by increasing the susceptibility of the individual to other genetic and environmental diabetogenic influences (Rao 1984).

Among other environmental factors cyanogenic food, especially Cassava and oxidative damage, have been implicated in the pathogenesis of FCPD. The disease was observed in areas where Cassava, which contains cyanogenic glycosides - linamarin and lotuslarin, are consumed as staples food in India and it has been suggested to play a crucial role in the causation of FCPD (McMillan and Geevarghese, 1979; Pitchumoni *et al.*, 1988). However, subsequent studies failed to support the initial findings. No direct relationship was established between consumption of Cassava and pancreatitis (Balakrishnan *et al.*, 1988; Yajnik *et al.*, 1989). Moreover, diabetes was absent in rural West Africa where Cassava is also eaten as staple food although this may depend on its preparation (Teuscher *et al.*, 1987). In animal models protein calorie malnutrition has been shown to produce a wide number of changes including in

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the pancreas and liver leading to glucose intolerance and insulin insufficiency. Short- and long-term experimental feeding of Cassava in animal models has produced conflicting results and lacks experimental support in the causation of diabetes and/ or FCPD (Kamalu 1991; Mathangi *et al.*, 2000).

Several subsequent studies strongly argued against malnutrition as a cause. The authors suggested that malnutrition at diagnosis of FCPD might be secondary to exocrine and/ or endocrine deficiency. In addition FCPD was not only prevalent among the poor, but also found among the middle- and upper economic strata of the society (Mohan *et al.*, 1998b; Azad Khan and Ali, 1997). It also suggested that not malnutrition but micronutrient deficiency might be involved in the pathogenesis of FCPD (Rao and Yajnik, 1996).

Chronic pancreatitis in white people has been linked to heightened oxidative detoxification induced by cytochrome P-450 within the pancreas and/ or liver (Braganza 1988). Faster theophyline clearance, which is an *in vivo* probe of the potentially toxic cytochrome-450I drug metabolism pathway, was observed in patients with chronic pancreatitis compared to controls. These patients had been revealed to high level of exposure to Xenobiotic (cigarette, alcohol, occupational chemicals, dietary corn oil etc) that are inducers of cytochrome-450I and/or yield reactive metabolites leading to oxidative stress (Chaloner *et al.*, 1990). Studies on antioxidant status of south Indian TCP showed low level of vitamin C and B-carotene; and it provided indirect evidence in support of the oxidative stress theory (Braganza *et al.*, 1993). Malnutrition has been postulated to induce a state of defective ability to scavenge free radicals and hence suggested that this could enhance the susceptibility for organ damage (Barman *et al.*, 2003). Direct evidence has been observed with a significantly higher number of patients demonstrating single stranded DNA (a marker of free radical mediated damage of double stranded DNA) compared to controls and other diabetic groups (McDonagh *et al.*, 1996). However, the free radical hypothesis has not yet been proven and merits further study.

1.7.2.2 Autoimmune status in FCPD

Autoimmunity has not so far been linked to the pathogenesis of FCPD. However, there are many a few studies where autoantibodies have been measured in this group of patients. The various studies are summarized in the table 1.8.

It is seen from the table the overall picture is therefore unclear as to whether there is an autoimmune component to FCPD.

Table 1.8:

Frequencies of autoantibodies in FCPD in various studies

Autoanti - bodies	Controls	FCPD	T1D	T2D	PDDM/ MMDM	References
GAD	0%	7%	47.5%	5.6%	-	Mohan *et al.*, 1998a
ICA	4.3%	6.3%	53.8%	9.9%	-	
ICA	-	0%	35%	-	13%	Dabadghao *et al.*, 1996
GAD	-	7%	40%	-	23%	
IA2	-	0%	22%	-	3%	
ICA	-	0%	41%		13%	Singh *et al.*, 2000
GAD	0%	7.1%	14.2%	0%	38%	Goswami *et al.*, 2001
IA2	0%	0%	2%	0%	0%	

1.7.2.3 Pathogenesis of FCPD

Chronic pancreatitis has been long been known to result from inflammatory process causing structural and functional damage resulting in exocrine and endocrine deficits. The most common etiological factor in the industrialized countries is long-term alcohol abuse; in 10-30% cases the cause is still remained remain, called idiopathic chronic pancreatitis.

In chronic idiopathic pancreatitis 'pancreatic autodigestion' has been implicated. Inappropriate pancreatic zymogens, especially trypsinogen, has been thought to play a key role in this process. There is experimental evidence that trace amount of trypsin may become physiologically active within pancreatic parenchyma causing autodigestion. Multiple mechanisms: pancreatic anatomy and secretion of trypsin inhibitor and ability of trypsin itself to start neutralization cascade, are known to prevent the premature uncontrolled activation of digestive enzyme cascade within the pancreas and thus protecting the organ from self-destruction. A well characterized trypsin inhibitor is serine protease kazal type 1 (SPINK1), also known as pancreatic secretory trypsin inhibitor (PSTI), which reversibly inhibits prematurely cleaved trypsin. The 'trypsin', in combination with trypsin-like enzymes such as mesotrypsins, hydrolyze trypsin and other proteases and maintain *milieu interior* (Truninger *et al.*, 2001).

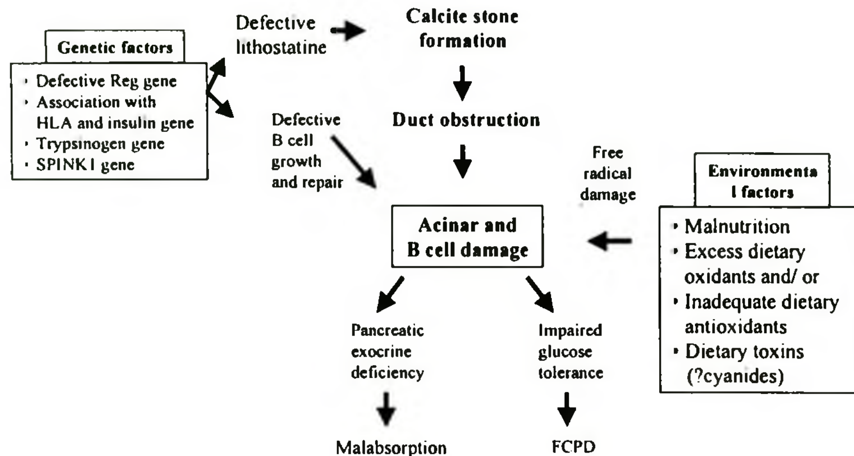

Figure 1.6: Schematic diagram showing the pathogenic mechanism of pancreatic damage leading to pancreatitis (Mohan *et al.*, 1998b).

1.7.3 Genetics of FCPD

Tropical calcific pancreatitis sometimes affects many members of the same family. In one study Pitchumoni (1970) found 17 families with two or more members having evidence of pancreatitis. Familial clustering has also been demonstrated Geevarghese (1985). This followed the report of Geevarghese as cited by Mohan and Premalatha (2002) who found that 8% of the total of 1700 cases of TCP had 'Familial pancreatitis' which also included three pairs of twins. In more recent study Mohan et al (1989) demonstrated familial aggregation of the disease and also evidence of vertical transmission. In their series they observed 12% of parents had chronic pacreatitis; of them one calcific type pancreatitis. Among the siblings 21% had pancreatitis, 10 of them was of calcific variety (Mohan *et al.*, 1989).

1.7.3.1 Candidate gene for diabetes in FCPD

In an attempt to explore genetic contribution to diabetes in FCPD associations of T1D and T2D predisposing genes has been sought. Kambo et al (1989) demonstrated that 40% of FCPD patients were positive for HLA-DQB, which is associated with T1D and 40% showed class 3 *INS* gene marker associated with T2D. Both markers were significantly more frequent in FCPD than in controls; 20% of FCPD and 1% Controls were positive for both the markers. Subsequently Sanjeevi et al (1992) have

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demonstrated association of FCPD with HLADR7 and DQ9 in a small number of patients with MRDM and claimed that MRDM is different from T1D. Later with larger number of patients they showed that FCPD is associated with DRB1*07 and DQ9 (A*80201-B*0303) and it is distinct from IDDM and PDDM (Sanjeevi *et al.*, 1999).

Since intraductal pancreatic stone and/ or pancreatic fibrosis is the hallmark of pancreatic pathology in FCPD isolation of pancreatic stone protein (PSP) gene and PSP protein created interest among researchers. The pancreatic stone protein and its secretory form (PSP-S) are inhibitors of $CaCO_3$ crystal growth, possibly involved in the stabilization of pancreatic juice. Giorgi et al (1989) have demonstrated that PSP-S mRNA was three times lower in CCP than in control, whereas the others were not altered. Terazono et al (1988) cloned and sequenced a cDNA, the REG gene, derived from pancreatic islets. It was termed REG on the basis of its induction during regrowth of the pancreas and its apparent origin from islets; the implication that the gene was involved in islet regeneration. Stewart (1989) found that the sequence was identical to that of pancreatic stone protein. Later on the REG gene was found to be part of a multigene family with 4 member REG family genes identified (Miyashita *et al.*, 1995).

Since FCPD is associated with both diabetes and pancreatitis, defective PSP or REG1A gene has been postulated to be associated with FCPD. However, no association was observed between REG1A gene variant and FCPD (Hawrami *et al.*, 1997).

1.7.3.2 Genes determining hereditary pancreatitis

Hereditary pancreatitis (MIM 167800) forms a small proportion of all pancreatitis. About 12.5% (Sibert 1978) of HP patients have been seen to develop diabetes and they generally have stones in major pancreatic ducts. Availability of multigenerational patients with pancreatitis provided a unique opportunity to carry out genetic analyses. Strong linkage was identified for HP to chromosome 7 and possible gene for pancreatitis mapped to 7q35 (Whitcomb *et al.*, 1996a). Two other groups also independently demonstrated linkage of HP to the chromosome 7q in French kindred (Le Bodic *et al.*, 1996; Pandya *et al.*, 1996).

Several genes mapped to chromosome 7q were considered to be candidate gene(s) for pancreatitis because these were already known to be expressed in exocrine

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pancreas and encode enzymes which could potentially activate digestive enzymes within pancreas. Rowen et al (1996) sequenced the whole 7q35 locus and identified family of identical 8 genes embedded within the T cell receptor B (TCRB) chain gene locus including the cationic trypsinogen gene 4 (Uni-Gene name: protease, serine 1; (PRSS1)) the precursor of the active trypsin enzyme.

Whitcomb et al (1996b) subsequently sequenced the cationic trypsinogen gene (Gene Bank Access No U66061) and identified the g.133283G→A (CGC to CAC) mutation resulting in R117H within the active peptide associated with pancreatitis. The R117H (previously called R122H in the chymotrypsin numbering system) (Chen and Ferec, 2000) mutation resides at cleavage site of protein deactivation. They have hypothesized a model that R117H mutation cause loss of its 'trypsin' ability to initiate neutralizing active trypsin in association with mezotrypsine such as zymogen (Figure 1.7) (Whitcomb *et al.*, 1996b).

Subsequently R117H and N21I were found to occur for disease in other pedigrees (Gorry *et al.*, 1997). Further functional analysis focused on interaction of R117H and N21I mutations. It was hypothesized that the N21I mutation results in withdrawal of amplifying properties on R122H fail-safe mechanism (Chen *et al.*, 2001).

The disease associated R117H and N21I mutations were also demonstrated in other populations with HP and also with chronic pancreatitis. Teich et al (1998) demonstrated g>131945A to T (AAC – TAC) mutation leading to N21I mutation, but not the G/A (R117H) in their series. A second mutation was identified in the exon2 of the trypsinogen gene by Witt et al (1999) and found to be associated with chronic pancreatitis with HP. The g131906>C to T (GCC – GTC) mutation results in A16V situated in the signal peptide cleavage site. A cohort of HP patients from UK were studied and found to be associated with R117H mutation (Bell *et al.*, 1998). The R117H mutation was also found to be associated with patients with idiopathic chronic pancreatitis (Creighton *et al.*, 1999). They found that 19% of ICP patients had trypsinogen gene G→A mutation (R117H). The high frequency of R117H mutation also in idiopathic pancreatitis patients strengthen the possibility of defective trypsinogen gene might be involved in the development of pancreatitis.

However, not all cases of HP could have been explained by mutations of the trypsinogen gene thus the existence of other factor leading to HP was suggested. A novel peptide had already been detected in the urine of a patient of ovarian cancer and hence termed as tumor associated trypsin inhibitor (TATI) by two groups (Huhtala *et al.*, 1982; Stenman *et al.*, 1982). The peptide was found to be identical or at least very similar to another peptide, pancreatic secretory trypsin inhibitor (PSTI) (Huhtala *et al.*, 1982). The peptide is also called the Kazal inhibitor, isolated as a crystalline trypsin inhibitor-anticoagulant protein from pancreas (Stenman 2002). Horii et al (1987) cloned and characterized the entire PSTI (Uni-gene name: serine protease inhibitor, Kazal type-1; SPINK1) gene and assigned (Gene Bank Access No: NM003122) to chromosome 5q32. Elevated serum and urinary levels of PSTI/SPINK1 occur particularly with mucinous ovarian cancer and may occur in nonmalignant diseases eg pancreatitis, severe infection and tissue destruction (Stenman *et al.*, 1991; Lukkonen *et al.*, 1999). Since PSTI/SPINK1 was also found in serum of various normal and malignant tissue diverse role of this peptide was postulated.

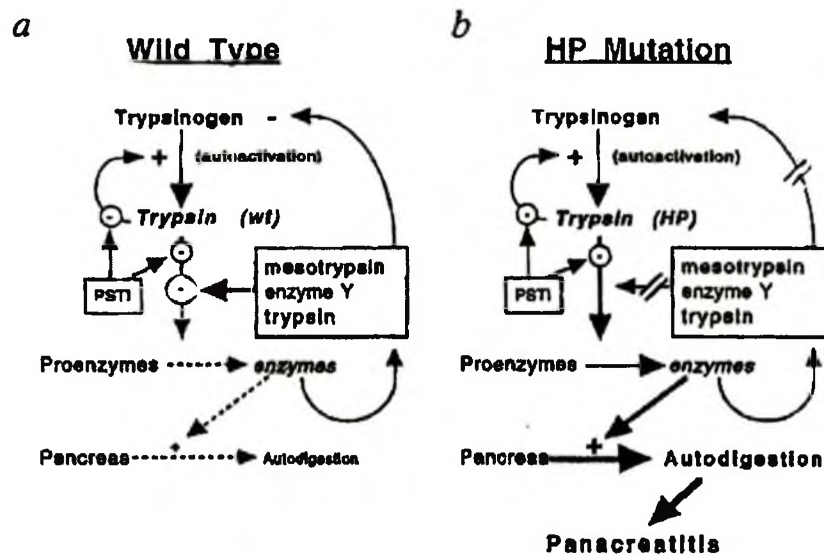

Figure 1.7: Model of trypsin self-destruct mechanism to prevent pancreatic autodisgestion. [a]Normal phenomenon and [b]model of mutant trypsin activation (Whitcomb *et al.*, 1996b).

The role of *SPINK1* to neutralize trypsin and its high level during pancreatitis suggested a link between pancreatic pathology and mutation of the *SPINK1* gene. SPINK1/TATI is strongly expressed together with trypsinogen by pancreatic acinar

cells. It is secreted into pancreatic juice where it constitutes 0.01-0.8% of total protein. The peptide is thought to protect pancreatic cells from destruction by inadvertent activation of trypsinogen and suggested to be capable of inhibiting 20% of potential trypsin (Truninger *et al.*, 2001). The postulated mechanism also been highlightened in a recent review by Liddle (2006).

Sequence analysis of *SPINK1* gene of patients with chronic pancreatitis and HP kindred from Germany and Austria demonstrated mutations in the coding and noncoding part of the gene (Witt *et al.*, 2000). They demonstrated significantly high rate of A→G (101A>G) mutation (N34S) in patients with chronic and hereditary pancreatitis. Out of 18 cases 6 were homozygous for the N34S variant. The A→G mutation, leading to N34S variation, is very close to the functionally active site of the polypeptide and thus the N34S variant suggested to lack the property to neutralize deduced trypsin within the aciner cells (Witt *et al.*, 2000).

Study of *SPINK1* gene involving Japanese patients with chronic pancreatitis and family history of disorder showed 5 only out of 32 (6.3%) individuals had the N34S variant (Kaneko *et al.*, 2001). In addition they found '-215G-A' mutation at translation initiation site in 2 individuals. Interestingly in a recent study of non-hereditary idiopathic pancreatitis an increased frequency of *SPINK1* mutations were found in Japanese patients (Kume *et al.*, 2005). They demonstrated that N34S mutation cosegregated with intronic variants (IVS1-37T>C) and it was present in 8 CP patients; its prevalence of (N34S; IVS1-37T>C) was 38% in familial pancreatitis and 13% in idiopathic pancreatitis where as 0.6% in the Controls.

Studies of the *SPINK1* gene on adult alcoholic, idiopathic and miscellaneous pancreatitis patients found that 12.2% of patients had mutations in the gene (6.9% in alcoholic pancreatitis, 20% in HP). Among the different mutations N34S was the most frequent. P55S was only present in 2 subjects whereas 13 had N34S variant; 11 out of 13 were heterozygous with N34S (Drenth *et al.*, 2002).

The *SPINK1* N34S mutation was screened in IP patients of European origin. Mutation was found in 9.3% of idiopathic pancreatitis patients compared to 1/100 in the Controls (Gomez-Lira *et al.*, 2003l). A low prevalence of *SPINK1* mutations in adult patients with chronic idiopathic pancreatitis has also been reported in another study (Ockenga *et al.*, 2001). Recently Masson et al (2006) have reported a pancreatitis family carrying a large genomic deletion in the *SPINK1* gene. The identification of this *SPINK1* gene lesion has not only expanded the SPINK1 mutational spectrum but also served to identify a novel mutational mechanism causing chronic pancreatitis.

Pancreatitis in TCP and/ or FCPD is unique and mainly prevalent in tropical developing countries. Significant association of *SPINK1* gene variant in HP and IP prompted the assumption for a role, if any, in the pathogenesis of pancreatitis in TCP/FCPD. In a pilot study with a small number of Bangladeshi TCP and FCPD patients the hypothesis for *SPINK1* gene variant was tested. In this series Rossi et al (2001) detected N34S *SPINK1* gene variant in 6 of 8 FCPD patients, but not in any of the TCP and healthy controls.

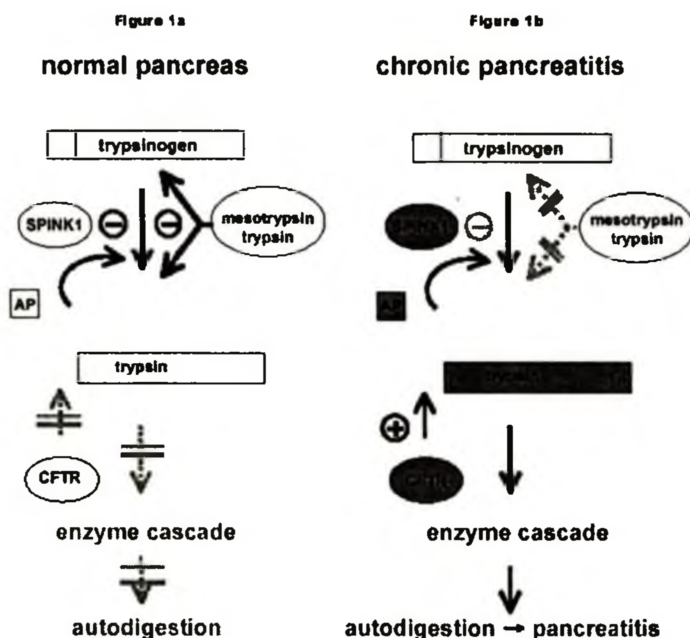

Figure 1.8: Model of chronic pancreatitis. [a]Condition in the normal pancreas: Natural defense mechanism prevents activation of pancreatic enzyme cascade within the pancreas and autodigestion. [b]Condition in chronic pancreatitis: Disruption of defense system leading to unopposed intrapancreatic activation of digestive enzyme. Dark boxes represent product of mutated genes AP, Activated peptide (Truninger *et al.*, 2001).

1.8 Diabetes in Bangladesh

Bangladesh is one of the tropical developing countries. Like other regions rapid urbanization is also taking place in this country and there is a change in the life style among the people. Diabetes is now seen as an epidemic in Bangladesh. A recent report has shown the global prevalence of diabetes for the year 2000 and estimated would be prevalence in 2030 (Wild *et al.*, 2004). In 2000 there were about more than three million diabetic patients in Bangladesh. They have estimated that by the year

2030 total number of diabetic patients will rise to 11.1 million. The proportional increase in Bangladesh seemed relatively higher compared to other Asian countries (Wild *et al.*, 2004).

Based on recent community based studies it appears that prevalence of diabetes in Bangladesh is around 5%. Studies involving rural and suburban population revealed the prevalence of T2D 4.3% and 4.1% respectively (Sayeed *et al.*, 1997 and 2003). In a recent study the same group has revealed that age adjusted prevalence of T2 DM was about 5.6% among the rural population, which is slightly higher compared to the previous report (Sayeed *et al.*, in press).

Diabetic Association Bangladesh (DAB) provides care to the diabetic patients through BIRDEM Hospital (the central institute of DAB), National Health Care Network (NHN) and its affiliated associations across the country. BIRDEM hospital provides diabetes care under an organized system. Presently there are about 350,000 diabetic patients registered with this institute. Of those registered cases about 7% belong to the age below 30 years.

1.8.1 YDM in Bangladesh

The prevalence of T1D is not known in Bangladesh. However, clinical experience suggests that the frequency of T1D has been increasing in the recent years. As mentioned earlier about 7% of all diabetic patients registered at BIRDEM are of young onset type (age of onset under 30 years). A substantial number of these young onset diabetic patients are lean (BMI <19) and usually present with moderate to severe hyperglycemia. A subset of these young patients present with pancreatic calcification and/ or fibrosis at diagnosis and according to the WHO (1995) criteria they fall to fibrocalculous pancreatic diabetes (FCPD) group. Those without pancreatic calcification were previously been classified as protein deficient diabetes mellitus (PDDM) group. Pathan (1994) has studied 453 young (<30 yrs age) newly diagnosed diabetic patients. In that series the proportion of type 1 DM was found to be 4.9%. However, diagnosis was done on the basis of clinical presentation. According to previous WHO classification (WHO 1985) around 50% of subjects were of MRDM and among them 14.8% and 35.2% were FCPD and PDDM respectively.

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

Autoantibodies have been estimated to evaluate their role in the pathogenesis of diabetes in this unique group of young diabetic patients. Hasin (2001) determined ICA, IAA and Anti-GAD antibodies in a group of newly diagnosed cases. She demonstrated that 11.4% of her series of diabetic patients were ICA positive compared to 6.7% of the controls. None of the diabetic and control subjects were positive for both IAA and GAD antibodies. The diabetic subjects did show reduced insulin secretory capacity, judged by C-peptide measurement, compared to control (0.855±0.441 vs 1.235±0.339 nmol/l respectively). The ICA autoantibody positive cases had low mean C-peptide levels (0.837±0.352, nnmol/l) compared to their negative counterparts (0.858±0.456), but the difference was not statistically significant. In another study diabetic patients with age range up to 18 years, were studied for anti-GAD and IA-2 antibody. In this study only 3% (1 of 33) were positive of IA2 and anti-GAD. The same individual was positive for both the antibodies. The diabetic individual was nonketotic at the time of venesection and subjects were recruited in the study within three months of diagnosis of diabetes (Rahman 2000). The study subjects, however, had very low insulin secretory capacity as judged by fasting C-peptide level (0.162±0.112 nmol/l, mean±SD). Crucial limitations of these studies were small number of samples and use of commercially

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available kits to determine autoantibodies. However, positivity of autoantibodies, even if, in a small proportion of study population did not overrule its possible role in the pathogeneis of diabetes in the young diabetes.

In a study Khan et al (1999) tried to explore the role of micronutrients in the etiopathogenesis of diabetes in the young. The study revealed that malnutrition itself might not be the cause of diabetes mellitus, rather deficiency of micronutrients. Measurement of other micronutrients e.g Cu and Zn also showed same trend (unpublished data).

1.8.2 T2D in Bangladesh

It was mentioned previously that the prevalence of T2D is around 5% in Bangladesh. Attempts have been made to explore the underlying pathophysiological mechanism involved in T2 diabetes in these patients. In the first study of this kind involving normal to over weight middle-aged T2 patients, B cell secretory capacity and insulin sensitivity (by ITT) was measured by short insulin tolerance test. The study revealed both deficiency of insulin secretory capacity and sensitivity in these patients (Al-Mahmood 2000). In a follow up study Insulin secretory capacity and defect were studied by 2-hour continuous infusion of glucose with model assessment (2-hCIGMA), in a group of lean type 2 patients, which also demonstrated both insulin secretory dysfunction and insulin resistance in the studied patients (Zinnat *et al.*, 2003). Findings of these studies suggested that both secretory dysfunction and insulin resistance are the dominant pathophysiology of T2D in the Bangladeshi population.

1.8.3 FCPD in Bangladesh

Fibrocalculus pancreatic diabetes (FCPD) and tropical calcific pancreatitis (TCP) is not uncommon in Bangladesh. FCPD has been found around 13% of the lean young onset type diabetes mellitus in two independent cohorts of newly diagnosed diabetic patients (Pathan 1994; Azad Khan and Ali 1997).

Islam et al (1998) studied a series of young onset diabetic patients to explore their insulin secretory capacity by glucagon stimulation test. Of the FCPD patients studied 70% had low insulin secretory capacity and 30% had intermediate level. Attempts were

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made to explore plausible environmental factor(s) responsible in the pathogenesis of pancreatitis in FCPD. Saha et al (2000) demonstrated that both tropical calcific pancreatitis (TCP) and FCPD had severe pancreatic ductal damage, but TCP were normoglycemic and had well preserved B cell secretory capacity. No particular environmental factor(s) were found to be associated with pancreatitis. Subsequently studies were carried out to explore the nature of exocrine pancreatic damage and its relation to endocrine pancreatic dysfunction. Ali et al (2001) evaluated exocrine pancreatic damage by measuring fecal elastase-1 and observed that both TCP and FCPD had moderate to severe exocrine damage, but only FCPD patients had compromised B cell secretory function where as TCP subjects had the function retained. Similar picture was also supported by the study of Rahman et al (2000). They evaluated exocrine pancreatic damage by ERCP and found that 93% TCP and 100% FCPD patients had severe pancreatic damage, but only FCPD had the endocrine dysfunction.

In Bangladesh Hereditary and idiopathic pancreatitis are not the common causes of chronic pancreatitis. FCPD and TCP contribute to the main bulk after infective and obstructive types of pancreatitis are excluded. Moreover, pathological features of pancreatitis of TCP and FCPD show some similarities with the pancreatitis of HP. Association of genetic variants in the trypsinogen (*TRYP*) and *SPINK1* genes with pancreatitis in HP patients and subsequently with idiopathic pancreatitis patients prompted the hypothesis that these genes might predispose to the pathogenesis of pancreatitis in TCP and FCPD.

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1.9 OBJECTIVES OF THE STUDY

General objective

The general objective of this research to study the etiological heterogeneity of young onset (<30 years) diabetic patients as a part of an attempt to explore the etiopathogenesis of diabetes mellitus in the young Bangladeshi population.

Specific objective

The specific objectives of the study were;

1. To investigate the physical and biochemical presentations of young onset diabetic subjects.
2. To study the autoimmune status of the young onset diabetic subjects.
3. To explore the presence of mutations in genes associated with pancreatitis of HP particularly in FCPD.
4. To study selected number of candidate genes (*SPINK1*, *INS*, *EIF2AK3* and a number of transcription factor genes involved in B cell differentiation and development) in the young onset diabetic subjects.

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CHAPTER 2

SUBJECTS AND METHODS

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2. SUBJECTS AND METHODS

2.1 Subjects

The study subjects consisted of a cohort of unrelated young diabetic patients, FCPD families and healthy Controls. Additional subjects from South India and UK immigrant Bangladeshi population were also analysed for candidate pancreatitis gene(s).

2.1.1 Unrelated Bangladeshi patients with diabetes

Young diabetic patients, disease diagnosed ≤30 years, were recruited from the Out- and In-patient Departments of the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Dhaka. FCPD and T1D were diagnosed and classified according to WHO recommendations (WHO 1999).

A total number of 423 young diabetic patients were enrolled into the study. The breakdown of these subjects was as follows:

Young diabetes mellitus (YDM) (n=372): Patients were non-ketotic and had no history of pancreatitis; thus on clinical criteria defined by WHO T1D and FCPD had been excluded.

Since frequency of T1D is found to be high at the age 14 years or below (Karvonen *et al.,* 2003) the 372 YDM subjects were further sub-classified; among the 372 YDM subjects 93 individuals had disease diagnosed at ≤14 years and were termed as $YDM14^-$ subjects and their counterparts were termed as YDM 14+ subjects (n=279).

Fibrocalculus pancreatic diabetes (FCPD) (n=51): The group consisted of diabetic patients presenting with chronic pancreatitis, as evidenced by pancreatic stone and/or fibrosis confirmed by X-ray and/ or ultrasonogram. FCPD subjects were also subdivided into FCPD 14^- subjects (n=12) and FCPD 14^+ subjects (n=39) like that of YDM subjects

Type 1 diabetes mellitus (n=11): Patients presenting with diabetic ketoacidosis without any precipitating cause were termed as type 1 diabetes mellitus (T1D). Because of the small number in the group no statistical comparisons were made with other groups.

The diabetic patients taking either oral hypoglycemic agents (OHA) or insulin were requested to withhold the evening dose of medicine on the day before and report to the Department in empty stomach. The treatment regimen of diabetic subjects are shown in the figure 2.1.

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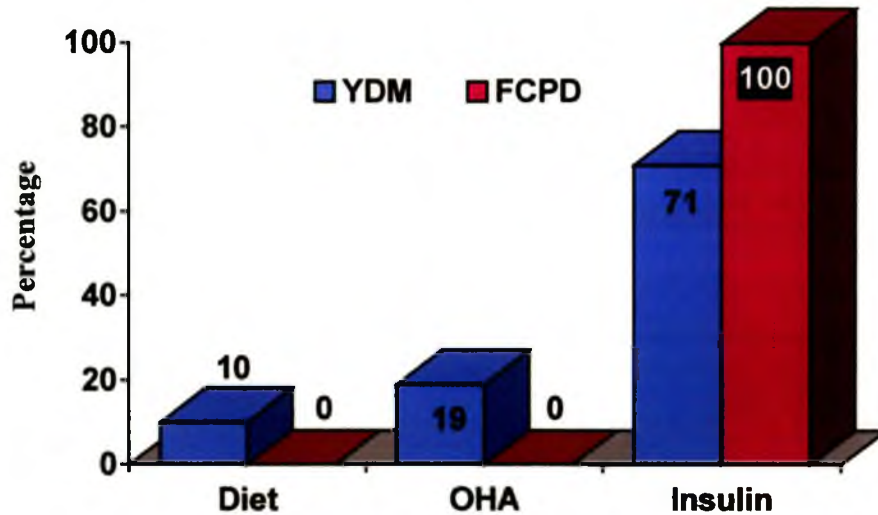

Figure 2.1: Proportion of subjects with different treatment regimen among the YDM and FCPD cases

2.1.2 Control subjects

A total number of 337 unrelated healthy subjects, aged up to 30 years, having no family history of diabetes up to second generation, served as Controls.

2.1.3 FCPD Families

Seventy simplex families, consisting of FCPD probands and both parents, were recruited from BIRDEM Out-patient Department. These 70 probands were additional to the unrelated FCPD cases. The parents were not known to have FCPD and/ or diabetes.

2.1.4 Additional subjects

The resources from South India consisted of unrelated FCPD patients (n=68) and 7 FCPD families collected in the MV Diabetes Research Centre (MV-DRC), Chennai, India (Hassan *et al.,* 2000). A cohort of 77 T2D, 56 IGT and 312 healthy controls, originating from an urban diabetes survey, were studied (Jackson *et al.,* 2004).

A cohort of 142 T2D patients and 156 healthy individuals from UK immigrants of Bangladeshi origin were studied. This group of subjects originated from northeastern region of Bangladesh (Sylhet) and therefore may be genetically distinct compared to the other Bangladeshi attending BIRDEM in general.

These additional resources were only tested for trypsinogen and *SPINK1* genes variants except those of the UK immigrant resources, who were analysed for *SPINK1* gene N34S variant only.

Further additional subjects consisting of young T2D (YT2D, n=235) and controls (n=152) of British Caucasoid origin from Andrew Hattersley's Lab were analysed for *SPINK1* gene N34S variant. The investigator was also involved in supervision of analysis of *SPINK1* N34S variant of 96 probands (30 MODY-X and 66 YT2D) of French origin.

2.1.5 Selection criteria

Diabetic patients fulfilling the recruitment criteria and willing to participate in the study were recruited.

2.2 METHODS

2.2.1 Blood collection and storage

Overnight fasting blood (12 ml) samples were collected following standard procedure. An 8 ml portion of the sample was taken into EDTA (1 mg/ml) tubes and preserved at -20°C freezer for future extraction of DNA. From the other portion serum was separated using refrigerated centrifuge and preserved at -70°C for future laboratory analyses.

2.2.2 Biochemical methods

Glucose was measured by glucose-oxidase method (Barham and Trinder 1972) using automated chemistry analyzer (Appendix 5.2). C-peptide was measured by ELISA (Yellow and Berson 1971) using a Kit from DRG Instruments, Germany (C-peptide, EIA-1293) (Appendix 5.3).

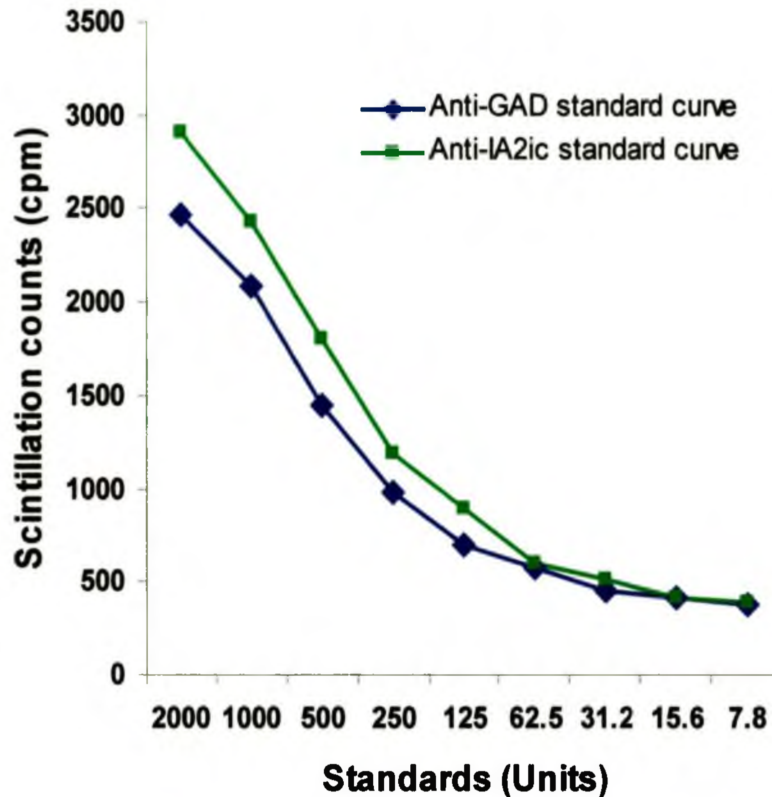

Figure 2.2: Standard curves for the determination of GAD and IA-2ic antibodies.

Calculation of results of unknown samples

All assays included a standard curve standardized to the Diabetes Antibody Standardization Program (DASP)-WHO standard as well as a positive and negative control sample. Samples were scored as positive based on the standard curve and mean+3SD of control samples.

Sensitivity and specificity of GAD and IA-2ic antibody assay

Sensitivity and specificity of GAD antibody assay of the laboratory are 87% and 95% respectively and of IA-2ic are 88% and 94% respectively. The quality control indices are in consistent with other laboratories.

2.2.3 Determination of anti-GAD and IA2-ic autoantibodies

Principle

Antibody against GAD and the putative intracellular fragment of IA2 (IA-2ic, amino acids 603-979) was measured by radioimmunoprecipitation assay method (Hawa *et al.*, 1997) (Appendix 5.4). The GAD and IA-2ic antibody assays employ coupled *in vitro* transcription and translation reaction for the two proteins. Separately human IA-2ic cDNA in pGEM-4Z vector (Payton *et al.*, 1995) and GAD65 cDNA in the pB1882 vector (Dr Thomas Dyrberg, Novo Nordisk Denmark) were *in vitro* transcribed and translated according to manufacturer instructions. About 0.8-1.0 μg cDNA of each GAD65 and IA-2ic was transcribed and translated with T7 and SP6 RNA polymerase respectively in a TNT coupled rabbit reticulocyte lysate system (Promega, Madison, WI) in the presence of ^{35}S methionine (0.8 mCi/ml) (Amersham, UK) (Appendix 5.4.3). Incorporated radioactivity was determined by precipitation with 10% trichloroacetic acid (TCA) and scintillation counting. For immunoprecipitation assay of anti-GAD and anti-IA-2ic antibody 50 μl translated GAD and IA-2ic protein(s), labelled with ^{35}S methionine (50,000–70,000 cpm), were incubated overnight with 2 μl serum at 4°C. The immunocomplexes were isolated on protein A Sepharose. The amount of immunoprecipitated GAD and IA2-ic was measured by scintillation counting using Microbeta Wallac Counter.

Assay procedure of anti-GAD and anti-IA-2ic antibodies

The protein translate (GAD and IA-2ic) was diluted in TBST to working strength, 50 μl of the mix gave radioactive count between 35000-50000 cpm, and used for GAD and IA-2ic assays respectively. Serum (2μl) and standard samples (2μl), equated to WHO reference standard (at 2000, 1000, 500, 250 125, 62.5, 31.25, 15.6 and 7.8), was dispensed in duplicate in 96 well Millipore Multiscreen plates (Appendix 5.4.6). In each plate a known positive and negative samples were included. To each well 50 μl working antigen solution was added, the plate was covered, placed on a shaker for brief period at 4°C and incubated over night at 4°C.

On the next morning already prepared and swelled protein sepharose A (50 μl/well) (Appendix 5.4.7) was added containing 1 mg/ml (approx) of PAS and incubated on a shaker for 90 minutes at 4°C. After the incubation the plate was washed with 300 μl TBST solution in total 8 times using a negative suction and left to dry on the bench for 15-20 minutes. The plate was then placed on Meltilex Scintillets Paper and counted using Wallac Microbeta Counter.

2.2.4 Extraction of DNA from peripheral blood

DNA was extracted from peripheral blood for this study using two different protocols, namely (i) Salt precipitation method, using PureGene DNA Kit and (ii) Silica gel-membrane DNA extraction kit, using QIAamp DNA Blood kit.

2.2.4.1 DNA extraction using PUREGENE kit

The PUREGENE extraction is based on salt precipitation of DNA. Cells lacking nucleus (red blood cells) were selectively lysed using lysis buffer (Appendix 5.5.1). White blood cells (WBC) were then recovered and its lysis was achieved by using WBC lysis buffer (Appendix 5.5.2). Cellular and sub-cellular proteins were precipitated using protein precipitation buffer (Appendix 5.5.3). DNA present in the solution was recovered using 100% isopropyl alcohol. Precipitated DNA was harvested, washed with 70% alcohol and resuspended using DNA hydration buffer (Appendix 5.5.4).

Procedure

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1. 300 μl EDTA-blood taken in a microcentrifuge tube.
2. 900 μl RBC lysis buffer was added, mixed thoroughly by repeated inversion and incubated at 37°C for 5 minutes.
3. Centrifuged at 13000g for 1 minute.
4. Repeated the step for 2/3 times, till lysis buffer become clear.
5. 20 μl RBC lysis buffer was added, the pellet resuspended by gentle pipetting and vortexing.
6. 300 μl WBC lysis buffer containing Proteinase K (450 μg/ml) was added, mixed thoroughly by vortex and spun briefly.
7. Incubated at 55°C for 60 minutes.
8. Reaction tubes left on the bench, to cool down at room temperature, 100 μl Protein Precipitation Buffer was added and vortexed for 20 sec.
9. Centrifuged for 5 minutes at 13000xg.
10. Supernatant transferred into tube containing 300 μl isopropyl (100%) alcohol. Tubes were inverted repeatedly, holding between fingers, to facilitate DNA precipitation.

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11. Centrifuged at 13000xg for 3 minutes.

12. Supernatant was decanted, pellet washed with 500 μl ethanol (70%). Centrifuged at 13000g for 2 minutes. Carefully poured the ethanol and drained the tube.

13. The tube was dried out in spin vacuum at 50 °C.

14. 100 μl hydration buffer was added, incubated 1 hour at 55°C and/ or left overnight at room temperature for DNA

15. Extraction was validated subjected to checking of the extract in 1% agarose gel.

16. Aliquots of DNA was diluted to 10 ηg/μl and preserved -20°C for future analyses.

2.2.4.2 High through-put DNA Extraction using QIAGEN Kit

Principle

The method is based on the silica-gel membrane DNA extraction procedure adapted into a spin column and 96-well plate format.

QIAamp 96 DNA blood kit (QIAGEN UK Ltd, Crawley, Sussex) is a high-through-put method simultaneously extract one 96 well plate in 2 hours. All centrifugation steps were carried out on a Sigma 6K-15 centrifuge (Sigma Laborzentrifugen GmbH).

Procedure

1. 25 μl of Qiagen Protease stock solution was added to each of the 96 wells of round-well block.

2. 200 μl aliquot of peripheral blood sample was added to each well.

3. 200 μl of chaotropic solution AL (QIAGEN) was added to each well. Wells were capped and the block shaken vigorously for 15 seconds.

4. The 96 well block was then spun briefly at 3000 rpm.

5. The block was incubated at 65°C for 10 minutes in an oven and spun briefly at 3000 rpm.

6. 210 μl of 100% ethanol was added to each well, the block was shaken vigorously for 15 seconds and spun briefly at 3000 rpm. Proper attention was paid to avoid any accidental swap of strip caps for wells.

7. QIAamp 96 column plate was placed on a plate receptacle and reaction mix (635 µl/well) transferred into the column. Plate was sealed with an adhesive strip and centrifuged for 6000 rpm for 4 minutes.
8. The 96 well column plate was washed with 500 µl of Qiagen buffer AW1. The plate was centrifuged at 6000 rpm for 2 minutes.
9. The second wash was done with 500 µl of Qiagen buffer AW2. The plate was centrifuged at 6000 rpm for 4 minutes. In each step of washing fresh sealing film was used to avoid any outside chance of cross contamination.
10. The QIAamp 96 column plate was removed from the waste receptacle block and placed on a 96 microtube collecting rack and incubated in an oven at 70°C for 10 minutes to dry the silica membrane.
11. Preheated (at 70°C) Qiagen AE Buffer 200 µl was added to 96 column membranes, sealed and incubated at room temperature for 5 minutes.
12. DNA elution into collecting tubules was done by centrifuging at 6000 rpm for 4 minutes. This step was repeated using 50 µl of preheated AE Buffer.
13. To maximise the DNA extraction a third elute was done using 100 µl Buffer AE in a separate collection block.
14. Random samples from the eluted DNA were electrophoresed on 1% agarose gel to assess the integrity, uniformity of yield. Approximate concentration of yield was assessed comparing with known quantity of molecular marker. DNA yield was approximately 30-40 ng/µl in the first elute.
15. Aliquot of DNA was diluted to 10 ng/µl in 96 deep well plates and preserved at 4°C.

Quantification of extracted DNA has been carried following standard procedure (Appendix 5.6)

2.2.5 Candidate gene(s) marker analyses

Candidate gene analysis was carried out by PCR-RFLP and direct DNA sequencing. Standard protocols for PCR (Appendix 5.7) and RFLP (Appendix 5.8) were followed throughout, if any necessary modification applied was mentioned in the respective sectlon. All the endonucleases used in the study are briefly mentioned in the appendix unless otherwise mentioned (Appendix 5.9). To approximate the size of the 100bp DNA ladder was used throughout (Appendix 5.10).

2.2.5.1 INS VNTR -23 bp A/T allele

The DNA fragment containing -23 A/T site was amplified using the following flanking primers set:

Forward 5'- AGC AGG TCT GTT CCA AGG GC -3'

Reverse 5'- CTT GGG TGT GTA GAA GAA GC -3'

The PCR was a 'Standard HotStart PCR' in a 25 μl reaction volume using HotStart Taq polymerase. Size of the amplified fragment was 360 bp.

PCR condition: Initial incubation at 95°C for15 minutes; followed by 35 cycles 'step of denaturation at 95°C for 30 sec; annealing at 58°C for 30 sec; extension at 72°C for 45 sec'; step of final extension at 72°C for 10 minutes.

PCR product was resolved on 2% agarose gel to ascertain the outcome. PCR product (7 μl) was digested with restriction enzyme, *Hph* 1 (Appendix 5.8.1). Enzyme digestion products were resolved on 4% agarose gel.

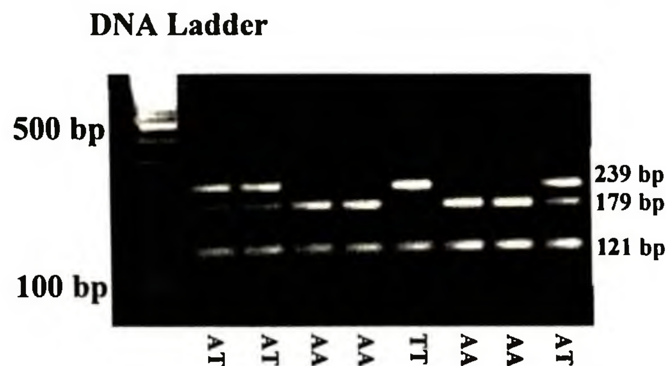

Figure 2.3: Image of agarose gel electrophoresis of *Hph*1 enzyme digest of insulin VNTR A/T allele assay

The polymorphism disrupts the restriction site. The amplified segment of DNA had an internal cutting site. The wild type genotype produced fragments of 60, 121 and 179bp; heterozygous genotype 60, 121, 179, and 239bp; homozygous polymorphic genotype 121 and 179bp. Fragments of 39bp could not be resolved in the agarose gel.

2.2.5.2 EIF2AK3 gene Indel15 AT^{+}/AT^{-} allele

DNA fragment containing *EIF2AK3* gene $AT^{+/-}$ deletion in the intron15 was amplified using the primer set:

Forward: 5'-TGTGGAATCTGTGGGATGTG-3'

Reverse: 5'- TGC TAAGGACCGCTTACGTT-3'

The PCR was a 'Standard HotStart PCR' in a 25 μl reaction volume using HotStart Taq polymerase. PCR product size was 356 bp.

PCR condition: Initial incubation at 95°C for 15 minutes; followed by 35 cycles of 'step of denaturation at 95°C for 30 sec; annealing at 58°C for 30 sec; extension at 72°C for 50 sec; and step of final extension at 72°C for 10 minutes.

PCR product was resolved on 2.5% agarose gel. PCR product of 8 μl was digested with restriction enzyme, *Nde* I (Appendix 5.8.2). Products of enzyme digestion were resolved on 4% agarose gel and fragments were sized up compared to undigested PCR product and DNA ladder.

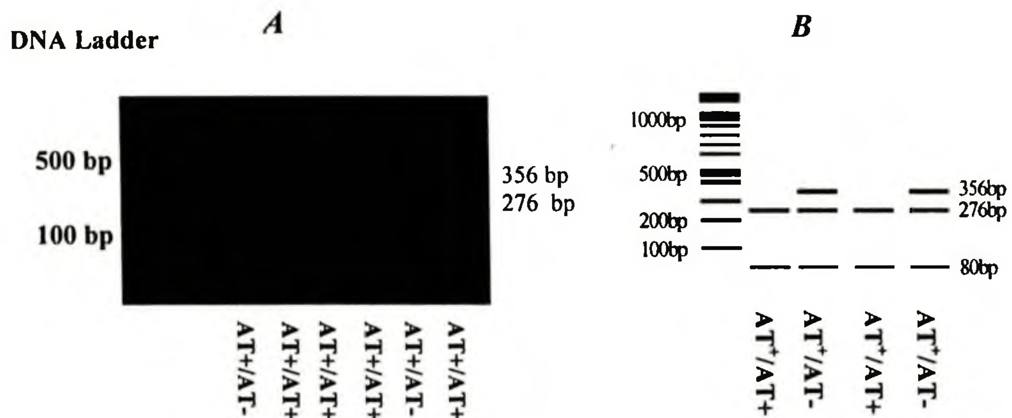

Figure 2.4: *(A)* Image of agarose gel electrophoresis of *Nde* I enzyme digest of *EIF2AK3* gene Indel15 At^{+}/AT^{-} allele assay. *(B)* Schematic presentation of the restriction enzyme digestion fragments.

The $AT^{+/-}$ variant of the gene abolishes the restriction site. The wild type AT^{+} genotype produced 80 and 276bp fragments; heterozygous variant TA^{+}/AT^{-} deletion genotype 80, 276 and 356 bp fragments; homozygous deletion ($AT^{-/-}$) will have no digest so resolved 356bp fragment.

2.2.5.3 *TCF1* gene C/T (A98V) allele

DNA fragment containing the *TCF1* gene C/T substitution leading to A98V was amplified using the following flanking primers set (Urhammer *et al.*, 1997b):

Forward: 5'-GGC AGG CAA ACG CAA CCC ACG-3'

Reverse: 5'-GAA GGG GGG CTC GTT AGG AGC-3

The PCR was a 'Standard HotStart PCR' in a 20 µl reaction volume. Size of the amplified portion of DNA was 483 bp.

PCR condition: Initial incubation for 15 minutes at 95°C; followed 35 cycles 'step denaturation at 95°C for 40 sec; annealing at 62°C for 40 sec; extension at 72°C for 45 sec; step of final extension at 72°C for 10 minutes.

PCR product was resolved on 2% agarose gel to check PCR yield. PCR product of 7 µl was digested with restriction enzyme, *Hae* III (Appendix 5.8.3). Products of enzyme digestion were resolved on 4.5% agarose gel and fragments were sized up compared to undigested PCR product and ladder DNA (100 bp DNA ladder from Promega).

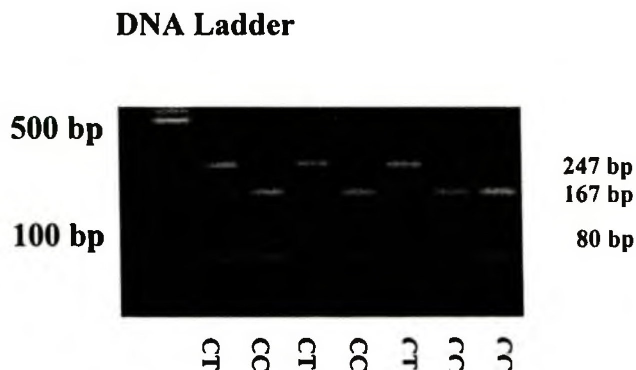

Figure 2.5: Image of agarose gel electrophoresis of *Hae* III enzyme digest of TCF1 gene C/T allele (A98V) assay

The amplified DNA segment had several restriction enzyme sites. The polymorphism abolishes the enzyme restriction site. The wild type CC genotype produced 80 and 167bp fragments; heterozygous variant CT genotype 80, 167 and 247bp fragments; homozygous variant TT genotype 247bp.

Enzyme digestion fragments like 8, 23 and 30 bp could not be resolved in the agarose gel. Three fragments 55, 59 and 61 bp resolved close to each other and appeared as a thick single band in the gel.

2.2.5.4 *NEUROD1* gene G/A (A45T) allele

DNA fragment containing *NEUROD1* gene G/A polymorphism (A45T) was amplified using the following primers set (Iwata *et al.*, 1999):

Forward: 5'-GACAAGAAGGAGGACGACCCTGAA-3'

Reverse: 5'-TCTCAATTTAAAACGCTCCAG-3'

The PCR was a 'Standard HotStart PCR' in a 20 µl reaction volume. Size of the amplified DNA fragment was 198bp.

PCR condition: Initial incubation at 95°C for 15 minutes; followed by 35 cycles of 'step of denaturation at 95°C for 30 sec; annealing at 57°C for 45 sec; extension at 72°C for 45 sec'; step of final extension at 72°C for 10 minutes.

PCR product was resolved on 3% agarose gel. PCR product of 7 µl was digested with restriction enzyme, *Mwo*I (Appendix 5.8.4). Products of enzyme digestion were resolved on 4% agarose gel and fragments were sized up compared to undigested PCR product and DNA ladder.

Figure 2.6: Image of agarose gel electrophoresis of *Mwo*I enzyme digest of *NEUROD1* gene G/A (A45T) allele assay.

In the forward primer contains 2 bp mismatch just proximal to 3' end (underlined in the primer sequence) such that 198 bp product contains a restriction site for the restriction enzyme *Mwo*I. The polymorphism prevented the PCR generated restriction site. The wild type GG genotype produced 31 and 157bp fragments; heterozygous variant GA genotype 31, 157 and 198bp fragments; homozygous variant AA genotype remained undigested (198bp).

2.2.5.5 *NEUROG3* gene variant

DNA fragment of the *NEUROG3* gene containing G/A (G167R) and C/T (S199F) polymorphisms was amplified using the following primers set (Jensen *et al.*, 2001):

Forward: 5'-GAC TCA AAC GCT GCG CAT AG-3'

Reverse: 5'-CTC TCC CTT ACC CTT AGC AC-3

The PCR was a 'Standard HotStart PCR' in a 25 µl reaction volume using HotStart Taq polymerase. Size of the amplified DNA segment was 288 bp.

PCR condition: Initial incubation at 95°C for 15 minutes; followed by 35 cycles 'step of denaturation at 95°C 30 sec; annealing at 59°C for 30 sec; extension at 72°C for 30 sec'; step of final extension at 72°C for 10 minutes.

2.2.5.5.1 NEUROG3 gene G/A (G167R) allele

The G/A nucleotide substitution (G167R) in the amplified DNA fragment was resolved by RFLP analysis using *Sma* I restriction enzyme digestion.

PCR product of 7 µl was digested using 10 units of *Sma* I enzyme (Appendix 5.8.5). The amplified DNA section has an internal cutting site. The G/A variation abolishes the enzyme restriction site. Products of enzyme digestion were resolved on 4.5% agarose gel and fragments were sized up compared to undigested PCR product and DNA ladder.

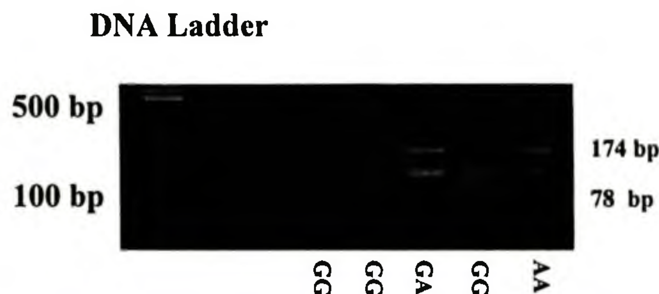

Figure 2.7: Image of agarose gel electrophoresis *Sma*I enzyme digest of *NEUROG3* gene G/A (G167R) allele assay.

The amplified DNA section has an internal cutting site. The G/A substitution abolishes the enzyme restriction site. The wild type GG genotype produced 78, 94 and 116bp fragments; heterozygous variant GA genotype 78, 94, 116 and 174bp fragments; homozygous variant AA genotype 116 and 171bp fragment.

2.2.5.5.2 NEUROG3 gene C/T (S199F) allele

The DNA fragment containing *NEUROG3* gene C/T polymorphism (S199F) was assayed by RFLP analysis using *Era* I restriction enzyme.

PCR product of 7 μl was digested with restriction enzyme, *Era* I (Appendix 5.8.6). Products of enzyme digestion were resolved on 4% agarose gel and fragments were sized up compared to undigested PCR product and 100bp DNA ladder.

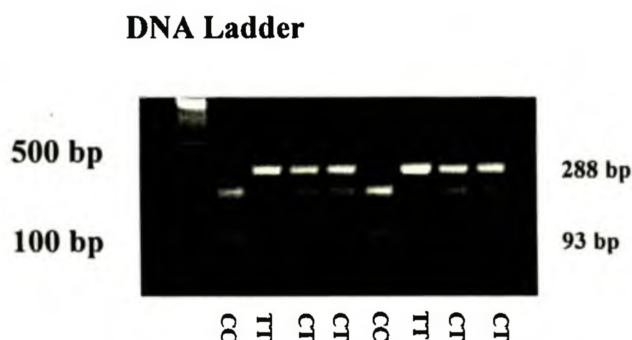

Figure 2.8: Image of agarose gel electrophoresis *Era*I enzyme digest of *NEUROG3* gene C/T (S199F) allele assay.

The C/T substitution abolishes the restriction site. The wild type CC genotype produced 93 and 195 bp fragments; heterozygous variant genotype 93, 195 and 288 bp fragments; homozygous variant genotype had undigested (288 bp) fragment.

2.2.5.6 Pancreatitis gene(s) assay

2.2.5.6.1 *SPINK 1* gene A/G (N34S) allele

DNA fragment containing *SPINK1* gene A/G mutation was amplified using the following flanking primers (Plendl *et al.,* 2001):

Forward: 5'-GAA GAA CGT GCC CCA AGA T-3'

Reverse: 5'-GTT TGC TTT TCT CGG GGT GAG-3'

The PCR was a 'Standard HotStart PCR' in a 25 μl reaction volume using HotStart Taq polymerase. The size of the PCR product was 595 bp.

PCR condition: Initial incubation at 95°C for15 minutes; followed by 35 cycles 'step of denaturation at 95°C for 30 sec; annealing at 58°C for 45 sec; extension at 72°C for 45 sec'; step of final extension at 72°C for 10 minutes.

PCR product was resolved on 2% agarose gel. PCR product (7 μl) was digested with restriction enzyme, *Taa* I (Appendix 5.8.7). Enzyme digestion resolved fragments were resolved on 4% agarose gel and fragments sized up compared to undigested PCR product and 100bp ladder DNA.

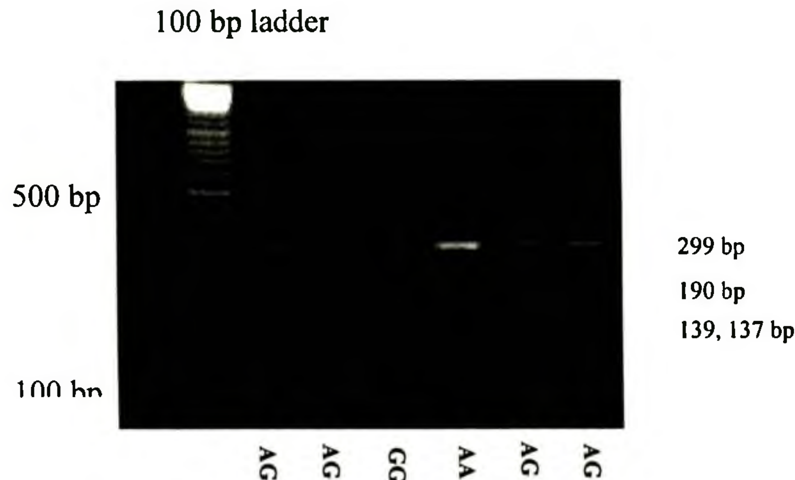

Figure 2.9: Image of agarose gel electrophoresis *Taa*I enzyme digest of *SPINK1* gene A/G (N34S) allele assay

The mutation created the restriction enzyme site. The amplified DNA segment had 3 internal cutting sites. So, the enzyme digestion yielded additional fragments of 20, 137 and 139bp. Wild type homozygous AA genotype had fragments 139, 137, 20 and 299bp; heterozygous AG genotype 20, 137, 139 and 109, 190, 299bp; homozygous variant GG genotype had 20, 137, 137, 109 and 190bs fragments. The 20bp fragment could not be resolved on 3.4% agarose gel.

2.2.5.6.2 Trypsinogen gene assay

2.2.5.6.2.1 Trypsinogen gene C/T (A16V) allele

DNA fragment containing C/T substitution in the trypsinogen gene exon 2 was amplified using the following set of primers:

The DNA segment was amplified using the following set of primers:

Forward: 5'-TTAGCAGAAAGCAATCACAGG-3'

Reverse 5'-ATCTTGGGGTGGTGAGAG-3'

The fragment also contains A/T mutation that does not disrupt any restriction enzyme site, hence studied by direct DNA sequencing.

Amplification was done by 'standard' PCR in a 25 μl reaction volume. Size of the PCR product was 495bp.

PCR condition: Initial incubation at 94°C for 3minutes; followed by 35 cycles 'step of denaturation at 94°C for 60 sec; annealing at 55°C for 60 sec; extension at 72°C for 60 sec'; step of final extension at 72°C for 10 minutes.

The PCR product was resolved in 2% agarose gel to check outcome and integrity of PCR program itself. Later 8 μl PCR product was digested with *Fnu4*HI enzyme (Appendix 5.8.9).

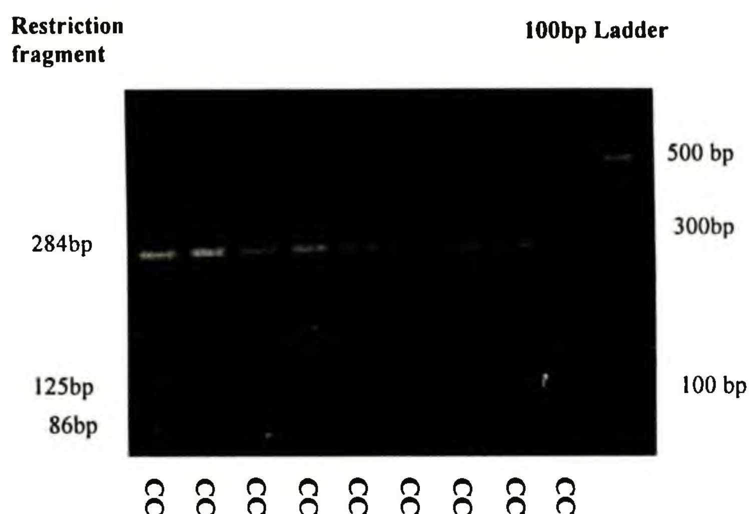

Figure 2.10: Image of agarose gel electrophoresis of *Fnu*4HI enzyme digest of trypsinogen gene C/T (A16V) allele assay

The DNA fragment amplified has an internal cutting site and produced additional fragment of 125bp. Product of enzyme digestion was resolved on 3.5% agarose gel. The wild type CC genotype had 86, 284 and 125bp fragments; heterozygous mutant CT genotype had 86, 284, 370 and 125bp fragments; mutant TT genotype would have 370 and 125bp fragment.

2.2.5.6.2.2 Trypsinogen gene G/A (R122H) allele

DNA sequence containing Trypsinogen gene G/A mutation (R122H) was amplified using following flanking primer (Whitcomb *et al.*, 1996b).

Forward 5'-GGTCCTGGGTCTCATACCTT-3'

Reverse 5'-GGGTAGGAGGCTTCACACTT-3'

Amplification was done by 'standard' PCR in a 25 μl reaction volume. Size of the PCR product was 911bp.

PCR cycle condition: Initial incubation for 3 minutes at 95°C; followed by 35 cycles 'step denaturation at 94°C for 45 sec; annealing at 59°C for 60 sec; extension at 72°C for 60 sec'; step of final extension at 72°C for 10 minutes.

PCR product was resolved on 2% agarose gel to check PCR integrity. PCR product of 7 µl was digested with restriction enzyme, Afl III. Products of enzyme digestion were resolved on 2.0% agarose gel and fragments were sized up compared with DNA ladder.

The mutation creates enzyme restriction site. The enzyme *Afl* III (Appendix 5.8.8) digest did not resolve any restriction fragment, G/A mutation was absent the study subjects.

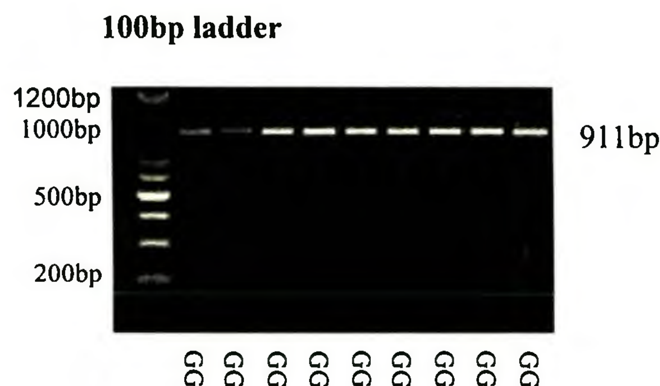

Figure 2.11: Image of agaorose gel electrophoresis of *Afl*III enzyme digest of trypsinogen gene G/A (R122H) allele assay

2.2.5.7 Candidate gene direct DNA sequencing

Trypsinogen gene exon2 and exon3 candidate variants were also assayed by direct DNA sequencing. The sequencing analysis was performed by ABI dye-terminator sequencing reaction method (Appendix 5.11) using ABI 373 Automated Sequencer.

2.2.5.7.1 N21I variant assay by direct DNA sequencing

Mutation causing A→T substitution resulting N21I in the exon2 of the trypsinogen gene did not disrupt any restriction enzyme site. So, N21I variant allele was examined by direct sequencing (Appendix 5.9).

Nested PCR was done using the same set of flanking primers (section 2.2.5.6.2.1) used for RFLP assay of C/T (A16V) allele assay to obtain clean amplification and cycle sequencing reaction. As a template DNA µl PCR product was used.

PCR condition: Initial incubation for 3 minutes at 94°C followed by 60 sec at 94°C x 60 sec annealing at 55°C x 60 sec extension at 72°C for 35 cycles and final step of final extension at 72°C for 10 minutes.

PCR yield was checked in 3% agarose gel. ABI dye terminator cycle sequencing reaction was carried out using 7 μl PCR product. Product of the cycle sequencing reaction was harvested and prepared, sequence gel was run and DNA sequence was compared to the reference sequence in the gene bank.

2.2.5.7.2 R122H variant assay by direct DNA sequencing

Mutation causing G→A substitution resulting R122H in the exon3 of the trypsinogen gene was analysed by RFLP and absence of mutation the studied population was confirmed by direct DNA sequencing in 25 probands.

To sequence the fragment of DNA a set the following set of 'sequencing primers were' designed and used for amplification and cycle sequencing reaction.

Forward: 5'-TGACCCACATCCCTCTGCTG-3'

Reverse: 5'-TCTCCATTTGTCCTGTCTCT-3'

PCR condition: initial incubation at 95°C for 3 min followed by 60 sec 94°C; 60 sec at 59°C; 60 sec at 72°C; for 25 cycles and then step of final elongation at 72°C for 10 mins.

The PCR yield was on agarose gel. The Cycle sequencing reaction using ABI dye terminator was carried (Appendix 5.9.4). For sequencing reaction 5 μl PCR product was used, product was harvested, sequence gel was run and the fragment of DNA sequenced was analysed using Sequence Navigator.

2.3 Statistical methods

2.3.1 Basic statistical analyses

Statistical analyses were performed using Statistical Package for Social Science (SPSS) for Windows Version 11.0 and a $p<0.05$ was taken as the level of significance throughout. Unpaired students 't' test, proportion test, non-parametric tests (eg Mann-Whitney test, Chi square test and Odds ratio) were applied where appropriate.

Data were initially analysed by means of contingency tables upon which a Chi square test of independence was performed. A Fisher Exact Test was employed for small numbers when indicated in 2 by 2 tables.

The odds ratio relates the odds of being a case to not being a case for those with the risk factor (a/b) to these same odds for those without the risk factor (c/d). When the odds in each group have equal risk, then odds ratio is equal to the unity.

An odds ratio of 1 implies that the event is equally likely in both groups. An odds ratio >1 one implies that the event is more likely in the first group. An odds ratio <1 implies that the event is less likely in the first group.

2.3.2 Transmission Disequilibrium Tests

Transmission disequilibrium test uses parents that are heterozygous or informative at the marker locus and compares the frequencies of the marker (M) alleles that are transmitted to the affected offspring with the frequencies of marker alleles that are not transmitted, using the test statistic $(n_{12}-n_{21})^2/(n_{12}+n_{21})$ with reference to table 3.8.1. An asymptotic chi-squared is generated with one degree of freedom when the disease and marker loci are unlinked. If an allele is transmitted on more than 50% of occasions to affected offspring then there is evidence for both linkage and linkage disequilibrium between marker and disease loci.

The standard TDT examines a single allele of a marker to determine whether it is transmitted from heterozygous parents to affected offspring more often than would be expected by chance, i.e. on more than 50% of occasions.

The extended TDT (ETDT) performs the transmission disequilibrium test for a marker with multiple alleles. The test has been modelled by Sham and Curtis (1995) to conduct allele-wise analysis.

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Table 2.1:

Basis of TDT statistic

Transmitted Marker (M) allele	Non-Transmitted Marker allele	
	M_1	M_2
M_1	n_{11}	n_{12}
M_2	n_{21}	n_{22}

This allele-wise analysis implements a parsimonious and biologically plausible model to test the hypothesis that different alleles may vary in the extent to which they are preferentially transmitted to affected offspring. The model fitted is appropriate when there is zero recombination between the disease and marker loci and when matings are random (which implies that subjects are unrelated). The model may fit less well when pedigree data are used, although in these circumstances it will still be unbiassed and not prone to produce false positives. This relates to the general considerations which apply to the tranmission disequilibrium test: when parents are unrelated then it is a pure test for linkage disequilibrium in the presence of linkage, whereas if all subjects come from a single large pedigree then the transmission disequilibrium test will tend to be positive if there is linkage even if there is no linkage disequilibrium. Thus although the TDT is not susceptible to produce false positive results when linkage is absent, the extent to which it provides evidence additionally for the presence of linkage disequilibrium can be difficult to assess if subjects are related.

2.3.5 Hardy-Weinberg Equilibrium

A large completely mixed (panmistic) population consists of a number overlapping generations or mating types, but because the genotypes of new members of the population are a function of the allele frequencies of the reproductive age class, the proportion of various genotypes at a given locus should remain unchanged from one generation to the next unless the population is subject to selection forces such as nonrandom mating, mortality, migration, or mutation rate that is not constant. Genotype frequencies should become homogenised across all age classes and sexes and this state is Hardy-Weinberg Equilibrium (HWE).

Under certain circumstances the frequencies of the genotypes at the locus under study can be a function of the allele frequencies alone, and underlying selection forces could stratify the population. Therefore it has become standard practice to cross-check the genotype frequencies in any given population with Hardy-Weinberg expectations as a departure from HWE could influence the validity of an association study. For example if the observed population allele frequencies significantly differ from the expected as a result of increased heterozygosity or homozygosity and if not due to genotyping error, it would indicate a degree of 'outbreeding' or 'inbreeding' respectively within the study population.

Table 2.2:

The Hardy-Weinberg equilibrium genotype frequencies for allele frequencies p (A_1) and q (A_2)

	Autosomal locus			**X-linked locus**				
				Male		***Female***		
Genotype	A_1A_1	A_1A_2	A_2A_2	A_1	A_2	A_1A_1	A_1A_2	A_2A_2
Frequency	p^2	2pq	q^2	p	q	p^2	2pq	q^2

Significant departure from HWE was assessed for all loci investigated using an MS-Excel sheet prepared by Dr. Curtis. This is incorporated in the HWE equation comparing observed and expected genotype frequencies and calculated by a Pearson chi-squared statistic with one degree of freedom (Sham 1998).

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CHAPTER 3

RESULTS

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3. RESULTS

3.1 Gender distribution of the study subjects

Among the 337 Controls 218 (65%) were male and 119 (35%) female. In case of 372 YDM subjects male and female distribution was 165 (44%) and 207 (56%) respectively and in case of 51 unrelated FCPD subjects the corresponding proportion was 19 (37%) and 32 (64%). There appeared to be male preponderance in the Controls and female preponderance in the diabetic recruits. This distribution showed significant difference ($p<0.001$). Among FCPD probands of the simplex families, out of 70 individuals 42 (60%) were male and 28 (40%) were female. In case of total FCPD subjects (unrelated FCPD and probands FCPD, n=121) 61 (50.4%) were male and 60 (49.6%) female (Table 3.1i).

3.2 Age (years) of the study subjects

Mean (±SD) age of the Controls was 22.5±4.9 and that of YDM 19.4±6.3 ($p<0.05$ vs Controls) and unrelated FCPD 19.9±6.0 ($p<0.05$ vs Controls). Mean (±SD) age of the YDM and unrelated FCPD subjects did not show statistically significant difference ($p=0.618$). Mean (±SD) age of onset of diabetes of the two diabetic groups was 18.8±6.1 and 19.1±5.7 respectively, which also did not show any statistical difference ($p=0.713$) (Table 3.1i).

Mean (±SD) age of the FCDP probands was 18±2.3. For total FCPD cases (unrelated FCPD cases and FCPD probands, n=121) mean (±SD) age was 18.8±5.2 years.

YDM14+ subjects had mean (±SD) age 22.0±4.9 years at diagnosis, almost similar to that of Controls (22.5±4.9, age at recruitment). FCPD14+ subjects had mean (±SD) age 22.1±5.2 years at diagnosis, which did not show any statistical difference compared to Controls (22.5±4.9) and also to YDM14+ subjects (Table 3.1i).

Mean (±SD) age of onset of diabetes in YDM and FCPD group was 18.8±6.1 and 19.1±5.7 respectively (Table 3.1). In YDM14+ and FCPD14+ subjects it was 22.1±5.2 and 22.5±4.9 years respectively (Table 3.1i).

Out of 372 YDM patents 296 were newly diagnosed and in FCPD it was 40 out of 51 cases. Duration of diabetes in YDM and FCPD subjects was 2.0 (0.1-12.3) and 3.6

(0.08-14.0) [median (range)] years respectively. There was no significantly statistical difference regarding the duration of diabetes between the two groups.

3.3 BMI of the study subjects

BMI was calculated for the subjects with age 18 years and above. Mean (±SD) BMI of the control (n=290) subjects was 20.1±3.1, YDM (n=195) 21.0±4.2 (p=0.008 vs Controls) and of FCPD (n=28) 18.1±3.1 (p=0.001). The YDM group was found to have significantly higher BMI compared to the Controls and FCPD (p=0.001) subjects (Table 3.1). For the subjects under the 18 years of age frequency of subjects at different BMI percentile was calculated. BMI percentile was calculated for those under the age of 18 years. Three were 47, 174 and 23 subjects under the age of 18 in controls, YDM and FCPD groups respectively. In the FCPD and YDM groups 87% and 69% respectively were at 5th BMI percentile compared to 34% in control group. The distribution of subjects at different percentile is shown in the table 3.1ii.

3.4 Demographic distribution of the study subjects

Among the 337 Controls 35%, 4% and 61% individuals came from rural, semi-urban and urban areas respectively. In case of 372 YDM subjects the proportions were 57%, 35% and 8%, and in the 51 unrelated FCPD 69%, 27% and 4% respectively (Table 3.2). High proportions of diabetic subjects were from the rural and semi-urban areas compared to more of the Controls from urban area. The distribution showed a statistically significant difference (overall $p<0.0001$) (Table 3.2).

3.5 History of diabetes in families of the study subjects

Out of the 372 YDM subjects 127 (34.14%) had relatives with diabetes mellitus. However, 87 (23.4%) subjects had diabetes among the first-degree relatives. Out of the 87 cases with first-degree relatives with diabetes 33 (8.9%) were fathers, 27 (7.3%) mothers, 16 (4.3%) both parents and sibs diabetic ($p<0.05$ vs fathers and mothers on with diabetes) and 11 (3.0%) sibs (Table 3.2).

In case of 8.9% fathers and 7.3% mothers with diabetes an overlap of presence of diabetes in siblings and, other second- and third-degree relatives was observed. Among the 33 fathers with diabetes, in 22 cases (5.9% among all YDM patients) fathers-only were diabetic and among other 11 cases, 2 sibs and, 6 second- and 5 third-degree relatives were diabetic with overlap among relatives. Among the 27 mothers with

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diabetes, in 13 cases (3.5% among all YDM) only mothers were diabetic, and among 14 others, 4 sibs and, 8 second- and 6 third-degree relatives were diabetic with marked overlap among relatives.

Forty (10.8%) YDM subjects had non-first degree relatives with diabetes. Among them 24 had second- and 21 third- degree relatives with diabetes, and 5 had more than one first- or second-degree relative with diabetes (Table 3.2).

Among 51 unrelated FCPD subjects 19.6% (10 individuals had relatives with diabetes and of the total 11.8% [6 had first-degree (3 fathers, 2 mothers and 1 sister)] with diabetes, and there was no overlap among themselves. Three individuals had second- and three with third-degree relatives with diabetes, and there were some overlap among them.

3.6 Anthropometric measurements of the study subjects

Anthropometric measurements were done in case of YDM14+ and FCPD14+ subjects and compared with those of Controls. Mean (±SD) of mid-arm circumference (cm) in the Controls (23.6±3.1) was significantly higher compared to YDM14+ (22.7±4.7, $p<0.05$ vs Controls) and FCPD14+ (21.0±4.2, $p<0.05$ vs Controls). Mean (±SD) MAC was significantly low in the FCPD14+ compared to YDM14+ subjects ($p<0.05$) (Table 3.3).

Mean (±SD) biceps skin-fold thickness (BSF) was 4.9±2.7in the Controls, 4.7±4.1 ($p<0.05$ vs Controls) in the YDM14+ subjects and 4.5±3.5 (p=ns, vs Controls) in the FPCD14+ subjects. BSF values between YDM14+ and FCPD14+ did not show statistical significant difference (Table 3.3).

Mean (±SD) of subscapular-triceps skin fold thickness ratio (STR) in YDM14+ subjects was 1.7±0.7 in the Controls, 1.5±0.6 in YDM14+ and 1.5±0.5 in FCPD 14+ subjects (Table 3.3).

Mean (±SD) of waist hip ratio (WHR) was 0.82±0.1 in the Controls, 0.85±0.1 ($p<0.05$ vs Controls) in YDM14+ subjects and 0.84±0.05 (p=ns, vs Controls) in the FPCD14+ subjects. WHR value between YDM14+ and FCPD14+ subjects did not show any significant difference (Table 3.3).

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Table 3.1i:

Clinical characteristics of the study subjects

Variables	**Controls** (n=337)	**YDM** (n=372)	**FCPD** (n=51)
Gender, [Male (Female)]	218 (119)	165 (207)	19 (32)
[a]Age (yrs)	22.5±4.9	19.4±6.3*	19.9±6.0*
[a]Age of onset of DM (yrs)	--	18.8±6.1	19.1±5.7
[a]Age (yrs) of DM subjects diagnosed after 14 yrs	--	22.1±5.2	22.5±4.9
[b]Duration of diabetes (yrs)	-	2.0 (0.10-12.3)	3.6 (0.08-14.0)
[c]BMI	20.1±3.1	21.0±4.2*	18.1±3.0*ᵗ

Results are expressed as [a] mean±SD and [b] median (range).

[c]BMI calculated for those subjects had age 18 yrs and above at the time of recruitment; it includes n=290, YDM n=195 and FCPD, n=28.

Statistical difference between groups was calculated by unpaired student's 't' test.

Significantly different from *controls and ᵗYDM.

Table 3.1ii:

Distribution BMI percentile of subjects on under 18 years of age of the study subjects

BMI percentiles	**Control (n=47)**	**YDM (n=174)**	**FCPD (n=23)**
5th percentile	16 (34.0%)	120 (69.0%)*	20 (87.0%)*
10th percentile	4 (8.5%)	6 (3.4%)	-
25th percentile	14 (29.8%)	16 (9.2%)	2 (8.7%)
50th percentile	8 (17.0%)	13 (7.5%)	1 (4.3%)
60th percentile and above	3 (10.6%)	19 (10.9%)	-

*Significantly different compared to controls.

Table 3.2:
Demographic distribution and history of diabetes in families of the study subjects

Variables	Controls (n=337)	YDM (n=372)	FCPD (n=51)
Demographic distribution [n (%)]			
Rural [n (%)]	137 (35)	211 (57)	35 (69)
Semi-urban [n (%)]	16 (4)	31 (8)	2 (4)
Urban [n (%)]	240 (61)	130 (35)	14 (27)
Family history of the study subjects [n (%)]			
Family history absent	337	245 (65.9%)	41 (80.4%)
Family history present	-	127 (34.1%)	10 (19.6%)
Up to 3rd degree relatives	-	21 (5.6%)	3 (5.9%)
Up to 2nd degree relatives	-	24 (6.5%)	3 (5.9%)
Up to 1st degree relatives	-	87 (23.4%)	6 (11.8%)
Father / Mother only	-	33 (8.9%) / 27(7.3)	3 (5.9%)/ 2 (3.9%)
Both parents	-	12 (3.2%)	0
Both parents and brothers and/ or sisters	-	4 (1.1%)	-
Brother/Sister/Both	-	6/5/0 (3.0%)	0/1/0 (2.0%)

Results are expressed as [a] mean±SD and [b] median (range).
[c]BMI calculated for those subjects had age 18 yrs and above at the time of recruitment; it includes 290 controls,195 YDM and, 28 FCPD subjects.
Statistical difference between groups was calculated by unpaired student's 't' test.
Significantly different from *controls and [t]YDM.

Table 3.3:

Anthopometric measurement of the Controls, YDM14+ and FCPD14+ subjects

Variables	Controls (292)	YDM14+ (n=277)	FCPD14+ (n=39)
MAC (cm)	23.6±3.1[a]	22.7±4.7*	21.0±4.2*
BSF (mm)	4.9±2.7	5.7±4.1*	4.5±3.5
STR	1.7±0.7	1.5±0.6	1.5±0.5
WHR	0.82±0.1	0.85±0.07*	0.84±0.05

N=number of subjects. Results are expressed as mean±SD; Statistical difference calculated by unpaired student's 't' test; *Significantly different compared to controls.

YDM14-, diabetes diagnosed at ≤14 years; YDM14+, diabetes diagnosed at ≥14 years; FCPD14-, FCPD diagnosed at ≤14 yeas; FCPD14+, FCPD diagnosed at≥14 years

MAC, mid arm circumference in centimeter; BSF, biceps skin fold thickness in millimeter; WHR, waist hip ratio; STR, subscapular triceps ratio.

3.7 Fasting blood glucose level of the study subjects

Fasting blood glucose (mmol/l, mean±SD) of the Controls was 5.04±0.64. Among the Controls 167 individuals had glucose levels <5.0 mmol/l, mean (±SD) was 4.5±0.42; 157 subjects had between 5.0-6.0 mmol/l [5.40±0.26, mean±SD] and 13 subjects had >6.0 mmol/l (IFG) [6.23±0.16, mean±SD].).

Mean (±SD) of fasting blood glucose (mmol/l) of the YDM and FCPD subjects were 15.2±6.6 and 16.7±7.7 respectively (Table 3.4i).

YDM14+ subjects had significantly lower fasting glucose level (14.1±6.0) compared to YDM14- subjects (17.1±6.2) (p<0.0001). In the FCPD subjects FCPD14+ also had significantly lower serum glucose level compared to FCPD14- subjects (20.71±6.75 vs 16.74±7.68, p=0.001) (Table 3.4ii). YDM14+ subjects (14.5±6.8) and FCPD14+ subjects (15.6±7.6) did not show any significant statistical difference regarding fasting blood glucose (Table 3.4iii).

3.8 C-peptide levels of the study subjects

C-peptide level (nmol/l, median-range) in the Control subjects was 0.39 (0.16-1.44), in YDM 0.20 (0.02-1.33) and in FCPD 0.12 (0.023-0.84) (p<0.0001 vs Controls for both the groups). Between the two diabetic groups, FCPD had significantly low C-peptide levels compared to YDM subjects (p<0.0001) (Table 3.4i).

Among YDM subjects YDM14- subgroup had significantly lower C-peptide 0.149 (0.022-1.054) compared to YDM14+ counterpart 0.29 (0.041-1.115) (p<0.0001). Among FCPD subjects FCPD14- subgroup had significantly lower C-peptide [0.149 (0.022-1.105] compared to FCPD14+ counterpart 0.140 (0.03-0.840) (p<0.008) (Table 3.4ii).

YDM14+ and FCPD14+ subjects were also compared with the Controls. C-peptide level in the YDM14+ subjects 0.23 (0.02-1.33) and FCPD14+ subjects 0.14 (0.032-0.84) were significantly low compared to the Controls 0.43 (0.16-1.44) (p<0.001 for both). YDM14+ subjects had significantly higher C-peptide levels compared to FCPD14+ subjects (p<0.0001) (Table 3.4iii).

Geometric mean of C-peptide (0.3899 nmol/l) value of the healthy Controls was taken as the cut-off level. Out of 362 YDM subjects 264 (73%) had C-peptide value below and

78 (29.5%) above the cut-off level. In the YDM14- subjects 29.5% had lower compared to 12.2% had higher C-peptide (p=0.001). Out of 49 unrelated FCPD 44 (92%) had low C-peptide and of them 27% were of FCPD14- and 72.7% FCPD14+ subjects (p=0.315) (Table 3.4iv).

3.9 C-peptide/glucose ratio of the study subjects

C-peptide/glucose [nmol/mmol (median-range)] ratio indirectly expresses the functional efficiency of the B cells. C-peptide/glucose ratio value in the Control subjects was 0.25 (0.1-0.81), in YDM subjects 0.044 (0.003-0.59) and in FCPD 0.02 (0.003-0.33) (p=0.0001 vs Controls for both the group). C-peptide/glucose ratio in FCPD subjects was also significantly lower compared to YDM (p<0.0001) (Table 3.4i).

Among YDM subjects YDM14- subgroup 0.024 (0.004-0.29) had significantly lower C-peptide/glucose ratio compared to YDM14+ counterpart 0.06 (0.003-0.59) (p<0.0001) (Table 3.4ii). Among FCPD subjects FCPD14- subgroup 0.01 (0.003-0.022) had significantly lower C-peptide/glucose ratio compared to FCPD14+ counterpart 0.03 (0.01-0.33) (p<0.0001) (Table 3.4ii).

YDM14+ and FCPD14+ subjects were also compared with the Controls. C-peptide level in the YDM14+ subjects 0.06 (0.003-0.59) and in FCPD14+ subjects 0.03 (0.01-0.33) was significantly low compared to Controls 0.25 (0.10-0.81). YDM14+ subjects had significantly higher C-peptide/glucose ratio compared to FCPD14+ subjects (p<0.01) (Table 3.4iii).

Table 3.4i:

Fasting glucose, fasting C-peptide, C-pep/glucose ratio of the study subjects

Variables	**Control** (n=337)	**YDM** (n=362)	**FCPD** (n=51)
F glucose (mmol/l)	5.04±0.64[a]	15.2±6.6	16.8±7.7
F C-peptide (nmol/l)	0.39 (0.16-1.44)	0.20 (0.02-1.33)[*]	0.12 (0.023-0.84)[*†]
C-pep/glucose ratio (nmol/mmol/)	0.25 (0.10-0.81)	0.044 (0.003-0.59)[*]	0.021 (0.003-0.33)[*†]

n=Number of subjects. Results are as expressed as mean±SD and median (range) as appropriate.
Unpaired student's t-test and Mann Whitney test were performed.
Significantly different compared to *controls and †YDM.

Table 3.4ii:

Fasting glucose, fasting C-peptide, C-pep/glucose ratio of the study subjects on the basis of age of diabetes onset

Variables	YDM Subjects		FCPD Subjects	
	YDM14- (n=93)	**YDM14+** (n=279)	**FCPD14-** (n=12)	**FCPD14+** (n=39)
F glucose (mmol/l)	17.0±5.5	14.5±6.8*	20.7±6.7	15.6±7.6*
F C-peptide (nmol/l)	0.15 (0.02-1.05)	0.23 (0.02-1.33)*	0.07 (0.02-0.28)	0.14 (0.03-0.84)*
C-Pep/gl ratio (nmol/mmol)	0.024 (0.004-0.29)	0.06 (0.003-0.59)*	0.01 (0.003-0.02)	0.03 (0.01-0.33)*

N=number of subjects. Results are expressed as mean±SD and median (range) where appropriate.
Statistical difference calculated by unpaired student's 't' test and Mann-Whitney test as appropriate.
YDM14-, diabetes diagnosed ≤14 years; YDM14+, diabetes diagnosed ≥14 years;
FCPD14-, FCPD diagnosed ≤ 14 yeas; FCPD14+, FCPD diagnosed ≥14 years

Table 3.4iii:

Fasting glucose, fasting C-peptide, C-pep/glucose ratio of the controls, YDM 14+ and FCPD 14+ subjects

Variables	**Control** (n=337)	**YDM14+** (n=279)	**FCPD14+** (n=39)
F glucose (mmol/l)	5.04±0.64[a]	14.5±6.8	15.6±7.6
F C-peptide (nmol/l)	0.39 (0.16-1.44)	0.23 (0.02-1.33)*	0.14 (0.03-0.84)*[†]
C-pep/glucose ratio (nmol/mmol)	0.25 (0.10-0.81)	0.06 (0.003-0.59)*	0.03 (0.01-0.33)*[†]

n=Number of subjects. Results are as expressed as mean±SD and median (range) as appropriate.
udent's unpaired 't' and Mann Whitney tests were performed where appropriate.
*Significantly different compared to *controls and [†] YDM.

Table 3.4iv:

Distribution of YDM and FCPD study subjects on the basis cut-off value of C-peptide

Groups	C-peptide cut off level=0.3899 nmol/l	
	Below [n (%)]	**Above** [n (%)]
YDM groups *(n=363)*		
YDM14$^-$ subjects	78 (29.5%)	12 (12.2%)
YDM14$^+$ subjects	186 (70.5%)	86 (87.8%)
	Chi Square = 11.45; p=0.001	
FCPD groups *(n=49)*		
FCPD14$^-$ subjects	12 (27.3%)	-
FCPD14$^+$ subjects	32 (72.7%)	5 (100)
	Fisher exact p=0.315	

C-peptide cut of level 0.3899 nmol/l derived from geometric mean of the controls. Below, C-peptide <0.3899 nmol/l; Above, C-peptide ≥0.3899 nmol/l.

3.10 Autoantibody status of the study subjects

Anti-GAD antibody and antibody against anti-islet antigen intracellular fraction autoantibodies were determined in 280 Controls, and 314 YDM and 41 FCPD patients.

Frequency of anti-GAD and IA-2ic positivity in the Controls was 3.2% (9 out of 280) and 0.4% (1/280). Among the YDM subjects positivity for anti-GAD and IA-2ic antibodies were 22.6% (71 out of 314) (p=0.001 vs Controls) and 11.8% (37 out of 314) (p=0.001 vs Controls) respectively. Among the FCPD subjects positively for GAD and IA-2ic antibodies were 19.5% (p=0.001) and 19.5% (p=0.0001) respectively (Figure 3.1).

Among 247 newly diagnosed YDM cases 60 (24.3%) were positive for GAD compared to 14.5% in established cases (p=0.061). Newly diagnosed and established cases had frequency of IA2-ic antibody positivity about 13.8% and 3.9% respectively (p=0.022). In the YDM and FCPD subjects 8.0% and 14.6% subjects respectively were positive for both GAD and IA2-ic antibodies (Table 3.5i).

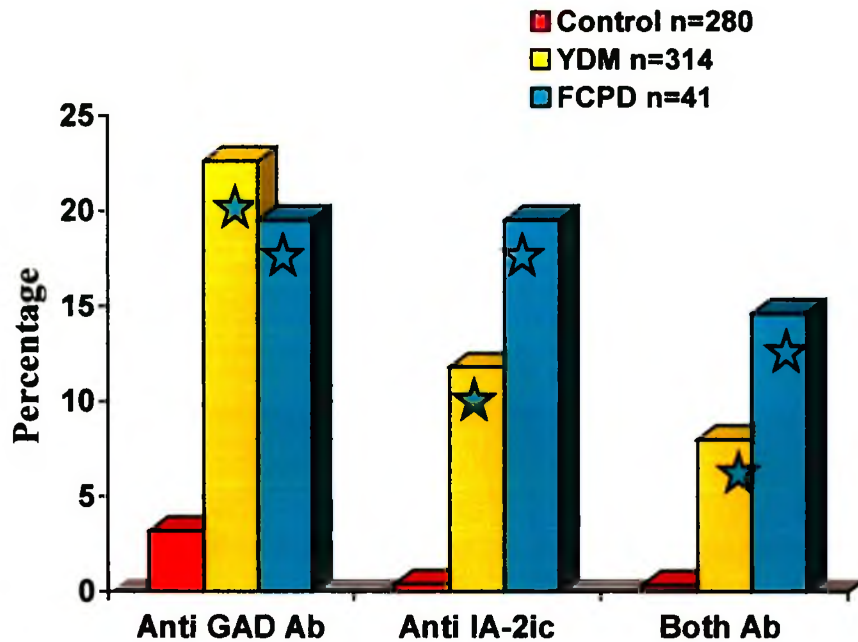

Figure 3.1: Autoantibody status (Anti-GAD and IA-2ic) in the study subjects

★ p values compared to controls 0.001-0.0001

In the FCPD group 7 out of 8 GAD positive cases and all 8 IA-2ic positive cases were of newly diagnosed. Six (14.6%) FCPD individuals were positive for both GAD and IA-2ic antibodies (Table 3.5i).

Antibody status was analyzed in relation to age of diagnosis of diabetes. Anti GAD was positive in 36.5% cases YDM14- subjects compared to 17.5% in YDM14+ subjects (p=0.001). IA-2ic Ab were positive in 20% subjects of YDM 14- subjects compared to 8.7% of YDM14+ subjects (p=0.010). Although total number of GAD Ab and IA-2ic positive cases in FCPD groups were 8 yet for both the antibodies 50% of FCPD14- subjects were positive against 9.7% of FCPD14+ subjects (p=0.013) Table 3.5i).

Among the YDM subjects 23% were positive for GAD Ab those have first-degree relatives with diabetes. In contrast 8.7% cases were positive for GAD those have no first-degree relatives with diabetes.

YDM subjects with GAD antibody positive cases were significantly of younger age at diagnosis compared to the antibody negative patients (16.2±6.3 and 18.9±5.8, yrs, respectively) (p=0.001) (Table 4.5i). Those positive cases had significantly higher blood

glucose (16.8±5.8 vs 14.8±6.7 mmol/l in negative cases, p=0.006) and lower C-peptide levels 0.14 (0.029-0.94) vs 0.23 (0.020–1.33) (p=0.001). The trend was also reflected in C-peptide/glucose ratio in positive and negative cases [0.03 (0.003-0.30) vs 0.055 (0.004-0.59), (p=0.001)]. The IA-2ic positive cases had similar trends, but the differences did not show any statistical difference (Table 3.5ii).

Between GAD Ab positive and negative cases in the FCPD Group there was no statistical difference regarding age of onset of diabetes, fasting glucose, fasting C-peptide and C-peptide glucose ratio (Table 3.5ii)

The IA-2ic positive subjects were younger at diagnosis (15.8±6.5) compared to negative counterparts (19.8±5.1, p=0.027). They had also higher fasting glucose level 19.9±3.3 compared to 15.7±8.7 (p=0.035) (Table 3.7). C-peptide and C-peptide/glucose ratios between IA-2ic antibody positive and negative cases did not differ (Table 3.5ii).

Table 3.5i:

GAD and IA-2ic autoantibody status in the new and established cases of DM, and on the basis of age of diagnosis of diabetes

Groups (n)	**GAD Ab Positive** N (%)	**IA-2ic Ab Positive** N (%)	**Both antibodies positive** N (%)
YDM subjects			
New cases (247)	60 (24.3%)	34 (13.8)	23 (9.3%)
Established cases (67)	11 (14.5%)	3 (3.9%)	2 (2.6%)
p value (Fisher exact)	*p=0.061*	*p=0.022*	
FCPD subjects			
New cases (33)	7 (21.2%)	8 (24.2%)	6 (18.8%)
Established cases (8)	1 (12.5%)	0	0
On the basis of age of diagnosis of DM			
YDM14⁻ cases (85)	31 (36.4%)	17(20%)	12 (14.0%
YDM14⁺ cases (229)	40 (17.5%)	20 (8.7)	13 (5.7%)
p values (Fisher exact)	*0.001*	*0.010*	
FCPD14⁻ cases (10)	5 (50%)	5 (50%)	4 (40%)
FCPD14⁺ cases (31)	3 (9.7%)	3 (9.7%)	2 (6.5%)
p value (Fisher exact)	*p=0.013*	*p=0.013*	

Results are expressed as number (percentage).
Fisher exact test was performed.

Table 3.5ii:

Onset of diabetes, fasting glucose, fasting C-peptide and C-pep/glucose ratio of the YDM and FCPD subjects on the basis of antibody status

Groups (n)	**Age of onset of DM** (yrs)	**F glucose** (mmol/l)	**F C-peptide** (nmol/l)	**C-pep/gl ratio** (mmol/nmol)
YDM subjects:				
On the basis of GAD Ab status				
Positive (71)	16.2±6.3	16.8±5.8	0.14 (0.029-0.94)	0.03 (0.003-0.30)
Negative (243)	18.9±5.8*	14.8±6.7	0.23 (0.02-1.33)*	0.055 (0.004-0.59)*
On the basis of IA2-icAb status				
Positive (37)	17.1±6.8	16.1±5.1	0.16 (0.04-0.95)	0.03 (0.006-0.37)
Negative (277)	18.4±5.9	15.1±6.8	0.21 (0.02-1.33)	0.05 (0.003-0.59)
FCPD Subjects:				
On the basis of GAD Ab status				
Positive (8)	17.5±3.8	19.4±3.8	0.10 (0.023-0.23)	0.015 (0.003-0.033)
Negative (279)	19.4±5.1	15.8±8.7	0.13 (0.024-0.84)	0.026 (0.006-0.33)
On the basis of IA2-ic Ab status				
Positive (8)	15.8±6.5	19.9±3.3	0.10 (0.023-0.24)	0.015 (0.003-0.037)
Negative (33)	19.8±5.1*	15.7±8.7*	0.13 (0.024-0.84)	0.024 (0.01-0.33)

N=number of subjects. Results are expressed as mean±SD and median (range) where appropriate.

Unpaired Student's 't' test and Mann-Whitney tests were performed where applicable.

*Statistically significant different compared to the counterparts

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3.11 Candidate gene markers analyses of the study subjects

3.11.1 INS VNTR –23bp A/T variant assay

The INS gene -23bp A/T variant had genotype (AA, AT and TT; homozygous wild, heterozygous and homozygous variant respectively) frequencies in the Controls were 0.753, 0.253 and 0.015; in YDM subjects 0.713, 0.258 and 0.029; and in the unrelated FCPD subjects 0.686, 0.216 and 0.098 respectively. Genotype frequency between Controls and YDM did not show any statistical difference p=0.374). The FCPD subjects had about 9.8% of AA genotype. Variant genotype frequency in FCPD did not show significant association compared to Controls (p=0.197; Fisher exact test). However, variant allele frequencies in unrelated FCPD subjects showed significant differences (p=0.038). Genotype and allele frequencies between YDM and FCPD did not show significant difference (p=0.129) (Table 3.6i). The power for INS gene VNTR polymorphism study is only 0.270 that suggest the need for inclusion of much larger number of subjects in the study to elucidate the role of the gene a risk for diabetes.

Hardy-Weinberg equilibrium for the INS VNTR genotype was tested. In the control (p=0.93) and YDM (p=0.523) groups there was no significant association. In the FCPD group the equilibrium showed significant (p=0.015) association. However, when YDM and FCPD subjects were tested together no significant association was observed (p=0.105).

FCPD probands had the frequencies 0.686, 0.30 and 0.014 for AA, AT and TT genotype respectively. At the genotype level there was no significant difference compared to the controls but comparing allelic frequency the 'A' was increased (p=0.038). When unrelated FCPD and probands FCPD patients were pulled genotype frequencies did not show significant difference compared to controls (p=0.115) but variant allele frequency was significantly difference than those of Controls (p=0.030) (Table 3.6i).

The YDM and FCPD subjects were also analyzed to compare the INS VNTR genotype by age of onset of diabetes. Among the YDM group YDM14+ subjects had relatively more frequency of AT and TT allele compared to YDM14- subjects, but the difference was not statistically significant (p=0.074). Among the unrelated FCPD group FCPD14- and FCPD14+ subjects had almost similar genotype frequencies (Table 3.6iv).

Study subjects were compared for clinical and biochemical parameters on the basis of wild versus variant (heterozygous and homozygous together) genotype. In the control group wild and variant types did not show significant difference regarding age, fasting glucose, C-peptide and C-peptide/glucose ratio (Table 3.7ii). YDM subjects with variant 'A' allele had significantly higher C-peptide level 0.23 (0.02-1.32) and C-pep/glucose ratio 0.056 (0.005-0.44) compared to those with wild 'A' allele 0.19 (0.03-1.13) (p=0.04) and 0.038 (0.003-0.59) (p=0.03). Among FCPD subjects with variant 'T' and wild 'A' allele did not show statistically significant difference regarding age, onset of diabetes, fasting glucose, fasting C-peptide and C-peptide glucose ratio (Table 3.7ii).

However, when YDM subjects analyzed by GAD Ab status for the possession of the INS VNTR 'T' allele 31.3% were GAD negative compared to 17% GAD positivity (p=0.018). But no association was observed between INS gene variant genotype and iA2-ic antibody (Table 3.6iii).

YDM and FCPD subjects with wild 'A' and variant 'T' genotype did not show significant statistical difference regarding age of diagnosis diabetes at 14- and 14+ years (Table 3.6iv).

Transmission of the INS gene VNTR A/T allele at –23bp upstream of the open reading frame was analyzed using extended transmission disequilibrium test (ETDT) in the FCPD probands. It was observed that from heterozygous parents variant allele was transmitted 20 times and non-transmitted 14 times to the probands. The ETDT analysis did not reveal any statistical significant difference for the transmission of candidate allele to the probands (p=0.3035), however, the power of such an analysis is low (Figure 3.2).

Table 3.6i:

INS VNTR genotype frequencies of the study subjects

INS gene −23bp A/T genotype	Controls (n=332)	YDM (n=372)	Unrelated FCPD (n=51)	Total FCPD[ŧ] subjects (n=121)
Wild AA	0.753 (250)	0.712 (265)	0.686 (35)	0.678 (82)
Variant AT	0.229 (76)	0.258 (96)	0.216 (11)	0.272 (33)
Variant TT	0.018 (6)	0.030 (11)	0.098 (5)*	0.050 (6)
p values*	-	-	0.197	0.115
Allele frequency	0.867/0.133	0.841/0.159	0.794/0.204	0.814/0.186
p values*	-	-	0.038	0.030

* Fisher xxact test was performed.

[ŧ]Total FCPD (n=121) stands for pooled unrelated FCPD (n=51) and FCPD probands (n=70).

Table 3.6ii:

Onset of age of DM, FSG, serum C-peptide and C-peptide/glucose ratio on the basis of INS VNTR wild and variant genotype in the controls and diabetic subjects

Genotype (n)	Age of onset of DM (yrs)	F glucose (mmol/l)	F C-peptide (nmol/l)	C-peptide/glucose (nmol/mmol)
Controls				
Wild type (250)	-	5.01±0.60	0.38 (0.16-1.44)	0.23 (0.10-0.81)
Variant type (82)	-	4.06±0.62	0.44 (0.17-1.36)	0.26 (0.11-0.76)
YDM Subjects				
Wild (265)	19.2±6.3	15.5±6.6	0.19 (0.03-1.13)	0.038 (0.003-0.59)
Variant (107)	19.8±6.1	14.4±6.5	0.23 (0.02-1.32)*	0.056 (0.005-0.44)*
FCPD subjects				
Wild type (36)	20.6±6.2	16.8±7.49	0.12 (0.02-0.84)	0.02 (0.003-0.28)
Variant type (15)	18.3±5.5	16.9±8.5	0.14 (0.06-0.59)	0.02 (0.01-0.34)

*Statistically significant at $p<0.05$ compared to the counterparts. Mann Whitney test was performed.

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Table 3.6iii:

Autoantibody status on the basis of INS VNTR wild and variant genotype of the YDM and FCPD subject

Groups and Genotype (n)	Anti GAD Ab		Anti-IA2-ic Ab	
	Positive (n=71)	**Negative** (n=243)	**Positive** (n=37)	**Negative** (n=277)
YDM subjects				
INS VNTR Wild (226)	59 (83%)	167 (69%)	30 (81%)	196 (71%)
INS VNTR Variant (88)	12 (17%)	76 (31%)	7 (19%)	81 (29%)
p values	χ^2 = 5.628, p=0.018		p=0.243	
FCPD subjects				
INS VNTR Wild (27)	5 (62.5%)	22 (67%)	4 (50%)	23 (70%)
INS VNTR Variant (14)	3 (27.5%)	11 (33%)	4 (50%)	10 (30%)
	p=1.00		p=0.411	

Fisher exact test performed

Table 3.6Iv:

Genotype frequencies of INS VNTR in YDM and FCPD subjects on the basis of age of onset of diabetes

GAD Ab status	INS VNTR genotype		
	AA	***AT***	***TT***
YDM14- subjects	73 (27.5%)	19 (20%)	1 (9%)
YDM14+ subjects	192 (72.5%)	77 (80%)	10 (91%)
	p=0.086		
FCPD14- subjects	8 (23%)	2 (18%)	2 (40%)
FCPD14+ subjects	27 (77%)	9 (82%)	3 (60%)
	p=1.0		

Fisher exact test done

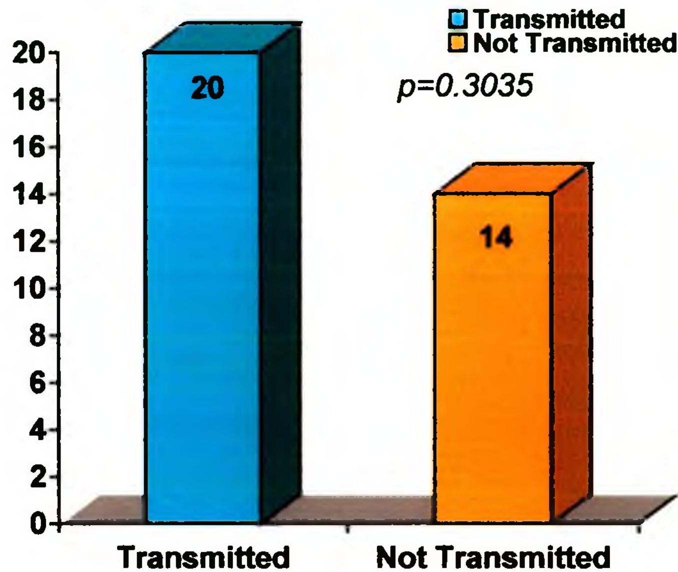

Figure 3.2: ETDT analysis of INS VNTR –23bp A/T variant in the FCPD simplex families

3.11.2 *EIF2AK3* gene Inel15 AT$^+$/AT variant assay

EIF2AK3 gene Indel15 (AT$^+$/-) had genotype (AT/AT, AT$^+$/AT$^-$ and AT$^-$/ AT$^-$; HZ Wild; Ht variant, and HZ variant respectively) frequencies of the study subjects were 0.95, 0.05 and 0 in the Controls, 0.94, 0.06 and 0 in the YDM, and 0.94, 0.05 and 0.01 in the FCPD subjects (Table 3.7i).

Hardy-Weinberg equilibrium for the *EIF2AK3* gene Indel15 genotype was tested. In the control (p=0.675) and YDM (p=0.538) groups there was no significant association. In the FCPD group the equilibrium showed significant (p=0.008) deviation. However, when YDM and FCPD subjects were tested together no significant association was observed (p=0.416).

Allele frequencies in YDM and FCPD subjects did not significant difference compared to controls (p=0.404 and 0.297 respectively). Allele frequency between YDM and FPCD subjects also did not show significant difference (p=0.553) (Table 3.7i).

EIF2AK3 gene Indel15 wild 'AT⁻' allele did not show statistically significant difference compared to variant 'AT⁺' allele for clinical and biochemical parameters in the Controls, YDM and FCPD group (Table 3.7ii).

In YDM subjects 22.6% with wild 'AT⁺' allele were anti-GAD positive against 23.5% with variant 'AT⁻' allele (p=1.0) and regarding positivity anti IA-2ic antibody the proportion was almost similar 11.8% for subjects with wild 'AT⁺' and variant 'AT⁻' allele (p=1.0). In FCPD subjects 17% (p=0.195) subjects with wild genotype were anti GAD and IA-2ic positive and only 1 individual with variant was positive for both antibodies (Table iii).

The frequencies for the wild 'AT⁺' and variant 'AT⁻' allele did not show statistically significant difference between the YDM14- and YDM14+, and FCDP14- and FCPD14+ subjects (Table3.7iv).

Table 3.7i:

Genotype frequencies of the EIF2AK3 gene Indel15 variant of the study subjects

Genotype	**Controls** (n=334)	**YDM** (n=371)	**Unrelated FCPD** (n=51)
Wild AT^+/AT^+	0.955 (319)	0.938 (348)	0.922 (47)
Variant AT^+/AT^-	0.045 (15)	0.062 (23)	0.059 (3)
Variant AT^-/AT^-	-	-	0.019 (1)
		p=0.404*	p=0.297*; p=0.553‡
Allele frequency	0.978/0.022	0.969/0.031	0.951/0.049

*vs controls and ‡vs YDM

Table 3.7ii:

Onset of age of DM, FSG, serum C-peptide and C-peptide/glucose ratio on the basis of EIF2AK3 Indel15 wild and variant genotype in controls and diabetic subjects

Genotype (n)	Age of onset of DM (yrs)	F glucose (mmol/l)	F C-peptide (nmol/l)	C-peptide/glucose (nmol/mmol)
Controls				
Wild type (319)	-	5.0±0.6	0.40 (0.16-1.44)	0.24 (0.10-0.81)
Variant type (15)	-	4.9±0.6	0.39 (0.18-0.70)	0.25 (0.11-0.38)
YDM subjects				
Wild (338)	18.7±6.1	15.2±6.5	0.20 (0.20-1.33)	0.05 (0.003-0.59
Variant (23)	19.0±6.3	15.5±7.6	0.17 (0.03-1.09)	0.04 (0.006-0.22)
FCPD subjects				
Wild (45)	19.2±5.8	16.6±7.4	0.12 (0.23-0.84)	0.02 (0.003-0.326)
Variant (3)	18.0±5.3	24.0±2.2	0.09 (0.09-0.23)	0.012 (0.010-0.028)

Unpaired student's 't' and Mann Whitney tests were performed where appropriate.

Table 3.7iii:

Autoantibody on the basis of EIF2AK3 gene Indel15 AT^{+}/AT^{-} allele in YDM and FCPD subjects

Group and Genotype (n)	Anti GAD Ab		Anti-IA2-ic Ab	
	Positive (n=71)	**Negative** (n=243)	**Positive** (n=37)	**Negative** (n=277)
YDM subjects				
Wild type	67 (94%)	229 (95%)	35 (95%)	261 (95%)
Variant type	4 (6%)	13 (6%)	2 (5%)	15 (5%)
Fisher exact p values	p=1.0		p=1.0	
FCPD subjects				
Wild type	7 (88%)	33 (100%)	7 (88%)	33 (100%)
Variant type	1 (12%)		1 (12%)	-
Fisher exact p values	p=0.195		p=0.195	

Table 3.7iv:

Genotype frequencies of EIF2AK3 gene Indel15 AT^{+}/AT^{-} allele of the YDM and FCPD subjects on the basis of age of onset of diabetes

GAD Ab status	INS genotype		
	AT++	***AT+/-***	***AT-/-***
YDM14- subjects	88 (25%)	5 (22%)	
YDM14+ subjects	260 (75%)	18 (78%)	
	p=0.086		
FCPD14- subjects	11 (23%)	1 (33%)	
FCPD14+ subjects	36 (77%)	2 (67%)	1 (100%)
	p=1.0		

Fisher exact test was performed.

3.11.3 *TCF1* gene C/T (A98V) variant assay

TCF1 gene C/T variant, resulting in A98V, had genotype (CC, CT and TT; Hz wild, Ht variant and Hz variant respectively) frequencies of 0.855, 0.142 and 0.003 in the Controls. In YDM subjects the frequencies were 0.821, 0.168 and 0.011 and in the unrelated FCPD subjects were 0.843, 0.137 and 0.020 respectively (Table 3.81).

Hardy-Weinberg equilibrium for the *TCF1* C/T variant genotype was tested. In the control (p=0.498), YDM (p=0.676) and FCPD (p=0.294) groups there was no significant association. However, when YDM and FCPD subjects were tested together also no significant association was observed (p=0.458).

Genotype frequency of the YDM and FCPD subjects did not show significant statistical difference compared to controls (p=0.262 vs YDM; p=0.302 vs FCPD) and between them (p=0.845) (Table 3.8i).

In both YDM and FCPD group subjects with *TCF1* gene wild 'C' allele did not show statistical difference regarding clinical and biochemical parameters compared to variant 'T' allele (Table 3.8ii). YDM subjects with wild 'C' and variant 'T' allele did not show statistical difference regarding anti GAD status (Table 3.8iii). In FCPD group also allele

variant did not show statistical difference regarding ant-GAD status. But 97% of FCPD cases with wild 'C' allele were IA-2ic Ab negative against 50% positive cases. Wild and variant allele frequencies in the YDM14- and YDM14+ subjects of YDM group, and FCPD14- and FCPD14+ subjects of FCPD group also did not show statistical difference between (Table 3.8iv).

Table 3.8i:

Genotype frequencies of *TCF1* gene C/T (A98V) genotype frequencies of the study subjects

Gene(s)	Controls	YDM	Unrelated FCPD
Genotype	337	370	51
Wild CC	0.855 (288)	0.821 (304)	0.843 (43)
Ht variant CT	0.142 (48)	0.168 (62)	0.137 (7)
Hz variant TT	0.003 (1)	0.011 (4)	0.020 (1)
		p=0.262*	p=0.302* p=0.845‡
Allele frequency	0.926/0.074	0.905/0.095	0.913/0.027

Fisher exact test was performed. Statistics compared to *controls and ‡YDM

Table 3.8ii:

Onset of age of DM, FSG, serum C-peptide and C-peptide/glucose ratio on the basis of *TCF1* gene C/T wild and variant genotype in controls and diabetic subjects

Genotype (n)	**Age of onset of DM** (yrs)	**F glucose** (mmol/l)	**F C-peptide** (nmol/l)	**C-peptide/glucose** (nmol/mmol)
Controls				
Wild type (288)	-	5.0±0.6	0.39 (0.17-1.44)	0.24 (0.10-0.81)
Variant type (49)	-	5.0±0.6	0.44 (0.16-1.36)	0.25 (0.11-0.76)
YDM Subjects				
Wild (304)	18.5±6.1	15.3±6.4	0.21 (0.022-1.33)	0.045 (0.003-0.588)
Variant (66)	19.8±6.2	14.3±7.1	0.181 (0.031-1.32)	0.051 (0.004-0.367)
FCPD subjects				
Wild (43)	18.9±5.4	16.4±8.1	0.107 (0.023-0.84)	0.021 (0.003-0.326)
Variant (8)	20.1±7.2	18.8±4.4	0.126 (0.06-0.240)	0.023 (0.010-0.037)

Unpaired student's 't' and Mann Whitney tests were performed where appropriate.

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Table 3.8iii:

Anti GAD and anti IA-2ic antibody status in relation to *TCF1* gene C/T genotype in YDM and FCPD subjects

Group and Genotype (n)	Anti GAD Ab		Anti-IA2-ic Ab	
	Positive (n=71)	Negative (n=243)	Positive (n=37)	Negative (n=277)
YDM subjects				
***TCF1* wild** (242)	56 (89%)	206 (85%)	29 (85%)	233 (70.6%)
***TCF1* variant** (50)	14 (20%)	36 (15%)	8 (15%)	42 (29.4%)
Fisher exact p values	p=0.355		p=0.345	
FCPD subjects				
***TCF1* Wild** (36)	6 (75%)	30 (91%)	4 (50%)	32 (9769.7%)
***TCF1* Variant** (5)	2 (25%)	3 (9%)	4 (50%)	1 (3%)
Fisher exact p values	p=0.246		p=0.003	

Fisher exact test was performed.

Table 3.8iv:

Genotype frequencies of *TCF1* gene C/T variant of the YDM and FCPD subjects on the basis of age of onset of diabetes

GAD Ab status	***TCF1* gene variant allele genotype**		
	CC	***CT***	***TT***
YDM14- subjects	77 (25%)	13 (21%)	3 (75%)
YDM14+ subjects	227 (75%)	49 (79%)	1 (25%)
	p=0.086		
FCPD14- subjects	19 (23%)	2 (29%)	
FCPD14+ subjects	33 (77%)	5 (71%)	1 (100%)
	p=1.0		

Fisher exact test was performed.

3.11.4 *NEUROD1* gene G/A (A45T) variant assay

The *NEUROD1* gene G/A variant, resulting in A45T, had allele (GG, GA and AA; HZ wild, Ht variant and HZ variant respectively) frequencies of 0.764, 0.209 and 0.027 in the Controls. In YDM subjects the frequencies were 0.764, 0.223 and 0.014 respectively. In the FCPD subjects the frequencies for wild type and heterozygous variant were 0.900 and 0.10 respectively. None was homozygous for the variant.

Genotype frequency of the YDM and FCPD subjects did not show significant statistical difference compared to controls (p=1.0 vs YDM; p=0.080 vs FCPD). Although no differences have been found between controls and YDM the frequency of the variant 'A' allele was decreased in FCPD, 0.100 compared to 0.23, p=0.028) (Table 3.9i).

Hardy-Weinberg equilibrium for the NeuroD/BETA2 gene G/A (A45S) variant genotype was tested. In the control (p=0.116), YDM (p=0.742) and FCPD (p=0.710) no significant association for equilibrium was observed. When YDM and FCPD subjects were tested together no significant association was also observed (p=0.786).

Subjects with *NEUROD1* gene wild 'G' and variant 'A' did not show statistically significant difference for clinical and biochemical parameters in the Controls, YDM and FCPD groups (Table 3.9ii). In the two diabetic groups the wild 'G' and variant 'A' genotype also did not show significant difference with antibody status (Table 3.9iii). There was also no significant difference of wild 'G' and 'A' genotype frequency between the YDM14- and YDM14+ subjects of the YDM group and FCPD14- and FCPD14+ subjects of the FCPD subjects (Table 3.9iv).

Table 3.9i:

Genotype frequencies of *NEUROD1* gene G/A (A45T) variant of the study subjects

Gene(s)	Controls	YDM	Unrelated FCPD
Genotype	330	364	50
Wild GG type	0.764 (252)	0.764 (278)	0.900 (45)
Ht variant GA type	0.209 (69)	0.223 (81)	0.100 (5)
Hz variant AA type	0.027 (9)	0.014 (5)	-
		p=1.0*	p=0.08*; p=0.028‡
Allele frequency	0.869/0.131	0.875/0.125	0.950/0.050

Fisher exact test was done. Statistics compared to *controls and ‡YDM.

Table 3.9ii:

Onset of age of DM, FSG, serum C-peptide and C-peptide/glucose ratio on the basis of *NEUROD1* gene G/A wild and variant genotype in Controls and diabetic subjects

Genotype (n)	Age of onset of DM (yrs)	F glucose (mmol/l)	F C-peptide (nmol/l)	C-peptide/glucose (nmol/mmol)
Controls				
Wild (252)	-	5.0±0.6	0.37 (0.16-1.36)	0.23 (0.11-0.81)
Variant (78)	-	5.1±0.6	0.46 (0.20-1.44)*	0.26 (0.10-0.59)
YDM subjects				
Wild (278)	18.5±6.4	15.5±6.8	0.193 (0.022-1.33)	0.043 (0.003-0.588)
Variant (86)	19.0±6.0	14.9±6.0	0.228 (0.04-1.17)	0.05 (0.006-0.445)
FCPD subjects				
Wild (45)	19.0±5.8	17.3±6.8	0.123 (0.023-0.84)	0.022 (0.003-0.326)
Variant (5)	19.5±4.4	13.5±12.2	0.15 (0.063-0.315)	0.033 (0.012-0.127)

Unpaired student's 't' and Mann Whitney tests were performed where appropriate.
*Significantly different compared to the counter parts

Table 3.9iii:

Autoantibody status on the basis of *NEUROD1* gene G/A (A45T) genotype of the YDM and FCPD subject

Group and Genotype (n)	Anti GAD Ab		Anti-IA2-ic Ab	
	Positive (n=71)	**Negative** (n=243)	**Positive** (n=37)	**Negative** (n=277)
YDM subjects				
***NEUROD1* Wild** (226)	54 (78%)	182 (76%)	27 (73%)	209 (77%)
***NEUROD1* Variant** (88)	15 (22%)	58 (24%)	10 (27%)	63 (23%)
	p=0.749		*p=0.680*	
FCPD subjects				
***NEUROD1* Wild** (27)	8 (100%)	29 (91%)	8 (100%)	29 (91%)
***NEUROD1* Variant** (14)	-	3 (9%)	-	0 (9%)
	p=1.0		*p=1.0*	

Fisher exact test was performed.

Table 3.9iv:

Genotype frequencies of *NEUROD1* gene G/A (A45T) variant of the YDM and FCPD subjects on the basis of age of onset of diabetes

GAD Ab status	INS genotype		
	GG	***GA***	***AA***
YDM14- subjects	71 (25%)	20 (25%)	2 (40%)
YDM14+ subjects	207 (75%)	61 (75%)	3 (60%)
	p=1.0		
FCPD14- subjects	11 (24%)	-	-
FCPD14+ subjects	34 (76%)	5 (100%)	-
	p=0.573		

Fisher exact test was performed.

3.11.5 *NEUROG3* gene variant assay

3.11.5.1 *NEUROG3* gene G/A (G167R) variant assay

The *NEUROG3* gene G/A variant, resulting in G167R, had genotype (GG, GA and AA; HZ wild, Ht variant and HZ variant respectively) frequencies of 0.94, 0.06 and 0% in the Controls. In YDM subjects the frequencies were 0.906, 0.086 and 0.008 and among the FCPD subjects these were 0.94, 0.06 and 0 respectively. Genotype frequency of the YDM and FCPD subjects did not show significant statistical difference compared to controls (p=0.093 vs YDM; p=1.0 vs FCPD) and between them (p=0.601) (Table 3.10i).

Hardy-Weinberg equilibrium for the *NEUROG3* gene G/A (G167R) variant genotype was tested. In the control (p=0.116) and FCPD (p=0.827) group no significant association was observed. The equilibrium showed significant deviation in the YDM (p=0.030) group. When YDM and FCPD subjects were tested together significant deviation was also was observed (p=0.034).

In all the three group subjects with wild 'G' and variant 'A' allele did not show significant statistical difference regarding age of onset of diabetes, fasting glucose, C-peptide and C-peptide/glucose ratio compared to their counterparts (Table 3.10ii). In YDM and

FCPD subjects those with wild 'G' and variant 'A' allele did not show statistical difference regarding autoantibody status (Table 3.10iii). Genotype frequency also did not show statistical differences between the YDM14- and YDM14+ subjects of YDM group, and FCPD14- and FCPD14+ subjects of FCPD group (Table 3.10iv).

Table 3.10i:

Genotype frequencies of ***NEUROG3*** gene G/A (G167R) variant of the study subjects

Genotype	**Controls** (n=335)	**YDM** (n=372)	**Unrelated FCPD** (n=51)
Wild GG	0.940 (315)	0.906 (337)	0.941 (48)
Ht variant GA	0.060 (20)	0.086 (32)	0.059 (3)
Hz variant AA	-	0.008 (3)	-
		p=0.093*	p=1.0*; p=0.601‡
Allele frequency	0.970/0.030	0.949/0.051	0.971/0.029

Fisher exact test was performed. Statistics compared to *controls and ‡YDM.

Table 3.10ii:

Onset of age of DM, FSG, serum C-peptide and C-peptide/glucose ratio on the basis of *NEUROG3* gene G/A allele wild and variant genotype in Controls and diabetic subjects

Genotype (n)	**Age of onset of DM** (yrs)	**F glucose** (mmol/l)	**F C-peptide** (nmol/l)	**C-peptide/glucose** (nmol/mmol)
Controls				
Wild (315)	-	5.0±0.6	0.39 (0.16-1.44)	0.24 (0.10-0.81)
Variant (20)	-	4.9±0.5	0.42 (0.22-0.90)	0.25 (0.12-0.59)
YDM subjects				
Wild (337)	18.7±6.1	15.2±6.5	0.193 (0.022-1.33)	0.046 (0.003-0.588)
Variant (35)	19.7±6.3	15.5±7.6	0.21 (0.029-0.729)	0.053 (0.006-0.357)
FCPD subjects				
Wild (46)	19.0±5.9	16.5±7.0	0.11 (0.023-0.84)	0.02 (0.003-0.326)
Variant (3)	20.1±0.19	22.5±15.8	0.13 (0.09-0.225)	0.028 (0.010-0.047)

Unpaired student's 't' and Mann Whitney tests were performed where appropriate.

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Table 3.10iii:

Autoantibody status on the basis of *NEUROG3* gene G/A (G167R) genotype in YDM and FCPD subjects

Group and Genotype (n)	Anti GAD Ab		Anti-IA2-ic Ab	
	Positive [n=71]	**Negative** [n=243]	**Positive** [n=37]	**Negative** [n=277]
YDM subjects				
Wild type	68 (96%)	216 (89%)	36 (97%)	248 (90%)
Variant	3 (4%)	27 (11%)	1 (3%)	29 (10%)
	p=0.107		P=0.104	
FCPD subjects				
Wild type	7 (88%)	31 (94%)	7 (88%)	31 (94%)
Variant	1 (12%)	2 (6%)	1 (312%)	2 (6%)
	p=0.488		p=0.488	

Fisher exact test was performed.

Table 3.10iv:

Genotype frequencies of *NEUROG3* gene G/A (G167R) genotype variant of the YDM and FCPD subjects on the basis of age of onset of diabetes

GAD Ab status	NEUROG3 gene G/A genotype		
	GG	***GA***	***AA***
YDM14- subjects	85 (25%)	7 (22%)	1 (33%)
YDM14+ subjects	252 (75%)	25 (78%)	2 (67%)
	p=0.84		
FCPD14- subjects	12 (25%)	-	-
FCPD14+ subjects	36 (75%)	3 (100%)	-
	p=1.0		

Fisher exact test was performed.

3.11.5.2 *NEUROG3* gene C/T (S199F) variant assay

The *NEUROG3* gene C/T variant, resulting in S199F, had genotype (CC, CT and TT; HZ wild, Ht variant and HZ variant respectively) frequencies of 0.179, 0.448 and 0.343 in the Controls. In YDM the frequencies were 0.167, 0.470 and 0.363 and in the FCPD subjects these were 0.255, 0.451 and 0.294. Genotype frequency of the YDM and FCPD subjects did not show significant statistical difference compared to controls (p=0.691 vs YDM; p=0.421 vs FCPD) and between them (p=0.122) (Table 3.11i).

Hardy-Weinberg equilibrium for the *NEUROG3* gene C/T (S199F) variant genotype was tested. There was no significant deviation for the equilibrium in the control (p=0.737), YDM (p=0.679) and FCPD (p=0.490). When YDM and FCPD subjects were tested together also no significant deviation was observed (p=0.492).

In all the three groups subjects with wild 'C' and variant 'T' allele did not show significant statistical difference regarding age of onset of diabetes, fasting glucose, C-peptide and C-peptide/glucose ratio (Table 3.11ii). In YDM and FCPD subjects those with wild 'C' and variant 'T' allele did not show statistical difference regarding autoantibody status (Table 3.11iii). Genotype frequency also did not show statistical differences between the YDM14- and YDM14+ subjects of YDM group, and FCPD14- and FCPD14+ subjects of FCPD group (Table 3.11iv).

Table 3.11i:

Genotype frequencies of *NEUROG3* gene C/T (S199F) variant of the study subjects

Gene(s)	**Controls**	**YDM**	**Unrelated FCPD**
Genotype	335	372	51
Wild CC	0.179 (60)	0.167 (62)	0.255 (13)
Ht variant CT	0.448 (160)	0.470 (175)	0.451 (23)
Hz variant TT	0.343 (115)	0.363 (135)	0.294 (15)
		p=0.691	p=0.421; p=0.122‡
Allele frequency	0.403/0.567	0.402/0.598	0.481/0.519

Fisher exact test was performed. Statistics compared to *controls and ‡YDM.

Table 3.11ii:

Onset of age of DM, FSG, serum C-peptide and C-peptide/glucose ratio on the basis of *NEUROG3* gene C/T allele wild and variant genotype in Controls and diabetic subjects

Genotype (n)	Age of onset of DM (yrs)	F glucose (mmol/l)	F C-peptide (nmol/l)	C-peptide/glucose (nmol/mmol)
NEUROG3 gene C/T (S199F) variant (wild, CC, Variant, CT and TT) genotype				
Controls				
Wild type (60)	-	4.9±0.6	0.42 (0.19-1.44)	0.25 (0.10-0.81)
Variant (275)	-	5.0±0.6	0.39 (0.16-1.36)	0.24 (0.11-0.76)
YDM subjects				
Wild (62)	19.0±6.6	14.9±6.6	0.190 (0.04-0.99)	0.053 (0.005-0.533)
Variant (310)	18.7±6.0	15.2±6.5	0.203 (0.022-1.33)	0.046 (0.003-0.585)
FCPD subjects				
Wild (13)	21.7±6.4	16.6±8.5	0.131 (0.032-0.84)	0.026 (0.005-0.282)
Variant (38)	18.2±5.2	16.9±7.5	0.097 (0.023-0.59)	0.018 (0.003-0.326)

Unpaired student's 't' and Mann Whitney tests were performed where appropriate.

Table 3.11iiI:

Antibody status on the basis of *NEUROG3* gene C/T (S199F) variant genotype of the YDM and FCPD subject

Group and Genotype (n)	Anti GAD Ab		Anti-IA2-ic Ab	
	Positive (n=71)	**Negative** (n=243)	**Positive** (n=37)	**Negative** (n=277)
NEUROG3 gene C/T (S199F) variant				
YDM subjects				
Wild (51)	7 (10%)	44 (18%)	5 (14%)	46 (17%)
Variant (263)	64 (90)	199 (82%)	32 (85%)	231 (83%)
	p=0.104		p=0.831	
FCPD subjects				
Wild (51)	1 (13%)	9 (27%)	2 (25%)	8 (24%)
Variant (263)	7 (87%)	24 (73%)	6 (75%)	25 (76%)
	p=0.653		p=1.0	

Fisher's exact test was performed.

Table 3.11iv:

Genotype frequencies of the basis of *NEUROG3* gene C/T (S199F) variant of the YDM and FCPD subjects on the basis of age of onset of diabetes

GAD Ab status	NEUROG3 gene C/T genotype		
	CC	*CT*	*TT*
YDM14- subjects	8 (29%)	43 (25%)	32 (24%)
YDM14+ subjects	44 (71%)	132 (75%)	103 (76%)
	p=1.00		
FCPD14- subjects	2 (15%)	7 (30%)	3 (20%)
FCPD14+ subjects	11 (85%)	16 (70%)	12 (89%)
	p=0.573		

Fisher exact test was performed.

3.11.6 *SPINK1* gene A/G (N34S) variant assay

Allele frequency in the Bangladeshi Subjects

SPINK1 gene A/G variant, leading to N34S, had genotype (AA, AG and GG; HZ wild, Ht variant and HZ variant respectively) frequencies of 0.943, 0.054 and 0.003 respectively in the Controls. In YDM subjects the frequencies were 0.879, 0.110 and 0.011 and in FCPD subjects these were 0.607, 0.275 and 0.118 respectively.

Genotype frequency of the YDM and FCPD subjects did not show significant statistical difference compared to controls (p=0.004 vs YDM; p=0.001 vs FCPD). Between YDM and FCPD group the genotype frequencies showed statistical significant differences (p<0.0001). Genotype frequencies in the FCPD probands were 0.739, 0.217 and 0.043 respectively (p<0.0001 vs Controls) (Table 3.12i).

Hardy-Weinberg equilibrium for the *SPINK1* gene A/G (N34S) variant genotype was tested. There was no significant deviation in the equilibrium of the controls (p=0.188). In the YDM (p=0.044) and FCPD (p=0.048) significant deviation in the equilibrium was observed. When YDM and FCPD subjects were tested together highly significant deviation was also was observed (p= <0.0001).

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ETDT analysis if the SPINK1 gene variant in the family

Transmission of the SPINK1 A/G variant (N34S) allele was analyzed using extended transmission disequilibrium test (ETDT) in the FCPD probands. It has been observed that from 22 heterozygous parents 20 alleles were transmitted against 2 non-transmissions to the probands offspring ($p<0.001$) (Figure 3.3). In this experiment there was 100% power to detecting p value of 0.05.

Effect of SPINK1 gene variant on onset of diabetes, fasting glucose level and B cell secretory capacity in the young Bangladeshi study subjects

Subjects with SPINK1 gene A/G (N34S) variant and wild type allele in the Controls did not show any statistical difference regarding their age, fasting glucose, fasting C-peptide and C-pep/glucose ratio (Table 3.12ii).

YDM subjects with SPINK1 gene wild 'A' allele was significantly younger (17.1±4.5 yrs) compared to those with variant 'G' variant allele (19.0±6.3, p=0.02). Subjects with variant 'G' allele had significantly higher blood glucose (17.6±6.8 vs 14.8±6.5 (p=0.008), lower C-peptide [0.124 (0.022-0.83) vs 0.212 (0.025-1.33) (p=0.0001)] and low C-pep/glucose ratio 0.05 (0-0.59) and 0.02 (0.01-0.28) (p=0.001) compared with those with wild genotype (Table 3.12ii).

FCPD subjects with wild 'A' allele did not show significant difference compared to those with variant 'G' allele regarding onset of diabetes (20.3±6.6 vs 18.2±5.0, p=0.233) and fasting glucose level (15.3±9.2 vs 17.8±6.4, p=0.245). Subjects with variant 'G' allele had higher C-peptide [0.184 (0.024-0.84) and 0.0.090 (0.023-0.45) (p=0.016)] and C-pep/glucose ratio [0.03 (0.01-0.33) and 0.016 (0.003-0.094) (p=0.006)] compared to those with wild 'A' allele (Table 3.12ii).

SPINK1 gene 'A' and variant 'G' allele frequencies did not show statistically significant difference with anti-GAD and anti-IA2-ic antibody status of the YDM and FCPD subjects (Table 3.12iii).

Wild 'A' and variant 'G' allele frequencies between YDM14- and YDM14+ subjects of YDM group, and FCPD14- and FCPD14+ subjects of FCPD groups did not show statistically significant differences (Table 3.12iv).

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Regression analysis

Since several different determinants influencing the insulin secretory capacity were identified in the Y2D group, hence a regression analysis was performed with 7 covariates namely age, sex, duration, BMI, GAD antibody, *INS* VNTR A/T and *SPINK1* gene A/G 'N34S' genotype and C-peptide as dependent variable (Table 3.12.v). In the model it was observed that C-peptide value was significantly independently influenced by age, sex, duration, BMI, GAD antibody positivity and *SPINK1* gene A/G 'N34S' genotype. It is of interest that significance for *INS* VNTR A/T genotype is lost although this may be related to the small number of subjects with the at risk genotype.

SPINK1 gene A/G variant genotype frequency in Bangladeshis living in UK

Among the Bangladeshi population, immigrated to Britain from Northeastern Sylhet district, the homozygous wild and heterozygous genotype frequencies were 0.968 and 0.032 in the nondiabetic subjects and 0.965 and 0.035 in T2DM subjects respectively (p=ns) (Table 3.12vi).

SPINK1 gene A/G variant genotype frequency in South Indian study subjects

The SPINK1 gene A/G (N34S) variant genotype frequencies were 0.965, 0.032 and 0.003 for homozygous wild, heterozygous variant and homozygous variant respectively in the nondiabetic Controls. Among the impaired fasting glucose/impaired glucose tolerance subjects the corresponding frequencies were 0.964, 0.036 (p=ns) and 0 and in the T2DM patients these were 0.961, 0.039 and 0 respectively (p=ns).

In the south Indian unrelated FCPD patients the frequencies were 0.647, 0.265 and 0.088 respectively (p<0.0001 vs Controls) (Table 3.12vi).

In the study among total subjects (Bangladeshi and southern Indian origin) the N34S variant was present in 33% of 188 subjects with FCPD, 4.3% of 857 (including 56 IGT cases) nondiabetic subjects (odds ratio 10.91, 95% CI 6.96-17.07; p<0.0001 compared with FCPD), 3.7% 219 subjects with T2D, and 12.1% of 372 subjects with young onset diabetes (YDM) (Figure 3.4).

SPINK1 gene N34S assay in FCPD families

SPINK1 gene N34S mutation was investigated in five South Indian families with FCPD (three families had more than one member with FCPD, and two had one member with

FCPD and at least one member with T2D). The variant was present in only one family. In this consanguineous family the father had FCPD and mother had TCP; both were homozygous for the N34S variant as well all the six children (two with FCPD, one with TCP, one with impaired glucose tolerance and with normal glucose tolerance).

SPINK1gene N34S analysis subjects of British and French Caucasoid origin

Out of 235 YT2DM patients (3.4%) 8 were positive for the *SPINK1* gene A/G mutation against 4.6% (7 out of 152) in the controls. All the mutant cases were heterozygous.

Mean (±SD) age of the mutant cases was 35.7±6.3, duration 11.6±4.3, BMI 30.7±7.2 and WHR 0.9±0.03

In the French cohort only 1 individual was found to have mutation. The individual was diagnosed at the age of 39 yrs, BMI 23.5 and negative for IAA and GAD antibody. Seven nondiabetic family members have variant positive and 2 other have homozygous wild genotype.

Table 3.12i:

Genotype frequencies of SPINK1 gene A/G (N34S) genotype of the study subjects

	Controls	YDM	Unrelated FCPD	FCPD probands
Genotype	333	372	51	69
Wild AA	0.943 (314)	0.879 (327)	0.607 (31)	0.739 (51)
Variant AG	0.054 (18)	0.121 (41)	0.275 (14)	0.217 (15)
Variant GG	0.003 (1)	0.011 (4)	0.118 (6)	0.044 (3)
P values[a]		*0.006*	*0.001*	*0.001*
Allele frequency	0.970/0.030	0.939/0.071	0.744/0.256	0.847/0.153

[a]For comparison with nondiabetic group. NS=Not significant.

Statistical significant association was shown compared to controls.

Table 3.12ii:

Onset of age of DM, FSG, serum C-peptide and C-peptide/glucose ratio on the basis of SPINK1 A/G (N34S) wild and variant genotype in controls and diabetic subjects

Genotype (n)	Age of onset of DM (yrs)	F glucose (mmol/l)	F C-peptide (nmol/l)	C-peptide/glucose (nmol/mmol)
Controls				
Wild (314)	-	5.0±0.6	0.39 (0.16-1.44)	0.24 (0.10-0.81)
Variant (19)	-	5.2±0.5	0.38 (0.22-1.22)	0.23 (0.11-0.74)
YDM subjects				
Wild (323)	19.0±6.3	14.8±6.5	0.212 (0.025-1.33)	0.05 (0.003-0.59)
Variant (45)	17.1±4.5*	17.6±6.8*	0.124 (0.022-0.83)*	0.02 (0.01-0.28)*
FCPD subjects				
Wild (31)	18.3±5.0	17.8±6.4	0.09 (0.023-0.45)	0.02 (0.003-0.094)
Variant (18)	20.3±6.6	15.3±9.2	0.18 (0.024-0.84)*	0.03 (0.01-0.33*

*Significantly different compared to the counterparts.
Unpaired students 't' test and Mann Whitney tests were performed.

Table 3.12iii:

Antibody status on the basis of SPINK1 gene A/G (N34S) genotype in YDM and FCPD subject

Group and Genotype	Anti GAD Ab		Anti-IA2-ic Ab	
	Positive (n=71)	**Negative** (n=243)	**Positive** (n=37)	**Negative** (n=277)
YDM subjects				
Wild type	66 (93%)	211 (87%)	35 (95%)	242 (87%)
Variant type	5 (7%)	32 (13%)	2 (5%)	35 (13%)
	p=0.310		p=0.310	
FCPD Subjects				
Wild type	6 (75%)	20 (61%)	5 (63%)	21 (64%)
Variant type	2 (25%)	13 (39%)	3 (37%)	12 (36%)
	p=0.687		p=1.0	

Fisher exact test was performed.

Table 3.12iv:

Genotype frequencies of SPINK1 gene A/G (N34S) variant on the basis of age of onset of diabetes in YDM and FCPD subjects

GAD Ab status	INS genotype		
	AA	*AG*	*GG*
YDM14- subjects	79 (24%)	12 (29%)	2 (50%)
YDM14+ subjects	248 (76%)	29 (71%)	2 (50%)
	p=0.358		
FCPD14- subjects	8 (26%)	4 (29%)-	-
FCPD14+ subjects	23 (74%)	10 (71%)	6 (100%)
	p=0.743		

Fisher exact test was performed.

Table 3.12v:

Regression analysis for C-peptide with age, sex, duration, BMI, GAD Ab, *INS* VNTR A/T variant and *SPINK1* gene N34S variant

Dependant variables	Unstandardized Coefficients		Standardized Coefficients	t	Significance	95% Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
Constant	*-1.368*	*.128*		*-10.711*	*0.000*	*-1.619*	*-1.116*
Age	0.0102	0.004	0.169	2.682	0.008	0.003	0.018
Sex	0.112	0.036	0.147	3.116	0.002	0.041	0.183
DUR	-0.0332	0.010	-0.168	-3.470	0.001	-0.052	-0.014
BMI	0.0310	0.005	0.380	6.171	0.000	0.021	0.041
GAD Ab	-0.155	0.045	-0.170	-3.431	0.001	-0.243	-0.066
SPINK1	-0.178	0.057	-0.148	-3.127	0.002	-0.289	-0.066
INS	0.0166	0.040	0.019	0.410	0.682	-0.063	0.096

Dependent Variable: C-peptide; R equals to 6.05;

Regression analysis of 372 YDM cases of C-peptide as dependent values with 7 covariates showed 6 covariates (age, sec, DUR, BMI, GAD Ab and *SPINK1*) independently significantly influences the C-peptide level (Sig ≤0.008) with the significant constant (Sig <0.000).

DUR, Duration of diabetes, GADAb, GAD antibody positivity; *SPINK1*, *SPINK1* gene A/G 'N34S genotype; *INS*, *INS* VNTR A/T genotype.

Table 3.12vi: Frequency of *SPINK1* gene A/G variant (N34S) in other studied populations

Groups (n)	*SPINK1 gene A/G allele frequency (n)*			
	AA	AG	GG	*p values*[a]
Bangladeshi Sylheti British immigrant subjects				
Nondiabetic [b]	0.968 (151)	0.032 (5)	0	
T2DM (142)	0.965 (137)	0.035 (5)	0	NS
South Indians subjects				
Nondiabetic (312)	0.965 (301)	0.032 (10)	0.003 (1)	
Impaired fasting glucose/ IGT (56)	0.964 (54)	0.036 (2)	0	NS
T2DM (77)[c]	0.961 (74)	0.039 (3)	0	NS
Unrelated FCPD (68)	0.647 (44)	0.265 (18)	0.088 (6)	<0.0001

[a]For comparison with nondiabetic group; [b]Defined by fasting blood glucose <6 mmol/l; [c]Defined by either random or fasting blood glucose < 6 mmol/l.
NS=Not significant; AA = Wild type; AG = Heterozygous mutant; GG = Homozygous mutant.

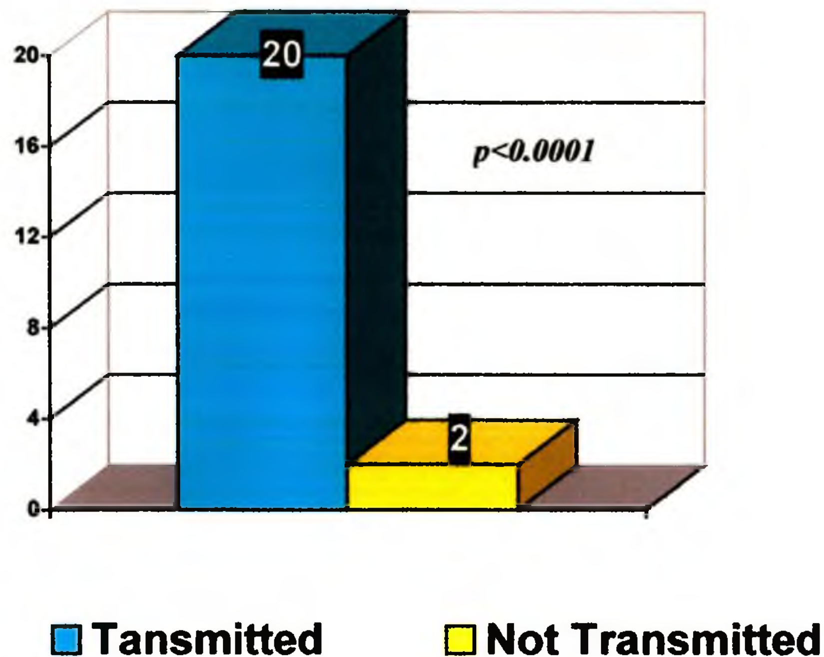

Figure 3.3: ETDT analysis of *SPINK1* gene A/G (N34S) allele in the FCPD probands.

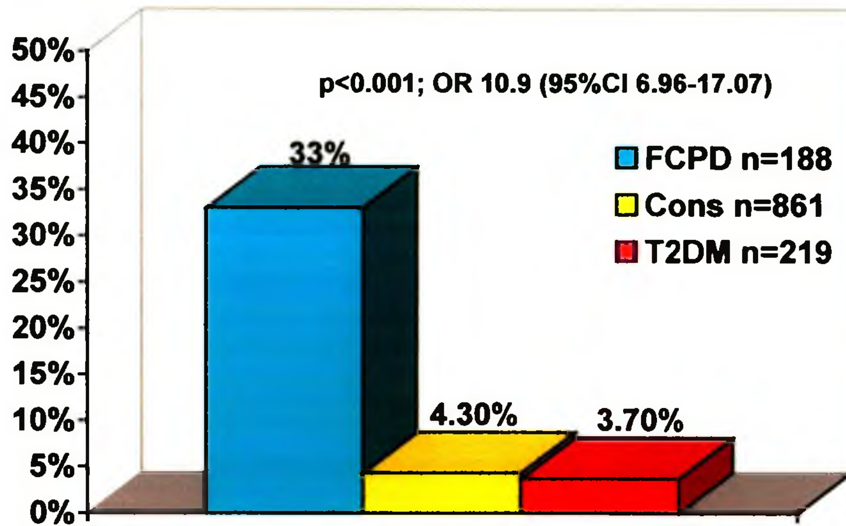

Figure 3.4: SPINK1 N34S gene variant and FCPD in the Indian Sub-Continent (Bangladeshi and South Indian subjects)

3.11.7 Trypsinogen gene mutations assay

3.11.7.1 Trypsinogen gene G/A (R122H) variant assay

Enzyme digestion of the amplified DNA fragment containing, G/A mutation site in the exon 3 of the trypsinogen gene, did not reveal restriction fragment(s). This indicated the absence of mutation in the gene of 70 FCPD probands, South Indian 50 unrelated FCPD and 7 FCPD families.

On direct DNA sequencing of 45 FCPD subjects and 14 FCPD family members the absence of mutation was confirmed (Hassan *et al.*, 2000).

3.11.7.2 Trypsinogen gene A/T (A16V) variant assay

Enzyme digestion of the amplified DNA fragment, containing A/T mutation site in the exon2 of the trypsinogen gene, did not reveal restriction fragment(s). This indicated the absence of A/T mutation in 70 FCPD probands, South Indian 50 unrelated FCPD cases and 7 FCPD families.

On direct DNA sequencing of 45 FCPD cases and 14 FCPD family members the absence of mutation was confirmed (Hassan et al 2000).

3.11.7.3 DNA sequencing of trypsinogen gene

The C/T mutation leading to N21I did not disrupt any restriction site. Hence the allele was analyzed by direct DNA sequencing. Thus direct DNA sequencing of both exon2 and exon3 containing A/T and C/T, and A/G mutations respectively confirmed lack of the mutations in 25 Bangladeshi FCPD probands, 20 South Indian unrelated FCPD cases and 14 available members of the FCPD families (Hassan et al 2000).

3.11.8 Candidate gene markers analyses in T1D

Using the criteria of ketoacidosis without any precipitating cause 11 individuals were typed as type 1 DM. Variables of these 11 patients (stated below) were not compared with other groups because of small number. Out of 11 cases 10 were newly diagnosed. The previously diagnosed individual had duration of diabetes about 6 months. None of these 11 cases had family history of diabetes. Their age at diagnoses was 13.1±4.0, fasting glucose 20.5±3.4, C-peptide 0.13±0.05 and C-peptide-glucose ratio 0.020±0.009.

Of these 11 individuals antibodies were determined in 9 samples. Out of 9 individuals 3 (33.3%) cases were positive anti-GAD and 1 for anti-IA2-ic antibody. None was positive for both the antibodies.

Genotype frequencies of these 11 individuals were shown in the following table (3.13).

Table 3.13: Genotype frequencies of T1D subjects

Gene variant	Genotype	Frequency
INS VNTR -23 A/T allele variant	AA - AT - TT	0.73 - 0.27 - 0
***EIF2AK3* Indel15 ($AT^{+/-}$) variant**	$AT^{+/+}$ - $AT^{+/-}$ - $AT^{-/-}$	0.91 - 0.09 - 0
***TCF1* gene C/T (A98V) allele**	CC - CT - TT	0.73 - 0.27 - 0
***NEUROD1* gene G/A (A45T)**	GG - GA - AA	0.64 - 0.36 - 0
NEUROG3 gene		
G/A (G167R) allele variant	GG - GA - AA	0.45 - 0.55 - 0
C/T (S199F) allele variant	CC - CT - TT	0.09 - 0.91 - 0
***SPINK1* gene A/G (N34S) allele**	AA - GA - GG	0.91 - 0.09 - 0

Genotype follows: homozygous wild, heterozygous and homozygous variant

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CHAPTER 4

DISCUSSION

4. DISCUSSION

4.1 GENERAL CONCLUSIONS

The data from the present study indicate that:

(i) Autoimmunity is involved in a substantial number of young Bangladeshi patients of both FCPD and YDM varieties, however, in contrast to classical T1D there is some preservation of B cell function in these patients. Based on autoantibodies alone T1D, as defined by the latest WHO and ADA Expert Committee criteria may be more common in Bangladeshi patients than generally thought.

(ii) *SPINK1* gene N34S mutation is strongly associated with pancreatitis of FCPD in Bangladesh (indeed in South Asia) and *SPINK1* gene (N34S variant) 'G' allele is preferentially transmitted to the FCPD probands from heterozygous parents. However, it is unclear whether this mutation has any direct association with diabetes in FCPD patients or simply is the etiological factor for chronic pancreatitis in this condition. Interestingly, an association has been found between the *SPINK1* N34S mutation and young onset diabetes (YDM) in the Bangladeshi population. It is yet to be established whether they are subclinical cases of FCPD.

(iii) Trypsinogen gene R122H, A16V and N29I mutations are not associated with pancreatitis either in Bangladeshi or in Southern Indian FCPD subjects.

(iv) INS VNTR A/T polymorphic 'T' allele is not associated either with FCPD or the YDM patients in Bangladeshi population. However, within the YDM patients those carrying the T1D associated allele genotype had a lower insulin secretory capacity than those carrying the variant allele.

(v) Common variant of transcription factor genes, linked to the neonatal and early childhood T1D and MODY, are not associated either with FCPD or with YDM in Bangladeshi population.

4.2 Diabetes in young Bangladeshi population

Diabetes in young Bangladeshi population poses a special problem as the patients are difficult to be grouped in classes following the criteria suggested by the Expert Committee of WHO (1999) and or ADA (1997). A small subset of this population can be grouped as Fibrocalculus Pancreatic Diabetes (FCPD) in a straightforward manner as they can be diagnosed to have chronic calcific pancreatitis by relatively simple radiological or imaging techniques. Even then diabetes in FCPD does not always fit to a simple picture of 'secondary diabetes' as proposed by both the WHO and ADA Expert Committees.

One of the major obstacles in grouping these patients is the remarkable heterogeneity in clinical and biochemical presentations. Due to the age and physical appearance of most of the patients many physicians are tempted to mark them as T1D. But, on closer analysis this simplistic view is not sustained. Typical T1D usually occurs in early childhood (Karvonen *et al.,* 2000). At diagnosis T1D patients are normal to under-weight and usually have moderate to severe glycemia. Endogenous insulin secretion in these subjects is usually very low to almost nil and in untreated state they are prone to develop ketosis. In contrast, most of the FCPD and a substantial number of YDM subjects present with moderate to severe glycemia but they are not prone to ketosis. They are usually normal to underweight and even in some cases cachetic. On the basis of clinical presentation and endogenous insulin secretion it appears that both T1D and T2D patients have been included in FCPD and YDM classes. Studies with FCPD and YDM subjects have revealed their preserved insulin secretory capacity (although blunted) in glucose stimulated (Rahman *et al.,* 2000), glucagon stimulated (Islam *et al.,* 1998) and arginine stimulated (Rossi *et al.,* 2004) states. The varying degree of this capacity - from residual to normal, confirms the heterogeneity of FCPD and YDM subjects regarding the endogenous insulin secretion. Heterogeneity has also been found in FCPD and YDM subjects of the present study regarding their clinical presentations (Table 3.1i), anthropometric features (Table 3.1ii and Table 3.3), biochemical parameters (Table 3.4i and Table 3.4iii) and genetic factors.

A total number of 423 young diabetic subjects were recruited of which 51 were FCPD and 372 were YDM. All patients were collected from the OPD of BIRDEM, a tertiary

care hospital. Against the conclusions regarding heterogeneous clinical and biochemical presentation of FCPD and YDM subjects and also regarding the conclusions that follows, one may always raise the question of representativeness of the sample and its size. However, being a free service provider the large OPD of BIRDEM (about 3000 patients per day) attracts patients of all socioeconomic and religious strata from all over the country, thus preventing a clustering of the samples.

Lack of epidemiological data makes it difficult to calculate an appropriate size of a sample although inclusion of more patients (particularly of the YDM group who are not as uncommon as the FCPD ones) could always be envisaged. There were also limitations in collecting the controls. They were screened by only one fasting blood glucose value thereby leaving the chance of possible inclusion of some IGT subjects, who could have been screened by OGTT. Also they could have been screened with repeated testing. In addition to the above fact the controls were recruited through personal contact rather than a randomized procedure. Notwithstanding these weaknesses or the necessity for community-based studies, the present data still render valuable insight regarding the etiopathological basis of FCPD and YDM by using some of the immunological and genetic tools available at present. Furthermore, it does not invalidate any positive results as the effect of inclusion of a few individuals in the control group, who might develop diabetes, will only lead to reduced power of such result.

4.3 Autoimmunity in FCPD and YDM

Autoimmune markers in background population

This is the first ever data on the GAD and IA-2ic antibody in a Bangladeshi population. Positivity for GAD Ab and IA2-ic Ab was 3.2% and 0.4% respectively. The data, favorably compare with the frequency of positivity of the two antibodies involving Caucasoid and non-Caucasoid populations (~4% for GAD and 0-2.5% for IA2 respectively) (Batstra *et al.*, 2001; Tsuruoka *et al.*, 1995).

Autoimmunity and FCPD

This is the first time GAD and IA2-ic antibodies have been measured in a cohort of FCPD patients of Bangladeshi origin. The autoantibody (anti-GAD and IA-2ic) data show that a substantial number of FCPD patients were positive for these T1D

susceptible markers: 19.5% for each of the GAD and IA2-ic antibody and about 14% for both. GAD antibody was found to be positive only around 7% of FCPD cases in a number of studies in different parts of India (Mohan et al., 1998a; Singh et al., 2000; Goswami et al., 2001); antibody against IA-2ic, however, was not determined in those studies. In one study ICA was found to be positive in 6.3% of FCPD cases (Mohan et al., 1998a). The presence of GAD and IA-2ic antibodies positivity in about 20% of cases is markedly lower compared to that of 50-70% in newly and recently diagnosed T1D cases (Velloso et al., 1993; Lan et al., 1996; Hawa et al., 1997) but it was significantly higher compared to the controls. Thus autoimmune process may be suggested to be involved in the pathogenesis of diabetes in a substantial number of FCPD cases of Bangladeshi origin. It is still needs to be explored why a larger proportion of FCPD patients in Bangladesh are autoantibody positive compared to the South Asian countries.

Autoimmunity and YDM

Among the YDM subjects about one quarter (22.6%) were found to be positive for anti-GAD and 11.8% for IA-2ic antibody. These proportion are much lower than those reported for newly diagnosed T1D cases which are usually between 50-70% (Velloso et al., 1993; Lan et al., 1996; Hawa et al., 1997). However, among Asian T1D patients GAD Ab was found to be positive in around 40% cases (Tuomi et al., 1995; Tsuruoka et al., 1995; Thai et al., 1997).

A relatively higher frequency of positivity for both anti GAD and IA-2ic have been observed in newly diagnosed cases compared to the established cases ($p=0.11$ and $p=0.022$ respectively, Table 3.5i). Since presence of autoimmunity is the hallmark of T1D and maximum frequency of T1D is found to occur in early second decade of life, ie usually at ≤ 14 years of age (Karvonen et al., 2003), positivity of the two autoantibodies were analyzed on the basis of cut-off age of 14 years. In 14- YDM subjects 36.5% were positive for GAD Ab against 17.5% in the relatively older age group ($p=0.001$). For IA-2ic the proportion was 20% vs 8.9% ($p=0.01$). GAD antibody positive and negative cases were compared regarding their clinical, biochemical and genetic data. Antibody positive cases had significantly lower insulin secretion as judged by C-peptide levels and also by C-peptide/glucose ratio. Moreover, GAD Ab positive cases were significantly younger than their counterparts (Table 3.6ii).

GAD Ab has been found to be positive about 10-34% among LADA patients (Turner et al., 1997; Scheil and Muller 2003). The lowest positivity of GAD Ab among Spanish LADA patients was found to be around 2% (Gambelunghe et al., 2000). In young ketosis resistant patients from India 38% were found to be positive for GAD Ab (Goswami et al., 2001) and 40% with ICA512/IA2 antibody (Sanjeevi et al., 1999).

Autoimmune phenomenon is involved in a substantial number of young Bangladeshi diabetic patients of both FCPD and YDM variety. However, preservation of at least residual B cell function in these patients, due to yet unidentified factor(s), modify their phenotypic expressions leading to ketosis resistance. Thus T1D, as defined by the latest WHO and ADA Expert Committee criteria, may be more common in Bangladeshi than generally thought so far and this is particularly the case for these patients diagnosed before the age of 14 years.

4.4 Genetic analyses in FCPD and YDM

4.4.1 *SPINK1* gene N34S mutation

Background population

In the present study *SPINK1* gene N34S mutation in Bangladeshi controls is 5.7% and that is in British Caucasoids is 4.6%. In other studies the frequency of the mutation are: 3.5% in healthy Bangladeshi UK immigrants and 3.2% in South Indian controls (Table 3.12v); 3.0% among another healthy Bangladeshi cohort (Schneider *et al.,* 2002). Previously Threadgold *et al.,* (2002) had demonstrated that 2.5% of Asian blood donors had *SPINK1* gene N34S mutation. The frequency of the mutation in the control subjects appears to be relatively higher compared to the observation of Schneider *et al.,* (2002). It may be noted that the age of the two groups of controls are different at the time of recruitment; in the present study they are of younger age group (22.5±4.9, yrs) who may have undiagnosed subclinical disease, compared to relatively older (mean age, 28 yrs) cohort of only 70 controls which left a chance of underestimation of the frequency.

It will be interesting to thoroughly examine and investigate those control subjects positive for *SPINK1* gene N34S mutation for any pancreatic pathology.

FCPD subjects

In unrelated Bangladeshi FCPD patients 39% had *SPINK1* gene variant 'G' allele leading to N34S mutations compared to the controls ($p<0.0001$). This high frequency of variant 'G' allele has also been observed in the unrelated FCPD patients (35.3%) from South Indian origin. In a pilot study Rossi et al (2001) first showed *SPINK1* gene N34S mutation in 6 out of 8 FCPD patients from Bangladesh, but in none iof their nondiabetic counterparts, ie TCP and controls. Subsequently Schneider *et al.,* (2002) found that 55% of their FCPD patients had *SPINK1* gene mutation leading to N34S variant. A study by Chandak et al (2002) reported *SPINK1* gene N34S mutation in 50% of FCPD cases of Eastern Indian origin (Table 4.1).

Table 4.1:

***SPINK1* gene N34S variant in FCPD, TCP and T2D**

FCPD (x/y)	TCP (x/y)	T2D (x/y)	Controls (x/y)	References
75% (6/8)	0% (0/4)	-	-	Rossi *et al.*, 2001
55% (12/22)	20% (3/15)	14% (6/43)	1.3% (1/76)	Schneider *et al.*, 2002
50% (12/24)	45% (20/44)	-	3% (3/100)	Chandak *et al.*, 2002
		2.2% (5/232)	3.3% (10/302)	Schneider *et al.*, 2005

x=number of variant positive cases in the study; y=number of subjects studied

SPINK1 gene N34S mutation has also been analyzed in idiopathic and alcoholic pancreatitis patients. Frequency of the mutation was found to be 9-20% in idiopathic pancreatitis (Table 4.2). Among alcoholic pancreatitis patients the mutation was found in 6% cases (Threadgold *et al.,* 2002) whereas in different studies (Table 4.1) frequency of positivity for the variant genotype was 50-75% in FCPD patients.

The high frequency of *SPINK1* gene N34S mutation in FCPD compared to idiopathic pancreatitis clearly suggests that mutant 'G' allele, possibly, confer an increased risk for development of pancreatitis in the tropical pancreatitis patients.

The Bangladeshi FCPD families showed significantly increased preferential ($p<0.0001$) transmission of the variant 'G' allele to the offspring from heterozygous parents. These

findings further indicate that the mutant gene 'G' allele may confer an increased risk for FCPD or has association with diabetes in these patients. However, this is not a fully penetrant mutation since none of the parents of the probands with *SPINK1* N34S variant had clinically apparent FCPD/TCP.

Table 4.2:
***SPINK1* gene N34S variant in alcoholic and idiopathic pancreatitis**

Alcoholic pancreatitis	Idiopathic , pancreatitis	Controls	References
-	9.3%	0.01%	Gomez-Lira *et al.*, 2003
6.9%	20%	1.67%	Drenth *et al.*, 2002
-	13%	0.6%	Kume *et al.*, 2005
6%	16%	2.5%	Threadgold *et al.*, 2002
2.4%	-	0%	Lee *et al.*, 2005

YDM subjects

In the YDM group *SPINK1* gene N34S variant was positive in about 12% subjects compared to the 5.7% of the age-matched controls. The YDM subjects were segregated from FCPD by clinical and radiological/ ultranology examinations. In one study the variant was found to be positive in 14% of typical T2D patients of Bangladeshi origin (Schneider *et al.*, 2002). Bhatia et al (2003) observed that *SPINK1* gene N34S mutation is not associated with T2D from Indian and German origin. But, it has been shown that the frequency of the *SPINK1* gene N34S variant has found to be 3.6% and 3.9% respectively in IGT and T2D subjects from South Indian origin (Hassan *et al.*, 2002). The present data also show that the frequency of the *SPINK1* gene N34S variant is 3.4% among the British Caucasoid young T2D subjects (section 3.11.6). The gene variant was observed in only one individual in a cohort of French MODY patients. *SPINK1* gene A/G mutation was also tested in T2D patients of US population and found to be playing no role in the pathogenesis of diabetes.

SPINK1 gene N34S mutation has been seen strongly ($p<0.001$) associated with FCPD subjects of South Asian (Bangladeshi and Southern Indian) and the gene variant (N34S) 'G' allele has been preferentially transmitted ($p<0.0001$) to the FCPD probands

of heterozygous parents. These findings strongly suggest an important role of *SPINK1* gene N34S variant in the pathogenesis of pancreatitis in FCPD. Since significantly high frequency of *SPINK1* gene N34S mutation was also observed in Bangladeshi YDM subjects ($p<0.006$) the gene variant one may argue that FCPD is secondary to YDM. In this regard it may be explained that pancreatitis has been excluded, clinically and radiologically, in the YDM and the mutation also has been identified in 14% of typical T2D patients of Bangladeshi origin (Schneider *et al.*, 2002). However, Bhatia et al (2003) have demonstrated lack of association of *SPINK1* gene N34S mutation in T2D patients from India and Germany. So, the *SPINK1* gene N34S mutation by itself or in combination with other gene(s) seems to be linked to the pathogenesis of diabetes in YDM and also of the T2D Bangladeshi subjects. In the Bangladeshi YDM patients with the N34S variant C-peptide was lower than in those without the variant supporting a pathological role of the variant in young onset diabetes. It would be interesting to study mutation positive patients with detailed exocrine pancreatic tests including fecal elastase analysis.

As already discussed the insulin secretory capacity was found to be decreased in YDM patients and it had an association with the *SPINK1* gene N34S variant. But, in contrast, the reverse was seen in FCPD although the interpretation has to be tempered by the small number of patients studied which increases the likelihood of a type 2 error (β error). Nonetheless there is a possible explanation that would be worthy of further investigation. It is known that the *SPINK1* gene N34S variant has a low penetrance since it is present in only 5.7% of young controls (Table 3.12i), in about 3.5% (Table 3.12v) of the general population of Bangladeshi and South Indian origin and in 4.6% of healthy British Caucasoids. The penetrance is also low in the parents of FCPD patients all of whom neither have overt diabetes or chronic pancreatitis. The variant is present in around 12% of YDM subjects without abdominal calcification. The etiology of chronic pancreatitis in FCPD is likely to be heterogeneous although one-third of cases possess the *SPINK1* N34S variant. This, however, is likely to represent the milder end of the spectrum whilst the other two-thirds are likely to be due to more penetrant gene mutations and/or environmental factors associated with a more severe form of disease leading to further decrease in insulin secretory capacity.

4.4.2 Trypsinogen (TRYP) gene mutations

FCPD subjects

TRYP gene mutations were investigated in unrelated FCPD patients from Bangladeshi and South Indian origin. The mutations were also investigated in small and multigenerational FCPD families. None of the common mutations (R122H, A16V and N29I), identified mainly among HP and chronic pancreatitis patients, were observed in the FCPD cases (Hassan *et al.,* 2000). These mutations were also absent in both small and multigenerational South Indian FCPD families (Hassan *et al.,* 2000). The absence of trypsinogen mutation also been supported by the pilot study carried out in the FCPD subjects of Bangladeshi origin (Rossi *et al.,* 1998).

Trypsinogen (*TRYP*) gene mutations, R122H and N29I, were originally identified in HP patients (Whitcomb *et al.,* 1996b). Subsequently those two mutations were identified in other HP kindred (Teich *et al.,* 1998; Bell *et al.,* 1998). The common *TRYP* gene R122H mutations, found in HP, were also identified in 19% of idiopathic pancreatitis patients (Creighton *et al.,* 1999). Since the nature of pancreatitis in FCPD, to some extent, shows some similarity to that in HP it was hypothesized that *TRYP* gene variations may also play crucial role in the pathogenesis of pancreatitis in FCPD.

The absence of trypsinogen gene mutations, particularly R122H, indicates that proposed mechanism for pancreatitis by Whitcomb et al (1996b) is unlikely to be responsible in the pathogenesis of pancreatitis in FCPD. Since the TRYP gene mutations were totally absent among FCPD patients, originating both from Bangladesh and South India, no further investigation was conducted in healthy controls and YDM subjects. Recent studies have demonstrated more mutations in different pancreatitis phenotypes, chronic pancreatitis including cystic fibrosis, both in the coding and noncoding regions of the *TRYP* and in particularly the *SPINK1* gene which have not been examined in the present study. In the view of above finding it is important to explore the putative mutations and genetic polymorphisms in these FCPD subjects to examine the possible pathological mechanism involved in pancreatitis and/ or diabetes.

4.4.3 INS VNTR –23bp A/T variant

Background population

In the present study a total number of 337 healthy subjects having no family history of diabetes up to second generation were studied. The healthy individuals were collected from the mainstream homogeneous population of Bangladeshi origin. The frequencies of INS VNTR A/T genotype in controls were 0.753, 0.229 and 0.018 for AA, AT and TT (wild, Ht and Hz variant) respectively. The wild 'AA' genotype frequency is found to be higher in the background population of Bangladesh compared to those of European origin (Table 4.3). However, wild AA genotype frequency was also found to be much higher in South Indian control subjects (Table 4.3).

Table 4.3:

INS VNTR –23bp A/T genotypes in healthy controls of different studies

INS VNTR –23bp A/T genotype frequency			**Reference**
1/1	1/3	3/3	
0.554	0.375	0.071	Owerbach *et al.*, 1982
0.636	0.364	0.034	Rotwein *et al.*, 1983
0.446	0.458	0.096	Bell *et al.*, 1984
0.480	0.474	0.112	Hitman 1994
0.905	-	0.095*	Kambo *et al.*, 1989
0.753	0.229	0.018	Present study

1/1=A/A, 1/3=A/T, 3/3 TT

*indicates genotype 1/3 and 3/3 frequencies in combination

FCPD subjects

INS VNTR class III allele 'T' frequency was found to be higher in FCPD subjects that yielded a RR of 1.27 (95% CI 0.81 - 2.0). For total FCPD subjects (unrelated FCPD and FCPD probands) the RR was 1.31 (95% CI 0.95-1.80). However, the variant 'A' allele is not preferentially transmitted in the probands (p=0.304; Fig 3.2) although this familial analysis is limited by the power (Chowdhury *et al.*, 2002). The weakness of power of INS VNTR marker analysis, which is 0.270, has already been mentioned in the result section. This increased frequency of class III allele in FCPD is also found to

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be consistent with the previous study by Kambo *et al.*, (1989) which was done in South Indian patients.

YDM subjects

A number of studies have previously demonstrated that INS VNTR class I 'AA' allele is significantly associated with T1D (Julier *et al.*, 1991; Hitman *et al.*, 1985). Although frequency of wild 'AA' genotype (0.753) in YDM subjects in this study is almost similar to those found by Julier et al (1991) and Hitman et al (1985) but the high frequency of 'AA genotype' in the controls suggest that the wild 'AA' genotype is not associated with young onset diabetes in Bangladesh.

Although INS VNTR wild genotype 'AA' is not associated with YDM but those with AA genotype had significantly lower ($p<0.05$) absolute C-peptide level compared to those with variant 'T' allele. The underlying phenomena need to be explored further.

INS VNTR genotype was analyzed in relation to GAD Ab positivity of the YDM subjects. It was observed that GAD positive subjects had significantly lower (17%) frequency of INS VNTR variant 'T' genotype compared to GAD negative cases (33%) (Table 3.6iii). The proportion of INS gene VNTR AA genotype was higher in GAD Ab positive group ($p=0.018$) confirming the reported association of class I INS VNTR polymorphism with autoimmunity. It reveals that 26% of YDM subjects having class I allele were GAD Ab positive and 14% of GAD Ab positive had class III allele. There was significantly higher C-peptide in GAD Ab negative cases (Table 3.5ii) and they have significantly higher frequency of INS VNTR variant 'A' allele (Table 3.6iii). This highlights the association of class I INS VNTR with autoimmune related diabetes and protective effect of class III INS VNTR on the insulin secretion in GAD Ab positive subjects. This is supported by the study of Matejkova-Behanova *et al.*, (2004) who observed that polymorphism of INS VNTR is associated with GAD Ab and postprandial C-peptide in diabetic patients with onset of diabetes after 35 years. They also showed that IA2-ic positive subjects did not show any association with INS VNTR in the YDM subjects.

HLA-DQB1* and TNF gene variant were studied previously in FCPD families. Increased transmission of HLA-DQB1 0302 allele, which is associated with T1D susceptibility, was observed in FCPD probands. However, HLA-DQB1 0202 allele has been observed to be significantly not transmitted (Chowdhury *et al.*, 2002). An association of HLA-DQB and TNF haplotypes with FCPD has also been observed.

Since, a subset of FCPD patients have some of the features of T1D investigation of the HLA- DQB1* and DRB1* genotypes in these subjects would have been useful. Therefore, it is important to carry out the HLA-DQB1* and DRB1* genotypes of these subjects which, in turn, may help to characterize them properly.

One of the major drawbacks of the study is the failure to carry out HLA-DQ genotyping. Since result of HLA-DQB1* ETDT analysis demonstrated increased transmission of HLA-DQB1* 0302 and decreased transmission of DQB1* 0202 allele (both being responsible for T1D susceptibility) to the offsprings of the healthy parents, and since biochemical and autoimmune data of the YDM and FCPD subjects clearly suggest that a substantial number of subjects belong to T1D subgroup (but may be phenotypically different from the classical type 1 variety), so analysis of HLA-DQB1* genotype would have been highly useful to characterize these subjects.

4.4.4 Transcription factor gene variant

4.4.4.1 *EIF2AK3* gene variant

The frequency of wild AT^{+}/AT^{+} genotype is 0.955 which is comparable to the data from South Indian origin (Allotey *et al.*, 2004).

Genotype frequencies of the *EIF2AK3* gene Indel15 AT^{+}/AT^{-} allele has not shown any association in FCPD, but AT^{-} allele is found to be significantly higher in the FCPD subjects. Genotype frequencies of the *EIF2AK3* gene Indel15 AT^{+}/AT^{-} allele has not shown any significant difference in YDM subjects.

The FCPD and YDM subjects with wild and variant genotype did not show any difference regarding the age of onset of diabetes and their absolute C-peptide levels.

EIF2AK3 gene variant has been found to cause WRS, a rare syndrome of neonatal and early childhood diabetes. The *EIF2AK3* gene has been studied in T1D patients of South Indian origin and a region around *EIF2AK3* gene has been found to create T1D susceptibility (Allotey *et al.*, 2004). Since *EIF2AK3* gene is an important transcription factor and is also involved in the modification of transcription of insulin gene, it was hypothesized that *EIF2AK3* gene mutation may play a role in the pathogenesis in these young patients. In the present approach only Indel15 AT^{+}/AT^{-} was studied. No association was observed between Indel15 AT^{+}/AT^{-} variant of *EIF2AK3* gene and

YDM. Since only Indel15 AT$^+$/AT$^-$ variant was analyzed in the present study which did not show any association with diabetes in FCPD and YDM, to exclude any influence of other variants of the *EIF2AK3* gene in the pathogenesis of diabetes in these YDM subjects the gene needs to be extensively analyzed.

4.4.4.2 *TCF1*, *NEUROD1* and *NEUROG3* genes variant

Background population

Data regarding the *TCF1* (A98V), *NEUROD1* (A45T) and *NEUROG3* (G167R and S199F) genes variant mainly obtained from the western populations. In present study for the first time these MODY related genes were investigated. The genotype frequencies (Table 4.4) observed of the control subjects in the Bangladeshi population are comparable with those of the South Indian population (Jackson *et al.*, 2004).

Table 4.4: Genotype frequencies of the *TCF1* (A98V), *NEUROD1* (A45T) and *NEUROG3* (G167R and S199F) in the present study

Wild	Heterozygous	Homozygous variant	Gene variant
0.855	0.142	0.003	*TCF1* gene C/T (A98V)
0.764	0. 209	0.027	*NEUROD1* gene G/A (A45T)
0.940	0.060	-	*NEUROG3* gene G/A (G167R)
0.179	0.448	0.343	*NEUROG3* gene C/T (S99F)

FCPD and YDM subjects

Genotype frequencies of the *TCF1 (A98V)*, *NEUROD1* (A45T) and *NEUROG3 (G167R and S199F)* gene variants do not show any significant difference compared to controls. Subjects with wild and variant genotypes do not show any difference regarding age of onset of diabetes, C-peptide levels and autoantibody status.

TCF1, *NEUROD1* and *NEUROG3* genes are involved in B cell differentiation and development. *TCF1 (A98V)*, *NEUROD1 (A45T)* and *NEUROG3 (G167R and S199F)* gene mutations have been found to be associated with different MODY phenotypes.

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These genes are also potential candidate genes for type 2 diabetes because they are part of transcriptional hierarchy and are involved in the B cell development. They are also capable of modifying the rate of transcription of insulin gene. Jackson et al (2004) observed that subjects with increased number of cumulative *TCF1*, *NEUROD1* and *NEUROG3* genes risk alleles had higher fasting glucose and higher proportion of subjects with diabetes. Hence it was assumed that those gene variants might play a crucial role in the pathogenesis of diabetes in FCPD and YDM. It is also important to note that in the present study the presence of increasing number of the *TCF1* (A98V), *NEUROD1* (A45T) and *NEUROG3* (A167G and S199F) has no effect on fasting blood glucose and C-peptide level in the FCPD and YDM subjects as well as in controls.

Since no significant difference was observed between FCPD and YDM subjects with wild and variant alleles the mutations may be excluded as a candidate gene markers for diabetes in FCPD and YDM. Since FCPD and YDM subjects also show heterogeneity regarding C-peptide levels investigation of other putative variants of these or different other genes may be useful to explore the pathological mechanism.

It is interesting to look at the molecular genetic data in parallel to the population genetics. Although the present study is not community based yet information derived regarding the presence of family history of diabetes may be pointed out. Presence of diabetes among first-degree relatives in FCPD patients was found to be around 10%. Among YDM patients 7.3% had their mothers and 8.4% had fathers with diabetes. This proportion of YDM subjects with either mother and father with diabetes is comparable to prevalence of family history of diabetes in 10% T1D patients. The presence of diabetes in mothers and fathers are much lower, around 20% or more, than that found in typical T2D patients (Karter *et al.,* 1999; Thomas *et al.,* 1994; Young *et al.,* 1995). Limitations in obtaining the family history of diabetes in the absence of reliable records, however, do not allow for a more meaningful conclusion in this regard.

The genetic studies, as discussed in the previous sections, have generated important findings, but their limitations must be mentioned. Introduction of PCR technique has revolutionized the research in the field of molecular biology in the early eighties and since then tremendous progress also have been achieved in the field of technology.

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Introduction of automation has expanded the robustness in DNA handling. Single nucleotide polymorphism (SNPs) and/or point mutation assay by PCR RFLP has been shifted to more robust and precise techniques of TaqMan assay, Pyrosequencing and HPLC-based screening by transgenomic wave analyzer. Even in direct DNA sequencing in place of cumbersome dye terminator DNA sequencing technique by easy to handle HPLC-based sequencing technique has been evolved which is also user friendly to analyze sequence after the run.

In the present study most of the laboratory works are based on PCR RFLP. This involves a number of manual steps giving rise to chance of human error. It is also subject to variability of enzyme of different sources and factors affecting its enzyme activity.

4.5 Nature of diabetes in FCPD and YDM

4.5.1 Diabetes in FCPD

Clinical and biochemical data; leanness, degree of hyperglycemia (usually moderate to severe) and positivity of autoantibody (anti-GAD) of FCPD subjects are more close to T1D; but, their lack of ketosis proneness differs from that of the classical form of diabetes. The FCPD subjects may have been showing some similarity with those with T1D patients at honeymoon phase, but they retain the residual B cell secretion for a longer period. Thus they do not show similarity with major bulk of T1D subjects who are totally deficient of insulin. The most important features of the FCPD patients are to have moderate to severe morphologic damage in the pancreas (Saha *et al.,* 2000) and corresponding compromise in the exocrine pancreatic function test (Ali *et al.,* 2001). On the basis of endocrine pancreatic functions and autoimmune status it may be said that a subgroup of FCPD subjects may actually belong to T1D, but they have an atypical phenotypic presentation.

Age of onset of diabetes in FCPD subjects is mostly similar to the range of young T2D patients. The FCPD subjects are lean to underweight (with cachetic appearance in some cases) in contrast to young T2D patients who are normal to mildly overweight. T2D is diabetes usually being diagnosed on routine examination and only in a minor number of the young T2D subjects the hyperglycemia may reach severe level; on the

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other hand, FCPD subjects show typical symptoms of diabetes and have severe degree of hyperglycemia. In spite of marked difference in glycemic status both groups show the lack of proneness to ketosis. Insulin may be required for the management of T2D, but in FCPD it is usually required. So far no data is available on the exocrine pancreatic morphology of T2D; however, there are reports on mild compromise of their exocrine pancreatic function (Rossi *et al.,* 2004). In contrast, FCPD is marked by a severe degree of morphological damage of the pancreas (Saha et el 2000; Rahman *et al.,* 2000) paralleled by a severely compromised exocrine function (Ali *et al.,* 2001).

Peripheral insulin level and insulin secretory capacity are major issues in characterizing the types of diabetes in a patient. Although typical T2D usually show normal to moderately high levels of plasma insulin, but HOMA analysis in a cohort showed almost decreased insulin secretory capacity (Zinnat *et al.,* 2003). A minor proportion of the FCPD subjects show normal plasma insulin levels (Table 3.4i), but in most of the cases they show markedly low to residual levels of plasma insulin. Previously it was shown that on stimulation with glucose (Parvin *et al.,* 1998), glucagon (Islam *et al.,* 1998) or arginine (Rossi *et al.,* 2004) B cell failure in FCPD becomes much more prominent with a range of mild to severe compromise. Thus, it seems that some of the FCPD patients may have T2D like insulin secretory capacities.

There is no report of antibody status among typical Bangladeshi T2D patients, but positivity of GAD Ab in almost 20% of FCPD subjects is in contrast to the assumption about these subjects that they have T2D which is superimposed with calcific pancreatitis.

Since B cell function related gene variants, so far studied, do not show any association with FCPD, the diabetes in FCPD is unlikely to be of T2D in the presence of pancreatic morphologic changes.

In the current classification of diabetes by both WHO and ADA Expert Committee FCPD has been termed as secondary to pancreatictis. The view was previously been contradicted (Azad Khan and Ali 1997) and the present data further support that reasoning: Firstly, FCPD has been found to occur in age group not different from the age of their nondiabetic counterparts, ie TCP (Azad Khan and Ali 1997; Saha *et al.,* 2000). Secondly, when compared with TCP, pancreatic morphological as well as pancreatic exocrine damage and B cell function fails to demonstrate a close relation (Ali *et al.,* 2001). The third evidence against a straightforward and secondary character of FCPD is the presence of almost normal glucagon levels in FCPD patients in

contrast to the severely compromised levels in the TCP group. In a recent elaborate study Rossi et al (2004) analyzed a series of FCPD patients and compared them with matched controls and TCP subjects who underwent arginine (endocrine pancreatic function) and secretin (exocrine pancreatic function) stimulation tests. Both TCP and FCPD had pronounced exocrine pancreatic dysfunction with beta-cell function differing significantly between the two groups. Compared to controls TCP showed normal plasma C-peptide levels at basal and after arginine stimulation state, while FCPD subjects demonstrated a typical diabetic pattern for plasma C-peptide levels. In contrast, pancreatic alpha-cell function (glucagon secretory response to arginine) was preserved in FCPD and TCP groups. It has been hypothesized that two factors, one eliciting chronic pancreatitis and the other selectively causing pancreatic beta-cell impairment leading to diabetes mellitus in FCPD, are involved (Rossi *et al.*, 2004).

The present data show that *TRYP* gene mutations (R122H, A16V and N29I) are absent in FCPD, but a substantial proportion (about 37%) of them have N34S mutation in the *SPINK1* gene (Hassan *et al.*, 2002). Similar findings for FCPD patients have been reported by other groups (Schneider *et al.*, 2002) and, in addition, TCP patients have been shown to have about 20% frequency of variant in one study (Schneider *et al.*, 2002) and 47% in another one (Bhatia *et al.*, 2002).

Thus, as presence of exocrine pancreatic dysfunction does not necessarily give rise to endocrine dysfunction and presence of a pancreatitis gene does not cause diabetes in straightforward way.

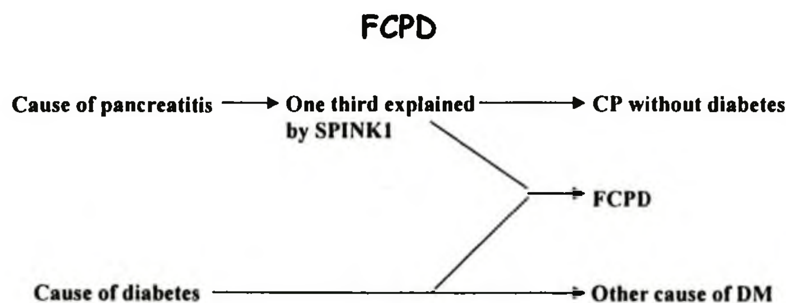

Figure 4.1: Overall relationship between pancreatitis and diabetes in FCPD

4.5.2 Diabetes in YDM

The YDM is likely to comprise of several different types of diabetes including T1D, T2D, subclinical FCPD and those what was previously classified as PDDM. Usually T1D occurs in the early second decade of life and onset of presentation is sudden. In YDM group as a whole the onset of diabetes is not as sudden as in T1D and it is characterized by the absence of ketoacidosis. At diagnosis or in an uncontrolled state T1D subjects are usually cachetic or lean and present with severe degree of hyperglycemia. On the other hand, in parallel situations, YDM subjects are lean to normal weight and they may present with variable (mild, moderate to severe) degree of hyperglycemia.

One of the major distinguishing features of T1D from T2D is the insulinemic status and insulin secretory capacity of the patients. T1D subject rapidly loses insulin secretory capacity due to complete destruction of B cells and eventually become total insulin dependent when all B cell function has been destroyed. In contrast, none of the YDM patients had undetectable level of plasma insulin level either in the present study (Table 3.2) or in previous studies (Ali *et al.,* 2003). The C-peptide levels of the YDM subjects have been shown to be quite variable, both in the basal and glucagon stimulated (which reflects insulin secretory capacity) states (Islam *et al.,* 1998), but a residual secretory capacity always remains (Table 3.2). A question then arises whether the YDM patients with only residual insulin secretory capacity are really T1D in honeymoon phase of B cell destruction. A more detailed evaluation of already existing data and generation of further data are required to address this issue. However, the presence of autoantibody may suggest this possibility.

As per the ADA (1997) and WHO (1999) criteria autoimmunity is the hallmark of T1D. GAD Ab has been found to be the single most useful autoantibody in this respect. It has also been found that combination of some or all of the antibodies (ICA, IAA, anti-GAD and IA2) can lead to an increased likelihood of complete insulin dependency and classic disease. ICA and IAA have not been measured in YDM cases, but measurement of anti-GAD shows about 23% positivity in this group. Thus, although the clinical and biochemical features of YDM, in general, have substantial difference, about one-quarter of them share autoimmune destruction with T1D patients. The findings regarding IA2-ic were not as useful as that of anti-GAD and the combination of

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the two antibodies did not provide any more advantage than only anti-GAD itself. Although many have identified as linked with the group for developed insulin dependency. The autoantibody positive group of patients might be classified as latent autoimmune diabetes in adult (LADA); however, in the present study, many subjects have an age of onset of diabetes less than 14 years making the term LADA inappropriate.

Many of the clinical presentations of YDM subjects raise the question whether they belong to a subtype(s) of T2D. Age of onset of diabetes (usually around 20 years), normal to mildly higher BMI, variable degree of hyperglycemia, absence of ketoacidosis, absence of any features of pancreatitis and no obligatory requirement of insulin for management - all these characteristics in YDM are indicative for a type of diabetes which is close to T2D in etiopathogenesis. However, presence of autoantibodies and certain diabetogenic genes, as discussed in the previous section, suggest that at least, a proportion of patients do not probably belong to T2D.

Analysis of biochemical data also supports this assumption. Basal serum insulin levels have previously been shown to be highly variable in these subjects and the picture became more prominent in glucose and glucagon stimulated states leading to a grouping as low, intermediate and normal insulin secretory capacity (Islam *et al.*, 1998; Ali *et al.*, 2003). Similar findings have been observed in glucose stimulated (Parvin *et al.*, 1998) as well as arginine stimulated states (Rossi *et al.*, 2004). The findings are compatible with the studies on Bangladeshi diabetic subjects where insulin secretory dysfunction has been found to be the major defect in all patients where as insulin resistance appears to play a role in higher degree of BMI (Zinnat *et al.*, 2003). In addition to insulin glucagon has been measured in YDM patients both in basal and arginine stimulated states (Rossi *et al.*, 2004) and the glucagon response have found to be preserved. Exocrine pancreatic function has also yielded slightly compromised results. Thus there is no indication that these patients may have secondary diabetes.

The YDM subjects have been excluded for pancreatic pathology by plain X-ray and/ or ultrasonogram of abdomen. It is interesting to observe that about 12% of them have mutation in *SPINK1* gene (Hassan *et al.*, 2002). The significance of these findings is yet to be clarified. However, it is of interest that YDM subjects, with the *SPINK1* gene

N34S variant, had lower insulin secretory capacity compared to those who did not carry the variant.

In conclusion, YDM is a heterogeneous disorder characterized by a decrease in insulin secretory capacity in contrast to the complete deficiency seen in T1D. In the present study several overlapping subsets of patients were identified which, within YDM, have even lower insulin secretory capacity. These groups of patients include, the autoantibody positive patients, those with the *INS* gene VNTR TT genotype (T1D associated class I allele) and those with subclinical FCPD characterized by presence of the *SPINK1* N34S variant. These groups of patients might have been classified as MRDM in the previous WHO diabetes classification (WHO 1985). The cause of diabetes in the remaining 50-60% of YDM subjects is yet to be elucidated but is likely to be due to genetic variants of the B cell genes as well as typical T2D.

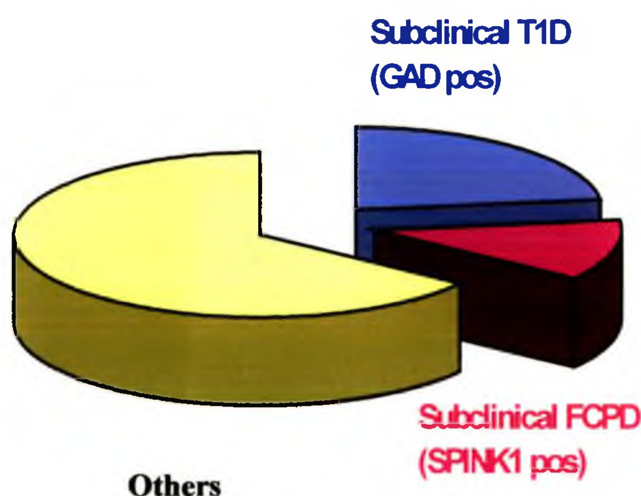

Figure 4.2: Plausible distribution of YDM subjects.

The present data also suggest that FCPD is a distinct entity. It is associated with a wide clinical spectrum from subclinical disease in YDM to overt disease in FCPD itself. In a third of cases the chronic pancreatitis is due to *SPINK1* gene variant, and in some, the diabetes that results has an additional genetic and autoimmune component associated with other forms of diabetes. In the latter cases the diabetes is more severe despite a similar degree of exocrine damage. Another unique feature of FCPD compared to other causes of chronic pancreatitis is preserved glucagon secretion.

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Results arising from this thesis and previously published data would therefore support FCPD being considered as a specific type of diabetes rather than simply a cause of secondary diabetes.

Further studies, particularly epidemiological data and more biochemical and genetic marker analyses, are needed before drawing conclusions regarding the etiopathological basis of diabetes in these young Bangladeshi patients; this will ultimately help in the proper classification leading to improved management and better prevention of this group of disorders.

5. APPENDICES

5.1 Historical perspectives of classification of diabetes mellitus

Diabetes mellitus has been classified on the basis of clinical presentation, age at diagnosis or need for insulin to treatment. In the later part of the past century mainly ADA and WHO made concerted efforts to diagnosis and classification of diabetes. A brief chronology of classification of diabetes is presented in the table 5.1.

Table 5.1 Historical perspectives of the classification of diabetes mellitus

A. Historical Background		
	I. Major classes	
600-400 BC Charaka and Sushruta	Severe, lean, thirsty, dehydrated (genetic)	Obese, indolence, fond of sweet and fatty food (environmental)
1875 Bouchardat	Young, sever	Older, obese
1880 Lancereaux (France)	Diabete mairge (small, lean)	Diabete gras (large fat)
1910-1920	Juvenile-onset type JOD Growth onset type, Ketosis prone, brittle	Maturity-onset type MOD Adult onset type, Stable, Ketosis resistant, Lypoplethoric
1936 Himsworth	Insulin sensitive	Insulin insensitive
1951 Lawrence	Type 1: Absolute insulin deficiency	Type 2: Relative insulin deficiency
1965 WHO	On the basis of age at recognition of DM; I) Infantile childhood (age 0-14 yrs), ii) Young DM (15-24 yrs), Adult DM (age 25-64 yrs), Elderly DM (65+ yrs)	
	II. Other Classes	
1946-51 Lawrence	Lipoatrophic diabetes	
1955 Hugh-Jones	J-type diabetes ketosis resistant diabetes in the young (KRYD)	
1955-1956 Zuidema	Pancreatic calcification and diabetes in malnourished young subjects (Z-type)	
1961-1964 O'Sullivan	Glucose intolerance during pregnancy, gestation diabetes	
1974-76 Tettersal & Fajans	Maturity-onset diabetes in the young (MODY); Mason type	

B. Current Developments		
	I. Major classes	
1979-80 NIDDG*, WHO**	Insulin dependent diabetes mellitus (IDDM), Type I	Noninsulin dependent diabetes mellitus (NIDDM), Type II
1985 WHO	IDDM	NIDDM
1997-99 ADA and WHO	Type 1	Type 2
	Type 1 and type 2 – non synonymous to IDDM and NIDDM	
	II. Other classes	
1979-80 NDDG and WHO	Gestational diabetes mellitus	
1998 WHO	Criteria updated	
1985 WHO	Malnutrition related diabetes mellitus (MRDM)	
	Fibrocalculous pancreatic diabetes (FCPD) (Corresponding to Z-type)	
	Protein-deficient pancreatic diabetes (PDDM) (corresponding to J-type)	
1987 Cuttack, India	Protein-deficient diabetes mellitus (PDDM) to replace protein deficient pancreatic diabetes (PDPD)	
1995 Cuttack	Malnutrition-modulated diabetes mellitus (MMDM) to replace PDDM	
	FCPD due to fibrocalculous pancreatopathy to be classed with 'Pancreatic disease' in the division 'type of diabetes associated with pancreatic pathology'.	
1997 The ADA Expert committee***	Deletion of MRDM. Shifting to FCPD to "disease of exocrine pancreas" section	
	Monogenetic defects in B cell function: phenotype - MODY	
1998 WHO	Deletion of MRDM and PDDM. May be MMDM in place of PDDM for which more studies are needed	

*NDDG - National Diabetes Data Group, USA; ** WHO - World Health Organization

***ADA - American diabetes association

5.2 Estimation of Glucose

Glucose was estimated by enzymatic-colorimetric (GOD-PAP) method using reagents of Randox Laboratories, UK (Barham and Trinder 1972).

Principle

Glucose in presence of enzyme, GOD, form gluconic acid and hydrogen peroxide. Under presence of caltalytic enzyme POD reacts with phenol and 4- aminophenazone to form a red to violet quinoneimine, which is determined spectophotometrically. Formation of quinoneimine is proportional to the concentration of glucose in the medium.

Reagents composition

Buffer: Phosphate Buffer (0.1 mol/l, pH 7.0) and phenol (11 mol/l)

GOD-PAP Reagent: 4-aminophenazone (0.77 mmol/l), Glucose oxidase ($\geq$1.5 kU/l) and Peroxidase ($\geq$1.5 kU/l).

Standard: Glucose (5.55 mmol/l)

Procedure and precautions

The estimation was carried out using an automated chemistry analyzer, AutoLab (Analyzer Medical System, Rome, Italy). The autoanalyzer was calibrated everyday. Utmost precaution was taken to dispense samples into the sample cups and place it in the sample holder. GOD-POD reagent was aliquoted in the reagent reservoir and placed on the reagent slot. Reaction cells were rightly placed and the AutoLab was run for the glucose estimation. The aspirated sample and reagent volume was 5 µl and 500 µl respectively. Incubation time was 10 mins at 37°C. The reading for the spectrophotometric measurement was done at 500 nm.

Quality control of the assay was ensured by intera- and interassay coefficient of variation. The intra- and inter-assay CV was 3.5% and 3.2% respectively.

5.3 Estimation of C-peptide

Principle

C-peptide EIA is based on the competitive binding of antigen and antibody and microplate separation (Yalow and Berson 1971). C-peptide present in the sample and C-peptide-conjugate compete for the binding sites of a polyclonal C-peptide antiserum (C-peptide antibody), which is coated onto the microwells. Enzyme-Complex binds to

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the C-peptide-Conjugate and antibody complex, and stabilized the antigen-antibody binding. The unbound Enzyme-Complex is washed off. Substrate solution when added binds to antigen-antibody-conjugate complex, which yields the color. Addition of stop solution fixes the developed color. Measurement of optical density provides the value of the unknown sample. Amount of C-peptide in the sample is inversely proportional to the optical density of final reaction.

Reagents

1. Microtiter Wells- 96 Wells coated with C-peptide antiserum.
2. C-peptide Conjugate; 25 ml. C-peptide Conjugate in Stabilizing Buffer Solution
3. Enzyme-Complex; 25 ml. Complex containing Horseradish Peroxidase
4. Standard Set; consisting of 0, 0.2 0.7, 2, 4 and 16 ng/ml
5. Specimen Diluent, 3 ml.
6. Substrate Solution - TMB, 25 ml.
7. Stop Solution, 0.5M H_2SO4, 14 ml.
8. Wash Solution, 40X concentrated, 30 ml

Assay procedures

- Microwellplate and other reagents were brought to room temperature.
- Reference standards were constituted using 1.0 ml of distilled water.
- Serum samples already thawed mixed thoroughly by short and gentle vortex and spun briefly.
- Standards and serum samples (100 μl) were dispensed into wells in duplicates. Pipetting for a full plate was done within 10 minutes.
- Incubated for 5 minutes at room temperature, around 22°C.
- C-peptide conjugate (200 μl) was dispensed into each well in the same order as that of standards and the samples pipetted into the wells. Conjugate was dispensed holding the pipette vertically ensuring complete mixing of standard solution and serum samples is achieved.
- The plate was secured with plate sealer and incubated over night (16 hours) at 4°C.
- Next morning briskly shaken out the contents of the wells.
- The microtitre wells were rinsed 3 times with 350 μl working Wash Solution (WS) using microplate washer. Removal of residual WS droplets was ensured.
- 200 μl enzyme-complex was dispensed into each well and incubated for 60 minutes at room temperature.

- After incubation biskly shaken off the contents of wells and rinsed the wells 5 times with 350 µl working WS. Removal of residual WS droplet was ensured.
- 200 µl substrate solution dispensed into each well and incubated for 30 minutes at room temperature.
- 100 µl stop solution was added into each well to stop the enzymatic reaction. The stop solution was added at the center of the well.
- Optical densities were measured at wavelength 450 nm using Microplate Reader (BIOTECK).

Calculation of C-peptide values

C-peptide values of unknown samples were calculated using KINETICAL Software. The Software calls the reading of the Microtitre plate into default template and map for the desired assay. A semi log standard curve was constructed and C-peptide values were obtained by interpolation from the standard curve.

Quality control of the measurement was assessed by coefficient of variation (CV) of the assay. Intra- and inter-assay CV of the assay was 3.2% and 7.8% respectively.

5.4 Determination of anti-GAD and IA2-Ic autoantibodies

5.4.1 *Transformation of GAD65 and IA-2ic cDNA*

Both GAD65 and IA-2ic cDNA were transformed using in Supercompetent Epicurian coli (Cat No 200236, Stratagene; describes below). An aliquot of Supercompetent Epicurian Cells were thawed on ice. Cells were mixed gently by hand and 100 µl of the cells suspension pipetted into a 50 ml falcon tube in duplicate. To the cells 1.7 µl B-mercaptoethanol (25 mM) was added and mixed gently by swirling, placed on ice for 10 minutes and swirled gently in every 2 minutes. To the cell suspension 1 µl (100 ng/µl) GAD65 or IA-2ic cDNA was added and in another tube 1 µl of pUC18 as positive control. The tube was incubated on ice for 30 mins and then heat pulsed at 42°C in a water bath for 45 sec and incubated on ice. Supercompetent cells suspension of 100 µl with 1.7 µl B-mercaptoethnoal but without cDNA was run as negative control. LB medium (0.9 ml) [LB Medium: 1 Liter contains Tryptone Bacto 10 g, Yeast Extract Bacto 5 g, NaCl 0.5 g, pH adjusted 7.5 autoclaved, cooled and Ampicillin 50 µg/ml] added was added to the tube and incubated at 37 °C for 1½ hour with shaking at 225-250 rpm in water bath. The transformed cells were plated using a spreader with 5, 10, 20, 50 100, 125 and 150 µl on LB agar plate with ampicillin. All the content of the tube containing

PUC18 plated on one plate. Plates were incubated over night at 37°C. On the next morning no growth in the plate containing PUC18 compared to other plates confirmed validity of the transformation procedure. A single colony was picked up and inoculated in 5 ml LB medium with ampicillin and grown on shaker for 2 hours or over night at 37°C. Cloudy appearance of the media indicated good growth of the colony.

Supercompetent Cells

Supercompetent Epicurian E coli (Catalogue No 200236, Stratagene) are Epicurian Coli XL1-Blue subcloning-grade competent cells and Epicurian Coli XL1-Blue competent cells are endonuclease deficient (*endA1*), recombination deficient (*recA*) and *hsdR*⁻. The mutation *hsdR*⁻ prevents the cleavage of clone DNA by EcoK (*hsdR*) endonuclease system, and the *recA* mutation helps ensure insert stability. The *endA1* mutation greatly improves the quality of plasmid miniprep DNA. The Epicurian Coli XL1-Blue subcloning-grade competent cells and competent cells contain the $lac^{q}\Delta M15$ gene on F' episome, which allow blue-white color screening of recombinant plasmids.

LB Plate Agar

LB medium: 1 litre

Agar 15 g in 1 liter H_2O

pH - 7.5 using NaOH

Autoclaved and cooled down to 50 °C, Ampicillin (50 μg/ml) was added, poured (30-35 ml of medium) on to 100 mm Petridish and allowed to hardened and finally stored at 4 °C.

Buffer and solutions needed for preparation and purification of plasmid DNA.

5.4.2 Isolation of the plasmid DNA

From the overnight culture 1.5 ml medium was transferred into to a sterile microcentrifuge tube, centrifuged at 10000g for 1 min and then supernatant discarded. Pellet was resuspended with 100 μl ice-cold resuspension buffer (Resuspension Buffer: Tris-HCL 25/50mM, EDTA 10mM, RNAse A 100μmol/ml, Glucose 50mM) and incubated at room temperature for 5 minutes. After the incubation freshly prepared lysis buffer was added to the tube (Lysis Buffer: NaOH 0.2 M, SDS 1%), mixed properly by inversion and again incubated on ice for 5 mins. Ice-cold potassium acetate solution (150 μl) (Potassium Solution: 11.5 ml glacial acetic acid in 28.5 ml H_2O gives K 3 M and Acetate 5 M, stored at 4°C) was added to the tube and mixed by inversion and/or gentle vortex

for 10 sec and again incubated on ice for 5 minutes. After the incubation centrifugation was done at 12000xg for 5 minutes. The supernatant was transferred into a fresh tube avoiding the precipitate. RNase A (20 μg/ml) was added and incubated at 37°C for 20 minutes.

One volume of TE (Tris-HCL 10mM, EDTA 1mM, pH7.5) saturated phenol/ Choloroform (24:1) mix was added into the tube and vortexed for 1 min, centrifuged at 12000xg for 2 minutes. The supernatant was again transferred into a fresh tube and added to it 2.5x volume of 100% ethanol and 10 it 1/10 th volume of 3M sodium acetate, pH 4 and kept on dry ice for 20 minutes and / or at -20 °C for over night, centrifuged at 4 °C for 1 min at 13000 g. Supernatant was discarded, the pellet was washed with 70% ethanol, centrifuged and supernatant decanted and the pellet was dried and reconstituted using nuclease-free H_2O. Concentration and purity was evaluated by spectrophotometry at 260/280 nm (Appendix 6.11). Working concentration (1 μg/μl) of both GAD and IA-2ic cDNA was prepared and stored at -20°C for future translation reaction.

5.4.3 Translation reaction

GAD65 and IA-2ic translation reaction set-up side by side. This is a coupled transcription-translation protocol developed by Promega (Promega, Madison, WI). The translation reaction volume was 50 μl. Components of translation reaction were pipetted into a 1.5 ml eppendorf tube, vortexed, spun it for 10 sec at 13000xg and incubated for 90 minutes at 30°C in water bath. After the translation reaction was completed, the reaction mix was then diluted to 250 μl using TBST (Trisma-Base 50 mM, NaCl 150 mM, Tween-20 1%) and translated protein was eluted using ion exchange chromatography column.

5.4.4 Purification of translated protein

The translated protein was purified using a Sephadex Gel Column (Appendix 6.4.4.1). The gel column was equilibrated with approximately 10 ml of TBST buffer (Trisma-Base 50mM, NaCl 150mM, Tween-20 1%). The equilibrated gel column was placed on a clamp. Diluted translate was added over the column carefully, allowed the equilibration buffer to enter the gel bed completely and the translated protein was collected, approximately 800-1000 μl, in a 1.5 ml tube. The protein fraction containing the protein of interest eluted with the haemoglobin fraction, which is easily identified by red color.

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Table 5.2:

Translation reaction protocol of GAD65 and IA-2ic

TNT Translation of GAD65		TNT Translation of IA-2ic	
Component	Volume	Component	Volume
T^{NT} Rabbit Reticulocyte	25 µl	T^{NT} Rabbit Reticulocyte	25 µl
T^{NT} Buffer	2 µl	T^{NT} Buffer	2 µl
T7 Polymerase	1µl	SP6 Polymerase	1µl
Amino acid mix except methionine	1 µl	Amino acid mix except methionine	1 µl
^{35}S Methionine	4 µl	^{35}S Methionine	4 µl
RNAse inhibitor	1 µl	RNAse inhibitor	1 µl
DNA template	1 µl	DNA template	1 µl
DNAse and RNAse free H_2O	14 µl	DNAse and RNAse free H_2O	14 µl

Sephadex column

Sephadex column (NAP™-5 Column) was obtained from Amersham Biosciences. The NAP-5 columns are prepacked disposable column contains Sphadex® G-25 Medium of DNA Grade for rapid and convenient desalting and buffer exchange of nucleic acids, protein and oligonucleotides (≥10 mers).

The column contains Sephadex G-25 Medium of DNA grade in distilled water containing 0.15% Kathone® CG/ICP Biocide as preservatives. The gel bed dimensions are 0.9x2.8 cm. Maximum volume may be eluted is 0.5ml and volume of eluted samples is 1 ml.

5.4.5 Determination of incorporation of ^{35}S Methionine into translated protein

From the translation (50 µl) reaction mix 2 µl was removed and added to 248 µl NaOH/2%H_2O_2 (1 M), mixed thoroughly by vortexing and incubated at 37°C for 10 mins. Here H_2O_2 removes the color and NaOH neutralizes the acidity of the mix. After the incubation 900 µl ice-cold 25% TCA was added to precipitate the translated protein and incubated on ice for 30 mins. Whatman GF/A glass filter was washed with 5% TCA. Precipitated translated protein was collected by vacuum filtering of 250 µl of reaction mix. The filter paper was first rinsed with ice-cold 5% TCA and then with 1-3 ml of acetone and allowed the filter paper to dry at room temperature. The filter was placed in 5 ml of scintillation fluid and counted in a liquid scintillation counter (a).

To determine total counts present in the reaction 5 μl from TCA mix directly spotted onto the filter and dried it for 10 minutes. The filter then placed in 5 ml scintillation liquid and counted in a liquid scintillation counter (b).

Percent (%) incorporation of ^{35}S methionine in translated protein was calculated as follows:

$$\textbf{Percent (\%) incorporation} = \frac{\text{cpm of washed filter (a)}}{\text{cpm of unwashed filter (b) x 50}} \times 100$$

5.4.6 Millipore MultiScreen 96 well plate

The 96-well Millipore MultiScreen 96 well plate is opaque, hydrophilic and low protein binding in nature. It is designed fro to scintillation counting. The plate has a Durapore membrane with a 0.45 microne (μm) pore size and a plastic under-drain to prevent cross contamination.

5.4.7 Protein A Sepharose medium

Protein A Sepharose CL-4B is a Protein A, derived from *Staphylococcus aurius,* immobilized to Sepharose CL-4B. Protein A binds to the Fc fragment of Immunoglobulins through interaction with heavy chain. It binds to IgG from a variety of mamaliam species and to some IgM and IgA. It acts as a powerful tool to isolate and purify classes and subclasses and fragments of from biological fluid and cell culture media. Since only the Fc region is involved in binding, the Fab region is available for binding antigen. Hence, Protein A Sepaharose CL-4B is extremely useful for isolating of immune complexes.

Protein A Sepharose CL-4B is supplied lyophilized in the presence of water soluble stabilization agents. These agents need to be washed away. Required amount of power weighed out (1 g of powder of Protein A Sepahrose CL-4B gives about 4-5 ml volume of medium), poured in buffer or distilled water, suspended by gentle swirling- no vortex. The medium swells immediately or may be kept over night at 4°C. The medium then washed twice using working (TBST) buffer. During washing rapid settling of the medium were achieved by low speed centrifuge (less than 500 g is recommended to avoid crushing the medium). Finally volume was adjusted to Protein A Sepharose concentration 1 mg per 50 μl of suspension.

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5.5 Reagents and buffer of PureGene Blood DNA Kit (Gentra System, Inc)

RBC lysis buffer

NH4Cl	155 mM
KCl	10 mM
EDTA	10 mM

WBC lysis buffer

Nacl	400 mM
Tris-HCl	10 mM
EDTA	10 mM
SDS	10 %

Protein precipitation buffer

Amoniumacetate	7 mM

DNA hydration buffer

Tris-HCl	10 mM
EDTA	1 mM

5.6 Quantification and assessment of the quality of DNA yield

To quantify the yield of a DNA extraction absorbance of 1 in 30 dilution of an elute (10 µl in 290µl) may be read at 260 and 280 nm. The OD_{260} of 1.0 gives an indication of the quantity, corresponding to a concentration of 50 ug/ml of double stranded DNA, consequently values were multiplied by 1.5 to get DNA concentration in µg/µl. Quality can be determined by the ratio of the OD at 260 nm and 280 nm (OD_{260}/OD_{280}). A ratio of 1.8 or greater indicates a pure preparation of DNA and a ratio significantly less than 1.8 indicates contamination with protein.

5.7 Polymerase chain reaction

5.7.1 Introduction

The polymerase chain reaction (PCR) is an *in vitro* method of nucleic acid synthesis by which a specific segment of DNA can be replicated exponentially (Saiki et al, 1985; Mullis and Faloona, 1987). The reaction requires a template, usually double-stranded genomic DNA, cDNA or mRNA. Synthetic primers (also called oligonucleotide and amplimers), anneal to opposite strands of the double stranded DNA flanking the target segment and direct the polymerisation to produce copies of complementary DNA. A

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DNA polymerase drives the synthesis in a reaction buffer with deoxynucleotide triphosphates providing nucleotides and magnesium ions provide essential monovalent cations for the enzyme to function. The resultant copies are also capable of binding the primers, and act as a template for further amplification. Thermally stable DNA polymerases allow repeated cycles of denaturation of the double-stranded DNA, annealing of the primers to their complimentary sequences, and extension of the annealed primers. Thus successive cycles theoretically double the amount of target DNA synthesised in the previous cycle. The target DNA segment is exponentially amplified at a rate equivalent to approximately 2^n, where 'n' is the number of cycles of amplification completed.

5.7.2 Standard PCR conditions

PCR reaction conditions were standardized, although sometimes target DNA segments required optimization reflecting the length of the target sequence, the magnesium ion concentration and primer annealing characteristics. Molecular applications downstream of the PCR also influenced adjustments in the reaction profile, cycle profile and choice of thermostable DNA polymerase used.

Standard PCR cycling condition comprises of an initial step of incubation at 95°C followed by 25-40 cycles of denaturation for 30-60 sec at 94°C, annealing for 30-60 sec at 50-68 °C elongation for 30-60 sec at 72°C followed by final step of elongation for 10 minutes at 72°C. The final extension period was followed by stand by at 4-6°C.

5.7.2.1 Adjustments to cycle profile

Denaturation: The temperature and time in for this most instances remained constant. Increase in temperature and time would only be necessary for very long target DNA fragments (>2Kb), or difficult sequences (long tracts of GC repeats). Prolonged higher temperatures (>94°C) are detrimental to processivity. The half-life of Taq DNA Polymerase is 60 minutes at 94°C, but only 10 minutes at 97°C.

Annealing: A melting temperature (Tm) based on base composition for each primer was either calculated by the design programme or available with the primer database. PCR reactions were initially performed at sub-optimal annealing temperatures, 4-6°C below primer's Tm. The temperature was then sequentially increased initially by increments of 2°C and then fine tuned by 1°C increments, until the specificity required was achieved. An annealing time of 30-45 seconds was adequate for all templates less than 1Kb.

Extension: Extension rate at 72°C is about 2-4 Kb per minute for thermostable DNA polymerases. Target DNA segments amplified in this works were usually less than 1000 bp, usually 30 second extension time was more than adequate.

Cycle number: The cycle number was adjusted according to the PCR downstream application and the efficiency of the primers/PCR reaction combination. Cycle numbers above 27 can generate 'junk ' non-specific DNA fragments so 'the fewer cycles the better'. For direct DNA sequencing PCR amplification for target DNA was done for 25 cycles. PCR for fluorescent micosatellite markers required fewer cycles (20-25 cycles) as sensitivity of detection was not a limitation and reduced problems of background interference. PCR-RFLP applications required sufficient cycles to generate enough DNA (usually 30-40 cycles), so that following restriction endonuclease digestion all resulting fragments could be visualized with ethidium bromide staining.

PCR blocks: PCR machines used included: MWG Biotech, Perkin Elmer.

5.7.3 Standard PCR reaction mix

Optimum concentration of components in PCR reaction is critical. Difference in reaction volume requires adjustment of components. So, components are calculated on the basis of reaction volume. For a standard PCR reaction in a volume of 25 μl the components are shown in the table 5.3.

5.7.3.1 Adjustments to PCR reaction mix

Reaction Buffer: Appendix I. Additionally a buffer containing $(NH_4)_2SO_4$ provide stringent primer-annealing conditions over a wider range of annealing temperatures and Mg^{2+} concentrations.

Mg2+: Monovalent cations are essential for the enzymatic action of DNA polymerases. Concentration of $MgCl_2$ in the buffer needs to be optimised for each primer set by titrating final concentrations of between 0.5 mM to 3.0 mM, (the concentration window most reactions tended to work at). Buffers based on $(NH_4)_2SO_4$ and containing 1.5 mM $MgCl_2$, required little to no adjustment.

dNTPs: (100 mM each dCTP, dTTP, dATP, dGTP). These were mixed at an equimolar concentration and used in sufficient quantity so as not to be rate limiting but without excess. Excess increased the amount of non-specific fragments and could compromise downstream applications.

Primers (oligonucleotides): Apart from primer design (Appendix 5.7.7), a minimalist approach was adopted. Primary reactions establishing PCR conditions contained excess of each primer, 20-50 pmoles/25µl reaction subsequently the amount necessary was titrated, usually less than 5 pmoles/reaction of each primer did not work efficiently.

Thermostable DNA polymerases: The standard thermostable DNA polymerase isolated from *Thermus aquaticus* (*Taq* DNA polymerase Promega UK), and recombinants of this (HotstarTaq, Qiagen), were used for most PCR experiments.

Table 5.3:

Standard protocol for PCR in 25 µl reaction volume

Components	Range	Volume
PCR Buffer	1/10th of reaction volume	2.5 µl
$MgCl_2$ (25 mM)	1.0-5 mM (1.5 mM)	~ 1.5 µl
dNTPs (25 mmol/l)	200 µmol/l	0.2 µl
Primers: Forward (10 pmol/µl)	5-20 pmoles	1.0 µl
Reverse (10 pmol/µl)	5-20 pmoles	1.0 µl
DNA *Taq* polymerase (5 units/µl)	1-5 units	0.2 µl
Template DNA	10-50 ng	5 µl
Water (DNAse free and UVied)		~ 14.5 µl

For a particular PCR a master mix was prepared first and added to DNA template.

5.7.4 Quality control of PCR

PCR is an extremely sensitive technique with the potential to produce 1 x 10^6 copies from a single copy of target DNA. There was always the possibility of contamination producing false-positive amplification results. Contamination from airborne human biological debris, such as skin cells or hair roots (>60% content of dust) was avoided by following good aseptic precautions in a designated work area. Materials such as plastics, tubes, microtitre plates, were virgin and as an extra precaution, exposed for 15 minutes at 254 nM UV irradiation to degrade any contaminating DNA. HPLC pure water also subjected to UV irradiation before use as water seen as the common source of contamination.

Chance of contamination was also controlled by using aerosol resistant pipette tips (filter tips) and where possible in association with multi-channel pipettes. Two PCR negative controls, using water as a substitute for the DNA template, were included in every assay.

5.7.5 Different modifications of PCR

HotStart PCR

This is a very simple modification to the standard PCR that was employed in most PCR reactions in the present study. This modification allowed to overcome the considerable amount of mispriming which used to occur at low reaction temperatures, when the completed reaction mix is ramped from room temperature to its first denaturing step of the first cycle. The problem was over come by development of 'HotStart' polymerase enzyme. Polymearse enzyme only released from the packing material after optimum incubation.

For most of the experiments HotStar *Taq*™, QIAGEN, UK Ltd was used. $MgCl_2$ is incorporated into the PCR Buffer of the HotStart Taq, which also has $(NH_4)_2SO_4$ in the buffer. All the steps were followed through and through including addition of *Taq*, in this case it was 'HotStart *Taq*. Instead of initial incubation for 3 minutes the reaction mix preheated for 15 minutes at 95°C to activate the thermostable polymerase prior to first cycle of standardized PCR profile above.

Nested PCR

Nested PCR uses two sets of flanking primers. The target DNA sequence of one set of primers (termed 'inner primers') is located within the target sequence of the second set of primers termed 'outer primers'. In practice, standard PCR reaction is first run with the source DNA using outer primers. Then a second PCR reaction is run using PCR product of the first run as template DNA with 'inner primers'. This procedure increases the sensitivity of the assay as it reamplifies the product of the first assay. It also increases the specificity of the assay because the inner primer' only amplify the product of the first PCR reaction.

PCR generated RFLP

The principle this modified PCR is 'designing of primers with one or two nucleotide inserted either in the forward or reverse primer so that a restriction endonuclease splicing site is generated', where in polymorphism does not disrupt any restriction site. PCR generated RFLP was followed in studying the NeuroD/BETA2 gene polymorphism.

5.7.6 Visualization of PCR product

In all experiments following completion of PCR, the product was run out on a 10 cm x 10 cm x 0.6 cm thick 2.0% agarose gel to assert the amplification of the target DNA or resolve RFLP fragments.

Preparation of 2% agarose gel: 2 gagarose weighed into polypropylene conical flask containing 100 ml working TBE (TBE working Buffer: Tris-Base 89mM, Boric Acid 89mM, EDTA 2mM). Agarose and buffer solution mixed by swirling of the flask. It was then heated to boiling point in a microwave oven with intermittent mixing. The gel was slightly cooled and ethidium bromide (0.5 μg/ml) was added. The gel was then poured into a horizontal gel mould, combs inserted, and allowed to polymerize. The gel was subsequently submerged in a horizontal electrophoresis tank filled with working TBE buffer.

PCR product of 5.0μl was mixed with 1-2 μl of loading buffer [DNA loading Buffer: Bromophenol blue 0.25%, Ficoll (Type 400) 15%, storing at room temperature] in strip tubes or microwell pate, as appropriate. Samples were then loaded on to the gel. Known DNA ladder was run in each lane of gel. Electrophoresis carried out at 80 V (6V per cm) for 30 minutes

The resolved DNA fragment(s) were then visualized using Gel-documentation system (Transilluminator and Alpha Imager) under UV and compared the target fragment with known DNA marker (Ladder DNA, mostly using a 100 bp ladder (Promega UK Ltd). Printed records were preserved as well as electronic version.

5.7.7 Design of primers /oligonucletides

Specific amplification of a DNA fragment (amplicon) by definition requires a unique set of primers (amplimers). Unique pairs of amplimers were designed using software such as PRIMER v3 at the Human Genome Mapping Project website (www.hgmp.mrc.ac.uk). Criteria for a good primer are as follows:

1. Optimum primer size 20 bases (min 18b ases,max 27 bases).
2. Optimum annealing temperature 60°C (min. 57°C, max 63°C)
3. Oligonucleotide melting temperature formula (Rychlik et al 1990)
4. Maximum Tm difference between primers, preferably 55-60 °C
5. GC content optimally around 50% (20%-80%)
6. Maximum complementarity, default 8 bases, maximum allowable local self-complementarity and between forward and reverse primers, and maximum 3' complementarity, default 3 bases, the most 3' part of primer most important for annealing specificity.

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Primer pairs identified were checked for cross homology with all other known human DNA sequences using the *BLASTn* search at HGMP resource Web site.

5.8 Restriction fragment length polymorphism

Majority of human DNA are not involved in coding sequence, non coding amino acids therefore are not subject to tight selection. This justifies that difference in DNA sequence between individuals is not a rarity. These natural variations usually take the form of simple base changes and are called polymorphism. These natural variations of DNA sequences are also often subject to alter restriction enzymes/endonuclease recognition site(s) of the DNA sequence, which in tern alter fragments of the DNA sequence on digestion with particular endonuclease, so is the term restriction fragment length polymorphism (RFLP) coined. In simple term RFLP has considered as simple point polymorphisms that disrupts, create or destroy, a restriction site. This property of DNA sequences was exploited to screen for variants, either coding or noncoding DNA in the population. The target region was PCR amplified from genomic DNA using specific primers, and then the product digested with the appropriate restriction enzyme(s).

5.8.1 Endonuclease

Endonuclease(s) are short stretch, usually 4-6 bp and very rarely more that 6bp, of double stranded DNA sequences which has a property to recognize the identical sequence and excise double stranded the DNA sequence at or close proximity, sometimes few base pairs away from recognition site. All most all the endonucleases were initially isolated from bacteria and later synthesized in the Lab.

5.8.2 Standard restriction enzyme digestion reaction

In general restriction enzyme digestion is carried out in a volume 15-20 µl. A standard protocol for restriction enzyme digestion is as follows:

Restriction enzyme digestions were carried out in long (20 µl) PCR plates. The plate top was heat-sealed with aluminium foil and incubated between 5-8 hours at recommended temperature for either in a heated water bath or dry-block.

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Table 5.4:

Standard protocol for restriction enzyme digestion

Component	Volume
PCR product	~5.0 µl
Restriction enzyme Buffer*	2.0 µl
Acetylated BSA (10 µg/ul)	0.15 µl
Spermidine (20 mM)	0.15 µl
Endonuclease (usually 5-10 units)	~ 1.00 µl
Sterile, deionised water	~ 7-12 µl

*Restriction enzyme buffer supplied in 10x of working concentration.

5.8.3 Visualization of the product enzyme digestion

After completion of digestion restriction fragments were size separated, visualised and scored on standard agarose gels or metaphor gels, with adjustment of agarose percentage according to size of digested fragments. To resolve shorter fragments higher percentage agarose gel, usually 4%, was used.

5.8.4 Adjustment to restriction digestion reaction

PCR product: Volume of PCR used in RFLP reaction depends on yield of PCR reaction. Checking of PCR product guides to calculate.

Amount of endonuclease(s): Use of enzymes requires some adjustment. Product guide provides information of calculated amount of DNA supposed to be digested by one unit of enzyme. The given information of the product database then cross-checked with band resolved for amplified DNA in PCR check. Usually 5-10 units of enzyme was sufficient for 5 µl of PCR product digestion. Enzymes are preserved in high concentration of glycerol, which prevents its activation. It is recommended that glycerol concentration should not exceed around 20% in the enzyme digestion mix. So, use of huge amount of enzyme may affect digestion reaction.

Bovine serum albumin: Some enzymes exert its maximum activity in the presence of BSA at 25mM concentration (BSA supplied 10 mg/ml; in 20 µl reaction volume 0.05 µl of stock was used). However, use of BSA invariably does not enhance the enzymatic activity.

Use of spermidine: Spermidine at 0.2 mM concentration is widely believed to accentuate enzyme activity.

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5.9 Endonucleases used in this study

Endonucleases used in this study were procured (unless otherwise stated) from New England BioLab (UK) Ltd unless otherwise stated.

***Hph* 1** Cat No: R0158 (S/L)
Recognition sequence:
5-'GGTGA$(N)_8$^-3'
3-CCACT$(N)_8$^-5'
Reaction Conditions: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9 @ 25°C).
Incubation: at 37°C. Thermal inactivation: at 65°C for 20 mins.

***Nde* I** Cat No: R0111 (S/L)
Recognition sequence:
5'-CA^TATG-3'
3'-GTAT^AC-5'
Reaction Conditions: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9 @ 25°C).
Incubation: at 37°C. Thermal inactivation: at 65°C for 20 mins.

***Hae* III** Cat No R0108 (S/L)
Recognition sequence:
5'-GG^CC-3'
3'-CC^GG-3'
Reaction Conditions: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9 @ 25°C).
Incubation: at 37°C. Thermal inactivation: at 37°C for 20 mins.

***Mwo* I** Cat No: R0573 (S/L)
Recognition sequence:
5'-GCNNNNN^NNGC-3'
3-CGNNNNN^NNCG-5'
Reaction Conditions: 150 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9 @ 25°C).
Incubation: at 60°C. Thermal inactivation: No

***Sma* I** Cat No: R0597 (S/L)
Resignation sequence:
5'-CCC^GGG-3'
3'-GGG^CCC-5'
Reaction Conditions: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9 @ 25°C).
Incubation: at 25°C. Thermal inactivation: at 65°C for 20 mins.

***Ear* 1** Cat No: R0528 (S/L)
Recognition sequence:
5'-CTCTTC(N)^-3'
3'-GAGAAG(N)^-5;
Reaction Conditions: 10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.0 @ 25°C).
Incubation: at 37°C. Thermal inactivation: at 65°C for 20 mins.

***Taa* I** Cat No: ER1361/1362
Fermentas AB (Helena BioSciences, Lithonia);
Recognition sequence:
5'-CAN^GT-3'
3'-TCN^CA-5'
Reaction condition: 33 mM Tris-acetate, 10 mM Magnesium acetate, 66 mM Potassium acetate, 0.1 mg/ml BSA (pH 7.9 at 37°C),
Incubation: at 65°C. Thermal inactivation: No

Afl III Cat No: R0541 (S/L)
Recognition sequence:
5'-A^CRYGT-3'
3-TGYRC^AT-5'
Reaction Conditions: 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.9 @ 25°C).
Incubation: at 37°C. Thermal inactivation: at 80°C for 20 mins.

***Fnu*4HI** Cat No: R0178 (S/L)
Recognition sequence
5'-GCN^GC-3'
3'-CGN^CG-'5
Reaction Conditions: 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9 @ 25°C).
Incubation temperature: 37°C. Thermal inactivation: 65°C for 20 mins.

5.10 DNA ladder (100 bp)

A 100 bp ladder DNA used as molecular weight standards for agarose gel electrophoresis. The DNA includes fragments ranging from 100-1,517bp, giving approximately 12 bands suitable for use. The 500 and 1,000 base pair bands have increased intensity to serve as reference points. The approximate mass of DNA in each of the bands is provided (assuming a 0.5 µg from 1 µl load from stock) for approximating the mass of DNA in comparably intense samples of similar size.

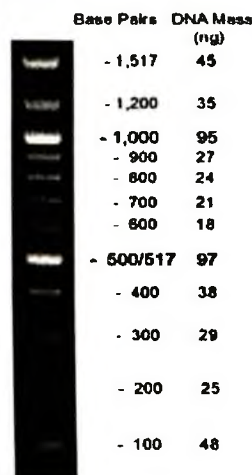

Figure 5.1: DNA ladder (100 bp), 1 ul (equals to 0.5 µg), visualized by ethidium bromide staining on 1.3% agarose gel.

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5.11 DNA sequencing

5.11.1 Principle

Direct sequencing of double stranded PCR generated templates were carried out using an ABI 373 Automated Sequencer. The cycle sequencing method used was quintessentially the chain termination method of Sanger and Coulson with modification. DNA sequencing uses a modified thermolabile T7 DNA polymerase to extend from a primer annealed to a DNA/RNA template. The substitution of this polymerase with a thermostable polymerase introduces an amplifying thermal cycling stage in the sequencing reaction. This offers a number of advantages over conventional sequencing.

1. A single oligonucleotide primer in the reaction results in the linear amplification of one strand of the double stranded template, reducing template requirement
2. High denaturation temperature during cycling circumvents the problems of rapid re-annealing of linear dsDNA templates.
3. High annealing temperatures during cycling increase the stringency of primer hybridisation.
4. High polymerisation temperature decreases the secondary structure of DNA templates allowing polymerisation through highly structured regions.

The ABI 373 Automated Sequencer sequencing technology is based on the sensitive laser detection of four different fluorescent dyes. The ABI Prism DyeDeoxy^{TM} Terminator Cycle Sequencing Kits uses each chain terminating dideoxyribonucleotide triphosphates, ddGTP, ddATP, ddTTP, and ddCTP labelled with a different wavelength permitting a single reaction tube single gel lane.

5.11.2 Considerations of DNA template for ABI 373

The double stranded DNA template was generated by PCR. The length and quality of the sequence was determined by a number of factors:

The ABI 373 Automated Sequencer, Well to Read gel plates of 36 cm has an ability to sequence a maximum of 400-500 bases.

The terminal sequences at both ends of the PCR template were sometimes difficult to interpret, often due to excess unincorporated dNTPS. This was generally avoided by amplifying a region in excess of the target sequence, and by overlapping contiguous templates.

In some circumstances the PCR Primers were inadequate for sequencing so 'nested' primers were utilised internal to the original PCR primers.

PCR product template had to be of sufficient quality and quantity. Some time template needs to be purified.

5.11.3 Fluorescent cycle sequencing of PCR template

Fuorescence-labelled cycle sequencing reactions were performed in 20 µl volumes using an ABI sequencing kit (Perkin Elmer). Each reaction contained 100 ng of PCR products template DNA, 8 µl of Terminator Ready Reaction Mix mix (containing AmpliTaq DNA polymerase, dNTPs, magnesium, fluorescent dichlirorhodamine dye Terminators, Tris-HCl) (PE Biosystems) and 3.2 pmole primer.

5.11.4 Dye terminator cycle sequencing reaction

In the cycle sequencing reaction the two strand of DNA are amplified separately ie for forward and reverse strand two separate reactions are set. This was a 20µl volume reaction. The dye terminator is ready kit containing four nucleotides, *Taq* polymerase in PCR buffer.

Table 5.5: Components of dye terminator cycle sequencing reaction

Components	Volume
Terminator Ready Reaction Mix (ABI)	8.0µl
PCR template 5.0µl	10-30ng/µl
Primer (Forward or reverse) 3.2µl	3.2 pmole
HPLC H_2O (ultra pure H_2O)	3.8µl
Total Reaction Volume	20.0µl

The cycle sequencing reaction was carried out in 0.5 ml thin wall tube. Reaction was over-layed with a drop of mineral oil. The reaction was performed in Cetaus thermal cycler.

The Reaction profile included rapid ram to 60°C and then step of denaturation at 96°C for 30 sec followed by step of annealing at 50°C for 15 sec and finally extension for 4 minutes at 60°C for 25 cycles.

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5.11.5 Purification of dye terminator reaction product

After PCR, extension products were purified from unincorporated dye-labelled terminators by ethanol/sodium acetate precipitation. It used to be a long procedure to purify cycle sequenced DNA template but use of new dye ABI terminator kit rather became simpler.

Procedure

In a microcentrifuge tube 2µl of 3 M sodium acetate (pH 4.6) and 50 µl of 95% ethanol was taken.

All 20 µl of the reaction contents were transferred to the Sodium acetate and ethanol containing tube, votexed and kept on ice for 30 minutes.

Centrifuged at 13000 rpm for 30 minutes.

Supernatant was carefully removed and pellet rinsed with 125 µl 70% ethanol and centrifuged at 13000 rpm for 5 minutes.

Supernatant decanted and tubes air-dried for 10-15 minutes and dried pellet resuspended in 5 µl auto-sequencing gel loading buffer (5:1 mix of Deionized Formamide: 25 mM EDTA, pH 8.0, Blue Dextran 50mg/ml), completely dissolved by vortexingand spun it down.

DNA samples were denatured at 95°C for 5 minutes and immediately placed on ice until samples were loaded on to sequencing gel.

5.11.6 Polyacrylamide sequencing gel

A 6% polyacrylamide (acrylamide/bisacrylamide 19:1) gel was prepared by dissolving 40 gm of urea (ICN Biomedical Inc.) in 28 ml of distilled water with 12 ml of 40% acrylamide/bis solution (19:1, Gradipore Ltd) and 8 ml of 10 x TBE. The gel solution was mixed in a beaker and stirred until completely dissolved. Meanwhile, the glass plates from a vertical 25 x 42 cm sequencing gel apparatus (Applied Biosystems, Perkin-Elmer) were thoroughly cleaned using glass plate washing solution and distilled water. Then, the gel solution was mixed with 400 µl of 10% (W/V) Aammonium persulfate (APS, Kodak Chemicals), and 40 µl of TEMED (N, N, N', N', - tetramethyl ethylene diamine) and immediately the solution was poured between the two glass plates separated by 0.4 mm spacer. Finally a well-forming comb (36-well sharks-tooth comb) was inserted immediately and the gel left at the room temperature to set for more than 2 hours.

5.11.7 Automated ABI 373 gel run

After 2 hours, when gel polymerised properly the gel plate loaded on to the ABI 373 Sequencer and scanned the gel for integrity. A pre-electrophoresis was done at 2000 volt, 40 watts for 20 minutes in working TBE (Tris-HCl 89 mM, Boric acid 89 mM and EDTA 2 mM). Sequencing sample(s) was loaded in the gel the Sequencing Gel Analysis Software was retrieved and sample sheet was prepared. A pre-run of 10 min was done and gel analysis started and run for 12 hours run at 2000 volts, 40 watts for 12 hours at 30°C.

5.11.8 Sequence gel analysis

The digitalised raw sequence was visually inspected and after attainment of correct and optimal base spacing (between 9-12 scans), samples files were imported in to the Sequence Navigator Software ABI Inc). The wild sequence corresponding to the template DNA, retrieved from Genbank (GDB), was imported to the Sequence Navigator program and designated as reference sequence. The multiple alignment comparisons using a Cluster algorythm were performed between the reference sequence and imported sequences. Forward and reverse (reversed and complementary) sequences were included for al template comparisons. The mismatch option of the program identified mismatched/mis-aligned sequence compared with the reference sequence within the sequence text file. The mismatched regions were examined on the electrophoregrams to establish whether they were genuine mutations or sequence artefacts. All potential mutations had to be confirmed by being present in both forward and reverse sequence data for each sample.

Potential mutations/polymorphism in individual sample was further confirmed by repeating the sample PCR sequencing reactions. This was necessary to eliminate false positives due to random non-template incorporation of dNTS by *Taq* DNA polymerase during PCR reaction.

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CHAPTER 6

ACKNOWLEDGEMENT

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6. ACKNOWLEDGEMENT

Some years have passed since I started working in BIRDEM and I got absorbed into biosciences. My time as a doctoral student has been shared between endless experiments in the laboratory and meeting many knowledgeable, inspiring and nice people. I would like to mention few names to acknowledge and express my sincere gratitude to them; without their encouragement and support completion of this thesis would not have been a reality.

Late National Professor M Ibrahim, founder of the Diabetic Association of Bangladesh (DAB) the parent organization of BIRDEM for his farsighted vision and dedicated effort that this enlightened institute has been built for fighting diabetes. I feel proud for the opportunity to work in this thriving institute and carrying out research in the field of diabetes.

Prof Graham A Hitman, my Supervisor, who introduced me in the field of genetics, for his supervision, encouragement and affection that he showed to me throughout the period. His professional attitude towards a scientific problem is inspirational.

Prof AK Azad Khan, my Co-supervisor, for his supervision, guidance, encouragement and moral support to me. I am always inspired by his positive attitude and dynamic personality and untiring working capability.

Prof Liaquat Ali, my Co-supervisor, for his supervision, critical suggestions and scientific approach. His untiring working capability has always been inspirational.

Prof SM Keramat Ali, for his valuable suggestions and guidance at the critical juncture of my carrier.

Prof Kishwar Azad and her group members - Dr Abu Hassan M Abdullah, Dr Fousia Mohsin and Dr Nahid; Dr Hassan Ali Chowdhury, Senior Medical Officer, Out-patient Department; and Omar Faruque and Nasima S Chowdhury, for their assistance in recruiting diabetic patients for the study. Kazal Biswas and Rafiqul Islam for their help during my study.

Prof MF McDermott, Dr Rebeca Allottey, Dr Ebun Aganna, Dr Paul G Cassell, Dr Khalid Barakat, Dr William Ogunkolade, Dr THC Going, Dr Jaydev Sudegana and Dr Bernard North, Dept of Diabetes and Metabolic Medicine, the Royal London Hospital, London, for their help in getting myself familiar in a foreign laboratory. Their theoretical input immensely helped me in getting into the subject and their assistance in the laboratory works, whenever needed, is highly appreciated.

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Prof David Lesslie and Mohammed I Hawa, Dept of Diabetes and Metabolism, St Barts Hospital, London, for their kind assistance in laboratory analyses and care taken for me at Bart's.

Dr Andrew Hattasley, Dr Tim Frayling and other members of the group are duly acknowledged for the supervision and assistance during laboratory analyses in the Dept of Pathology, Exeter University, Exeter, UK.

Dr Martine Vaxillaire, Good Pasteur Lab, Lille France for her hard work in the analysis of French MODY patients.

Dr Begum Rokeya, Dr Sabrina Murshed, Dr Soheli Sattar, Dr Nur-e-Alam, Dr SH Khan, Ms Monika, Dr Q Nahar, Dr Rahelee Zinnat, Ms Samira Habib and Research Fellows of the BMRG for their moral support and friendly environment. Dr MA Sayeed, Dr Faruque Pathan, Dr Tofail Ahamed, Dr Reza Bin Zaid and PA Khanam for never-ending encouragement.

Mr Sultan Mahmud and his team, Information Management Unit, BIRDEM for all sorts of help. Technical staff of the BMRG for their assistance during the study period and BIRDEM Library staff for their ever-smiling-welcome in the reading room and help whenever needed.

Saima and Samiha- my two daughters, whom I deprived of their time, but they have been a source of warm welcome at home after a long day and they enabled me to forget all the pain and stress during the whole study period. Dr Rosy Sultana, my wife, who has always motivated me to keep going. I owe a lot to her. I am also thankful to my dear and near ones for their forgiveness in my failure of keeping social commitments.

Proprietors and staff of the Star Garments and Rupa Sweater for their help in recruiting Control subjects. I feel obliged to extend my special thanks to Mr Abul Hassan Md Obayedullah for his active role in recruiting controls and his inquisitiveness in science, which touched my heart.

International Program in the Chemical Sciences (IPICS) for the Fellowship and other financial support during the study period and Diabetic Association of Bangladesh (DAB) for logistic support.

All the diabetic subjects and healthy volunteers for their consent to participate in the study, without their participation the study would have not been possible.

(Dr Md Zahid Hassan, MBBS)

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CHAPTER 7

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7. BIBLIOGRAPHY

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