GENETIC CHARACTERIZATION OF TROPICAL CALCIFIC PANCREATITIS IN PATIENTS WITH AND WITHOUT DIABETES MELLITUS

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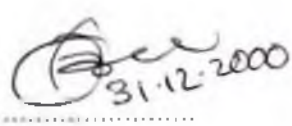
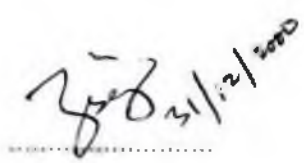
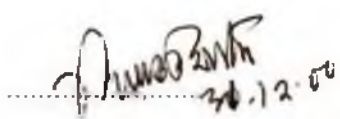
Declaration

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Philosophy (MPhil) in Biochemistry, University of Dhaka, Bangladesh. The work was carried out in the Research Division, BIRDEM, Dhaka. No part of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other University or other Institutes of learning.







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To

The memory of my son Asif

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Summary

A type of chronic calcific pancreatitis (CCP), distinct from alcoholic pancreatitis, occurs in many tropical countries including Bangladesh. This type of CCP is known as Tropical Calcific Pancreatitis (TCP) and has a group of counterpart patients who, in addition, develop a variety of diabetes known as Fibrocalculus Pancreatic diabetes (FCPD). Although, so far, FCPD has been thought to develop simply due to nonspecific damage of the pancreas which causes TCP (ie FCPD is secondary to TCP), recent evidences on Bangladeshi subjects indicate that the diseases may not be related with such a straightforward process. Thus etiopathogenesis of exocrine and endocrine abnormalities, both in TCP and FCPD, require further investigation with particular focus on gene-environment interactions. The present study was undertaken to characterize some biochemical characteristics (particularly insulinemic status), to explore the involvement of oxidative and autoimmune damage, and to find out whether some common genetic variations found in similar diseases are involved in the development of pancreatitis and diabetes in chronic pancreatitis.

In total 7 TCP and 11 FCPD subjects were studied. Of them 5 TCP and 5 FCPD were from simplex and 2 TCP and FCPD were from multiplex families. The remaining 4 FCPD cases were individual patients. Glycemic status was assessed by oral glucose tolerance test (glucose measured by glucose-oxidase method); lipidemic status by plasma triglyceride, total cholesterol, HDL-c and LDLc (by enzymatic-coloriemtric methods); and insulinemic status was determined by the measurement of fasting and postprandial C-peptide and insulin (by ELISA methods). Insulin secretory capacity was judged by C-peptide-glucose and Insulin-glucose ratios. Response to oxidative damage was determined by total antioxidant status (by enzymatic-colorimetric method) and autoimmune involvement was assessed by determining islet cell antibody (ICA), insulin autoantibody (IAA) and anti-GAD (ELISA techniques). Mutations (A16V in exon 2 and R117H in exon 3) in trypsinogene gene, known markers of hereditary pancreatitis (HP), was analyzed [(using restriction fragment length polymorphism (RFLP) technique (by *Fnu*4H1 and *Aff*III endonucleases)] for possible association with TCP and FCPD. HLA-DQB1 alleles, found to be associated with type 1 diabetes, was also analyzed (using sequence specific primers called SSP-PCR) to explore possible association with the diseases.

Mean (±SD) BMI of the Control subjects was 23.62±2.4, TCP- 18.15±3.3 and FCPD-18.45±4.0. TCP and FCPD had almost similar BMI and both values were significantly (p= 0.005) lower than the Controls. Fasting plasma glucose level (mmol/l, mean±SD) of the FCPD group (15.4±6.3) was almost 3.5 times higher (p=0.001) compared to the Control (4.4±0.4) and TCP (4.9±1.0) subjects. Corresponding postprandial values were 27.2±7.4, 5.2±0.6 and 5.9±1.0 respectively. In contrast, fasting C-peptide level (nmol/l, median-range) of the Controls (0.70, 0.39-1.78), was about 3 times higher compared to FCPD (0.23, 0.10-0.30), (p= 0.006) and 1.4 times higher than those of TCP subjects (0.52, 0.21-0.56) (p=0.040). Fasting C-peptide level of the TCP subjects was more than 2 times higher than the FCPD subjects (p=0.008). Postprandial C-peptide levels of the Control subjects (2.23, 1.99-3.38) was about 6 times higher than the FCPD group (0.29, 0.11-0.51) (p=0.003). The TCP subjects had nearly similar postprandial value (1.92, 0.68-3.09) compared to Controls (p=0.165) but the value was about 5 times higher compared to FCPD subjects (p=0.042). Fasting and postprandial insulin level showed similar trend like that of C-peptide.

Insulin secretory capacity was judged by secretion of unit C-peptide and insulin in response to unit mole of glucose (C-peptide:glucose and insulin:glucose ratio respectively) and hepatic utilization of insulin was estimated by C-peptide-insulin ratio. Although the absolute levels of C-peptide and insulin in FCPD patients showed that the patients still have some capacity of insulin secretion, the C-pep/glucose (nmol/l, median-range) in the fasting [C-peptide:glucose- 0.15, 0.09-0.41 in Controls; 0.011, 0.003-0.015 in FCPD and 0.09, 0.06-0.12 in TCP) and postprandial (0.44, 0.36-0.78, 0.012, 0.005-0.077 and 0.36, 0.12-1.42) states reveal that their capacity of increasing insulin secretion to meet the demands of hyperglycemia is almost nil. The insulin-glucose ratios further substantiated the finding.

Fasting C-peptide/insulin ratio of the FCPD subjects 0.009, 0.005-0.019 was similar to Controls (0.012, 0.009-0.019) and TCP subjects (0.017, 0.005-0.018). However, the ratio in the postprandial state of the FCPD (0.003, 0.001-0.005) subjects was significantly lower than the Controls (0.008, 0.005-0.010). Mean (±SD) total antioxidant level (mmol/l) of the TCP subjects (0.83±0.07) was significantly lower than that of the Controls (1.08±0.16) and FCPD subjects (1.01±0.09) (p=0.001). Determination of ICA, IAA and anti-GAD antibody revealed that autoimmunity is not related with diabetes in FCPD.

PCR-RFLP analyses of the exon 2 and exon 3 of the trypsinogen gene preclude the relationship of mutations in the gene with pancreatitis. HLA-DQB1 typing of the simplex families showed high polymorphism of this region. Both in TCP and FCPD subjects allele 0202 seemed to be transmitted equally and that seem to be the commonest allele compared to other transmitted alleles. Alleles 0303, 0302/0307 and 0301/0304 appeared almost in the same frequency in both TCP and FCPD subjects.

The results suggest that: a) from the sociodemographic and clinical characteristics and from glycemic and insulinemic status it appears that TCP and FCPD are different diseases with possible overlapping (and interactions) of features related to pancreatitis and diabetes. Thus, diabetes in FCPD may not be merely secondary to TCP; b) absence of common mutations in the trypsinogen gene attributed for the pancreatitis of HP confirm that both TCP and FCPD are genetically different from HP; c) HLA DQB1 typing precludes the presence of this type 1 diabetes related allele in TCP and FCPD subjects and d) other genes for diabetes and pancreatitis need to be studied for a better understanding of the etiopathogenesis of pancreatitis and diabetes in chronic pancreatitis patients.

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CCP	Chronic calcific pancreatitis
TCP	Tropical calcific pancreatitis
FCPD	Fibrocalculus pancreatic diabetes
IP	Idiopathic pancreatitis
HP	Hereditary pancreatitis
AP	Alcoholic pancreatitis
TRYP	Trypsinogen gene
CFTR	Cystic fibrosis transmembrane conductance regulator gene
A16V	Alanine 16 Valine
R117H	Arginine 117 Histidine
N21I	Asparagine 21 Isoleucine
cm	Centimeter
mm	Millimeter
mm-hg	millimeter of mercury
Kg	Kilogram
g/l	gram per liter
mmol/l	millimol per liter
nmol/l	nanomol per liter
pmol/l	picomol per liter
ICA	Islet cell antibody
IAA	Insulin autoantibody
GAD	Glutamic acid decarboxylase

INTRODUCTION

1. INTRODUCTION

Chronic calcific pancreatitis (CCP) is a unique type of pancreatitis mostly prevalent in the tropical developing countries including Bangladesh [1]. It is distinct from alcoholic pancreatitis. The patients have a history of abdominal pain from childhood and the disease follows an aggressive course. It has been termed differently from time to time namely, tropical juvenile pancreatic syndrome, tropical nutritional pancreatitis, tropical calcific pancreatitis and non-alcoholic pancreatitis [2]. The condition is most prevalent in the tropical countries (30° North and South of the Equator) and as pancreatic calcification is a prominent feature, it has been termed as 'tropical calcific pancreatitis' (TCP). Although it predominantly affects in early childhood, young adults and even elderly people often suffer from TCP, pancreatic calcification develops in almost all cases and it is not purely a nutritional disease[3]. The average age of onset of symptoms in TCP has been 12.5 years in one series[3] in contrast to 38.4 years in alcoholic pancreatitis as reported by Sarles[4].

A substantial proportion of TCP patients present with diabetes, termed as fibrocalculus pancreatic diabetes (FCPD), was earlier classified as a subclass of malnutrition related diabetes mellitus (MRDM) by the WHO Study Group on Diabetes Mellitus[5]. The latest classification proposed by WHO Study Group[6] and ADA Expert Committee categorized FCPD as a subclass of Exocrine Pancreatic Diseases[7].

Diabetes associated with calcific pancreatitis was reported in the tropics in the early twentieth century but it attracted greater attention only after its description by Zuidema in 1959[8]. Geevarghese[9] highlighted its significant contribution to diabetes among the young in South India. He succinctly summarized the natural history of TCP as 'recurrent abdominal pain in children, diabetes around the age of puberty and death at the prime of life'.

The relative frequency of diabetes in calcific pancreatitis is higher in the tropics than subtropics. In the tropics, diabetes was diagnosed in 82.2 percent of 45 cases in Nigeria and 90 percent of 1700 cases in India[3]. In the subtropics, the average incidence of diabetes in 404 cases of pancreatic calcification from 118 reports was

34 percent[10]. There is also high prevalence of calcific pancreatitis among diabetics in the tropics: 7.6 percent in Uganda[11], 8.6 percent in Nigeria[12,13] and 14.8 percent in Kerala (India)[3]. In the subtropics, the above prevalence varies from 0.03 to 2.25 percent among diabetics. No population based survey on the incidence of prevalence of TCP or FCPD has yet been made in Bangladesh. The Outpatient Department of the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), the central Institute of Diabetic Association of Bangladesh (DAB), takes care of the highest number of diabetic patients (>240000 registered by June 2000) in one center. Of these patients 4 percent belong to the under-30 age group and among these young patients, 13 percent had pancreatic calcification. Thus, as per this calculation, 0.52 percent of total diabetics have FCPD. In the Institute of Postgraduate Medicine and Research or IPGMR (presently known as Bangabandu Sheik Mujib Medical University or BSMMU) 48 cases of TCP were recorded during the period of 1993-96 among whom 14 cases (29%) were found to have diabetes[14].

FCPD patients comprise of a unique group of diabetic population. They are young and have lower BMI at presentation. Usually they have history of chronic abdominal pain since early childhood, which gradually become more frequent with advancement of age. They present with high blood glucose and invariably require insulin to control of blood glucose; however, they rarely develop ketoacidosis[15]. In view of clinical features and marked insulinopenia some investigators believe that FCPD along with PDDM may be a variant of type I diabetes[16].

Although FCPD is a widely recognized straightforward condition to TCP, its etiopathogenesis is still not clearly understood. It seems to be a multifactorial disease. Both environmental and genetic factors are implicated in its etiopathogenesis, however, environmental factors are thought to play the major role[14-17]. Both TCP and FCPD predominantly come from poor socioeconomic background. However, economic conditions vary between two groups with higher income groups mostly in TCP patients[18].

Protein calorie malnutrition and cassava (tapioca) ingestion have been implicated[19] as a possible environmental factor in the etiopathogenesis of diabetes

in FCPD. Geographical overlap between the areas of cassava consumption and the distribution of TCP and FCPD supported the cassava hypothesis[20-30], but the occurrence of the disease in areas (those in Bangladesh) where cassava is neither grown nor imported has been cited as an evidence against the etiological role for cassava. However, other cyanide containing foodstuffs can not be excluded.

Protein calorie malnutrition as the probable cause of pancreatic fibrosis with the calcification seen in juvenile diabetes have been suggested by many authors[8,20-23]. The WHO classification of FCPD as one of the subclass of MRDM implied that malnutrition is etiologically and diagnostically important in this disease. However, many workers in Asia and Africa[24-28] have presented arguments against protein malnutrition as the primary or initiating factors of TCP or FCPD. Others have claimed that micronutrient deficiency (ß-carotene, vit C, selenium) may play a role in making the pancreas vulnerable to toxic agent[29], but the hypothesis still remains to be proven. Thus the relationship between FCPD and malnutrition is debated. In a recent Workshop on the Diabetics in Tropics in Cuttack, India[30], it was recommended that FCPD should not be considered as one of the subclass of MRDM; rather it should be classified as an entity by itself.

To elucidate the etiopathogenesis of MRDM, a series of studies have been undertaken at BIRDEM during the last few years. Environmental factors seem to play a more important role in this disease[1,31]. Most of the subjects in this center are of younger age group, coming predominantly from rural areas with poor socioeconomic background. Family history of diabetes in FCPD subjects was less positive in this group compared to the NIDDM subjects of similar age group. The above findings indicate that environmental factors may be implicated in the etiopathogenesis of FCPD.

A collaborative study between BIRDEM and Department of Gastroenterology, BSMMU was conducted to investigate the etiological factors and relationship between exocrine and endocrine pancreatic damage in TCP and FCPD[14]. Supporting the previous findings, similar age, family history and socioeconomic status were observed. Both the fasting and postprandial serum glucose values clustered at the higher end in case of FCPD and those were clearly demarcated from TCP where clustering was seen at the lower end. C-peptide is produced in

equimolar concentration with insulin during splitting of proinsulin and it has a longer half-life than insulin. C-peptide level reflects pancreatic secretory activity and thus is a clinical method in conditions where insulin antibodies in the circulation prevent accurate assessment of insulin by the standard insulin immunoassay. Serum fasting and postprandial C-peptide level was remarkably less in FCPD than in TCP subjects studied at BIRDEM Hospital. Although the endocrine pancreatic function was severely compromised in FCPD subjects, exocrine pancreatic morphology, evaluated by ERCP was almost similar in both groups. Nutritional status of these subjects, evaluated by body mass index (BMI), was found to be significantly lower in FCPD group than TCP. But it remained unanswered whether under nutrition in FCPD cases was a cause or consequence of diabetes[18].

Increased oxidative damage is associated with low level of both endogenous and exogenous antioxidant molecules. Subnormal levels of plasma vitamin C and selenium, β carotene and α-tocopherol have been found associated with chronic pancreatitis in black African population with pancreatitis[32]. Deficient levels of β-carotene and vitamin C levels were observed in tropical pancreatitis patients by Braganza et al[33] and role of oxidative stress has been proposed in the etiopathogenesis of FCPD. One study conducted by BIRDEM group provided evidence regarding the involvement of oxidative damage[34] through significantly higher single stranded DNA (a marker of free radical mediated damage of double stranded DNA) compared to Controls. Until recently endogenous antioxidants could only be evaluated individually. Recently a commercially available kit measures status of total antioxidants in the biological samples, but no report has yet been published on Total Antioxidant Status (TAS) in TCP subjects.

Diabetic patients often have autoantibodies against islet cell antigens that are considered as markers of beta cell damage. At the time of diagnosis almost all children and adolescent with diabetes show antibody positivity[35]. However, little is known about the persistence of these antibodies after clinical diagnosis. Islet cell antibodies (ICA) and antibodies to the glutamic acid decarboxylase (anti-GAD antibody) have been observed to persist after the clinical diagnosis of type I diabetes. Immunological markers like islet cell antibody (ICA) and other antibodies

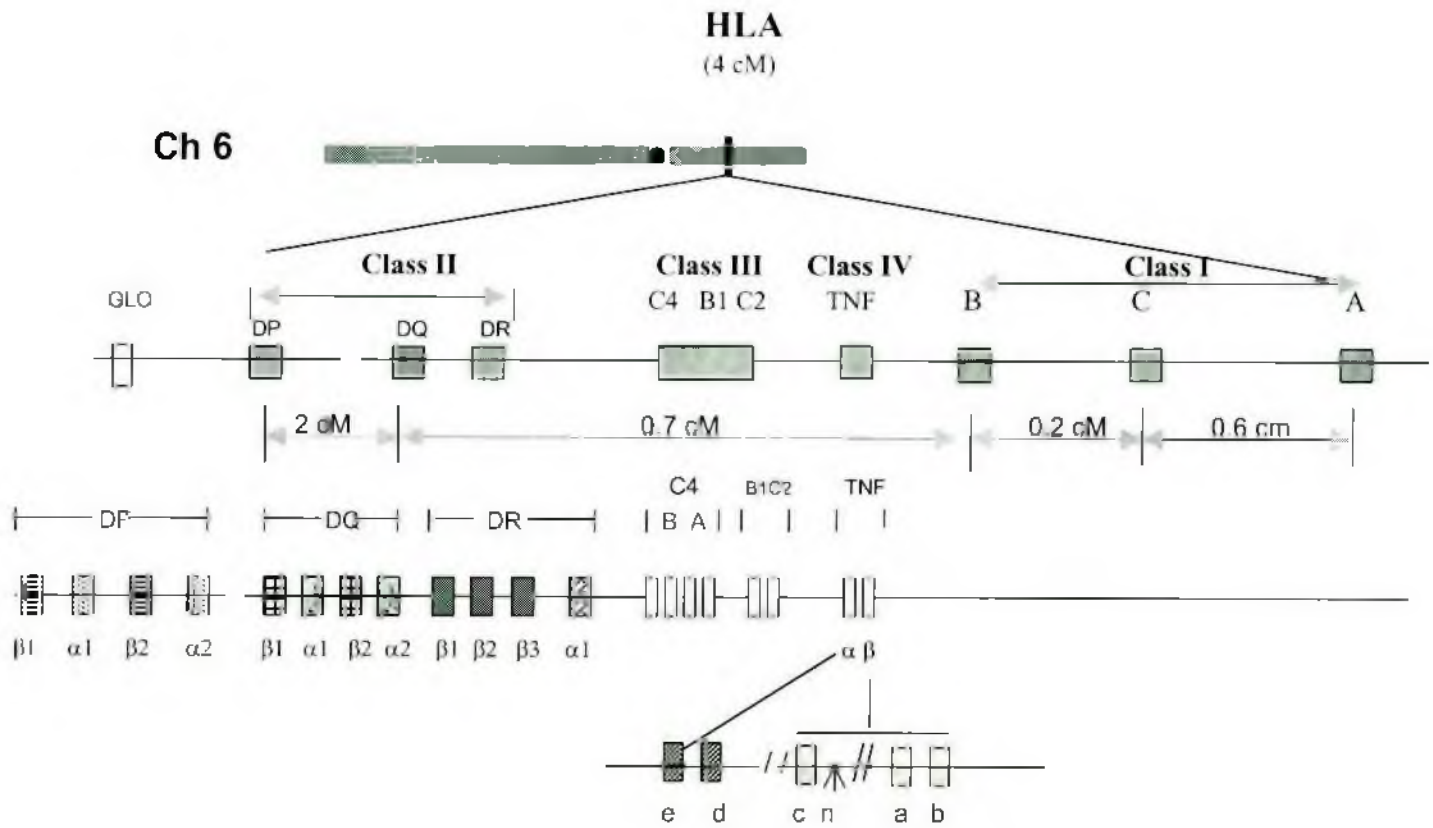


Fig 1: Organisation of HLA regions in Chromosomes 6

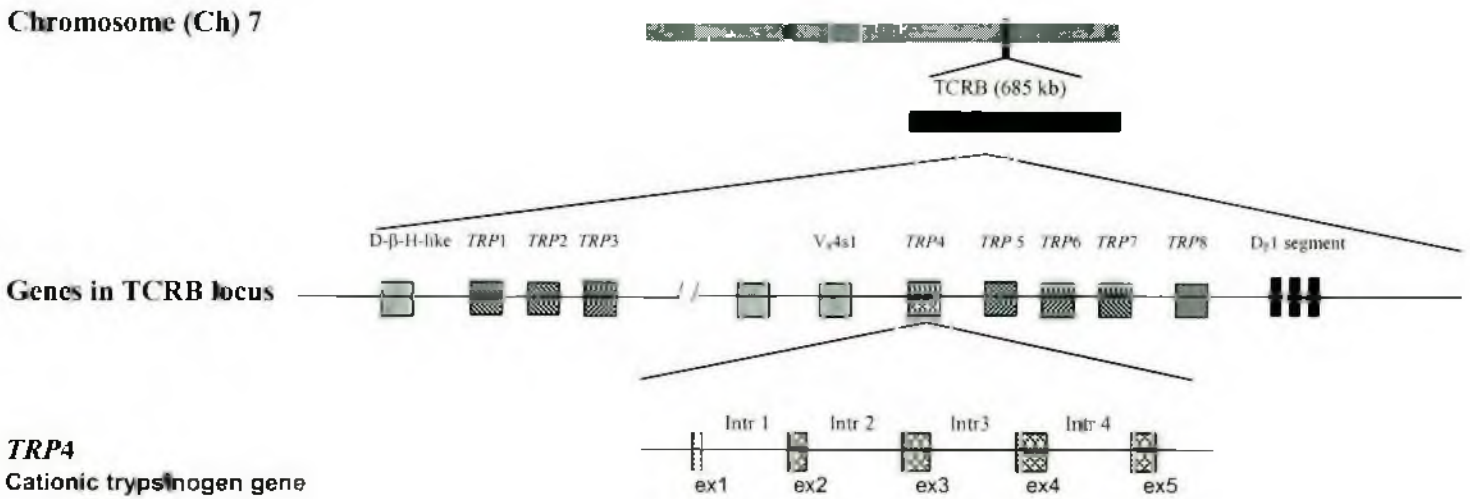


Fig 2: Schematic diagram of the trypsinogen (*TRYP*) gene organisation

were seen to be less important in Nigerian diabetics[36], which is in contrast with the findings in India, where ICA was found in 37.5% of patients with diabetes compared to that in classical IDDM[37]. Although antibodies (ICA and IAA) have been measured (unpublished data) in young diabetic patients in BIRDEM including FCPD and TCP subjects but no association was observed. However, this does not exclude other antibodies like anti-GAD antibody and IA-2, the antibody against tyrosine kinase.

Evidence of increased oxidative damage has been found in FCPD cases[18] but the role of genetic susceptibility underlying this damage has not yet been explored.

One of the idiopathic form of chronic pancreatitis, known as hereditary pancreatitis (HP), is mostly prevalent among the Caucasoid descents. The clinical features and pathophysiology of this type of pancreatitis has close similarity with FCPD as well as TCP. However, prevalence of diabetes in HP cases has been observed to be much less than that in chronic pancreatitis. Also the nature of diabetes in HP has been shown to be much more aggressive[38]. Some 2-3 kindred with HP have so far been recorded[39,40]. Recently genetic linkage has been identified between cationic trypsinogen gene on chromosome 7 in the TCRB locus, locus 7q35[41]. A mutation (G to A) in the exon 3 of trypsinogen gene causing substitution of arginine to histidine (R117H) has been reported by Whitecomb et al[42] and it was later confirmed in independent family[43]. A second mutation, C to T in the exon 2 of the TRYP gene leading to A16V (alanine to valine) substitution in the *Fnu*4HI restriction site of trypsinogen has been reported in the another kindred[44].

To address the involvement of genetic component in the etiopathogenesis of diabetes in FCPD attempts have been taken by different groups. In 1970 Pitchumoni CS[45] observed aggregation of diabetic patients in families with chronic pancreatitis. This finding was further substantiated by another study by Mohan V et al in 1989[46]. Kambo et al has reported an association of HLA-DQB1 and class III allele of insulin gene[47] with FCPD. In another study Sanjeevi et al has shown increased frequency of HLA-DQ allele A0201-B*0303 alleles in Eastern Indian population[48]. But no data effort has so far been reported to compare FCPD and TCP patients for biochemical and genetic components.

In the above context it appears that TCP and FCPD are complex disorders both from the viewpoints of pancreatitis and diabetes. For Bangladeshi patients it has now become important to further characterize these patients biochemically and genetically. In particular, their insulinemic status and insulin secretory capacity, total antioxidant status, autoimmune involvement, common variations in the trypsinogen gene and association of HLA conferring diabetes susceptibility need to be explored.

1.1. Objectives

The objectives of the present study were to:

1. Investigate clinical and biochemical characteristics of TCP and FCPD subjects with particular focus on insulin secretory capacity.
2. Evaluate the total antioxidant status in TCP and FCPD subjects.
3. Explore autoimmune involvement in the pathogenesis of diabetes in pancreatitis.
4. Find out whether common mutations found in HP are present in TCP and FCPD subjects
5. Explore the association of HLA (which is a marker of genetic susceptibility to type 1 diabetes) with diabetes in FCPD.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURES

2.1. Definition

2.1.1. Chronic pancreatitis (CP)

Chronic pancreatitis is a chronic inflammatory disease of the pancreas characterized by irreversible morphological changes with destruction of exocrine parenchyma and fibrosis and at least in the late stages, the destruction of endocrine parenchyma[49]. It is frequently complicated during its early stages by attacks of acute pancreatitis, which are responsible for recurrent pain that may represent the only clinical symptoms. However, after some years exocrine and endocrine insufficiency developed and acute attacks decreases and disappears.

2.1.2. Chronic calcifying pancreatitis (CCP)

It is the most frequent form of chronic pancreatitis characterized by development of intraductal calcification visible on plain abdominal radiographs[49]. There is different form of chronic calcific pancreatitis. Alcoholic (ACP) and tropical (TCP) calcific pancreatitis are the most common form. Hereditary (HP) and hypercalcimic chronic pancreatitis are also seen less frequently. Tropical calcific pancreatitis is a form of chronic calcific pancreatitis observed mostly in children and young adults in many tropical countries of Asia and Africa[50, 51]. It is distinct from alcoholic calcific pancreatitis (ACP).

2.2. History and incidence of pancreatitis

The first case of pancreatic calculus in the tropics was published by Kini from India in 1938. In 1952 Prof R Kesavan Nair presented a paper in his experience of operating 23 cases of pancreatic calculi at the Medical College Hospital, Trivandrum in Kerala State. In 1954, Elizabeth and Stephen reported 9 cases of pancreatic calculi from Vellore. In 1959 Zuidema reported on a series of 45 cases with pancreatic calcification in non-alcoholic young Indonesian from Java[8]. In 1961, Mahadevan[51] from Madras, India published 17 operated cases of pancreatic lithiasis. In 1963, Kinner[13] reported 30 cases from Nigeria. 1964 Shaper collected 48 cases of pancreatic calculi with diabetes from Uganda[11]. A very similar syndrome of chronic pancreatitis with pancreatic calculi and

diabetes mellitus was subsequently reported from Malawi, Zaire, Uganda, Srilanka and Nigeria[53].

The single largest series of cases of tropical pancreatitis reported to date is from the southwestern state of Kerala in India. Geevarghese and pitchumoni observed this disease in endemic proportion in two major medical college hospital of the state[22,54]. More than 400 cases of chronic pancreatitis associated with pancreatic calculi were seen in the diabetic clinics of the two hospital Trivandrum Medical College Hospital and Kottay Medical College Hospital. The incidence of pancreatic diabetes was 1 percent of the Medical college Hospital Trivandrum whereas at Kottayam it was 1.5% and 16% respectively.

No population based survey has yet been made on the incidence or prevalence of tropical calcific pancreatitis or fibrocalculus pancreatic diabetes in Bangladesh. Tropical calcific pancreatitis is not a common disease but is not found uncommonly in gastroenterological practice in Bangladesh. Among 1449 under 30 diabetic patients registered at BIRDEM between January 1990 to May 1992, 809 (55%) were diagnosed to suffer from MRDM of 179 (13%) had calcific pancreatitis[55]. In the subtropics pancreatic calcification was reported to be rare up to 1938 after its first description by Graaf in 1667. In 1938 Haggard and Kirtley collected 166 cases of pancreatic calculi from the literature over the preceding 258 years including 64 cases of their own Johson and Zintel found 677 additional cases collected up to 1962 making a total of 843 cases till then[3].

In some of the developed countries of the subtropics alcoholic calcific pancreatitis (ACP) is the more common type as in France, South Africa, Australia, USA, Rasa, Japan, Switzerland, Finland, Rumania and Zimbabwe.

Alcoholic calcific pancreatitis is less common where alcoholic consumption is comparatively low as in northern part of India. Alcoholic calcific pancreatitis is also very uncommon in Bangladesh.

2.3. Pathology

The pathology of the pancreas as noted by observers in India and other countries, is remarkably similar[56]. Macroscopically the pancreas is firm, fibrous and gritty to touch. Its

consistency varies in different regions of the pancreas. Homogenous areas, early fibrosis, advanced fibrosis, cystic dilatation of the gland and advanced stages of calculi formation can be seen in the same specimen of pancreas. Occasionally the pancreas becomes indistinguishable from surrounding fat owing to replacement by adipose tissue. The pancreatic duct may be eccentrically placed as a result of destruction of the pancreas. Pancreatic calculi of different sizes and shapes are distributed throughout the duct system[51]. The dilatation of the main pancreatic duct and its afferent ducts is due to abnormally high pressure of the pancreatic juice resulting from obstruction. Dilatation of the duct of Wirsung is accompanied by ectasia of its afferent ducts, the caliber of which is also irregular. In some cases the main ducts are so dilated that a bulge develops, resulting in the formation of ductal cyst initially communicating with the ducts and appended to them like "grapes on a bunch" as well demonstrated by pancreatography[47].

Microscopically, dilatation of the ducts, pancreatic lithiasis, chronic inflammatory cell infiltration and fibrosis and atrophy of the parenchyma are seen. The main ducts, collecting ducts and small ductules show marked dilatation. Denudation of the ductal epithelium and squamous metaplasia are seen. The dilated duct contain pancreatic calculi and/or inspissated mucoid material which stains metachromatically toluidine blue and is PAS positive.

The characteristic cellular infiltration of the pancreas is composed by lymphocyte, and plasma cell distributed mostly around the ducts. Acute inflammation is not usually observed. In less than 10 percent of the cases, eosinophilic infiltration may be seen. Patient with very advanced disease, inflammatory changes in the pancreas may be absent.

Interlobular fibrosis is characteristic in early cases and focal segmental or diffuse interacinar fibrosis in more advanced cases special stains have shown peri acinar and peri insular proliferation of reticulum fibers.

The acini show varying degrees of atrophy. Destruction of the parenchyma and replacement with fibrous tissue can be seen side by side with relatively normal looking parenchyma.

The islets are virtually absents in many specimens. Aldehyde fuchin stain of β cell granules shows a reduction in β cell. Rarely hypertrophy of the islet cell seen.

Vacuolation, ballooning and glycogen infiltration of the islets characteristic of juvenile diabetes are seldom noted. Carcinoma is seen in four cases from 27 patient operated in one institution[54].

2.3.1. Pancreatic Calculi: Patients with tropical calcific pancreatitis have intraductal stone. Parenchymal calcification is not seen. Multiple stone of varying size, some as small as sand particles and others as big as 3 cm in diameter are distributed throughout the main pancreatic duct wedged in the ductules. The calculi have an unusually knobby and irregular surface, some are staghorn shaped and few are smooth and rounded. They are hard in consistency and grayish white in color. Solitary calculi are occasionally seen near the ampulla of rater. The larger stones are usually located in the head of the pancreas.

In patients who had no radiographically demonstrable calculi in antimortem X-ray of the abdomen postmortem examination of the pancreas showed multiple tiny radiopaque material which was shown to consist of intraductal calculi. Analysis of pancreatic calculi by X-ray diffraction using power diffractometry showed that stones were almost pure calcium carbonate. The cut surface in some showed a central amorphous material might be protein plugs reported by Sarles and associates in alcoholic pancreatitis[57]. Similar studies on stones suggesting that the thermodynamics of stone formation in the pancreas irrespective of etiology alcoholism, malnutrition, idiopathic) are the same.

In one study, a microsection from decalcified stones showed ghost-like remnants of cell, exhibiting a columnar arrangement mixed with mucoproteinous material, probably from the ductule epithelium[56].

Pancreatic stones were also analysed by Prof. Geevarghese[3]. The chief constituent was found to be calcium carbonate 95.5 percent. Calcium phosphate may be present up to 2.5 percent. Trace of magnesium, urate and oxalate has been reported in few instances.

The frequency of pancreatic calculi in tropical calcific pancreatitis is higher than in alcoholic pancreatitis seen in western countries. Over 90 Percent of patients with tropical calcific pancreatitis may eventually developed pancreatic calculi[3,51].

2.4. Clinical presentation

The clinical presentation of tropical calcific pancreatitis varies from one patient to another. Geevarghese succinctly summarizes the natural history of tropical calcific pancreatitis as recurrent abdominal pain in childhood, diabetes around the age of puberty and death at he prime of life[9].

2.4.1. Age and sex incidence

Tropical calcific pancreatitis mostly occurs in younger age group. The average age of onset of symptoms Geevarghese[3] series was 12.5 year whereas in alcoholic pancreatitis it was 38.4 years reported by Sarles[4]. Olurin and Olurin[53] from Nigeria reported 45 patients, 31 were 20 years old or less and 5 were over 35 years. The average age was 20.4 year. Zuideme[8] recorded that most of his Indonesian patients were young adult. In Geevarghese sseries[9] 38 percent of the patients were below 13 years and 95.5 percent of the patient were below the age of 40 years. In the United States of America pancreatic calcification is a disease of middle age; Wirts and Snape[58] found an average age of 38.6 years in 24 patient. This pancreatic calcification occurs in younger age groups in tropical countries whereas it is a disease of middle aged people in Western Europe and the USA. The youngest patient was a girl of 7 years and oldest was a man of 62 years.

Regarding sex incidence of the tropical pancreatitis most hospital based studies have reported male predominance. The male to female ratio of cases in tropical calcific pancreatitis is 1.6:1 in Kerala[22], 1.5:1 in Bangladesh, 3:2 in Nigeria[53], 1.7; 1 in Geevarghese series[9] and 5:1 in Congo[59]. In Indonesia male and female were almost equally affected[8] and Balaj found a slight female predominance in his population[60].

2.4.2. Symptoms

The clinical picture of a well established case of tropical pancreatitis with diabetes and pancreatic calculi is characteristic enough to make on the spot diagnosis. The presenting complaints of most patients are abdominal pain and the effects of diabetes mellitus. Very rarely obstructive jaundice, hyperglycemic comma, peptic ulcer disease or appendicitis is the mode of presentation.

2.4.2.1. Abdominal pain

Abdominal pain is the most prominent symptom of chronic calcific pancreatitis. However in children, abdominal pain often ignored or attributed to parasitic infestation, which are quite common in developing nations. Age of onset of pain usually in the second and third decade in the tropical calcific pancreatitis whereas in alcohol induced pancreatitis initial attack of pain at the age of 35-40 years.

The frequency of abdominal pain varies from 30-95 percent in different series[51,56]. Patient presented without abdominal pain, diagnosis depends upon the presence of pancreatic calculi on a plain film of the abdomen.

The intensity of abdominal pain varies from relatively mild to severe in nature. The more typical form is marked by recurrent attacks of pain occurring in intervals of months or years. The intervals between attacks may progressively shorten until pain is almost persistent or the attacks may diminish in frequency and severity as pancreatic fibrosis developed and advances. Abuse of alcohol or ingestion of heavy fatty meals may precipitate attacks of pain. The onset of the attack is usually fairly sudden. However, the attack may start with a mild discomfort and become progressively more severe over a period of few hours before attaining maximum intensity. Vomiting usually associated with severe pain. The intensity, site, character and duration of the pain vary considerably. The pain may be relatively mild but is offer sufficiently severe to necessitate hospital admission. Although generally diffusely distributed over the upper abdomen, the pain may be maximal in the epigastrium, in the right or left upper quadrantor even the back. Radiation directly through to the back is common. Less frequently, pain may be referred to the lower anterior chest and the left renal angle. Shoulder pain may occur with subphrenic extension of inflammation. The pain is usually persistent, relentless and deep seated. Occasionally it is describe as a heaviness, a "gnawing" discomfort and every now and then as "cramp like" or burning. Antacid or food does not afford relief. Non steroidal anti inflammatory drugs may be helpful but many patient require narcotics. The patient may adopt certain characteristic posture, such as, sitting up in bed, leaning forward, with the palm of the hand pressing on the site of the abdominal pain.

Pain of acute episodes of chronic pancreatitis ordinary persists for days rather than hours, the usual attack lasting for 3 to 7 days unless a complication occur. Indeed, the relentless, persistent nature of the pain and the tendency of the patient to seek postural relief are perhaps the most characteristic feature of the pain. In addition to nausea and vomiting, there may be constipation and mild jaundice with the passage of dark urine.

The events that trigger the pain of chronic pancreatitis are poorly understood. One hypothesis proposed that the pain of chronic pancreatitis be due to ductal hypertension resulting from pancreatic duct obstruction by stricture or stone. This is supported by reports indicating an increase in pancreatic duct pressure in patient with chronic pancreatitis and dilated cuts and a correlation between the intraductal pressure and pain relief following duct-decompressive surgery[61]. Other studies in which ductal pressure have not been measures have not shown a correlation between the amount of pain experienced by patients and with degree of pancreatic duct abnormalities observed on ERCP[62]. Another line of investigation has focused on pancreatic neural inflammation as an important source of pain in chronic pancreatitis.

2.4.2.2. Steatorrhea

Clinical steatorrhea is generally not a striking feature. Zuidema concluded that external secretory function of the pancreas was not distributed enough to cause steatorrhea[8]. Fat and protein are not malabsorbed until at least 90 percent of the secretory function is lost[63]. However, lack of obvious steatorrhea appears to be due to the very low consumption of fat in the diet. Interestingly on a 100g fat diet, nearly 70 percent of the patient studied had stool fat of more than 6g in 24 hours[64]. Pancreatic secretory studies performed as duodenal aspirates have confirmed a marked decrease in lipase activity in the large majority of patient[58]. In another study performed in a larger number of patients a decrease in enzyme and volume was noted but in addition, in nearly 50 percent of patients the bicarbonate output was also suppressed[64].

Steatorrhea was found in 33 percent of patient reported from Nigeria[11], 38.8 percent patient reported by Howwat[65], Ban[66] reported 25-30 percent incidence of steatorrhea. In view of the intact absorptive capacity and enterohepatic circulation of the bile salts the

malabsorption of chronic pancreatitis is rarely associated with vitamin, iron or calcium deficiency.

About 40 percent of patients with pancreatic insufficiency have demonstrated malabsorption of cobalamin (vit B_{12}), although over cobalamin deficiency rarely developed. In this setting cobalamin malabsorption appear to be attributed to reduced degradation by pancreatic protease of cobalamin R protein complexes, resulting in decreased availability of cobalamin to bind to intrinsic factor in the small intestine[67].

2.4.2.3. Loss of weight

Loss of weight varies with the frequency of relapse. Patient loses weight during and just after attack. Weight is regained when remission is long lasting and is lost when relapses follow closely one on another. The reason for the weight loss is complex. Malabsorption is one of the several important cause for it. Many patient limit their food intake because of the pain caused by eating or because of anorexia. The glycosuria of untreated diabetes, goitrogenic food deprivation during hospitalization and insufficient fund for food may also contribute to malnutrition[68]. Olurin and Olurin[53] reported weight loss in 40 patients out of 45 patients (89%).

2.4.2.4. Physical findings

Physical findings are obviously dependent on the severity of the attacks. Systemic findings may be absent, but typical features include mild fever, tachycardia, slight jaundice, and rarely transient hypertension.

Patients with advanced disease and untreated malabsorption are under weight and have varying degrees of malnutrition. Extreme emaciation, peculiar cyanotic hue of the lips, bilateral parotid gland enlargement, multiple vitamin deficiency, skin infection and distension of upper abdomen are seen in most of the patient with tropical calcific pancreatitis[51]. Abdominal examination may be unrewarding in the milder attacks. Even in the more severe ones a discrepancy exist between the intensity of pain and paucity of physical findings. Upper abdominal tenderness is almost invariable but guarding and rigidity are less common. A vague fullness or a mass in the epigastrium or right upper quadrant is sometimes found.

2.5. Diagnosis

The medical history usually leads to suspicion of the disease, the physical examination often less helpful. Chronic calcific pancreatitis should be considered in every patient with recurrent attacks of upper abdominal pain, persistent pain after a solitary attack of abdominal pain, diabetes mellitus or steatorrhea.

The definitive diagnostic criteria of chronic pancreatitis are pancreatic calcification or histologic evidence of the disease. The diagnosis is commonly made on the basis of other considerations: (a) recurrent attack of pain, (b) development of steatorrhea in a diabetes with a previous history of abdominal pain, and (c) the nature of the attacks with classic ERP changes of chronic pancreatitis or grossly abnormal pancreatic function test.

The diagnosis of chronic calcific pancreatitis should be linked if at all possible, with same etiologic basis for the condition. Thus young individual with episodes of pain with diabetes mellitus raise possibility of tropical calcific pancreatitis as finding of hyperlipidemia, hypercalcemia or alcohol abuse in a patient with abdominal pain in western countries.

In Indian State of Kerala, chronic calcific pancreatitis is so endemic that diagnosis is not difficult. So in an editorial it is said that "in fact, physician in Kerala are so familiar with the clinical picture of a malnourished and usually under developed young person with enlarged parotid, cyanotic hue of the lips and potbelly, that chronic calcific pancreatitis is often a matter of spot diagnosis, further demonstration of diabetes mellitus and pancreatic calculi on abdominal X-ray complete the picture[60]".

2.6. Etiology and pathogenesis of pancreatitis

Despite repeated attempts to elucidation, chronic calcific pancreatitis in the tropics continues to be a fascinating and enigmatic disease. The etiological factors differ from alcoholic calcific pancreatitis in western countries.

The potential causes of chronic calcific pancreatitis to be considered are those associated with alcoholism, TCP, gallstones, hyperparathyroidism, hereditary pancreatitis, hyperlipidemia and cystic fibrosis[3].

2.6.1. Tropical calcific pancreatitis

Etiology and pathogenesis of tropical pancreatitis are still largely unknown. There are many hypothesis and many likely candidate. Probably the etiology is multifactorial. Geographical distribution in the tropical belt mostly in the developing countries suggests a significant role for environmental factor(s)[20]. Childhood infection and diarrhea changes in pancreatic secretion have also been suggested as significant factors. Recently oxidant stress damage has been proposed as an important mechanism in pancreatic damage.

2.6.2. Malnutrition

Protein malnutrition has been suggested as one of the probable causes of pancreatic calcification in tropical countries like Indonesia, Nigeria, Uganda, Malawi, Sri Lanka and some part of Brazil[22]. Epidemiological evidence in support of this hypothesis is the high prevalence of the disease in these countries. The incidence of tropical calcific pancreatitis varies in adjoining countries with a low and high protein diet. For example in some parts of Uganda where the staple food is low protein cassava, the disease is more common then in Kenya where the staple food is maize.

Severe malnutrition is a causative factor for many of the tropical calcific pancreatitis patient has also been suggested by Zuidema[8] and Shaper[11]. There is substantial accumulation of data to suggest that the pancreas is quite vulnerable to protein malnutrition[22]. Profound pancreatic functional changes have been noted in severely malnourished children[70]. The pancreas in postmortem examination of children dying of Kwashiorkor shows a picture similar to that in tropical pancreatitis. In Kwashiorkor the pancreas is shrunken and fibrotic[71]. A number of studies in experimental animal have shown morphological changes in the pancreas very similar to those describe above[22]. Children with Kwashiorkor and adults from famine areas shows pancreatic acinar atrophy, reduced enzyme output and fibrosis of the gland. Endocrine pancreatic function (insulin secretion) is also known to be diminished in malnutrition[72]. Recent animal work has suggest that even mild to moderate protein malnutrition in utero or in childhood or even later in life can lead to β cell dysfunction and hyperglycemia in later life[73-75]. Similar long-term studies are not available in human. It is possible that a pancreas

weakened by early life malnutrition could be more susceptible to many toxic influence to which it might be exposed in later life.

An international comparative study of dietary factors suggest that high protein intake was associated with alcoholic pancreatitis and low fat intake with tropical calcific pancreatitis[76]. This observation was confirmed recently in Kerala and Marseille[77]. Nwokolo and Oli suggested that recurrent infection and a high carbohydrate, low protein diet (mostly from cassava) in children in the tropics leads to pancreatic stasis, lamination of secretion and inspissation of mucus ultimately leading to calcification in the pancreatic duct and chronic pancreatitis[20]. Bearing such data in mind and also that cases of tropical calcific pancreatitis in all the reports were from lower socio-economic group of poor, developing countries we are able to understand that protein deprivation in childhood emerge as a major factor in the etiopathogenesis of tropical calcific pancreatitis.

Undernutrition appears to be involved at several levels in the pathogenesis of diabetes mellitus. These include; (1) primary etiological role with progressive β cell damage from malnutrition leading to β cell failure and overt diabetes. (2) A secondary role with additional nutritional insult to the β cell is being responsible for unmasking a latent diabetic state. (3) A permissive role with the protein deprived β cell being rendered more vulnerable to other β cytotoxic influences such virus, autoimmune destruction and toxin. (4) A modulating influence on the clinical course of diabetes resulting in the atypical presentation seen in malnourished subjects. It may also influence the course of the over all diabetic syndrome by increasing the susceptibility of the individual to serious infections as a result of depression of immunity. Finally it may lead to more rapid deterioration of β cell function in a diabetic subjects[78]. The data against protein malnutrition as the primary or initiating cause of tropical calcific pancreatitis are quoted by many workers in the field from Asia and Africa[25,26,27,79]. Although in the state of Kerala the disease is endemic, in many parts of India including the neighboring state of Tamil Nadu (Madras), protein malnutrition and Kwashiorkor occur more often but pancreatitis is seldom seen[28]. No past history of failure to thrive or a generalized edematous state was noted in the patient of Kerala. The serum albumin of more than 75% of patients was above 3.5 g/dl in initial pancreatitis is not seen. In Nigeria the incidence

of the disease is higher in the south than in the north. More strikingly in India, the prevalence of the disease only in some parts of the country could not be explained[82]. Moreover in the state of Kerala, there is highest rate of literacy and lowest infant mortality, the growth and development of their children are considered among the best of the India. If these facts can be considered as indices of a better socioeconomic condition, it is an enigma, why the disease, if attributable solely to protein malnutrition, should be endemic in Kerala. Malnutrition at presentation of tropical calcific pancreatitis/fibrocalculus pancreatic diabetes patient could be secondary to severe exocrine and endocrine pancreatic deficiency; a case for pancreatitis related and diabetes related malnutrition rather than malnutrition related diabetes.

In view of the above observation in India and similar discrepancies noted in African countries, it has been emphasized the need for considering other factors that might play an important role, if not an initiating one in the etiopathogenesis of tropical pancreatitis.

2.6.3. Cyanogenic alkaloids

Epidemiological data in support of cassava as a cause of tropical calcific pancreatitis show that the disease is more prevalent in Nigeria, Uganda, India and in some parts of Brazil where cassava intake is high. Even in Nigeria it is more common in the cassava eating north[19]. Causative role of cassava in the etiology of tropical calcific pancreatitis also suggested by Mcmillan[19,80]. Deficiency of dietary sulfur amino acid (methionine, cystine) could hamper detoxification of cyanide to thiocyanate and lead to high level of free cyanide, which might be toxic to β cells. Acute cyanide poisoning is known to be associated with hyperglycemia and McMillan produced hyperglycemia in rats by feeding cyanide[19]. Toxicity of cyanogenic alkaloids might operate through increased oxidant stress[81]. However studies in Africa failed to find tropical calcific pancreatitis or fibrocalculus pancreatic diabetes in areas where cassava is eaten as a staple food[82]. These reports cast doubt over the etiological role of cassava in tropical calcific pancreatitis but the absence of a susceptibility factor in this population could also explain the result. Occurrence of tropical calcific pancreatitis in areas where cassava is not eaten is also

cited as evidence against an etiological role for cyanide. However other cyanide containing foodstuffs (ragi, sorghum) could be responsible[17,29].

2.6.4. Genetics of pancreatitis

Pancreatitis in cystic fibrosis has been extensively studied. So far 600 mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene have been reported to be implicated in the pathogenesis of pancreatitis of cystic fibrosis. Of the huge number of variations reported Δ F508 was found accounted in 70% cases[83]. Recently some of the common mutations of the CFTR gene have been found in the patients with idiopathic pancreatitis[84]. This interesting finding has created interest among the geneticist for extensive study to explore genetic component in the pathogenesis of pancreatitis in IP.

Although the geographical distribution of tropical calcific pancreatitis is highly suggestive of an environmental etiology, familial clustering has also been reported from south India. Pancreatitis has been observed in successive generation by Pitchumoni in 1970[38]. Families with more than one tropical calcific pancreatitis patient, sometimes in successive generation have been described[85]. A systemic study from south India in families of 38 fibrocalculus pancreatic diabetes patient showed evidence of exocrine pancreatic involvement in 12 percent parents and 21 percent of sibling and 21 percent parents and 11 percent of siblings revealed previously undiagnosed type 2 diabetes[46].

Unless a specific marker is available it is difficult to decide whether these results reflect genetic transmission or a strong environmental influence. However, consanguinity was common in families with tropical calcific pancreatitis in successive generation and this might indicate a genetic connection. Genetic marker for type 1 and type 2 diabetes (HLA DQ gene and the insulin gene) have been studied in the same population which showed presence of an HLA DQB marker in 40 percent fibrocalculus pancreatic diabetes patient, class-3 insulin gene marker in 40 percent and both marker in 20 percent fibrocalculus pancreatic diabetes patient but 1 percent in control[47].

The relevance of these markers in the pathogenesis of fibrocalculus pancreatic diabetes is debatable specially if diabetes is secondary to pancreatitis. Markers of autoimmune islet cell damage (Islet cell antibody) are however absent in fibrocalculus pancreatic diabetes and histology does not show insulinitis[86].

Hereditary pancreatitis (HP) has also been described in USA by Gross and Arechlogan and they proposed non sex linked Mendelian dominant transmission[87,40] but other postulated that genetic abnormality in HP encompasses a wide variety of structural and anatomical defect of pancreatic ductal system which may be amenable to surgery[88,89].

Recently in Germany a mutation in the cationic trypsinogen gene was detected in 5 patients: in 2 patients with a family history of chronic pancreatitis (CP) and in 3 patients with idiopathic CP[44]. In one patient of this study the well known R122H (R117H) mutation was detected and in four patients a hitherto unknown mutation was found at the signal peptide cleavage site leading to an alanine to valine exchange in codon 16 (A16V). These results indicate that a significant percentage of patients with idiopathic CP may have a genetic basis for their disorder; therefore, genetic testing should be included in the diagnostic evaluation of these patients.

2.6.5. Oxidant stress and antioxidant deficiency

Free radical mediated damage: The human body is constantly under attack of free radicals. Everyday human body undergoes oxidative damage mainly due to the formation of free radicals (oxidants), which are highly reactive, unstable compounds[90]. Free radicals also occur as a part of normal cell metabolism and by exposure to environmental factors such as UV light, cigarette smoke, and environmental pollutants and gamma radiation. Some toxic compounds can result in the production of free radicals, which includes anti-cancer drugs, anesthetics, analgesics etc. Free radicals cause cellular damage, disrupt and deactivate the cell ability to function properly. Ultimately leading to the damage of vital molecules like DNA, proteins, lipids and carbohydrates. Fortunately human body can protect itself against these unfavorable oxidation with the aid of antioxidants. Antioxidants neutralize or deactivate the undesired free radicals produced in the body and protect the body from the harmful consequences of free radicals[91].

Diabetes is associated with an increased incidence of vascular complications. Oxidative damage by free radicals has been implicated in the development of vascular disease in patients with diabetes[92]. Serum total antioxidant activity was significantly lower in patients with type I diabetes than in controls. Vitamin C, vitamin E and urate are the major contributors to serum total antioxidant activity. Lower total antioxidant status in normal individuals was found with family history of heart diseases[93]. Increased lipid

peroxidation and reduced antioxidant status may contribute to the development of complications in diabetes[94]. There are one hundred diseases in which free radicals have been implicated. Total antioxidant status measures the antioxidant protection of an individual.

Oxidant stress in pancreatitis: Braganza suggested that tropical calcific pancreatitis is a result of (i) heightened and unmitigated oxidative detoxification reaction in pancreas and liver coupled with (ii) exposure to xenobiotic biotransformed by cytochrome P-450 and (iii) a relative deficiency of antioxidant[33]. In preliminary studies she found evidence of increased exposure to xenobiotics specially polycyclic aromatic hydrocarbon (cigarette and fire wood smoke and vehicular fumes) in patient of fibrocalculus pancreatic diabetes compared to controls[95].

2.6.6. Rare causes of calcific pancreatitis in tropics

In tropical countries where parasitic infestation is common one would expect a high incidence of helminthic pancreatitis. The worm that infest the pancreas and its duct systems *are Ascaris lumbriccoides, Schistosoma mansoni* and *haematobium, Clonorchis sinensis, Echinococcus granulosus* and *Taenia saginata*.. Only schistosomiasis and hydrated diseases causes pancreatic calcification[53,,96] and others cause acute pancreatitis[97,98]. Pancreatitis in Th cases appeared to be due to ampullary or pancreatic ductal obstruction[99].

2.6.7. Other causes of chronic calcific pancreatitis

2.6.7.1. Alcohol

Alcohol consumption is considered to be the main cause of chronic calcific pancreatitis in the temperature areas of the world including Europe, USA, Argentina, Chile, South Africa and Japan but also in Latin America - Brazil and Mexico and in some African states: Senegal, Ivory coast, Madagascar and Uganda[100]. Alcoholic pancreatitis in the developed countries constitutes 70-80 percent of calcific pancreatitis[3]. There is a linear relationship between the logarithm of the risk of developing chronic calcific pancreatitis and the mean daily consumption of alcohol[101]. The risk also increases with duration of

consumption. The type of alcohol beverage and the pattern of alcohol consumption have no significant influence on the risk of developing chronic calcific pancreatitis[102]. Chronic calcific pancreatitis is frequently observed in people who drink more alcohol and eat more protein and fat than controls[100] but in other studies there was no significant difference between the two groups[103].

The mechanism by which alcohol induces chronic pancreatitis is still a matter of dispute. The generally accepted theory for the mechanism of alcoholic chronic pancreatitis is the intraductal formation of protein plugs with or without calcification leading to obstruction and secondary damage and destruction of the acinar tissue which gives rise to an inflammatory reaction with fibrosis. This theory is substantiated by changes in the pancreatic juice in chronic alcoholics which include elevated protein and calcium concentration leading to hypersaturated pancreatic juice[104,105]. Pancreatic lithogenesis seems to comprise precipitation of calcium salt and degraded from of a special protein called pancreatic stone protein (PSP). PSP prevents calcium precipitation in saturated solution suggests that a deficiency of pancreatic stone protein in pancreatic secretion could play an important part in the disease. This is likely since the messenger RNA responsible for pancreatic stone protein biosynthesis is significantly decreased in the pancreas of patient with CCP (whether alcoholic or non alcoholic)[106]

Allan and White[107] give alternative mechanism for the formation of intraductal plugs. They postulated abnormal enzyme activation in the ducts giving rise to abnormal protein. which probably flocculate.

In contrast Harada et al[108] postulated the necessity of clusters of desquamated epithelial cells and a high concentration of mucopolysaccharides for the formation of intraductal plugs. Harada et al. also postulated the high viscosity of the pancreatic juice to be essential. It is also known that the pancreatic juice of pancreatic stone protein patient has low bicarbonate, increased amount of lactoferrin, an increased ratio of lysosomal to digestive hydrolases and reduced pancreatic trypsin inhibitor[109].

2.6.7.2. Metabolic abnormalities

Hyperparathyroidism and hyperlipidemia are established etiologic factors for chronic pancreatitis. The proportion of cases of chronic pancreatitis varies between 0 and 5 percent in different studies. Hyperparathyroidism (hypercalcimia) induces a high concentration of calcium in the pancreatic juice, which probably is the basis for intraductal precipitation and calcification[110]. The mechanism for the pancreatic damage in hyperlipidemia is obscure.

2.6.7.3. Hereditary pancreatitis

Hereditary pancreatitis (HP) describe first by Comfort and Steinberg[39] in 1952 and later by Gross et al[87] in 1962 from the Mayo clinic has provided some information regarding the congenital anomalies of pancreatic duct system. There are more than 92 families with 482 of hereditary pancreatitis in the literature up to up to 1976. In Gevarghese's series of 1700 cases of tropical calcific pancreatitis incidence of familial pancreas was 8 percent in father and son; mother and daughter, brother, sister and three pair of twin[3]. The precise nature of the inherited defect is still unknown. The most probable mechanism of hereditary pancreatitis is pancreatic ductal anomaly. It is suggested that a disease with non-sex linked autosomal dominant inheritance, as hereditary pancreatitis should manifest itself with anatomic defect rather than with biochemical abnormalities. It is postulated that the defect may be at the level of the sphincter of Oddi or in the pancreatic duct itself[88,89].

It has also been suggested that decreased concentration of pancreatic stone protein in pancreatic juice may genetically determine protein precipitation and stone formation in hereditary pancreatitis[3].

Hereditary pancreatitis (HP) is a rare, early onset genetic disorder characterized by epigastric pain and often more serious complications. Recently it has been suggested that an Arg-His substitution at residue 117 of the cationic trypsinogen gene is associated with the HP phenotype[42]. This mutation was observed in all HP affected individuals and obligate carriers from 5 kindreds, but not in individuals who married into the families or in 140 unrelated individuals. They have proposed a fail-safe mechanism. Trypsinogen is the products of trypsinogen gene. Acinar cells of pancreas produces this zymogen which

activates outside the pancreas when intestinal enterokinase hydrolyses it to active trypsin. As trace amounts of trypsin normally become activated within pancreatic acinar cells, two protective mechanisms are available to prevent the digestive enzyme activation cascade and pancreatic autodigestion----

1) Pancreatic secretory trypsin inhibitor (PSTI)

2) Trypsin like proteases--- mesotrypsin and enzyme Y, resistant to PSTI and rapidly inactivates trypsin.

In addition Trypsin itself catalyses trypsin degradation.

Substitution mutation of trypsinogen gene in exon 3 at R117H would render trypsin resistant to hydrolysis at this site causes autodigestion of pancreas which results pancreatic sclerosis associated with pancreatic exocrine failure, pain, ductal dilatation, calcifications, pseudocyst and/or diabetes mellitus.

In the eastern and mid western regions of United States a single A to T transversion mutation was identified in the second exon of cationic trypsinogen gene in the affected members of the hereditary pancreatitis (HP2) family[38] resulting in an asparagine to isoleucine transition mutation at position 21 (N21I). The identification of a second mutation in the cationic trypsinogen gene (HP2) suggests a dominant role of trypsin in premature protease activation-mediated forms of acute and chronic pancreatitis.

2.6.7.4. Cystic fibrosis (CF)

Cystic fibrosis with an incidence of 1 in 2000 live birth is the most common cause of pancreatic insufficiency in the first 3 decades of live in the USA. It is an inborn error of metabolism with autosomal recessive inheritance.

The incidence of pancreatic calcification in cystic fibrosis is 1:165 to less than 1:1000. According to Blanc calcification in cystic fibrosis is rather high[3].

The mechanism of pancreatic calcification in cystic fibrosis is shown to be the mucous plugs formed as a result of an excess quantity of viscid mucous secreted from histologically normal mucous gland, in the pancreatic duct due to an enzyme defect. Another explanation offered for the formation of protein plugs in cystic fibrosis is hypersecretion of zymogen granules[111]. The mucous plugs may contain even 10 times more calcium than in normal controls. Calcium combine with mucous anion and leads to

dehydration and inspissation of intraductal secretion and ductal obstruction. The resulting acinar over distention tends to break the continuity of their walls and cause leakage of pancreatic enzyme into the interstitial tissue. This is followed by local inflammation, tissue necrosis, interlobular saponification of fat and fibrosis finally the islet cells choked in fibrous tissue causing overt diabetes in almost 15 percent of cystic fibrosis individuals above 18 years of age[3].

It was unknown whether genetic factors predispose patients to idiopathic pancreatitis. In patients with cystic fibrosis, mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene typically cause pulmonary and pancreatic insufficiency while rarely causing pancreatitis. In a recent study it has been found a strong association between chronic pancreatitis and *CFTR* mutations among 27 patients who do not have lung disease of cystic fibrosis and were referred for endoscopic evaluation of idiopathic chronic pancreatitis[84]. In these patients, the frequency of a single CFTR mutation was 11 times the expected frequency and the frequency of two abnormal alleles was 80 times the expected frequency.

2.6.7.5. Congenital anomalies

Chronic calcific pancreatitis has been described in a number of patients with different anatomical abnormalities. Calcific pancreatitis in pancreas divisum has been reported by Sahel *et al*[112]. Geevarghese found calcific pancreatitis in annular pancreas, anomalies of the accessory duct (accessory duct does not open into duodenum, hypertrophy of sphincter). Other anatomical abnormalities which may be associated with chronic pancreatitis includes juxtapapillary duodenal diverticula, duodenal enterogenous cyst and duodenal polyp[3,113].

2.7. Genetics of Diabetes

Diabetes mellitus is not a single disease, but a genetically heterogeneous group of disorders that share glucose intolerance in common[114-116]. It was widely recognized the diabetic individuals have an increased family history of disease[117]. The prevalence of diabetes varies in different ethnic groups and this is probably due to genetic rather than environmental causes in most instances. This is highlighted in the communities, which

exhibit a 'founder effect'. In the Pacific Island of Nauru the over all prevalence of diabetes is about 30%.

Progress in the identifying genetic markers was greatly hampered due to extreme heterogeneity of different chromosome and locus association of different types of diabetes.

Type 1 diabetes

Sixty to seventy percent of the genetic susceptibility to type one diabetes can be accounted for by associations with the HLA loci on short arm of chromosome 6. In population surveys of American and European Caucasoid subjects with type 1, associations were found HLA-A1-B8-DR3, B18-DR3 and DR4. In different racial population the DR association are different: in Chinese DR3 is associated with type 1 and in Japanese with DR4[118,119].

HLA gene studies

The HLA complex is comprised of the Class I region (A, B, C and E loci), the class III region (TNFα, TNFβ, complements) and Class II region (HLA-Dr, DQ and DP). The total complex spans 3500 kb. The Class II region (1000 kb) encodes class II molecules, which consists of non-covalently bound α and β chains, and is subdivided into the DR (3-4 DRβ genes and one a chain) DQ (1 DQ α, DQ β 1 DX α and 1 DX β gene).

Non-MHC associations in type 1

The most consistent non-MHC association with type 1 has been that of class I allele in 5'-flanking region of insulin gene[120,121]. This has been reported by groups in the UK, America and Denmark but not found in Black populations, Japanese and Indian type 1 diabetics.

Type 2 diabetes

Despite the demonstration that Type 2 diabetes is a predominantly genetic disease the genes involved in its pathogenesis are far from being elucidated. The two loci most studied are the insulin gene and insulin receptor. Till date defecting of the following genes have been identified for the development of type type 2 diabetes[122]: Insulin gene, Insulin receptor gene, Insulin receptor substrate-1 gene, Glucagon like peptide-1 receptor gene, Glucose transporter genes (Glut-1, Glut-2, Glut-4) Glucokinase gene, HNF 4α in

chromosome 20, HNF 1 α, in Chromosome 12, HNF 1 β in chromosome 17, IPF 1 in chromosome 13, the HLA region on chromosome 6, Genes involved in lipid metabolism, Glycogen synthase gene, Beta3-Adrenergic receptor gene and genes of the mitochondrial DNA.

SUBJECTS AND METHODS

3. SUBJECTS AND METHODS

3.1. Type of study

This was family based prospective study.

3.2. Place of study

The study was carried out at the Department of Cell & Molecular Biology, Research Division, Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic disorders (BIRDEM).

3.3. Subjects

A total of 11 FCPD and 7 TCP subjects were recruited from Gastroenterological practices in Dhaka City and BIRDEM. Five parents from each TCP and FCPD group were incorporated in the study, which comprises TCP and FCPD simplex families. Two multiplex (Fig 1 and 2) families, TCP and FCPD members of which were incorporated as individual members, were also studied.

Patients with history of abdominal pain were first screened for pancreatic calcification by plain X-ray abdomen and / or ultarsonogram. Subjects were then subjected to oral glucose tolerance test and diabetes was diagnosed on the basis of WHO study group criteria. Patients with pancreatic calcification without diabetes were entered into the study as TCP and patients with pancreatic calcification having diabetes were included as fibrocalculus pancreatic diabetes (FCPD).

Control (n=10) subjects without any family history of diabetes up to second generation were collected from a garments factory near Rampura area. Informed consent was obtained from each subject before entry into the study.

Family I

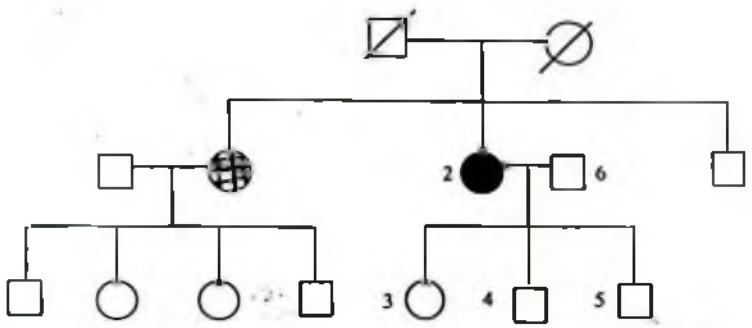


Fig 3: Pedigree of F1 multiplex family

Family II

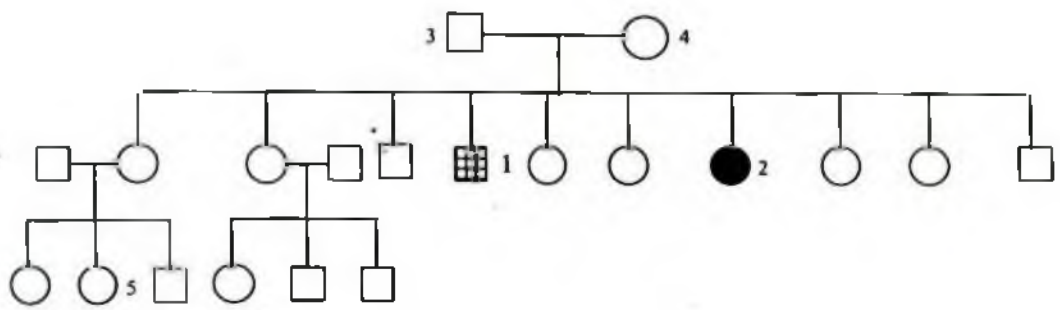


Fig 4: Pedigree of F2 multiplex family

FCPD ■ TCP ▤

3.3.1. *Exclusion criteria*

- Patients presenting pancreatitic calcification with acute complication of diabetes.
- Patients suffering from systemic illness on the basis of history and clinical examination.
- Females who were pregnant.

3.4. Methods

Detailed sociodemographic data, pedegree of the proband, family history of diseases and medical history were taken using a predesigned Case Record Form. Physical and clinical examinations of the patients were done on the very first day of the visit. Detail of each of the parameters are given below:

3.4.1. Annual family income: Annual income in the family was expressed in Taka per annum.

3.4.2. Personal history: Subjects were asked about smoking, alcohol drinking, eating of panta rice and dry fishes.

3.4.3. Relevant past history: History of measles, mumps, pox, jaundice, respiratory tract infection in the past were taken from the subjects and accompanied relatives. Special esquire was done about abdominal pain suggestive of pancreatitis and large bulky loose stool suggestive of steatorrhea. History of malnutrition was noted with specific question about edema in the past.

Family history of diabetes mellitus

History of diabetes in the family was taken up to second generation and second degree relative which included father, mother, sibling, grandparents from both paternal and maternal sides, uncle and aunts from both paternal and maternal sides and cousins. Diabetes in first degree relatives was considered when diabetes was present in father or mother or both along with siblings.

3.4.4. Physical examination: In general examination special attention was given about stigmata of malnutrition, vitamin and mineral deficiencies.

3.4.4.1. Anthropometric measurements

The body weight, in kilogram was measured in patients wearing light cloths and height in

centimeter, by using appropriate scales on bare foot (Detect-Medic, Detect Scales INC, USA). Skin fold thickness (biceps, triceps, sub-scapular) in millimeter were measured by using the Harpenden Skinfold Calipers.

Body mass index of the subjects was calculated as weight in kg divided by height in square meter

3.4.4.2. Measurement of blood pressure and pulse

Pulse and blood pressure of each patients were recorded by the same physician. Blood pressure was measured with a sphygmomanometer (Turf Trading Co, Ltd. Tokyo, Japan) following standard procedure.

3.4.4.3. Radiological and imaging study

Plain X-ray abdomen in supine position in empty stomach in the morning after meticulous preparation with tablet ultracarbon and laxative was done to detect pancreatic calcification.

3.4.5. Preparation of the subjects

After selection of the subjects, the experiments to be performed were explained to individual patients and informed consents were taken from each of them. Prior appointments were given for the subsequent experiments subjects were requested to fast overnight (12 hours) and advised not to take any kind of medicine or smoke on the previous day. Oral glucose tolerance test was performed between 8:00 - 9:00 am in the next morning. After taking fasting blood sample of 6 ml in EDTA containing test tube, patient was swallowed 1.75 g/kg body weight glucose dissolved in 200 ml of water. After 2 hours of glucose load another 8 ml blood was drawn in another tube.

Blood samples were drawn for estimation of fasting and 2h after glucose, lipid profile, total protein, albumin, total antioxidant status, C-peptide, insulin, ICA, IAA and anti-GAD antibody.

3.4.5.1. Laboratory wares

Glass wares and accessories were washed properly using laboratory quality detergent.

(a) The materials were first washed with detergent.

(b) Then rinsed with double distilled deionized water several times.

(c) It was then dried in drier at 60°C.

3.4.5.2. Collection of blood samples

Blood samples drawn were allowed to settle for 15 mins leaving the test tubes on ice. Blood samples were then centrifuged for 15 minutes at a rate of 3000 rpm at 4°C. Plasma separated was allequoted and preserved immediately at - 70°C for future analyses.

For the genetic analysis 5 ml of the blood (EDTA 1 mg/ ml) was preserved at -70°C for future DNA extraction and genetic analyses.

3.4.6. Laboratory techniques

3.4.6.1. Estimation of plasma glucose

Plasma glucose was estimated by Glucose Oxidase (GOD-PAP) method (SERA PAK, Bayer USA)[123].

3.4.6.1.1. *Principle*

The aldehyde group of β-D-glucose is oxidized by glucose oxidase to give gluconic acid and hydrogen peroxide. Hydrogen peroxide is further broken down to water and oxygen in presence of peroxidase and in presence of an oxygen acceptor i.e. phenol, a color compound is produced. The reaction of (GOD-PAP) reagent with glucose produces 4-aminophenazone, a colored compound.

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconate} + H_2O_2$$

$$2H_2O_2 + \text{4- aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{4-(P- benzoquinone-mono- imino) phenazone} + 4H_2O$$

3.4.6.1.2. *Preparation of standard solution*

A sample of 36 mg of glucose (MW, 180.16, E.MERCK Limited, Bombay, India) was taken in a clean stopper test tube and then dissolved in 10 ml of water to make a stock solution of 20 mmol/l. From the single stock solution different concentration (1, 2, 4, 8, 12 and 16 mmol/l) of standard solutions were prepared with distilled deionized water. The

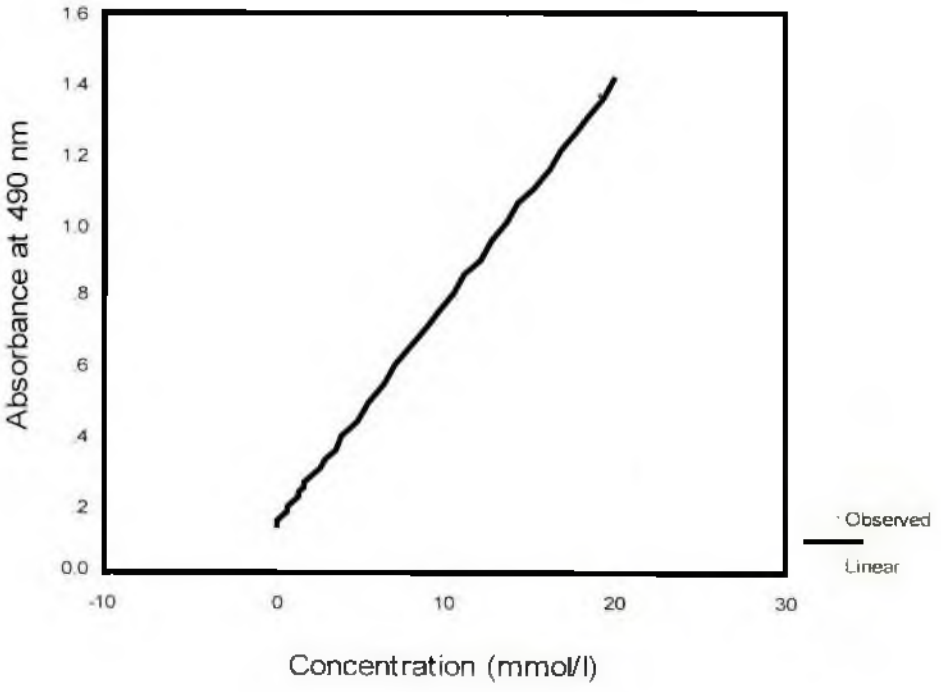
concentration of standard solutions were 1, 2, 4, 8, 12,16 and 20 mmol/l respectively.

3.4.6.1.3. *Procedure*

Standard glucose solution (5 µl) of each concentration were taken in the initial 8 wells of the microplate. Then plasma (5 µl) was taken in the remaining microwells of the plate and in all the well GOD-PAP reagent (200 µl) was added. The mixture was then incubated for 15 minutes at 37°C and then absorbance of the solution was measured. All absorbance were measured at 490 nm with the help of Microplate Reader (Bio-Tek EL-340, USA). Two parallel experiments were carried out for each sample. Thus a calibration curve was obtained for the absorbance vs concentrations of the standard solutions against a reagent blank. On the basis of the calibration curve, the unknown concentrations of glucose in plasma sample were measured maintaining the same mixing and incubation conditions as for the standard solutions. The standard curve was drawn on every experimental day.

3.4.6.1.4. *Calculation of Results*

Concentration was calculated by using Kinetic-Calculation program for the microwell plate Reader.



Standard curve for glucose

3.4.6.2. Estimation of Plasma C-peptide

Serum C-peptide was estimated by an ELISA method (DRG International Inc, Germany[124].

3.4.6.2.1. *Principle*

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

3.4.6.2.2. *Reagents*

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

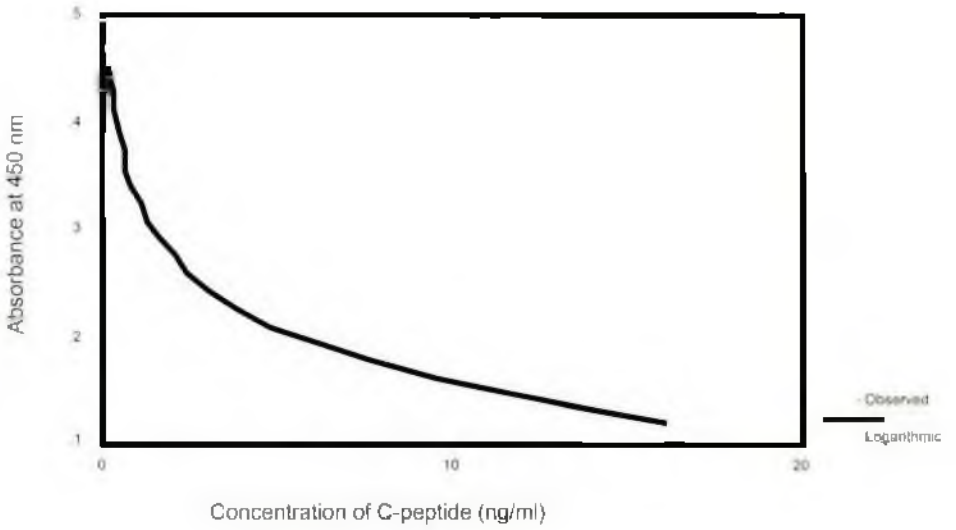
3.4.6.2.3. *Materials Required*

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

3.4.6.2.4. *Procedure*

1. Desired number of coated microtiter wells were placed in the holder.
2. 100 µl of C-peptide standards were dispensed into wells.

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



Standard curve for C-peptide

3.4.6.2.5. *Calculation of Results*

Optical densities were fed into a computer and calculation was done using the software program (Kinetic calc). Values for the unknown samples were calculated by extrapolating the log-fit standard curve for the standard.

3.4.6.3. Estimation of Plasma insulin

Plasma insulin was measured by IMX method (Abott Laboratories, Tokyo, Japan)

3.4.6.3.1. *Principle*[135]

The Abott IMX insulin assay is a microparticle absorbed Fluorescence Enhanced Enzyme Immunoassay (MEIA). It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample react with alkaline phosphatase-conjugate anti-insulin antibodies and anti-insulin antibodies bound to microtritration well. A simple washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 4-methylumbelliferyl phosphate and the fluorescent product is measured by the MEIA optical assembly. The IMX insulin assay shows no cross-reactivity with pro-insulin.

3.4.6.3.2. Reagents

Reagent pack

IMx Insulin Reagent Pack, 100 Tests (No. 2a 10-20)

1. 1 bottle (7 ml) Anti-Insulin (Mouse, Monoclonal) Coated Microparticles in buffer with protein stabilizers. Preservative: Contains Sodium Azide and Antimicrobial agents.
2. 1 bottle (9 ml) Anti-Insulin (Mouse, Monoclonal): Alkaline Phosphatase Conjugate in buffer with protein stabilizers. Minimum concentration: 3 g/m l. Preservative: Contains Sodium Azide and Antimicrobial agents.
3. 1 bottle (10 ml) 4-Methylumbelliferyl Phosphate, 1.2 mM, in buffer. Preservative: Contains Sodium Azide.

4. 1 bottle (14 ml) Assay Buffer in calf serum. Preservative: Contains Sodium Azide and Antimicribial agents.

IMx Insulin MODE 1 Calibrator

1 bottle (4 ml) Mode 1 Calibrator (D). Concentration: 30 μU/ml. Insulin (porcine derived human)[11] in buffer. Preservative: Contains Sodium Azide and Antimicrobial Agents.

CALIBRATORS

IMx (R) Insulin Calibrators (No. 2A 10-10)

6 bottles (4 ml each) of IMx Insulin Calibrators are referenced against WHO Insulin 1st IRP at 92.5% of the WHO concentration. The IMx Insulin Calibrators contain insulin (porcine derived human) prepared in buffer at the following concentration:

1	0 μU/ml
2	3 μU/ml
3	10 μU/ml
4	30 μU/ml
5	100 μU/ml
6	300 μU/ml

Preservative: Contains Sodium Azide and Antimicrobial Agents.

CONTROLS

3 bottles (8 ml each) of IMx Insulin Controls contain insulin (porcine derived human) prepared in buffer to yield the following concentration ranges:

1	8 μU/ml	6-10 μU/ml
2	40 μU/ml	32-48 μU/ml
3	120 μU/ml	96-144 μU/ml

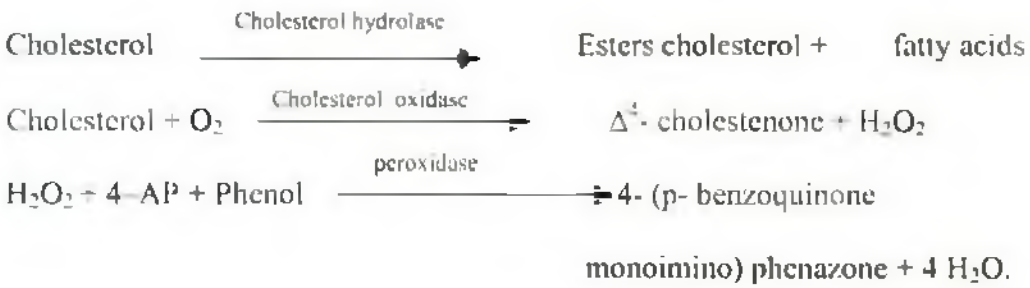
Preservative: Contains Sodium Azide and Antimicrobial Agents.

3.4.6.4. Estimation of plasma total cholesterol

Total cholesterol was measured by enzymatic colorimetric (Cholesterol Oxidase/ Peroxidase) method (SERA PAK, Bayer USA).

3.4.6.4.1. *Principle* [126,127,128]

Cholesterol esters are hydrolyzed by cholestserol hydrolase to free cholesterol and fatty acids. Existing free cholesterol together with that produced by this reaction is oxidized by cholesterol oxidase (CHOD, EC 1.1.3.6) to Δ^4- cholestenone and hydrogen peroxide. The latter, in the presence of peroxidase (EC 1.11.1.7) oxidizes the chromogen system 4-aminophenazone (4-AP) / 2- hydroxyphenylacetic acid to a red quinoneimmine with a maximum absorbance at 490 nm. The principle is based on the following reaction system:



3.4.6.4.2. *Reagents*

1 Buffer /2- hydroxyphenylacetic acid /surfactants

1A Buffer /Enzymes / 4-Aminophenazone /Potassium Ferrocyanide

Standard: Cholesterol 200 mg/dl (5.17 mmol/l).

Preparation of working solutions

30 ml of reagent 1 was added to one vial 1A and mixed gently to dissolve the contents. The solution was stable for two weeks at 2° to 8°C.

Components and concentrations of working solution:

Phosphate buffer	100 mmol/l, P^{H} 7.2
Cholesterol oxidase	≥170 U/l
Cholesterol ester hydrolase	≥400 U/l
Peroxidase	≥400 U/ l
2- hydroxyphenylacetic acid	9 mmol/l
4- aminophenazone	0.5 mmol/l
Potassium ferrocyanide	7 µmol/l
Surfactants	6 g/l

3.4.6.4.3. *Procedure*

A series of standard cholesterol solution (0, 25, 50, 100, 125, 150 mg/dl) were prepared by diluting a stock standard solution of cholesterol (200 mg/dl). Standard cholesterol solution (5 µl) of each concentration were taken in the initial 6 microwell of the plate. Then the plasma (5 µl) was taken in the remaining microwells of the plate and in all well the working reagent (200 µl) was added. The mixture was then incubated for 5 minutes at 37°C and then absorbance of the solution was measured. All absorbance were measured at 490 nm with the help of microplate Reader (Bio-Tek EL-340, USA). Two parallel experiments were carried out for each sample. Thus a calibration curve was obtained for the absorbance vs. concentrations of the standard solutions. On the basis of the calibration curve, the unknown concentrations of cholesterol in plasma sample were measured maintaining the same mixing and incubation conditions as for the standard solutions. The standard curve was drawn on every experimental day.

3.4.6.4.4. *Calculation of results*

Concentration was calculated by using kinetic-Calculation program.

4.4.6.5. Estimation of plasma Triglycerides

Plasma Triglycerides was measured by enzymatic colorimetric (GPO-PAD) method (SERA PAK,Bayer USA)

3.4.6.5.1. *Principle*[179]

Sample triglycerides incubated with a lipoprotein lipase liberate glycerol and fatty acids. Glycerol is converted to glycerol-3-phosphate by glycerolkinase and ATP. Glycerol phosphate oxidized to dihydroxyacetone phosphate by glycerol phosphate oxidase in the presence of peroxidase, hydrogen peroxides oxidizes the chromogen 4-aminophenazone/N-ethyl-N-(3-sulphopropyl)-m-anisidine to a violet colored compound. The formed hydrogen peroxide is detected by a chromogenic oxygen acceptor, phenolampyrone, in the presence of peroxidase (POD). The red quinone formed is proportional to the amount of triglycerides present in the sample. The principle is based on the following reaction system:

$$\text{Triglycerides} + H_2O \xrightarrow{\text{lipase}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + ATP \xrightarrow{\text{glycerol kinase}} \text{Glycerol-1-P} + ADP$$

$$\text{Glycerol-1-P} + O_2 \xrightarrow{\text{glycerol-1-P oxidase}} \text{Dihydroxyacetone-P} + H_2O_2$$

$$H_2O_2 + \text{phenol} + \text{Ampyrone} \xrightarrow{\text{peroxidase}} \text{Quinone} + 2H_2O$$

3.4.6.5.2. *Materials*

- Micropipettes and pipettes
- Incubator for maintaining 37°C temperature.
- Photometer for measurements at 500 nm (±10 nm).

3.4.6.5.3. *Reagents*

1 N-ethyl-N- (3-sulphopropyl)- anisidine / Surfactant / Buffer

1A Enzymes /ATP /4-Aminophenazone /Potassium Ferrocyanide

Standard: Glycerol (equivalent to 200 mg/dl of triolein).

3.4.6.5.4. Preparation of working reagents

30 ml of reagent 1 was added to one vial 1A and mixed gently to dissolve contents.

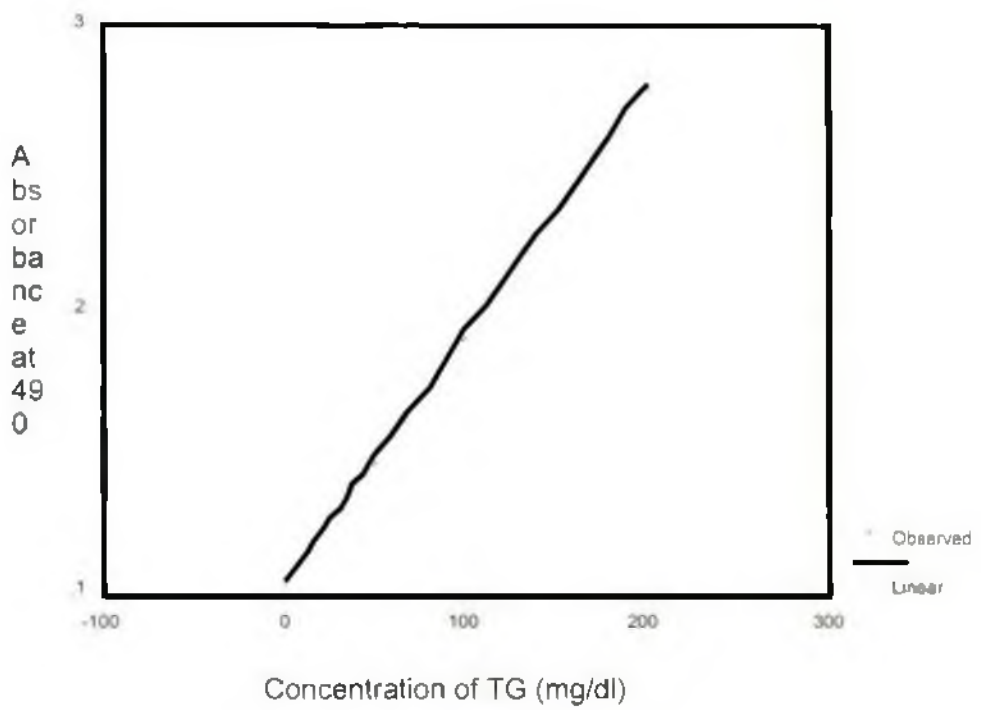
Component and concentration of working solutions:

Buffer	50 mmol/l, P^H 7.0
Lipoprotein lipase	≥ 50 U/ml
Glycerolkinase	≥ 0.05 U/ml
Glycerolphosphate oxidase	≥ 2.0 U/ml
Peroxidase	≥ 0.3 U/ml
Adenosine 5- triphosphate	0.7 mmol/ l
4- aminophenazone	1.0 mmol/l
Potassium ferrocyanide	7.0 µmol/l
Magnesium salts	0.6 mmol/l

N-ethyl- N –(3-sulphopropyl)-m-anisidine	1.2 mmol/l
Surfactant stabilizers	2.0 g/l

3.4.6.5.5. *Procedure*

Series of standard triglyceride solutions (0, 25, 50, 100 and 150 mg/dl) were prepared by diluting a stock standard solution of triglyceride (200 mg/ dl). Standard triglycerides solutions (5 µl) of each concentration were taken in the initial 6 microwell of the plate. Then the plasma (5 µl) was taken in the remaining microwells of the plate and in all well the working reagent (200 µl) was added. The mixture was then incubated for 5 minutes at 37°C and then absorbance of the solution was measured. All absorbance were measured at 490 nm with the help of microplate Reader (Bio-Tek, EL-340, USA). Two parallel experiments were carried out for each sample. Thus a calibration curve was obtained for the absorbance vs concentrations of the standard solutions. On the basis of the calibration curve, the unknown concentrations of triglyceride in serum were measured maintaining the same mixing and incubation conditions as for the standard solutions. The standard curve was drawn on every experimental day.



Standard curve of triglyceride

3.4.6.5.6. *Calculation of results*

Concentration was calculated by using kinetic-calculation program for microwell plate Reader.

3.4.6.6. Estimation of Plasma High density Lipoprotein (HDL)

Plasma High density Lipoprotein (HDL) was measured by enzymatic colorimetric (cholesterol Oxidase/ Peroxidase) method (SERA PAK, Bayer, USA).

3.4.6.6.1. *Principle*[130]

HDL (High Density Lipoproteins) are separated from chylomicrons, VLDL (Very Low Density Lipoproteins) and LDL (Low density Lipoprotein) by the addition of a precipitating reagent (phosphotungstic acid-magnesium chloride) to serum or plasma. After centrifugation, the cholesterol contents of HDL fraction which remains in the supernatant are determined by the enzymatic- colorimetric method using cholesterol esterase, cholesterol oxidase, peroxidases and a chromogen system.

3.4.6.6.2. *Reagents*

1 Buffer / 2-hydroxyphenylacetic acid / surfactants

1A Buffer/ Enzymes /4-Aminophenazone/ Potassium Ferrocyanide

Standard: Cholesterol 50 mg/dl (1.29 mmol/l).

3.4.6.6.3. *Preparation of working reagents*

Precipitating Reagent: The precipitating reagents contains

Phosphotungstic acid	7.0 g/l
Magnesium chloride	39 mmol/l

Solution (1)

30 ml of reagent 1 (code 6684) was added to one vial 1A (code 6687) and mixed gently to dissolve contents.

Components and concentrations of working solutions:

Solution (1)	
Phosphate buffer	100 mmol/l, P^{H} 7.2
Cholesterol oxidase	≥170 U/l
Cholesterol ester hydrolase	≥400 U/l
Peroxidase	≥400 U/ l
2- hydroxyphenylacetic acid	9 mmol/l
4- aminophenazone	0.5 mmol/l
Potassium ferrocyanide	7 µmol/l
Surfactants	6 g/l

3.4.6.6.4. *Procedure*

Samples (150 µl) and precipitating reagents (150 µl) were taken in a centrifuge tube and mixed was homogenezed by shaking and was left at 20 to 25 in a water bath. Then it was centrifuged at 4000 rpm for 10 minutes. The supernatant was used as sample for colorimetric assay. A series of standard cholesterol solutions (0, 10, 20, 25, 30, 40, 45 mg/dl) were prepared by diluting a stock standard solutions of cholesterol (50 mg/dl). Standard cholesterol solutions (15 µl) of each concentration were taken in the initial 6 microwell of the plate. Then the plasma (15 µl) was taken in the remaining microwells of the plate and in all well the working reagent (200 µl) was added. The mixture was then incubated for 5 minutes at 37°C and then absorbance of the solution was measured. All absorbance were measured at 490 nm with the help of microplate Reader (Bio-Tek EL-340, USA). Two parallel experiments were carried out for each sample. Thus a calibration curve was obtained for the absorbance vs concentrations of the standard solutions. On the basis of the calibration curve, the unknown concentrations of cholesterol in plasma sample were measured maintaining the same mixing and incubation conditions as for the standard solutions. The standard curve was drawn on every experimental day.

3.4.6.6.5. Calculation of results

Concentration was calculated by using kinetic-cal program.

3.4.6.7. ESTIMATION OF LDL-CHOLESTEROL

The LDL-Cholesterol level in plasma was calculated by using Friedewald formula:[131]

Formula

LDL cholesterol = Total cholesterol– (HDL cholesterol + 1/5 x Triglycerides).

4.4.6.8. Determination of plasma total protein

3.4.6.8.1. Test principle[132]

Proteins give an intense violet-blue complex with copper salts in an alkaline medium. Intensity of the coloration is a measure of the quantity of the total protein present in the sample tested. The test was adjusted to microwell plates in Research Division, BIRDEM.

3.4.6.8.2. Apparatus

Microplate reader

Adjustable pipette (0.5-10 µl) and adjustable multichannel pipette

Vortex
Centrifuge

3.4.6.8.3. Procedure

Serial dilution of given standard was done to construct a standard curve.

	Blank	Unknown	Standard
Plasma	-	5 µl	-
Standard	-	-	5 µl
Reagents	200 µl	200 µl	200 µl

The plate was put on to a vertical shaker to mix properly and allowed to stand at room temperature for 15 min. Absorbance was taken using microwell plate reader at a wave length 550 nm.

3.4.6.8.4. Calculation of results

Optical densities were fed into a computer and calculation was done using the software program (Kinetic calc). Values for the unknown samples calculated extrapolating the standard curve using kinetic package.

3.4.6.9. Estimatiuon of plasma albumin by bromocresol green method[132]

3.4.6.9.1. *Principle*

Formationof an albumin/bromocresol-green complex at pH 4.2 and photometric measurement of absorbance.

3.4.6.9.2. Reagents

	Initial concentrations of solutions
Succinate buffer	74 mmol/l, pH 4.2
Bromocresol green	0.15 mmol/l
Brij 35	

3.4.6.9.3. Preparation stability of reagent solution

Dilute contents of one bottle with 650 ml redistill water. Rinse bottle with an aliquot of this dilution and return to working solution.

Stable for three months at +15 to +25^{0}C.

3.4.6.9.4. *Procedure*

Measure against reagent blank (RB). One reagent blank and one standard are sufficient for each assay series

	Blank	Standard	Unknown
Reagent solution (ml)	3.00	3.00	3.00
Sample (ml)	-	-	0.01
Precinorm U (ml)	-	0.01	-

Mix, incubate for 5 minutes at 20-25^{0}C, then measure absorbances of sample and standard against reagentblank: A_{sample} and $A_{standard}$

if the albumin concentration exceeds 6g/dl, dilute the sample and repeat the assay.

3.4.6.9.5. Calculation of the concentration © of albumin in the sample

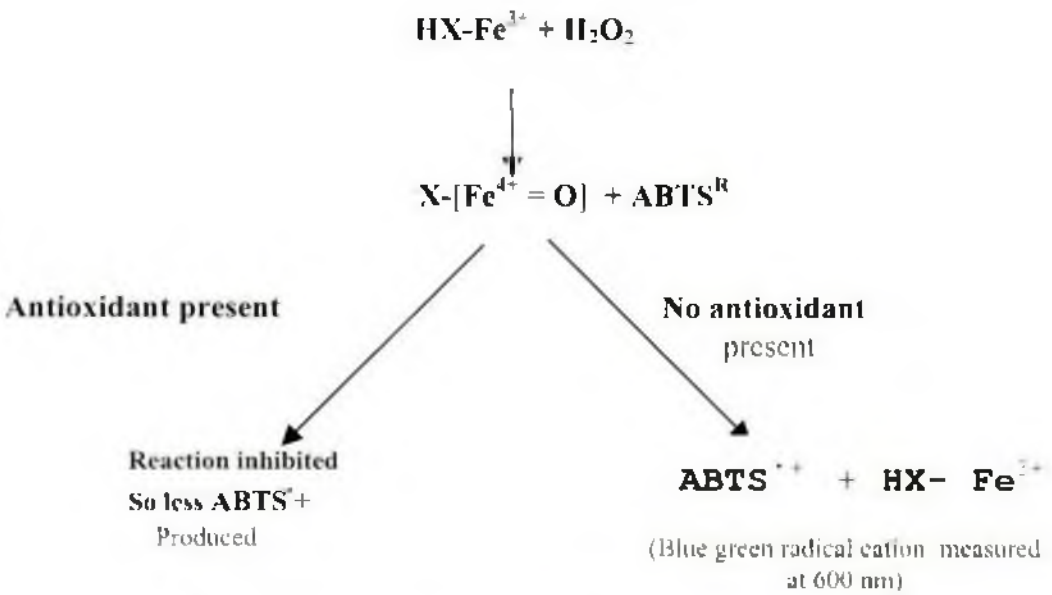
C = Actual value precinorm U x (A_{sample} / $A_{standard}$) g/dl

The actual value is specified in the precinorm U pack insert in the section 'Proteins'.

3.4.6.10 . Estimation of Plasma Total Antioxidant Status (TAS) by enzymatic Colorimetric method (*RANDOX,UK*)

3.4.6.10.1. Principle[133]

$ABTS^R$ (2,2′-Azino-di -[3-ethylbenzthiazoline sulphonate]) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the redical cation $ABTS^{\cdot}$. This has a relatively stable blue green color, which is measured at 630 nm. Antioxidants in the added sample cause suppression of this color production to the degree which is proportional to their concentration. This principle is



$HX\text{-}Fe^{3+}$ = Metmyoglobin

$X\text{-}[Fe^{4+} = O]$ = Ferrylmyoglobin

$ABTS^R$ =2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate]

3.4.6.10.2. Reagents

1. **Buffer**

Phosphate buffer saline	5 mmol/l, pH 7.4

2. **Chromogen**

Metmyoglobin	6.1 µmol/l
ABTS	610 µmol/l

3. **Substrate**

Hydrogen peroxide (in stabilised form) 250 µmol/l

4. **Standard**

6-hydroxy- 2,5,7,8-tetramethylchroman -2-carboxylic acid 1.66 mmol/l

3.4.6.10.3. ***Preparation of working solutions***

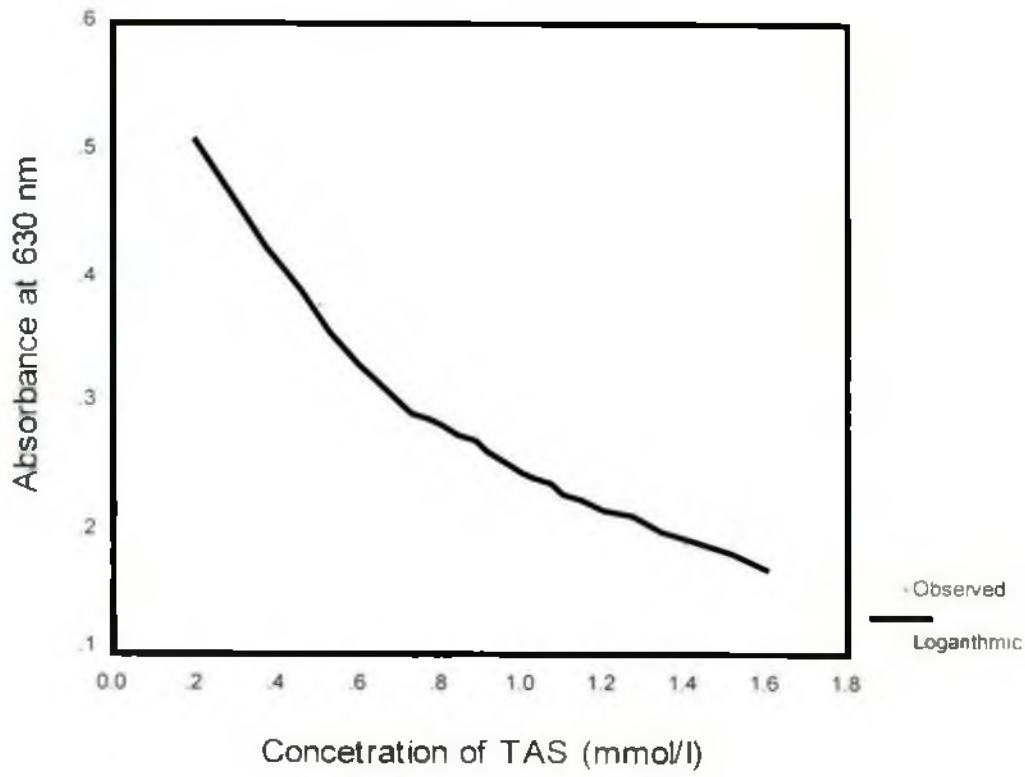
Chromogen: One vial of chromogen was reconstituted with 10 ml of buffer

Substrate: 1 ml of substrate was diluted with 1.5 ml of buffer

Standard: One vial of Standard (1.66mmol/l) was reconstituted with 1.0 ml of double deionized water.

3.4.6.10.4. Procedure

A series of standard solutions (0.0, 0.2, 0.6, 0.8, 1.0 and 1.2 mmol/l) were prepared by diluting a stock standard solution of 1.6 mmol/l. Standard solutions (5 µl) of each concentrations were taken in the initial 7 microwells of the microplate. Then plasma (5 µl) was taken in the remaining microwells of the plate and in all the well chromogen reagent (200 µl) was added. Then the solution was mixed well and the initial absorbance (A_1) of the solution was measured at 630 nm. Then the substrate solution (50 µl) was added to each microwell . By mixing well the final absorbance (A_2) of the solution was measured after exactly 3 minutes. All absorbance were measured at 630 nm with the help of microplate ELISA Reader (Bio- Tek EL- 340, USA). The difference of the absorbance ($\Delta A = A_2 - A_1$) was then calculated. Two parallel experiments were carried out for each sample. Thus a calibration curve was obtained for the absorbance (ΔA) vs. concentrations of the standard solutions. On the basis of the calibration curve, the unknown concentrations of TAS in plasma were measured maintaining the same mixing and incubation conditions as for the standard solutions. The standard curve was drawn on every experimental day.



Standard curve for TAS

3.4.6.10.5. Result

Optical densities were fed into a computer and calculation was done using the software program (Kinetical calculation) for microwell plate ELISA Reader (Bio- Tek, EL- 340, USA). Values for the unknown samples were calculated by extrapolating the log-fit standard curve for the standard.

3.4.6.11. Estimation of islet cell antibody (ica)

3.4.6.11.1. *Principle*[134]

The highly purified mixture of pancreatic antigens immobilized into microwells. The positive control, negative control and one dilution of patient plasma are added to the appropriate microwells. Antibodies in the plasma samples are allowed to react at room temperature with the antigen molecule on microwells. After washing off excess or unbound plasma materials, the bound antibodies are determined by an enzyme lebelled goat antibody specific to human IgG. After through washing a substrate is added and the

color generated is measured spectrophotometrically. The intensity of the color is directly proportional to the concentration of ICA in the sample.

3.4.6.11.2. Isletest reagents provided

Microwell Strips (with holder)

IgG Enzyme Conjugate (concentrate)

Sample Diluent (concentrate)

Conjugate Diluent

Positive Control

Negative Control

Substrate tablet buffer

Washing Buffer (concebtrate)

Substrate Tablets

Stopping Solution (1N NaOH)

3.4.6.11.3. Additional Materials

Deionized water

Absorbent paper towels to blot drey the strips after washing steps and plastic wraps to cover strips during incubations.

Glass test tubes (5 ml)

Micropipette with disposable trips to deliver 25 µl, 50 µl and 100 µl.

A microtiter plate washer.

Pipette (10 ml) for substrate buffer and conjugate diluent delivery.

500 ml graduated cylinder

microtiter plate reader with 405 nm absorbance capability.

Plastic label tapoe, to tape-up unused wells before assay.

3.4.6.11.4. Specimen collection

Samples were taken out of freezer 1 hr before and allowed to thaw and later on vortexed for 5 sec.

3.4.6.11.5. Reagent preparation

Isletest - IgG enzyme conjugate reconstitution: Accurately transferred 5 ml of the Isletest conjugate diluent into one bottle containingIsletest - IgG enzyme conjugate. Bottle was cleaned and mixed thoroughly by inversion.

*Isletest - sample diluent buffer:*Sample diluent buffer diluted according to the need. Calculated amount of concentrated isletest sample diluent transfer to a clear cylinders and added certain amount of deionized water and mix thoroughly by gentle inversion.

Isletest wash: Isletest wash concentrate is for 500 ml of isletest wash solution. Calculated volume of wash concentrate was taken in a clean cylinder, desired amount of deionized water added to it and mix throughly by inversion.

Plasma sample preparation: 25 µl of sample pipetted into 2.5 ml of the sample diluent

Isetest substrate reagent: The substrate reagent prepared immediately prior to use. keep it at room temperature for 15 min and shaking periodically.

3.4.6.11.6. Assay procedure

Calculated number of streps assembled in the holder. The microwell strip snapped in place firmly with its square side.

100 µl of islets negative control was dispensed into microwells C1 and D1.

100 µl of islets positive control was pipetted in microwells E1 and F1.

100 µl of diluted plasma was pipetted onto microwell in duplicate.

The plate was covered with a plate sealer and left at room temperature (25±1^{0}C) for 1 hr.

After 1 hr of incubation solution in the microwell was discarded and the microwells were washed with washing buffer using automatic plate washer, except the A1 and B1 in the

first column. Afterwards the plate was blotted by tapping on a paper towel.

100 µl of Isletest IgG-enzyme conjugate reagent was added into all wells except wells A1 and B1.

The plate was covered with a plate sealer and left it stand at 25±1^{0}C for 1 hr.

Substrate reagent was prepared.

After incubation washing step was repeated and the microwells were blotted dry.

100 µl of freshly prepared substrate was pipetted into all microwells including wells A1 and B1 at a rapid and steady pace without any interruption.

The plate was left in the dark for 30 min at 25±1^{0}C

After 30 min swiftly added 50µl of the stopping solution into each well B1 at a rapid and steady pace without any interruption.

Reading was taken using microwell plate reader at the wavelength 405 blanking the reader with wells A1 and B1.

3.4.6.11.7. Calculation

Average of reading of samples and both the positive and negative control done in duplicate calculated and recorded. The average reading of the negative control data is **N**, positive control data is **P** and sample data is **S.** The cut-off point of each run was calculated as follows.

Cut off point = **N**x 2.5

The average OD values (S) now compared with the cut-off value and value of samples above the cut-off value is taken as a positively.

3.4.6.12. Estimation of insulin autoantibody (iaa)

3.4.6.12.1. *Principle*[135]

Human insulin is immobilized into microwells. The positive control, negative control and one dilution of patient plasma are added to the appropriate microwells. Human IgG specific antibodies to insulin in the plasma sample and control s bind to the insulin molecules on the microwells. After washing off excess or unreacted plasma materials, an enzyme labeled goat antibody specific to human IgG is added to the antigen-antibody complex. After through washing to remove the unbound enzyme a substrate solution is added and the color is directly proportional to the concentration of IAA in the sample. Two quality controls (positive and negative) are provided to monitor and validate assay results.

3.4.6.12.2. Reagents provided

Microwell Strips (with holder)

Anti-Human IgG Enzyme Conjugate (concentrate)

Sample Diluent (concentrate)

Antibody conjugate Diluent

Positive Control

Negative Control

Substrate buffer

Washing Buffer (concentrate)

Substrate Tablets

Stopping Solution (1N NaOH)

3.4.6.12.3. Additional Materials

Deionized water

Absorbent paper towels to blot dry the strips after washing steps and plastic wraps to cover strips during incubations.

Glass test tubes (5 ml)

Micropipette with disposable trips to deliver 25 µl, 50 µl and 100 µl.

A microtiter plate washer.

Pipette (10 ml) for substrate buffer and conjugate diluent delivery.

500 ml graduated cylinder

Microtiter plate reader with 405 nm absorbance capability.

Plastic label tape, to tape-up unused wells before assay.

3.4.6.12.4. Specimen collection

Samples were taken out of freezer 1 hr before and allowed to thaw and later on vortexed for 5 sec.

3.4.6.12.5. Reagent preparation

IAA - IgG enzyme conjugate reconstitution: Accurately transferred 5 ml of the IAA conjugate diluent into one bottle containing IAA - IgG enzyme conjugate. Bottle was cleaned and mixed thoroughly by inversion.

IAA - sample diluent buffer: Sample diluent buffer diluted according to the need. Calculated amount of concentrated IAA sample diluent transfer to a clear cylinders and added certain amount of deionized water and mix thoroughly by gentle inversion.

IAA Wash: IAA wash concentrate is for 500 ml of IAA wash solution. Calculated volume of wash concentrate was taken in a clean cylinder, desired amounts of deionized water added to it and mix thoroughly by inversion.

Plasma sample preparation: 25 µl of sample pipetted into 2.5 ml of the sample diluent

Substrate reagent: The substrate reagent prepared immediately prior to use, keep it at room temperature for 15 min and shaking periodically.

3.4.6.12.6. Assay procedure

Calculated number of strip assembled in the holder. The microwell strip snapped in place firmly with its square side.

100 µl of IAA negative control was dispensed into microwells C1 and D1.

100 µl of IAA positive control was pipetted in microwells E1 and F1.

100 µl of diluted plasma sample was pipetted into microwell in duplicate.

The plate was covered with a plate sealer and left at 2-^{0}C temperature for overnight.

In the next morning solution was discarded and the microwells were washed with washing buffer using automatic plate washer, except the first two well in the first column. Afterwards the plate was blotted by tapping on a paper towel.

100 µl of IAA-IgG-enzyme conjugate reagent was added into all wells except wells A1 and B1.

The plate was covered with a plate sealer and left it stand at 25±1^{0}C for 1 hr.

Substrate reagent was prepared.

After incubation washing step was repeated and the microwells were blotted dry.

100 µl of freshly prepared substrate was pipetted into all microwells including wells A1 and B1 at a rapid and steady pace without any interruption.

The plate was left in the dark for 30 min at 25±1^{0}C

After 30 min swiftly added 50µl of the stopping solution into each well B1 at a rapid and steady pace without any interruption.

Reading was taken using microwell plate reader at the wavelength 405 blanking the reader with wells A1 and B1.

3.4.6.12.7. Calculation

Average of reading of samples and both the positive and negative control done in duplicate calculated and recorded. The average reading of the negative control data is **N**, positive control data is **P** and sample data is **S**. The cut-off point of each run was

calculated as follows.

Cut off point = **N**x 2.5

The average OD values (S) now compared with the cut-off value and value of samples above the cut-off value is taken as a positively.

3.4.6.13. Estimation of Glutamic Acid Decarboxylase (GAD) Autoantibodies

3.4.6.13.1. Principle[136,137]

ELISA technique was used for the quantitative *in vitro* determination of Glutamic acid decarboxylase autoantibodies in plasma with streptavidin pre-coated microtiter plates. In the first step GAD-biotin is bound by streptavidin to the microtiter plate and in a second step after washing it is bound by the GAD specific autoantibody from the sample. Following the washing step, the peroxidase (HRP) labeled anti-IgG binds to GAD autoantibodies. The HRP bound in the complex is developed by ABTS (2,2'-Azino-di-[3-ethylbenzthiazolinesulfonate] / di-ammonium salt) as substrate and determined photometrically. The color intensity is proportional to the concentration of anti-GAD.

3.4.6.13.2. Reagents

Reagents from Roche Diagnostics GmbH, Germany, were used for the determination of Glutamic acid decarboxylase autoantibodies.

I. GAD-biotin: Reconstituted the lyophilizate in 0.5 ml double distilled water (stock solution) for 10 min at room temperature and then allowed to stand for further 30 min before use. 0.5 ml stock solution was added to 15 ml solution 2, which resulted a clear, slightly yellowish solution.

II. Coating buffer: Slightly yellowish, clear solution and ready to use.

III. Sample buffer: Slightly yellowish, clear solution and ready to use.

IV. Anti-GAD standards: Slightly yellowish, clear solutions of 5 standards (0, 140, 360, 1100 and 2580 mmol/l) were used. Ten micro-liter of each standard was diluted with 250 µl of solution 3.

V. Anti-GAD positive control: It was diluted as anti-GAD standards.

VI. Washing buffer: The washing buffer concentrate is a turbid solution which clears after prolonged standing at room temperature and dilution (10 times) with double distilled water.

VII. Conjugate buffer: Ready to use, clear solution.

VIII. Anti-h-IgG-HRP: One ml double distilled water was added to the supplied lyophilysate and rolled for 15 min, then 1 part stock solution was added to 29 parts conjugate buffer and mixed thoroughly.

IX. ABTS substrate solution: 2,2'-Azino-di-[3-ethylbenzthiazolinesulfonate] / di-ammonium salt was used as substrate which was light green clear solution.

3.4.6.13.3. Assay Procedure

i. 100 µl of the solution was added into the appropriate wells and cover the microtiter plate (MTP) tightly with an adhesive cover foil and incubated under constant shaking at 300 rpm for 60 min.

ii. Washed 3 times with solution 6. After the last washing, tap the MTP face downward onto an absorbent paper towel to completely remove washing solution.

iii. Appropriate well-diluted 100 µl of each of the standards, samples and positive control were added into each well and incubate under constant shaking at 300 rpm for 60 min.

iv. Washed 3 times as step ii.

v. 100µl of the solution 8 was added into each well and incubated under constant shaking at 300 rpm for 60 min.

vi. 100µl of the solution 9 was added into each well and incubate under constant shaking at 300 rpm for 30 min. Absorbance was measured at 405 nm against blank.

3.4.6.13.4. Calculation

Average of reading of samples and positive control done in duplicate calculated and recorded. The average OD values of samples now compared with the positive control and value of samples above the positive control value is taken as a positively.

3.4.6.14. DNA extraction[138]

DNA was extracted form whole blood. This is salt precipitation method.

3.4.6.14.1. Reagents

RBC Lysis buffer

Ammonium Chloride (NH_4CL)	155 mM
Potassium hydrocarbonate ($KHCO_3$)	10 mM
Ethylenediamine tetraacetic acid (EDTA)	10 mM

Cell lysis buffer

Tris-HCL pH 7.4	10 mM
Saodium chloride	400 mM
Ethylenediamine tetraacetic acid (EDTA)	10 mM
Sodium dodecyl Sulphate (SDS)	1%

Protein precipitation buffer

Ammonium acetate	7 mM

Hydration buffer (TE)

Tris-HCL pH 7.4	10 mM
Ethylenediamine tetraacetic acid (EDTA)	1 mM

3.4.6.14.2. Procedure

Blood samples were taken out of the freezer and allowed to thaw. After thawing it was homogenized by repeated gentle inversion of the tube for several times.

Then the following extraction procedure followed:

1. 300µl of whole blood was taken in a microcentrifuge tube
2. 900 µl of RBC lysis buffer was added
3. Inverted several times to mix thoroughly by gentle inversion
4. Incubated at room temperature for 10 - 15 min and mixed occasionally by inversion
5. Then the mixture was centrifuge for 30 sec in a refrigerated microcentrifuge (13000 rpm)

6. The supernatant was Eliminated with a micropipette or pour off carefully
7. Then 900 µl of RBC lysis buffer was added
8. Step 4-6 was repeated one or two times more till the supernatant was clear
9. The pellet of white blood cells was resuspended in RBC lysis buffer (≤20 µl) by vortexing / pipetting.
10. 300 µl of Cell lysis buffer containing 300-500 µg/ml of proteinase K was added
11. Inverted the tubes to mix (avoid lumps of cells)
12. The tube was incubated for 1 hr at 55^{0}C (can be left overnight at this temperature0
13. Cooled the sample to room temperature.
14. Protein precipitated buffer (100µl) was added.
15. The sample was vortex (20 sec) to mix and centrifuged 13000 rpm for 5 min.
16. Poured the supernatant into a new tube containing 300 µl of 100% isopropanol.
17. Mixed by inversion several times and centrifuged at 13000 rpm for 3-5 min.
18. The supernatant was discarded and 500 µl of 70% ethanol was added.
19. Inverted several times to mix and centrifuge 13000 rpm for 1 min.
20. Ethanol was drained carefully
21. Dried the tube in a Laminar Air Flow
22. 100 µl of hydration buffer (1X TE) was added
23. The DNA was allowed to hydrate 1 hr or overnight at 65 ^{0}C
24. The DNA was stored at 4 ^{0}C for further analysis.

3.4.6.15. Trypsinogen gene analysis

Exon 2 and exon 3 of the trypsinogen gene were analysed for the detection of A16V and R117H mutation.

3.4.6.15.1. Exon 2

3.4.6.15.1.1. Polymer Chain Reaction (PCR)

A 495 base pairs (bp) fragment includes exon 2 of trypsinogen gene was amplified using the following primers[40]: Forward: 5' – TTA GCA GAA AGC AAT CAC AGC – 3' and reverse: 5' – CTC TCA CCA CCC CAA CAT – 3'. PCR was done in total 35 µl reaction volume containing 3 µl unmeasured DNA, 3.5 µl x10 Mg free thermophilic buffer (contains 50 mM KCl, 10 mM Tris-HCl, pH 8.3 at 25 ^{0}C) 1% Triton X-100, 5 pmol of each primers, 0.2 mM of each dNTPS (dATP, dCTP, dGTP and dTTP), $MgCl_2$ 1.5 mM

and 0.8 Unit of *Taq* DNA polymerase. Conditions for PCR were: 4 min initial denaturation step at 95 ^{0}C followed by 35 cycles at 94 ^{0}C x 1 min, 55 ^{0}C x 1 min and 72^{0}C x 1 min and a final elongation step of 10 mins at 72 ^{0}C. 5 µl of DNA dye was added to the PCR amplified DNA. After a momentary spin the PCR products were loaded in wells of 2% agarose gel containing ethidium bromide (10 mg/ml). Electrophoresis was accomplished at 60 volts and the PCR products were visualized under automated Gel Documentation system.

3.4.6.15.1.2. RFLP (For the detection of A16V):

The A16V mutation abolishes restriction site of endonuclease *Fnu* 4H1 (5' – G↓CNGC – 3') in the 16th position of the trypsinogen gene. PCR product of the exon 2 was digested over night at 37 ^{0}C with *Fnu* 4H1. Digested products were analyzed using 3% Metaphore Agarose Gel and were visualized under the same Gel Documentation System.

3.4.6.15.2. Exon 3

Exon 3 of the human cationic trypsinogen gene was amplified using a polymerase chain reaction according to Whitcomb DC[46] et al., 1996. A 911 base pairs (bp) fragment includes exon 3 of trypsinogen gene was amplified using the following primers: Forward: 5' – GGT CCT GGG TCT CAT ACC TT – 3' and reverse: 5' – GGG TAG GAG GCT TCA CAC TT – 3'. PCR was done in total 35 µl reaction volume containing 3 µl unmeasured DNA, 3.5 µl x10 Mg free thermophilic buffer (contains 50 mM KCl, 10 mM Tris-HCl, pH 8.3 at 25^{0}C) 1 % Triton X-100, 5 pmol of each primers, 0.2 mM of each dNTPS (dATP, dCTP, dGTP and dTTP), $MgCl_2$ 1.5 mM and 0.8 Unit of *Taq* DNA polymerase. Conditions for PCR were: 4 min initial denaturation step at 95^{0}C followed by 40 cycles at 94^{0}C x 1 min, 59^{0}C x 1 min and 72^{0}C x 1 min and a final elongation step of 10 min at 72^{0}C. 5 µl of DNA dye was added to the PCR amplified DNA. After a momentary spin the PCR products were loaded in wells of 1.5 % agarose gel containing ethidium bromide (10 mg/ml). Electrophoresis was accomplished at 60 volts and the PCR products were visualized under automated Gel Documentation system.

RFLP (For the detection of R117H):

The R117H mutation in the exon 3 creates a restriction site 5' – A CG↓TGT – 3' (arrow indicates splice site) is recognized by endonuclease *Afl*III. PCR product was digested

overnight at 37°C by *Afl*III. Electrophoresis was accomplished at 60 volts and the digested products were visualized under automated Gel Documentation system.

3.4.6.16. Human Leukocyte Antigen (HLA) Analysis

A PCR based method using sequence-specific primers, so termed PCR-SSP, was used for HLABQB1 typing. This is a powerful method for detecting genetic variability, including single base pair mismatches. The technique is based on the principle that a completely matched primer will be more efficiently used in the PCR reaction than a primer with one or several mismatches in the 3' end. Fourteen set of primers were used which picked up 28 alleles of the HLA-DQB1 region (0201, 0202, 0203, 0401, 0402, 0501, 0502, 0503, 0504, 0601, 0602, 0603, 0604, 0605, 0606, 0607, 0608, 0609, 0610, 0611, 0612, 0613, 0301, 0302, 0303, 0304, 0305, 0307) were analyzed using PCR -SSP[139].

For HLA DQB1 typing, each sample was amplified by a first set of eight PCR primer pairs, followed in some cases by two to six additional PCR reactions.

The PCR reaction mixtures (10 ul) consisted of 75 ng genomic DNA, PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$ 10 mM Tris-Cl, pH 8.3, 0.001% gelatin), 200 μM each of dNTPs, 0.25 μM of the allele and group specific DQB1 primers and 0.2 unit of *Taq* polymerase. Thirty amplification cycles were carried out, each cycle consisted of denaturation at 94 °C for 20 seconds, annealing at 65 °C for 50 seconds and extension at 72°C for 20 seconds. Absence or presence of PCR products was visualized by 2% agarose gel electrophoresis.

3.4.6.17. Statistical analysis

Details medical history was taken and clinical findings of the subjects were recorded in a predesigned case record form. All analyses were done using the SPSS (Statistical Package for Social Science) package for windows. Experimental values were expressed as mean ±SD (standard deviation) or median (range) as appropriate. To compare results between two groups students t-test or Mann-Whitney tests were performed where appropriate. Statistical significance was considered to be indicated by a p value of less than 0.05 in all cases.

RESULTS

4. Results

4.1. Clinical and Sociodemographic characteristics (Table I)

Mean (±SD) age (in years) of Controls, TCP and FCPD subjects were 26±3, 28±6 and 22±7 respectively. FCPD subjects had significantly lower age compared to Control ($p<0.05$) and TCP ($p<0.05$) subjects.

Mean (±SD) BMI of the Control subjects was 23.62±2.4. BMI of TCP (18.15±3.3) and FCPD (18.45±4.0) subjects were almost similar and both had values significantly lower ($p= 0.005$) than the control subjects.

Male-female ratio of the Control group was 1:2.3. A male preponderance was observed in TCP (M:F=4:3) and female preponderance in FCPD (4:7). This increased number of female subjects in FCPD cases may be due to small number of study subjects.

Diabetes mellitus in other family members was found in 14.3% of the TCP subjects and 27.3% of the FCPD subjects. History of abdominal pain in the past was present in 100% of the TCP and 54.5% of the FCPD subjects.

4.2. Relevant past history (Table II)

History of viral diseases like measles was found in 28.6% of TCP patients and 18.2% of FCPD subjects. In 36.4% and 18.2% of the FCPD subjects had past history of mumps and jaundice respectively.

4.3. Anthropometric Indices and Blood Pressure (Table III)

Mean (± SD) waist-hip ratio (WHR) were 0.85±0.07, 0.80±0.08 and 0.93±0.16 in Control, TCP and FCPD groups respectively. WHR values did not differ statistically among the three groups.

Mean (±SD) subscapular-triceps ratio (STR) of the three groups was Controls-1.85±0.73; TCP-1.29±0.32 and FCPD- 1.22±0.37. The STR value of TCP group significantly differ compared to Controls (p=0.039) and in FCPD it showed difference but did not reach to level of significance (p=0.063). The value did not differ between FCPD and FCPD cases.

Both the systolic and diastolic blood pressures of the three groups were almost similar among each other. Systolic blood pressure (mean±SD, mmHg) was 101±20, 113±9 and 101±7 and diastolic 68±8, 72±4 and 67±8 respectively in Control, TCP and FCPD groups.

4.4. Glycemic and insulinemic status (Table IV)

Fasting plasma glucose level (mmol/l, mean±SD) of the FCPD group (15.4±6.3) had almost 3.5 times higher (p=0.001) compared to the Control group (4.4±0.4) and 3 times higher (p=0.001) than the TCP group (4.9±1.0). Fasting blood glucose level in TCP did not differ with the Controls. Similarly plasma glucose 2 hrs after 75 g oral glucose drink in FCPD subjects (27.2±7.4, p=0.001) was about five times higher (p=0.001) compared to the Control (5.2±0.6) and TCP groups (5.9±1.0).

In contrast to glycemic status, fasting C-peptide level (nmol/l, median-range) of the Control group (0.70, 0.39-1.78), was about 3 times higher compared to FCPD (0.23, 0.10-0.30), (p= 0.006) and 1.4 times higher than the TCP subjects (0.52, 0.21-0.56) (p=0.040). On the other hand fasting C-peptide level of the TCP subjects was more than 2 times higher than the FCPD subjects (p=0.008). Similarly postprandial C-peptide after 2 hours of 75 g oral glucose in Control subjects 2.23 (1.99-3.38) was 6 times higher than the FCPD group (0.29, 0.11-0.51) (p=0.003) but the value did not differ compared to TCP group (1.92, 0.68-3.09) (p=0.165). Postprandial C-peptide level of the TCP subjects was about five times higher than the FCPD group (p=0.042).

Paralleling the fasting C-peptide values fasting plasma insulin level (pmol/l, media-range) of the Control group (48, 32-124), was more than 2 times higher compared to the FCPD (21, 13-48) (p=0.008) and 1.5 times higher than the TCP subjects (32, 29-47) (p=0.028). Fasting plasma insulin of the TCP subjects was 1.5 times higher than FCPD subjects, however, the difference did not reach statistically significant level. Postprandial insulin levels showed a pattern almost identical to postprandial C-peptide values (Controls- 328, 224-380; TCP- 270, 194-337 and FCPD- 101, 88-142; p=0.001 vs Controls, p=0.007 vs TCP).

4.5. Insulin secretory capacity (Table V)

C-peptide-glucose (nnom/mmol) and insulin-glucose ratios (pmol/mmol) (median-range) in both fasting and postprandial states of Control subjects (C-peptide-glucose: 0.15, 0.09-0.41 and 0.44, 0.36-0.78; insulin-glucose: 11.27, 6.78-28.94 and 60.88, 38.06-88.21 respectively in fasting and postprandial state was significantly higher than FCPD [C-peptide-glucose: Fasting-0.011, 0.003-0.015 and Postprandial-0.012, 0.005-0.077; insulin-glucose: Fasting-1.30, 0.66-5.12 and Postprandial-3.65, 2.67-5.73], and TCP subjects [C-peptide-glucose: Fasting-0.09, 0.06-0.12 and Postprandial-0.36, 0.12-1.42; insulin-glucose: Fasting- 6.44, 5.38-13.14 and Postprandial-43.61 38.36-58.92].

Hepatic extraction of insulin (Table V)

C-peptide-Insulin (nmol/pmol, median-range) ratio of fasting state of the FCPD subjects (0.009, 0.005-0.019) was similar with the Control (0.012, 0.009-0.019) and TCP subjects (0.017, 0.005-0.018). This ratio in the postprandial state of the FCPD subjects (0.003, 0.001-0.005) was significantly lower than the Control (0.008, 0.005-0.010) but not significantly different with the TCP subjects (0.008, 0.002-0.011).

4.6. Lipid Profile (Table VI)

Mean (±SD) triglycerides (mg/dl) of the Controls, TCP and FCPD subjects was 110±73, 100±45 and 135±43 respectively. Total cholesterol (mg/dl, mean±SD) was

121±47, 126±19 and 140±32 the three groups. HDL-cholesterol (mg/dl, mean±SD) was 32.3±8.6, 27.8±13.4 and 27.1±14.4 the three groups and LDL-cholesterol 66.7±44.1, 78.5±18.1 and 86.6±26.4 three groups respectively. The lipid levels did not differ statistically among the three groups.

4.7. Plasma Proteins and Total Antioxidant status (Table VII)

Total plasma protein levels (g/dl, mean±SD) in Control subjects (7.50±0.70) was similar with FCPD subjects (7.11±0.49) but significantly higher than TCP (6.70±0.43) (p=0.036). Plasma albumin level (g/dl) in FCPD subjects (4.47±0.47) was significantly lower than the Control (5.10±0.57) (p=0.023) subjects.

Mean (±SD) of total antioxidant status (mmol/l) in TCP subjects (0.83±0.07) was significantly (0.001) lower than the Control (1.08±0.16) and FCPD subjects (1.01±0.09).

4.8. Autoantibodies (Table VIII)

In the FCPD and TCP subjects 18.2% and 28.6 % respectively shown ICA positivity. On the other hand 27.3% of the FCPD subjects IAA positivity. All of the subjects negative for anti-GAD antibody.

4.9. Genetic Analysis

Gene associated with diabetes

HLA-DQB1 typing: HLA-DQB1 typing of the simplex families showed high polymorphism of this region (Table IX, X). Both in TCP and FCPD groups allele

0202, which seem to be more common alleles compared to others transmitted alleles, was transmitted equally. Alleles 0303, 0302/0307 and 0301/0304 appeared almost in the same frequency among the TCP and FCPD subjects.

Gene associated with pancreatitis

Trypsinogen Gene Analysis (Table XI): PCR-RFLP analysis of the exon 2 and exon 3 of the trypsinogen gene was done to study it's variations reported for HP patients. Mutation (C : T) in the exon 2 abolishes a cutting site (5'–GC^NGC–3') for endonuclease *Fnu*4HI. Restriction fragments of the exon 2 PCR product yielded 3 distinct digested products for the individual subject and parents (: : .) indicating that mutation was absent in gene of interest (Fig 5 and Fig 6). Mutation (G : A) in the exon 3 creates a mutation site (5'–A^CG TGT–3') for endonuclease *Afl*III. Gel analysis of PCR-RFLP of the exon 3 of TRP gene revealed no mutation as evaluated by single band in the 2% agarose gel (Fig 7) for the individual patients and parents.

Table I: Clinical and socio-demographic characteristics of the study subjects in different groups

Parameters	Controls (n=10)	TCP (n=7)	FCPD (n=11)
Age (in years)	26±3	28±6	22±7*
BMI (Kg/m^2)	23.62±2.4	18.15±3.3**	18.45±4.0**
Male : Female	1:2.3	4:3	4:7
Rural : Urban	0:10	4:3	5:6
Annual income, median (range) in thousand Taka	49 (30-84)	45 (30-80)	48 (12-100)
FH of DM	0	1 (14.3%)	3 (27.3%)
FH of TCP	0	1 (14.3%)	2 (18.2%)
History of Abdominal pain	0	7 (100%)	6 (54.5%)

* $p < 0.05$ compared to TCP, **$p<0.005$ compared to control
Results are expressed as mean±SD unless otherwise sated.
FH, family history; DM: Diabetes mellitus

Table II: Presence of relevant past history of different diseases in three groups

Groups	Jaundice		Measles		Mumps	
	No	(%)	No	(%)	No	(%)
Control (n=10)	1	10	3	30	0	0
TCP (n=7)	1	14.3	2	28.6	0	0
FCPD (n=11)	2	18.2	2	18.2	4	36.4

Table III: WHR, STR and blood pressure of the study subjects

Group	WHR	STR	SBP (mmHg)	DBP (mmHg)
Control (n=10)	0.85±0.07	1.85±0.73	101±20	68±8
TCP (n=7)	0.80±0.08	1.29±0.32	113±9	72±4
FCPD (n=11)	0.93±0.16	1.22±0.37	101±7	67±8
t/p values				
Control vs TCP	*-1.36/ 0.19*	*-1.62/ 0.070*	*1.21/0.25*	*1.0/0.33*
Control vs FCPD	*1.26/ 0.23*	*-0.65/ 0.53*	*0.89/0.38*	*-1.12/0.28*
TCP vs FCPD	*-1.65 / 0.12*	*0.35/ 0.73*	*2.52/0.027*	*1.14/0.27*

Values were expressed as mean ±SD. Mean differences between groups were calculated using unpaired Students 't' test.

Table IV: Glycemic and insulinemic status of different groups in the study subjects

Groups	Plasma glucose (mmol/l)		Plasma C-Peptide (nmol/l)		Plasma insulin (pmol/l)	
	0 min	120 min	0 min	120 min	0 min	120 min
Control (n=10)	4.4±0.4	5.2±0.6	0.70 (0.39-1.78)	2.23 (1.99-3.38)	48 (32-124)	328 (224-380)
TCP (n=7)	4.9±1.0	5.9±1.0	0.52 (0.21-0.56)	1.92 (0.68-3.09)	32 (29-47)	276 (194-337)
FCPD (n=11)	15.4±6.3	27.2±7.4	0.23 (0.10-0.30)	0.37 (0.11-0.51)	21 (13-48)	101 (88-142)
t/p value			*U/p value*			
TCP vs Control	*1.46/ 0.169*	*1.872/ 0.084*	*8.00/ 0.040*	*13.0/ 0.165*	*7.0/ 0.028*	*15.0/ 0.254*
FCPD vs Control	*4.186/ 0.001*	*3.998/ 0.0001*	*12.0/ 0.006*	*10.0/ 0.003*	*13.0/ 0.008*	*4.0/ 0.001*
TCP vs FCPD	*-3.60 / 0.001*	*-8.49/ 0.001*	*10.0/0.008*	*7.0/ 0.042*	*11.00/ 0.071*	*3.0/ 0.007*

Results were expressed as mean±SD and median (range). Difference between groups was calculated using unpaired students 't' test and Mann-Whitney test where appropriate.

Table V: Insulin secretory capacity of the study subjects

Groups	C-Peptide/glucose		Insulin/glucose		C-Peptide/Insulin	
	0 min	*120 min*	*0 min*	*120 min*	*0 min*	*120 min*
Control (n=10)	0.15 (0.09-0.41)	0.44 (0.36-0.78)	11.27 (6.78-28.94)	60.88 (38.06-88.21)	0.012 (0.009-0.019)	0.008 (0.005-0.010)
TCP (n=7)	0.09 (0.06-0.12)	0.36 (0.12-0.42)	6.44 (5.38-13.14)	43.61 (38.36-58.92)	0.017 (0.005-0.018)	0.008 (0.002-0.011)
FCPD (n=11)	0.011 (0.003-0.015)	0.012 (0.005-0.077)	1.30 (0.66-5.12)	3.65 (2.67-5.73)	0.009 (0.005-0.019)	0.003 (0.001-0.005)
U/p value						
TCP vs Control	*4.00/0.008*	*11.00/0.099*	*8.0/0.040*	*8.00/0.040*	*22.0/0.77*	*23.0/0.859*
FCPD vs Control	*0.000/ 0.000*	*0.000/ 0.000*	*1.000/ 0.000*	*0.000/ 0.000*	*21.0/0.091*	*0.000/0.001*
TCP vs FCPD	*1.000/ 0.003*	*0.00 / 0.002*	*4.000/ 0.019*	*0.000/ 0.002*	*13.0/ 0.354*	*9.0/0.127*

Results were expressed as median (range). Differences between groups were calculated by Mann-Whitney Wilcoxan rank Sum test.

Table VI. Lipid Profile in different groups

Groups	TG (mg/dl)	T Chol (mg/dl)	HDL-c (mg/dl)	LDL-c (mg/dl)
Controls (n=10)	110±73	121±47	32.3±8.6	66.7±44.1
TCP (n=7)	100±45	126±19	27.8±13.4	78.5±18.1
FCPD (n=11)	135±43	140±32	27.1±14.4	86.6±26.4
t/p values				
Control vs TCP	*-0.28/0.78*	*0.24/0.82*	*0.79/0.44*	*0.63/0.54*
Control vs FCPD	*-0.32/0.75*	*1.09/0.29*	*036/0.72*	*1.15/0.27*
TCP vs FCPD	*-1.42/0.18*	*-0.92/0.38*	*0.096/0.93*	*-0.69/0.51*

All the values were expressed as mean±SD. Difference of mean between groups calculated using unpaired Students 't' test.

Table VII: Plasma total protein, albumin and total antioxident status (TAS) of the study subjects

Groups	Total protein (g/dl)	Albumin (g/dl)	TAS (mmol/l)
Control (n=10)	7.50±0.70	5.10±0.57	1.08±0.16
TCP (n=7)	6.7±0.43	4.86±0.37	0.83±0.07
FCPD (n=11)	7.36±0.88	4.47±0.47	1.01±0.09
t/p values			
TCP vs Control	*-2.34/0.036*	*-0.83/0.421*	*-4.28 / 0.001*
FCPD vs Control	*-0.95/0.355*	*-2.518/0.023*	*1.29 / 0.408*
TCP vs FCPD	*-1.56/0.144*	*0.093/0.058*	*-4.49/0.001*

Results were expressed as mean ±SD. Difference between means of the three groups were calculated using unpaired students 't' test.

Table VIII: Islet Cells Antibody (ICA) and Insulin Autoantibody (IAA) among the study subjects

Groups	ICA		IAA		Anti-GAD Ab	
	No	(%)	No	(%)	No	(%)
Control (n=10)	0	0	1	10.	0	0
TCP (n=7)	2	28.6	1	14.3	0	0
FCPD (n=11)	2	18.2	3	27.3	0	0

Table IX: HLA DQB1 of the TCP families

DQB1 allele	0202	050x 0503	060y	0301 0304	0302 0307	0303
T	2	1	2	2	1	2
NT	5	1		-	1	1

T= Transmitted, NT = Not Transmitted,
050x = 0501, 0502, 0503, 0504; 060y = 0601, 0602, 0610, 0611, 0613

Table X: HLA DQB1 Typing of the FCPD families

DQB1 allele	0202	050x 0503	060y	0301 0304	0302 0307	0303
T	2	1	1	1	1	1
NT	2	2	1	2	1	1

T= Transmitted, NT = Not Transmitted,
050x = 0501, 0502, 0503, 0504; 060y = 0601, 0602, 0610, 0611, 0613

Table XI: PCR RFLP assay of the exon 2 and exon 3 of the trypsinogene gene

Families	Exon 2			Exon 3		
	FF	Ff	ff	AA	Aa	aa
Simplex TCP (n=5)	0	0	5	5	0	0
Simplex FCPD (n=5)	0	0	5	5	0	0
Multiplex families (n=2)	0	0	2	2	0	0

Exon 2 assed by *Fnu* 4H1 and exon 3 by *Afl* III restriction enzyme digestion.

FF, Ff and ff stand for homogygot uncut, heterogygot and homogygot cut respectively for endonuclease Fnu 4H1 digestion assay.
AA, Aa and aa stand for homogygot uncut, heterogygot and homogygot cut respectively for endonuclease Afl III digestion assay.

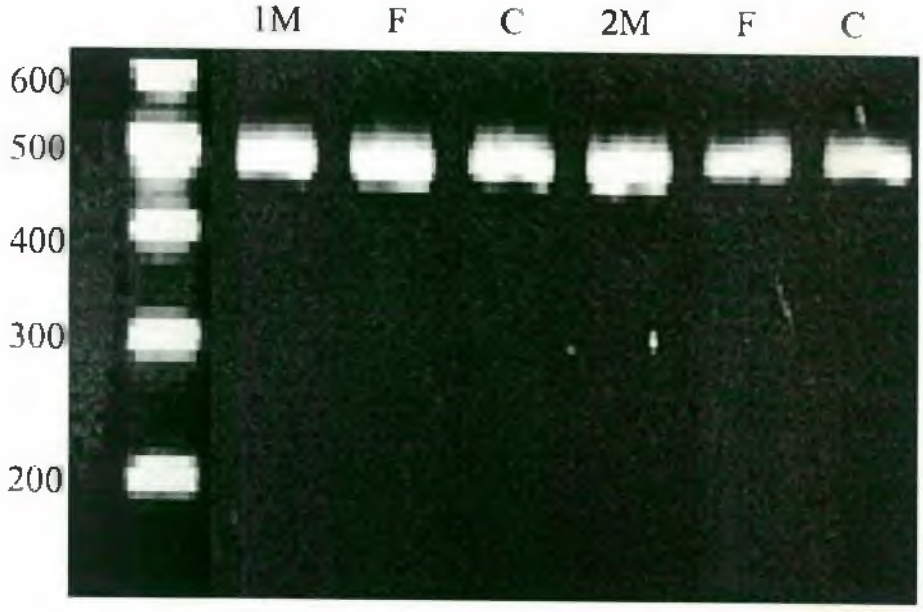


Fig 5: PCR product of the exon 2 in 2% agarose gel

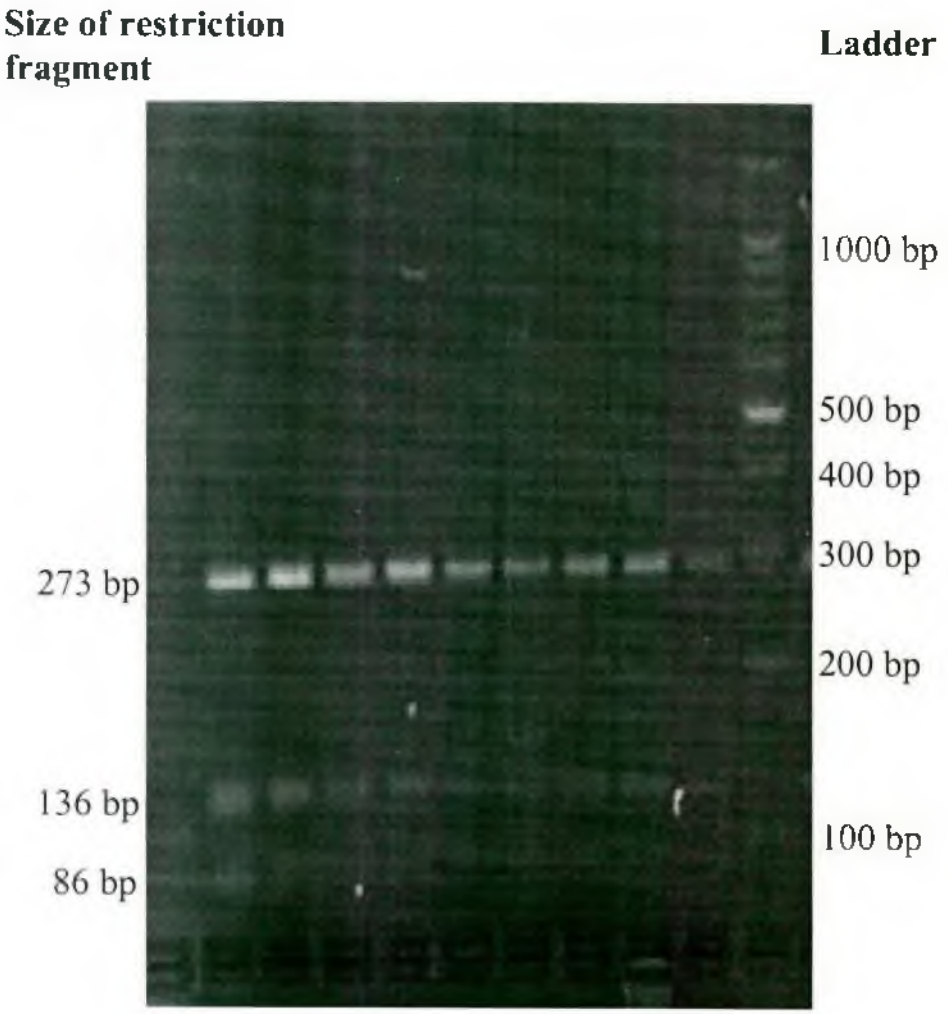


Fig 6. Fnu4H1 restriction enzyme digestion of exon 2

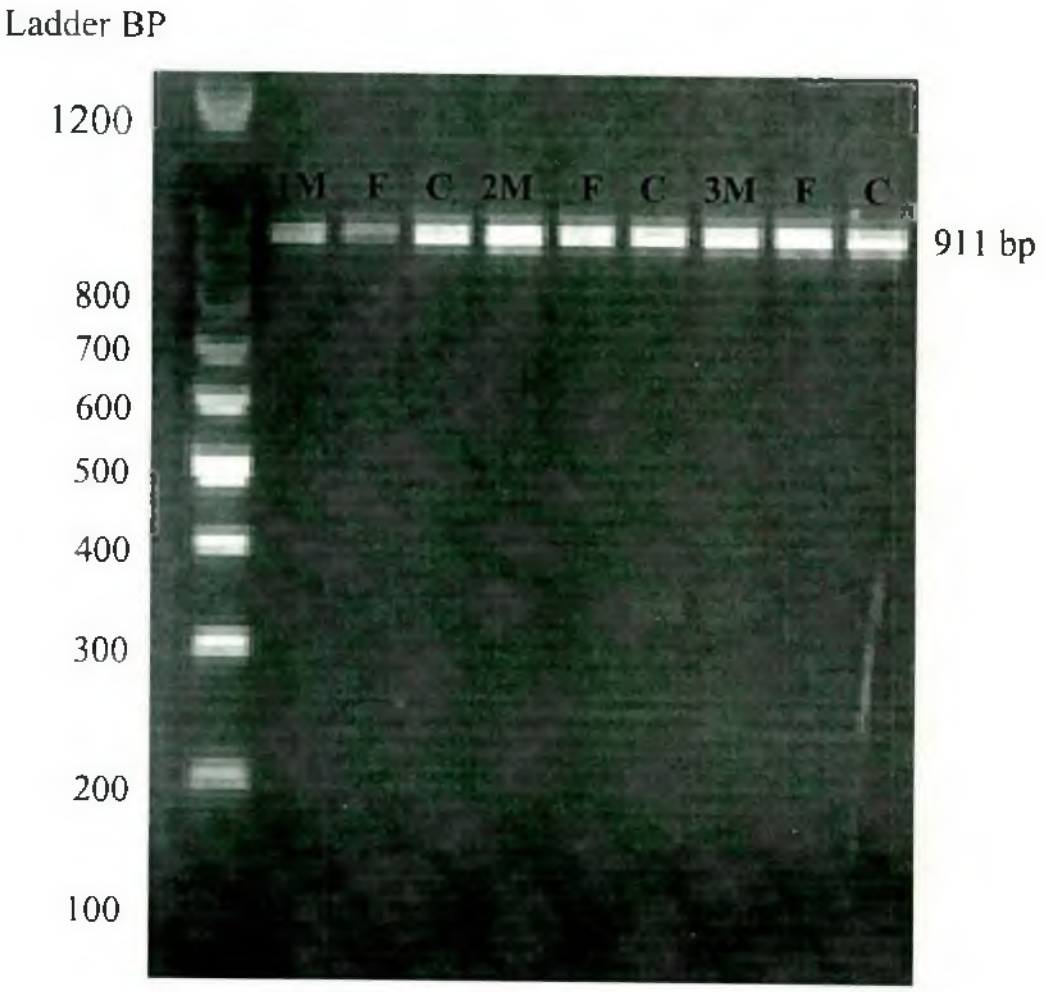


Fig 7 *Afl* III restriction digestion of exon 3 of TRYP gene

DISCUSSION

5. Discussion

5.1. Age and sex distribution

Mean age of the TCP subjects in the present study was found to be significantly higher than the FCPD group. The finding contradicts the suggestions of the ADA[7] or WHO Committee[6] regarding FCPD as a secondary type of diabetes. The data is compatible with previous results in the same population and it reinforces the view that diabetes in FCPD may not be just a progression of TCP[14,18,140].

There was a female preponderance in FCPD subjects (M:F=4:7) in the present study. In previous reports in the same population a male predominance in both TCP and FCPD subjects was found. However, this was attributed to the health care seeking pattern between sexes rather than real differences[18]. Males, particularly from rural areas, usually come more for treatment than females. The apparently reverse finding in the present study may indicate that all the results on the sex distribution may be incidental rather than real, possibly due to small number of cases.

5.2. Sociodemographic features

Both TCP and FCPD patients came equally from rural and urban background.

Annual family income of the patients of both the groups shared a wide range of income status (Tk. 30,000 - 80,000 in TCP and 12,000 - 100,000 in FCPD). Majority of the subjects came from lower middle class income (Tk 30001-60000) group (57% in TCP and 63% in FCPD). The rest of the subjects came from low and middle or higher income groups almost at equal proportions. The data, in line with the previous findings in the same population[140], suggest that both TCP and FPCD patients originate from diverse socioeconomic classes and evident clustering (which could point towards some environmental factor(s) for the etiopathogenesis of the diseases) is lacking.

5.3. Dietary habits

Dietary habit, like ingestion of cyanogenic alkaloids, was implicated in the etiopthogenesis of diabetes[19]. In a study at BIRDEM it has been shown that no particular food, like cassava (which is neither grown nor imported in Bangladesh), is associated with diabetes in this population[141]. In the present study food habit of the subjects of the present study was examined. Like the previous finding no particular food item was found to be associated with diabetes and /or pancreatitis. However, food items containing toxic materials other than cyanogen, which might be related with the diseases, may not be excluded.

5.4. Anthropometric indices

The BMI of TCP and FCPD cases spread from underweight to normal range. Mean (±SD) BMI of the TCP and FCPD cases was significantly lower than the Control subjects. Although BMI was almost similar in TCP and FCPD groups but 2 (28.57%) and 4 (36.36%) subjects respectively had BMI between 19.1 - 25 level indicating that both the diseases are not necessarily accompanied with underweight. In earlier studies BMI value was found lower in FCPD compared to TCP[18]. If FCPD had been secondary to TCP, BMI values would have been consistently lower in FCPD. No difference in BMI between TCP and FCPD groups again points against a simple theory of secondary diabetes in FCPD. It remains uncertain whether the low BMI in FCPD is a cause or consequence of diabetes[9,139, 142-149].

Waist-hip ratio was found similar in all three groups which suggests similar degree of wasting in the TCP and FCPD cases.

5.5. Other biochemical parameters

Lipid levels of the FCPD did not differ compared to controls. Usually diabetes is associated with higher atherogenic lipid levels. However, the present finding is in agreement with earlier studies where lipid levels were within normal levels in FCPD subjects[14,18]. FCPD subjects were also shown to have low NEFA levels compared to their age-matched NIDDM counterparts[150]. Though there was no

statistical difference but atherogenic lipids (total cholesterol, TG and LDL) in the FCPD patients had consistently higher values compared to the TCP and Control subjects. TCP patients also had tendency to higher atherogenic lipids.

Since the clinical stigmata of malnutrition are almost absent in these patients it is difficult to ascertain whether they really suffer from malnutrition. This has also been reflected in the plasma total protein and albumin levels in all the groups where values were within normal both in FCPD and TCP groups.

5.6 Glycemic and insulinemic status of the study subjects

FCPD subjects had high fasting and postprandial (2 hrs after 75g oral glucose load) plasma glucose levels. In contrast the TCP subjects showed normal ranges of plasma glucose values. Similar findings were observed in FCPD subjects in other studies[25,142,143,147,148,151,152]. Such a clustering of glycemic indices at extreme ends for diabetic and nondiabetic CP subjects argues against a simple scheme of secondary diabetes in FCPD subjects. Nonspecific destruction of endocrine cells due to gradual damage in exocrine tissue should lead to glucose intolerance of varying severity in some cases of TCP subjects.

FCPD subjects had significantly lower fasting and postprandial C-peptide values than those of TCP subjects. They had also lower insulin levels in response to oral glucose which is in agreement with other studies[24,151,153].

Fasting C-peptide levels in the TCP subjects were significantly lower than those of control subjects. However, when postprandial C-peptide and insulin were considered, TCP subjects did not differ statistically with Controls. Thus B cell capacity in response to increased demand for insulin is preserved in TCP but not in FCPD.

To evaluate secretory capacity in the 3 groups of study subjects C-peptide-glucose and Insulin-glucose ratios were calculated. It was observed that stimulated C-peptide secretion per mole of glucose in TCP subjects does not differ statistically compared to Controls ($p=0.099$) where as in FCPD subjects C-peptide:glucose ratio

value is extremely low indicating already compromised insulin secretory capacity (p=0.0001 vs Controls and p=0.002 vs TCP). Postprandial Insulin-glucose ratio also showed similar trend. The finding further strengthens the argument that FCPD is not simply a progression to TCP and the two groups may represent two different subsets of population.

C-peptide-insulin ratio was calculated to evaluate hepatic utilization of insulin. The Controls and TCP subjects had almost similar C-peptide: insulin ratio values but the value in FCPD subjects was significantly lower compared to Controls (p=0.001). The difference between TCP and FCPD did not reach statistical significance, but there was a definite tendency of lower ratio in FCPD compared to TCP . This lower ratio in FCPD was reflect the existence of insulin resistance in hepatic tissues in case of FCPD subjects

5.7 Total antioxidant status in the study group

Oxidative stress has been suggested in the etiopathogenesis of FCPD[18]; however, the assumption was not substantiated. Recently the BIRDEM group has reported some evidence of oxidative damage. It has been shown that FCPD subjects had significantly higher level of single stranded DNA compared to Controls. It was for the first time that direct evidence of oxidative damage was reported in FCPD[34].

Measurement of total antioxidant status (TAS) reveals the resultant of the defensive responses against oxidative stress *in vivo*. Excessive consumption of antioxidants usually leads to a fall in TAS. Total antioxidant status (TAS) was measured in FCPD, Controls and, for the first time, in TCP patients. It was observed that TAS in TCP was significantly lower compared to Controls and FCPD (p=0.001). Interestingly FCPD subjects had almost similar levels of TAS compared to Controls. In an earlier study also FCPD subjects showed nearly same level of TAS compared to Controls and type 2 diabetes[154]. This finding suggests that oxidative stress may be particularly severe in TCP. It also indicates that pathogenesis of pancreatitis may differ in TCP and FCPD, and oxidative stress in FCPD may not lead to imbalance of TAS at the initial stage.

5.8. Autoantibodies

ICA, IAA and anti-GAD are important tools for differentiating type 1 from other classes of diabetes. ELISA methods were used in this study to measure the three antibodies. Using these techniques 70% for ICA, 20-50% for IAA and 20-81% for anti-GAD positivity were found in type 1 diabetic subjects of European origin. However, when ICA and IAA in combination were used, chance of positivity went up to 50-70% only. So, measurement of anti-GAD became important along with ICA which, in combination, identify positive cases more correctly.

In the present study autoantibodies including anti-GAD were measured in FCPD and, for the first time, in TCP patients. The results showed a positivity of 18.2%, 27.3% and 0% in FCPD and 28.6%, 14.3% and 0% in TCP compared to 0%, 10% and 0% in Controls for ICA, IAA and anti-GAD antibody respectively. A relatively higher percentage of positive cases were observed for ICA and IAA in TCP and FCPD cases but none of the cases was found positive for anti-GAD antibody in the two groups. More over all the control subjects were negative for anti-GAD antibody. The positivity for ICA and IAA may be due to presence of nonspecific proteins in our population and thus, immune mediated mechanism may not necessarily be involved in the etiopathogenesis of diabetes in FCPD subjects.

5.9. Genetic variations

Genes of pancreatitis

Hereditary pancreatitis (HP) a unique form of chronic pancreatitis which has been found associated with mutations in trypsinogen (TRP) gene[38, 41, 42]. In the present study we have studied TRP gene mutations in the exon 2 and exon 3. The result demonstrate that the established genetic mutations, A16V in exon 2 and R117H in exon 3 of the trypsinogen gene, related to pancreatitis in HP, are not associated in the pancreatitis in TCP and FCPD. Trypsin plays a central role in pancreatic exocrine physiology by serving as the trigger enzyme responsible for activation of all other pancreatic zymogens. Normally trypsin remains inactive as the

trypsinogen state until it reaches the duodenum where it is activated by enterokinases, thereby initiating an activation cascade. A series of protective mechanisms are used by the body to prevent premature activation and limit zymogen activation, including incorporation of an R117 fail-safe autolysis site in the trypsinogen[42,155-158]. The R117H mutation leads to deletion of a trypsin-cutting site from the deduced protein. This protein, in turn, stabilizes active trypsin within the pancreas, and a defect in this mechanism has been suggested to propagate or even initiate the development of pancreatitis[42,156,159]. Mutation A16V involves the cleavage site of the signal peptide that has been postulated to be involved in release of pre-protein resulting in disturbed intracellular transport and formation of active trypsin[44]. The absence of two common mutations does not exclude implication of other reported mutation, N211 in the Tryp gene in the etiopathogenesis. Further studies are needed to test association of TCP and FCPD withN211 mutation, serine protease kazal type 1 (SPINK 1) or pancreatic secretory trypsin inhibitor (PSTI) mutations (found in IP cases[160]), common CFTR mutations (found in idiopathic pancreatitis[161]) and other possible genetic variations.

Genes for diabetes

To explore genetic markers in the pathogenesis of diabetes a lot of works has so far been done. Type 1 diabetes has been found to be strongly associated with MHC region in the chromosome 6 and insulin gene polymorphism and numerous locus in different chromosomes have been found to be associated with type 2 diabetes. HLA Class II region- HLA-DQB1, DR3, DR4 antigens, TNF (presently termed HLA Class IV) and '5-flanking region of insulin genes are of interest for type 1 diabetes and 3'-region insulin gene, insulin gene receptor, glucagon like peptide-1 gene, insulin receptor substrate-1 gene, glucokinase, genes and HNF in chromosome 12 HNF in chromosome 17 for type 2 diabetes[122].

FCPD has been found to be a multifactorial disease. Studies so far been conducted showed that environmental factors plays a crucial role in the etiopathogenesis of diabetes in genetic susceptible subjects. It has already been mentioned that

clinically FCPD belongs to be a special class of diabetes. There are still arguments whether this is really a unique class of diabetes or it is just a variant of type 1 or type 2 diabetes. Most cases of type 1 diabetes mellitus is caused by an autoimmune process that destroys pancreatic B cells. Like other autoimmune diseases, autoimmune B-cell destruction is influenced by HLA molecules. FCPD has been found to be associated with HLA Class II alleles by different investigators[46, 47] using case control studies. Allele 0201 in HLA-DQB1 was found to be mostly associated with type 1 form[120,121]. However, HLA-DR3 and DR4 antigens were also found to be positively associated with type 1 diabetes. To explore the genetic contribution for diabetes and pancreatitis in FCPD and TCP approach from various angle are needed.

We have studied HLA for exploring it's possible association with diabetes and trypsinogen gene for possible role in the development of pancreatitis using a simplex family based study. In the present study HLA-DQB1 typing was done and it was observed that none of the of the individuals of 5 TCP and FCPD families had the 0201 DQB1 allele which was transmitted in equal proportion in the probands. However, to elucidate the association more clearly further works with more families including other markers like HLA-DR3, DR4, insulin gene, and glucokinase gene are needed.

Limitations: Sample size of this study is small. It seemed difficult to motivate patients to make them understand about the experiment. Moreover, prevalence of TCP and FCPD is less. In the original plan estimation of serum amylase and HbA_{1c} was not included. Determination of serum amylase and HbA_{1c} would have given valuable information.

Conclusions:

1. From the sociodemographic and clinical characteristics and from glycemic and insulinemic status it appears that TCP and FCPD are different diseases with possible overlapping (and interactions) of features related to pancreatitis and diabetes. Thus, diabetes in FCPD may not be merely secondary to TCP.

2. Absence of common mutations in the trypsinogen gene attributed for the pancreatitis of HP confirm that both TCP and FCPD are genetically different from HP.

3. HLA DQB1 typing precludes the presence of this type 1 diabetes related allele in TCP and FCPD subjects.

4. Other genes for diabetes and pancreatitis need to be studied for a better understanding of the etiopathogenesis of pancreatitis and diabetes in chronic pancreatitis patients.

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Case Record Form

Case No: Date:

Name: Age yrs Sex

Father's Name

Address

Residence: U / R / SU Family Member: Annual income:

Height m Weight Kg

Anthropometry	Right	Left
MAC (cm)		
Skinfold thickness (mm):		
Triceps		
Biceps		
Subscapular		
Hip circumference (cm)		
Waist circumference (cm)		

Past Medial History	Yes	No
Measles		
Mumps		
Chickenpox		

Pancreatic disease	Y	N
History of acute pancratitis		
History of chronic abdominal pain		

BLOOD PRESSURE	
Systolic	mmHg
Diastolic	mmHg

Past medical history

Family History of Diabetes	Total	ND	D
1. Father			
2. Mother			
3. Brother			
4. Sister			
5. Cousins - M			
6. Cousins- P			
7. Uncle/Aunt- M			
8. Uncle/Aunt- P			
9. GF - M			
10. GF - P			
11. GM - M			
12. GM - P			
13. Sons			
14. Daughters			