GENETIC AND BIOCHEMICAL CHARACTERIZATION OF TROPICAL CALCIFIC PANCREATITIS (TCP) AND FIBROCALCULUS PANCREATIC DIABETES (FCPD) IN BANGLADESHI POPULATIONS

PhD Thesis

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Genetic and Biochemical Characterization of Tropical Calcific Pancreatitis (TCP) and Fibrocalculus Pancreatic Diabetes (FCPD) in Bangladeshi Populations

The Thesis is submitted in Partial Fulfillment of the Requirement for the Degree of Doctors of Philosophy

Declaration

This thesis entitled 'Genetic and Biochemical Characterization of Topical Calcific Pancreatitis (TCP) and Fibrocalculus Pancreatic Diabetes (FCPD) in Bangladeshi Populations'' is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD), under the Faculty of Postgraduate Medical Science and Research, University of Dhaka. Laboratory analysis for the thesis were carried out in the Biomedical Research Group (BMRG) Laboratory, Dhaka: Laboratory of Endocrine and Metabolism, University of Basel, Switzerland; Zurich University Hospital, Switzerland: Viollier Laperatories, Basel, Switzerland: Center for Genomic Sciences, University of Pittsburgh, PA, USA. Any part of result of this work has not been submitted for any degree from any University at home or abroad.

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Dedicated to

The memory of my Maternal Grand Father Late Prof AFM Nurul Islam

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(Dr Soheli Sattar)

Student

Abbreviations

ACR - Albumin-creatinine ratios

ADA – American Diabetes Association

Bp – base pair

BIRDEM - Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders

BMI - Body mass index

BSF - Biceps skin-fold thickness

BSMMU - Bangobandhu Sheikh Mujib Medical University

CCP - Chronic calcific pancreatitis

CP - Chronic pancreatitis

CI - Confidence interval

Cu- Copper

CF - Cystic fibrosis

CFTR - Cystic fibrosis transmembrane conductance regulator

DAB - Diabetic Association of Bangladesh

DNA – Deoxyribonucleic acid

EDTA – Ethylinediaminetetraacetic acid

ERCP - Endoscopic Retrograde Cholangio Pancreatography

FCP - Fibrocalculous pancreatitis

FCPD - Fibrocalculus pancreatitic diabetes

GFR- Glomerulous Filtration Rate

HbA1c - Glycosolyteted hemoglobin

GAD- Glutamic Acid Decarboxylase

HP - Hereditary pancreatitis

HDL-High Density Cholesterol

ICA- Islet Cell Antibody

IA-2 – Islet antigen 2

IAA- Insulin Auto Antibody

ICP - Idiopathic chronic pancreatitis

IL-18 – Interleukin-18

LDL- Low Density Lipoprotein

MRDM - Malnutrition-related diabetes mellitus

MAC - Mid-arm circumference

min,- minute

Mg- Magnatium

OGTT - Oral glucose tolerance test

OR - Odds ratio

PRSS1- Protease, serine, 1

PSTI - Pancreatic secretory trypsin inhibitor

PABA - p-aminobenzoic acid

RIA - Radio immune assay

SPSS - Statistical Package for Social Science

SPINK1- Serine protease inhibitor, Kazal type 1

TCP - Tropical calcific pancreatitis

T1D - Type 1 diabetes

TPD - Tropical pancreatic diabetes

T2DM - Type 2 Diabetes Mellitus

TG - Tryglyceride

TP-Tropical Pancreatitis

Transferrin-creatinine ratios- (TCR)

Waist hip ratio (WHR)

WHO – World heleath organization

Zn-Zinc

SUMMARY

Tropical calcific pancreatitis is a form of chronic calcific non alcoholic pancreatitis, seen almost exclusively in developing countries of the tropical world including Bangladesh. Fibrocalculous pancreatic diabetes (FCPD) is a unique form of diabetes secondary to Tropical calcific pancreatitis It has been observed that among all diabetic patients registered in BIRDEM, the central diabetic centre of Diabetic Association of Bangladesh (DAB), about 7% belonged to the group whose onset of diabetes is under 30 years of age. In a cohort of these young (<30 yrs) diabetic patients 13% were found to be constituted by the FCPD patients. To explore the genetic and biochemical characteristics of TCP and FCPD Bangladeshi population the study has been performed at the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Dhaka, Bangladesh, University of Basel, Switzerland and University of Pittsburg, USA between 2001 and 2008. The study protocol was approved by the local Ethics Committee. Type 2 Diabetes mellitus (T2DM) and Fibrocalculus pancreatic diabetes (FCPD) patients were prospectively selected from the outpatient department of BIRDEM. Tropical calcific pancreatitis (TCP) was chosen from the gastroenterology units at BIRDEM, Bangobandhu Sheik Mujib medical university (BSMMU) in Dhaka and from local gastroenterologists. All the patients were selected within an age band of 30-55 years. 34 TCP, 82 FCPD and 48 Type 2 diabetic subjects have been included in the study. After routine examinations (patient history, physical examination, fasting and 2 hour after breakfast glucose, Glycosolyteted hemoglobin (HbA1c), plain abdominal X-ray) patients with diabetes mellitus and pancreatic calcifications on abdominal X-ray were classified as FCPD and T2DM according to the 1985 WHO classification, which was valid at the time of this investigation. After providing oral informed consent, additional screening investigation was done (anthropometric measurements). The same screening procedures were used for TCP. A modified set of Case Record Form was filled out for each patients and detailed history was taken. Glycemic status has been assessed by fasting glucose and HbA1c. Lipid profile, serum creatinine and serum ALT have been assessed by enzymetric colorimetric method. at BIRDEM. Routine and microscopic examinations of the stool

were done to exclude fat, parasites and blood. Arginine stimulation test has been performed at BIRDEM and sample for serum glucose, serum C-peptide and serum glucagon, ICA, IA2, GAD antibody and sample for fecal elastase 1 has been frozen at -20^0c and later transported to the university of Basel, Switzerland for analysis. The parameters have been analysed by RIA method. Samples for DNA analysis has been also frozen at -20^oc and transported to University of Pittsburge, USA. Statistical analysis was performed using Statistical Package for SocialScience (SPSS) for Windows Version 11.0 and $p<0.05$ was taken as the level of significance throughout. Unpaired't' test. Proportion test, non-parametric tests (eg, Mann-Whitney test, Chi square test and Odds ratio) were applied where applicable. There appeared to be male preponderance in all the groups. Diabetes appears relatively earlier in FCPD than the T2DM subjects which is statistically significant ($p<.000$). High proportions of TCP and FCPD subjects are from the rural compared to more of the T2DM from urban area ($X^2=10.842$, $p= 0.028$). Significant difference ($p=.945$) had been found in WHR value between FCPD and T2DM subjects In TCP subjects, fasting blood glucose (mmol/l, mean$\pm$SD) and hbA1c (%) is normal as aspected. FCPD subjects show higher fasting blood glucose level than the T2DM which is statistically insignificant. After 2 hours after breakfast blood glucose level in TCP is within normal value, also as expected. FCPD subjects show insignificantly higher blood glucose level than that of T2DM. Age of inset of diabetes (yr) in FCPD is higher than T2DM were showing significant difference statistically ($p=.142$). Significant difference has been found regarding total cholesterol level in TCP vs FCPD (t/p value=-.004/.997). TCP and FCPD subjects significantly differs in low density lipoprotein (LDL-c) level (mg/dl) (t/p value=1.588/0.120). Regarding ALT levels, TCP differs significantly with T2DM ($p=.019$). GAD antibody and IA-2 antibody are absent in all the three groups. ICA anibody has been detected in all the groups. All the groups show severe pancreatic exocrine insufficiency.During Arginine infusion has not altered plasma glucose levels in any of the groups. Arginine stimulation test revealed almost 2 fold increase in serum C-peptide level (nmol/ml) in TCP from base line to 30 min and FCPD subjects showed near 1.5 fold increase of the same compared to that of T2DM which remained un changed. In TCP and FCPD, basal serum level of C-peptide did not differ significantly. The basal value of serum glucagon

(pg/ml) revealed the same in TCP, FCPD and T2DM during arginine stimulation test. After 30 minutes of arginine infusion, serum glucagon level showed almost 2 fold increase in both TCP and FCPD with out any significant difference in between confirming preserved cell function. The association between the SPINK 1N34S gene and TCP and FCPD subjects have been found as expected. The study reports for the first time, the association of CFTR gene in TCP and FCPD subjects. CFTR ex22 gene mutation is not found in any of the TCP subjects. Out of 60 FCPD subjects, 58 (96%) are of wild homozygous CFTR ex22 genotype and 1 (1.7%) heterozygous mutant has been detected and no homozygous mutant have been detected. Among the 33 T2DM subjects, 31 (94%) are of wild homozygous type and no CFTR ex22 mutant have been detected. A number of groups reported the association of CFTR gene mutation in idiopathic chronic pancreatitis but not in TCP and FCPD subjects in particular (Chon et al., 1998; Sharer et al., 1998; Audrezet et al., 2002; Noone et al., 2001; Cohn et al., 2005). The study also reports for the first time, the association of IL-18 gene in TCP, FCPD and T2DM subjects. Although, a significant percentage of IL-18 -607 gene mutation have been found, none of the subjects showed presence of helminth infestation (in regards to the absence of any larva in the stool). This scenario might be due to a wide line of practice of prescribing anthelminthic drug in a regular interval to the poor of rural as well as urban population. The study reports for the first time the correlation of the biochemical parameters and other genetic status of TCP and FCPD subjects with SPINK 1 N34S mutant. Among the biochemical parameters only fecal elastase 1 concentration is significantly lower (p=0.006) compared to the wild genotype. A significant negative correlation is detected between IL-18 -607 CC genotype and the SPINK1 (N34S) haplotype in the subgroup of patients with FCPD (r=.49 ; p=0.02). 15 TCP and 22 FCPD has either the IL-18 CC genotype and/or the SPINK1 n34S haplotype.

2. INTRODUCTION

2.1 An introduction to the topic

Tropical calcific pancreatitis (TCP)

Tropical calcific pancreatitis is a juvenile form of chronic calcific non alcoholic pancreatitis, seen almost exclusively in developing countries of the tropical world (Barman *et al*, 2003). In 1959 Zuidama from Indonesia first drew attention to this peculiar type of chronic pancreatitis. In the most simple of term, tropical calcific pancreatitis has been described as a disease with “pain in childhood, diabetes in puberty and death at the prime of life” (Geevarghese, 1985). TCP patients in former years were mostly children, adolescents, or sometimes young adults, who had common characteristics of malnutrition, deficiency signs, a cyanotic hue of enlarged lips, bilaterally enlarged parotid glands, a pot belly, and sometimes pedal edema. However, the clinical features and presentation of tropical pancreatitis have changed over the past 50 years with an older age of onset; severe malnutrition being uncommon with many patients being of ideal body weight which is attributed to improved nutritional status (Viswanathan, 1980; Zuidema, 1955; Pichumoni *et al*., 2004; *Mohan et al.*, 2003).

Characteristically, the disease presents as recurrent episodes of severe abdominal pain starting in childhood when the patients are between 5 and 15 years of age. After several years, signs of exocrine and endocrine dysfunction arise, such as steatorrhea and diabetes mellitus (Rossi *et al*., 2004). However, overt steatorrhoea is only present in about 20% of patients with TCP. The low frequency of steatorroea is attributed to the low fat intake in the diet (Barman *et al*., 2003). People living in or migrating from, tropical regions are affected. Known causes of chronic pancreatitis, such as alcohol intake and biliary disease, are not associated with this disorder. Findings with ERCP are characteristic and consist of grossly enlarged pancreatic ducts and intraductal calculi. Chronic pancreatitis of the tropics is a type of idiopathic chronic pancreatitis that is clinically similar to hereditary pancreatitis but without the

hereditary factor, and additionally shows findings of obstructive pancreatopathy (Rossi *et al*., 2004).

Fibrocalculus pancreatitis (FCPD)

Fibrocalculous pancreatic diabetes (FCPD) is a unique form of diabetes secondary to nonalcoholic chronic calcific pancreatitis seen in tropical, developing countries of the world associated with either overt protein–calorie malnutrition or more likely with deficiency of certain micronutrients. FCPD affects young individuals and runs an aggressive course to reach the endpoints of diabetes, pancreatic calculi and exocrine pancreatic dysfunction (steatorrhoea) in the majority of cases (Mohan *et al*., 1998). It is a condition in which, in addition to diabetes being present, there is also evidence of chronic pancreatitis of unknown origin with large intraductal pancreatic stones (Mohan *et al*., 1998). Patients frequently have a low body mass and a history of chronic abdominal pain and require insulin treatment, although, unlike in type 1 diabetes (T1D), they are not prone to ketosis (Hassan *et al*., 2000). There are characteristic features of FCPD radiologically, ultrasonographically, on endoscopic retrograde cholangiopancreatography and on histopathology which distinguish it from chronic pancreatitis of other aetiologies seen in temperate zones, e.g. alcoholic chronic pancreatitis.

Several terms have been proposed for this syndrome, though, for sake of uniformity and international agreement, it is advisable to adopt the term fibrocalculous pancreatic diabetes proposed by the WHO Study Group Report on Diabetes on 1985, when this entity was introduced as a subtype of malnutrition-related diabetes mellitus (MRDM). In the recent Expert Committee on Classification of Diabetes (1998) the entity known as “malnutrition-related diabetes mellitus” was deleted and FCPD is now classified as a “disease of exocrine pancreas” under the category of “Other types of diabetes”. The commonly used suffix “tropical” may not be appropriate as the disorder has been recently reported from temperate zones in migrants from tropical

countries (Chong *et al*., 1990). We propose that the term "fibrocalculous pancreatitis" (FCP) be used when one refers to this unique form of chronic pancreatitis restricted to developing countries of the world and the term fibrocalculous pancreatic diabetes when one refers to the diabetes secondary to FCP.

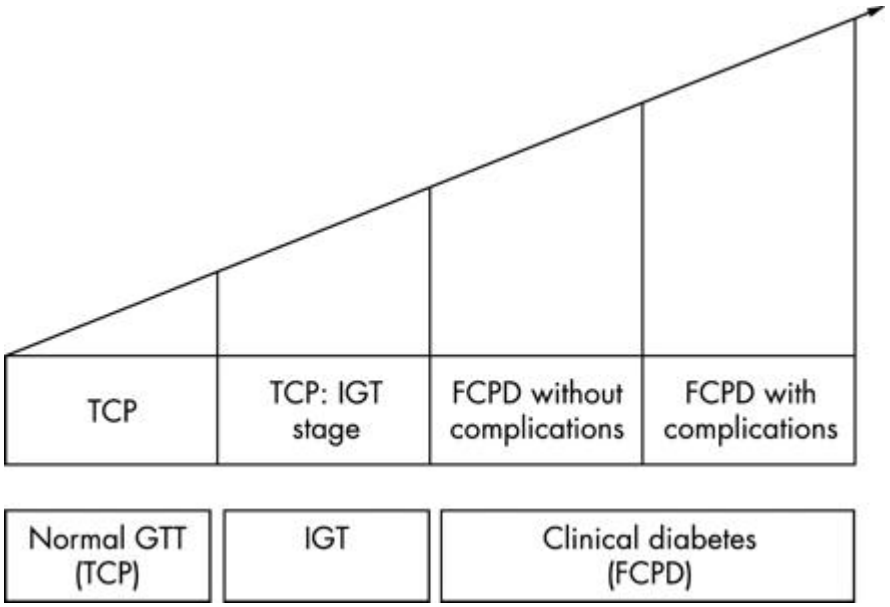

Figure 1: Natural history of tropical chronic pancreatitis (TCP); FCPD, fibrocalculous pancreatic diabetes; GTT, glucose tolerance test; IGT, impaired glucose tolerance.

Type 2 Diabetes Mellitus (T2 DM)

Diabetes is a leading cause of morbidity and mortality. Prevention of diabetes and its associated burden, primarily cardiovascular morbidity and mortality, has become a major health issue worldwide (Narayan *et al*., 2000). Recent estimates indicate there were 171 million people in the world with diabetes in the year 2000 and this is projected to increase to 366 million by 2030 (Wild *et al*., 2004) In the developing countries diabetes occurs at a younger age than in the developed countries, where it generally occurs in individuals aged 65 and above. Therefore, developing countries such as Bangladesh and India are expected to confront an enormous health care burden due to a large number of the population suffering from this chronic disorder and its sequelae. It is observed that India has the largest number of diabetes than any other country.

It is predicted that by 2025 Indian will harbor >60 millions diabetic patients and that cardiac diseases would be the leading cause of death i.e., 1 out of 4 individuals will be an Indian diabetic in the world while 3 out 4 will be from the developing countries (Wild *et al*., 2004) .

It has been estimated that the total diabetic patients in Bangladesh was more than 3 million in 2000, and this number would rise to 11.1 million by the 2030. The proportional increase in Bangladesh seemed relatively higher compared to other Asian countries (Wild *et al*., 2004). A recent epidemiological study in Bangladesh revealed that the prevalence of DM had increased exponentially in urban and rural populations. The study shows that in Bangladesh the prevalence of diabetes in urban areas is double than in rural areas (8% vs. 4%) and rises with affluence (Ghaffar et *al*., 2004). In a recent report it has been shown that age adjusted prevalence of T2DM was about 5.6% among the rural population (Sayeed *et al*., 2007). This creates a great challenge to the health care system in the developing country like Bangladesh, since diabetes is a lifelong disease requiring daily treatment.

According to American Diabetic Association 2005, Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels.

Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia.

Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia. Acute, life-threatening consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome. Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot arthopathy; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes.

The vast majority of cases of diabetes fall into two broad etiopathogenetic categories. In one category, type 1 diabetes, the cause is an absolute deficiency of insulin secretion. Individuals at increased risk of developing this type of diabetes can often be identified by serological evidence of an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers. In the other, much more prevalent category, type 2 diabetes, the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. In the latter category, a degree of hyperglycemia sufficient to cause pathologic and functional changes in various target tissues. The progressive deterioration of pancreatic insulin secretion has been implicated as the proximate cause of the progressive increase in plasma glucose level (Taylor *et al*,. 1994), but without clinical symptoms, may be present for a long period of time before diabetes is detected. During this asymptomatic period, it is possible to demonstrate an abnormality in carbohydrate metabolism by measurement of plasma glucose in the fasting state or after a challenge with food or an oral glucose load.

In short, Diabetes is a condition primarily defined by the level of hyperglycaemia giving rise to risk of microvascular damage (retinopathy, nephropathy and neuropathy). It is associated with reduced life expectancy,

significant morbidity due to specific diabetes related microvascular complications,increased risk of macrovascular complications (ischaemic heart disease, stroke and peripheral vascular disease), and diminished quality of life.

2.2 Tropical calcific pancreatitis (TCP) and fibrocalculus pancreatic diabetes (FCPD): Prevalence, epidemiology and etiopathogenesis

2.2.1 Prevalence and epidemiology

Patients with tropical pancreatitis and diabetes was first reported by Zuidema in 1959 from Indonesia. Following this first report, Shaper in 1966 demonstrated presence of the similar condition in Uganda. Since then, many reports have been published establishing TCP as a distinct form of chronic pancreatitis that is present in many developing countries in the tropics (Geevarghese, 1968; Pitchumoni, 1985; Kini, 1937;).The first case of pancreatic calculi from India was reported by Kini in 1937 and this was followed by reports of pancreatic calculi observed at postmortem from Vellore in southern India (Elizabeth, 1954). Reports from several tropical parts of the world including Nigeria (Kinnear, 1963), Uganda (Shaper, 1960), other parts of Africa (Mngola, 1982), Brazil (Dani *et al*., 1976), Thailand, (Vannasaeng, 1998), Bangladesh,(Azad *et al*., 1991) and Sri Lanka (Illangovekara, 1995) have subsequently confirmed the existence of TCP. However, it was also reported in 1967 from Southern India by Geevarghese, one of the pioneers in the field, documented one of the largest series in the world from Kerala state in Southern India that TCP attracted international attention (Pitchumoni, 1985). Large series of TCP and FCPD patients have also been reported by a number of workers from various states in India (Augustine, 1997; Balakrishnan, 1987; Mathew, 1996; Pai, 1974; Tripathy, 1987; Nagalotimath, 1986; Viswanathan, 1980; Moses, 1976; Mohan *et al*., 1985; Pendescy *et al*., 1990; Bhattachariya, 1990; Anand, 1987; Mohan, 1999). At the M V Diabetes Specialties Centre, Chennai (formerly Madras), a

large referral centre for diabetes in south India, about 50 patients with FCPD are registered annually, which constitutes about 1% of all diabetic patients seen at the centre.2 Unfortunately most of the available data are clinic based and hence subject to referral bias. There is very little information on the prevalence of TCP in the population. One survey done in Kerala reported a prevalence of 125/100 000 population (Rao, 1984). However this was done in an area that is endemic for TCP and the frequency is probably much lower in other parts of India. In a recent study from, World Health Organisation (WHO, 1999) the condition was termed as tropical pancreatic diabetes (TPD) since the unique condition was mostly found in the tropical countries. It attracted the attention of diabetologists and physicians when a WHO Study Group Report on Diabetes Mellitus in 1985 recognized the condition and termed it as fibrocalculus pancreatic diabetes (FCPD) (WHO 1985). FCPD has subsequently been reported from several tropical developing countries including Brazil, Congo, Nigeria, Madagasker, Kenya, Zambia, Zimbabwe, Sri Lanka, India, Bangladesh, Singapore, Thailand and New Guinea (Abu-Bakare *et al.,* 1986).

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

diabetic patients 13% were found to be constituted by the FCPD patients (Azad Khan and Ali 1997).

2.2.2 Etipathogenesis

Little is known about the etiology of pancreatitis in TCP and FCPD. To date, no etiologies have been identified (Khan and Ali, 1997; Yajnik *et al*, 1992; Sidhu *et al*, 1995; Sarles *et al*., 1994; Balaji *et al*., 1994; Mohan and Alberti, 1997; Mohan *et al*., 1998). The etiopathogenitic mechanism of TCP and FCPD still remain unclear. There is no experimental model for TCP and FCPD. The following hypotheses have been proposed based on epidemiological data (Mohan *et al*., 2003).

1. Malnutrition theory
2. Cassava hypothesis and other dietary toxins
3. Familial and genetic factors
4. Oxidant stress hypothesis and trace element deficiency states

In western industrialized countries the most common etiological factors in chronic pancreatitis is long-term alcohol abuse. However, in 10-30% of chronic pancreatitis patients there is no apparent underlying cause and these are classified as idiopathic chronic pancreatitis (ICP). Small percentage, less than 5%, of chronic pancreatitisis is due to hereditary pancreatitis (HP) and cystic fibrosis (CF), and others (Andren-Sandberg, 2003). In the tropical developing countries both HP and cystic fibrosis are rare and the main bulk of patients with chronic pancreatitis is contributed by TCP. About 90% of the patients with TCP, of all ages, ultimately develop diabetes. However, the proportion of TCP patients among the young becoming FCPD is still to be ascertained.

Chronic pancreatitis due to alcoholic abuse occurs at 4th and 5th decades of life; on the other hand TCP and FCPD occur at much earlier age. Since the condition is mostly restricted in tropical developing countries and the earlier reports mainly involved poorer section of people. Environmental factors being implicated in the etiopathogenesis., so was the name chosen malnutrition

related diabetes mellitus (MRDM) (WHO 1985). Chronic under nutrition has been suggested to be important determinant of diabetes in an individual, either by progressively impairing beta cell function or by increasing the susceptibility of the individual to other genetic and environmental diabetogenic influences (Rao 1984).

Among other environmental factors cyanogenic food, especially Cassava and oxidative damage, have been also implicated. These diseases were observed in areas where Cassava, which contains cyanogenic glycosides - linamarin and lotuslarin, are consumed as staples food in India and it has been suggested to play a crucial role in the causation of FCPD (McMillan and Geevarghese, 1979; Pitchumoni *et al.,* 1988). However, subsequent studies failed to support the initial findings. No direct relationship was established between consumption of Cassava and pancreatitis (Balakrishnan *et al.,* 1988; Yajnik *et al.,* 1989). Moreover, diabetes was absent in rural West Africa where Cassava is also eaten as staple food although this may depend on its preparation (Teuscher *et al.,* 1987). In animal models protein calorie malnutrition has been shown to produce a wide number of changes in the pancreas and liver leading to glucose intolerance and insulin insufficiency. Short- and long-term experimental feeding of Cassava in animal models has produced conflicting results and lacks experimental support in the causation of diabetes and/ or FCPD (Kamalu 1991; Mathangi *et al.,* 1996).

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

Chronic pancreatitis in white people has been linked to heightened oxidative detoxification induced by cytochrome P-450 within the pancreas and/ or liver [Rao and Yajnik, 1996].Faster theophyline clearance, which is an *in vivo* probe of the potentially toxic cytochrome-450I drug metabolism pathway, was

observed in patients with chronic pancreatitis compared to controls. These patients had been revealed to high level of exposure to Xenobiotic (cigarette, alcohol, occupational chemicals, dietary corn oil etc) that are inducers of cytochrome-450I and/or yield reactive metabolites leading to oxidative stress (Chaloner *et al.,* 1990). Studies on antioxidant status of south Indian TCP showed low level of vitamin C and B-carotene; and it provided indirect evidence in support of the oxidative stress theory (Braganza *et al.,* 1993). Malnutrition has been postulated to induce a state of defective ability to scavenge free radicals and hence suggested that this could enhance the susceptibility for organ damage (Barman *et al.,* 2003). Direct evidence has been observed with a significantly higher number of patients demonstrating single stranded DNA (a marker of free radical mediated damage of double stranded DNA) compared to controls and other diabetic groups (McDonagh *et al.,* 1996). Several lines of evidence suggest that genetic factors may be important. However, too little is known about these problems to make any specific recommendations. Major insights into the two forms of tropical pancreatitis are likely in the future. However, the free radical hypothesis has not yet been proven and merits further study.

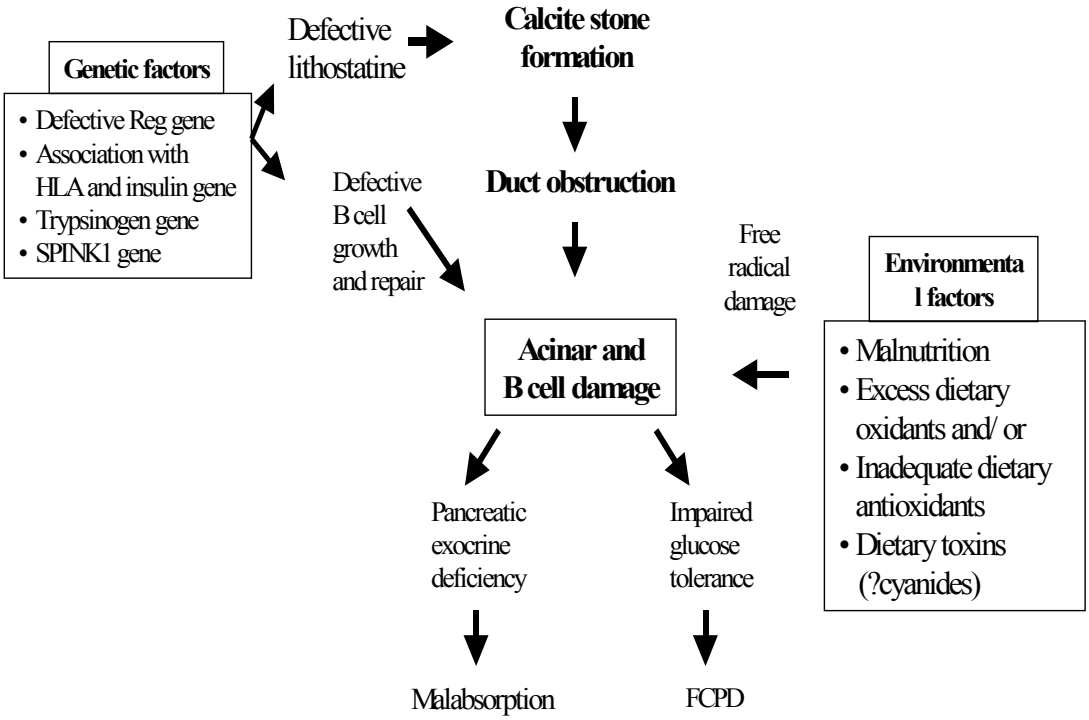

Figure 2: Schematic diagram showing the pathogenic mechanism of pancreatic damage leading to pancreatitis [Mohan *et al.,* 1998b].

2.2.3 PANCREATIC EXOCRINE – ENDOCRINE INTERRELATIONSHIP: FOCUS ON DM, TCP AND FCPD

Exocrine pancreatic dysfunction often potentiate endocrine pancreatic dysfunction and vice verca because the endocrine and exocrine pancreas are anatomically and functionally interrelated. Disorders of exocrine pancreas, such as, chronic and acute pancreatitis and pancreatic adenocarcinoma, can induce endocrine pancreatic disorders, such as diabetes mellitus and islet cancer. In turn, diabetes and islet cancers are often associated with exocrine pancreatic insufficiency.

2.2.3.1 Exocrine pancreatic disorders in Diabetes Mellitus

Diabetes is an endocrine disorder characterized by a fall in plasma insulin and an increase in blood glucose level. In tandem, exocrine pancreatic secretion of amylase, trypsin, lipase and bicarbonate also falls (Kang and Go, 1999). Diabetes is also characterized by in somatostatin-secreting, glucagon-secreting, and pancreatic polypeptide-secreting cells, all of which can contribute to inhibiting exocrine secretion (Kang and Go, 1999).

2.2.3.2 Endocrine pancreatic disorders in Pancreatitis and Exocrine Pancreatic Cancer

The incidence of diabetes mellitus secondary to chronic pancreatitis varies from 40% and 70% with a frequency as high as 90% in chronic calcific pancreatitis (Bank *et al*, 1975). The number of β cells is reduced by more than 60%, and their optimal responsiveness to glucose in substantially diminished. Although the number of β cells decrease in chronic pancreatitis, the number of α cells increases. This α cell hyperplasia produces inappropriately high glucagon release for the circulating glucose concentrations (Kloppel *et al*., 1978). Once insulin-dependent diabetes developes secondary to chronic pancreatitis, however, no significant increase in plasma glucagon occurs. (Larsen *et al*., 1988). Moreover, basal glucagon levels in pancreatic diabetes resulting from chronic pancreatitis are significantly lower than those in primary diabetes (insulin-dependent diabetes mellitus, non-insulin-dependent diabetes mellitus).

2.2.4 Autoimmune status in TCP and FCPD

Islet cell antibody (ICA), Insulin auto antibody (IAA), Glutamic acid decarboxylase (GAD) and Islet antigen 2 (IA-2) are the important auto antibodies related to diabetes. So far no study has been done to see the status of auto antibodies in TCP subjects. Not many studies have been done regarding autoimmunity in FCPD. Autoimmunity has not so far been linked to the pathogenesis of FCPD. However, there are many a few studies where autoantibodies have been measured in this group of patients.

The various studies are summarized in the table 1.

Table 1: Frequencies of autoantibodies in FCPD in various studies

Autoant - bodies	Controls	FCPD	T1D	T2D	PDDM/ MMDM	References
GAD	0%	7%	47.5%	5.6%	-	Mohan *et al.,* 1998a
ICA	4.3%	6.3%	53.8%	9.9%	-	
ICA	-	0	35%	-	13%	Dabadghao *et al.,* 1996
GAD	4%	0	-	-	32%	Sanjeevi *et al.,* 1999
GAD	0	7%	-	-	23%	Singh *et al.,* 2000
IA2	-	0	22%	-	3%	
ICA		0	41%		13%	
GAD	0	7.1%	14.2%	0	38%	Goswami *et al.,* 2001
IA2	0	0	2%	0	0	

2.2.5 Genetic and Familial factors in TCP and FCPD

Whatever the nutritional or toxic factor that predisposes to TCP and FCPD, it is clear that only a minority of people exposed to the risk seem to get the disease, suggesting a possible role for genetic factors in the causation of the diseases.

The 1996 discovery that mutations in cationic trypsinogen gene (UniGene name: protease, serine, 1; PRSS1) cause hereditary pancreatitis (Whitcomb et al., 1996) opened a new chapter in the book on chronic pancreatitis including TCP and FCPD. The recognition of frequent CFTR mutations (Sharer et al., 1998; Cohn et al., 1997) and serine protease inhibitor, Kazal type 1 (SPINK1) mutations (Witt et al., 2000; Pfutzer et al., 2000) in patients with idiopathic chronic pancreatitis has heightened awareness of the importance of genetic mutations in the disease. These discoveries not only provide insights into the molecular mechanisms of pancreatitis, but present the possibility of powerful diagnostic tools.

FCPD shares common susceptibility genes with type 1 and type 2 diabetes (Kombo *et al*.,1989). Many recent studies have looked for genetic abnormalities in all forms of chronic pancreatitis following the discovery of genetic mutations in hereditary pancreatitis (Whitcomb *et al*., 1996; Witt *et al*., 2000; Pfutzer *et al*., 2000). The most genetic mutations studied in relation to hereditary pancreatitis (HP), and to a lesser extent in other forms (alcoholic, TCP and FCPD) of pancreatitis, are those involving the cationic trypsinogen gene (PRSS1). serine protease inhibitor (SPINK 1) and cystic fibrosis transmembrane conductance regulator (CFTR) (Noone *et al*., 2001). In two recent studies, one from India and one from Bangladesh, it was noted that pancreatitis was highly associated with the SPINK 1 N34S mutation. Whitcomb *et al*. in 1996 found that an Arg-His substitution at residue 117 (R117H) or the cationic trypsinogen gene is associated with the HP phenotype, and Gorry *et al*. in 1997 reported that an

N211 mutation in the same gene also is associated with HP in some families. A study with TCP and FCPD patients conducted by Rossi *et al*.in 1998 first have shown a lack of the R117H mutation in the cationic trypsinogen gene responsible for HP in these patients. Later Hassan et al (Hassan *et al*., 2000) also confirmed the same view.

2.2.5.1 Cationic trypsinogen gene mutations: - An autosomal dominant disorders

Cationic trypsinogen (Uni-Gene name: protease, serine 1; PRSS1) is among the most abundant molecules produced by pancreatic acinar cells (Rossi *et al*., 1998) Cationic trypsinogen plays a central role in hydrolyzing dietary proteins at lysine and arginine amino acid residues and also plays the key role in activating all other digestive proenzymes (Rossi *et al*., 1998). Premature activation of trypsinogen within the pancreas, with subsequent activation of other enzymes leading to pancreatic autodigestion, is believed to be central to the development of acute pancreatitis. Recurrent attacks of acute pancreatitis, as in hereditary pancreatitis, eventually lead to chronic pancreatitis. Mutations in codons 29 (exon 2) and 122 (exon 3) of the cationic trypsinogen gene cause autosomal dominant forms of hereditary pancreatitis.(Gorry *et al*., 1997; Whitcomb *et al*., 1996; Whitcomb, 1999; Whitcomb, 2000). The codon 122 mutations usually result in a R122H substitution, older nomenclature R117H (Whitcomb, 2000; Pfutzer, 1999; Antonarakis, 1998), which eliminates a fail-safe trypsin hydrolysis site in the side chain of trypsin that connects the two halves of the molecule. Elimination of this site causes a gain-of-function mutation because prematurely activated trypsin cannot be in-activated by autolysis (Whitcomb *et al*.,1996; Whitcomb, 1999; Varallyay *et al*., 1998). The N29I mutation (older nomenclature N21I) causes a clinical syndrome identical to the R122H mutation syndrome, although the molecular mechanism causing the gain of function continues to be debated (Sahin-Toth, 1999; Whitcomb, 1999) Other less common mutations at codon 29 and 122 have also been identified

(Howes *et al*., 2001). The common N29I and R122H mutations occur in patients from the North America (Gorry *et al*., 1997; Whitcomb *et al*., 1996), Europe (Finch *et al*., 1997; Ferec *et al*., 1999; Teich *et al*., 1999), Japan (Nishimori *et al*., 1999) and likely elsewhere. The prevalence of cationic trypsinogen mutations in various populations varies widely, ranging from 0% to 19% among patients presumed to have idiopathic chronic pancreatitis (Creighton *et al*., 1999; Teich *et al*., 1999; Bohm *et al*., 1999; Cohn *et al*., 2000). This observation may reflect the settlement patterns of the descendants of early disease founders. Mutations at codons 16, 22, and 23 in exon 2 of cationic trypsinogen appear in some patients, resulting in A16V (Pfutzer *et al*., 1999; Witt *et al*., 1999; Chen *et al*., 1999), D22G (Teich *et al*., 2000), and K23R (Ferec *et al*., 1999) amino acid substitutions. The D22G and K23R mutations appear to be gain-of-function mutations by facilitating activation of trypsinogen to trypsin (Teich *et al*., 2000). They do not result in the high-penetrance, autosomal dominant pancreatitis as seen with codon 29 and 122 mutations. Indeed, to our knowledge, only 2 patients with chronic pancreatitis and D22G mutation (Teich *et al*., 2000). and 1 or 2 patients with chronic pancreatitis and a K23R mutation (Ferec *et al*., 1999) have been identified and confirmed worldwide. The reason for the low incidence of pancreatitis in patients with activation-facilitating mutations may be because the highly effective fail-safe R122 autolysis mechanism remains intact. The pancreatitis-predisposing mechanism of the A16V mutation remains unknown. However, more than a dozen patients with the A16V mutation and chronic pancreatitis have been reported (Pfutzer *et al*., 1999; Witt *et al*., 1999; Chen *et al*., 1999).

2.2.5.2 Serine protease inhibitor Kazal type 1 (SPINK1) gene mutation in TCP and FCPD

Pancreatic secretory trypsin inhibitor (PSTI, Uni-Gene name: serine protease inhibitor, Kazal type 1; SPINK1) is a 56 amino acid peptide that specifically inhibits trypsin by physically blocking the active site. SPINK1 is synthesized by

pancreatic acinar cells along with trypsinogen, and it colocalizes with trypsinogen in the zymogen granules, in the mechanistic models of pancreatic acinar cell protection, SPINK1 acts as the first line of defense against prematurely activated trypsinogen in the acinar cell (Whitcomb *et al*., 1996; Witt *et al*., 2000; Pfutzer *et al*., 2000; Rossi *et al*., 1998; Rinderknecht *et al*., 1988). However, because of a 1:5 stoichiometric disequilibrium between SPINK1 and trypsinogen (Rossi et al.,1998). SPINK1 is only capable of inhibiting about 20% of potential trypsin. Thus within the pancreas SPINK1 appears to act as the first line of defense against prematurely activated trypsinogen. Because gain-of-function trypsin mutations cause acute pancreatitis and chronic pancreatitis, it was hypothesized that loss of trypsin inhibitor function would have similar effects. In 2000, the role of SPINK1 mutations in chronic pancreatitis emerged (Witt *et al*., 2000; Pfutzer *et al*., 2000; Chen *et al*., 2000), SPINK1 N34S and P55S mutations are relatively common, being present in: 1% of alleles tested and therefore: 2% of the western general population (Pfutzer *et al*., 2000; Chen *et al*., 2000). Families affected with pancreatitis in whom trypsinogen mutations were excluded often have SPINK1 mutations, but the mutations do not segregate with the disease (Pfutzer *et al*., 2000; Chen *et al*., 2000).Thus, SPINK1 mutations are not sufficient to cause hereditary pancreatitis in an autosomal dominant inheritance pattern. However, the frequency of SPINK1 mutations in populations with idiopathic chronic pancreatitis is markedly increased (23% to 25%) (Witt et al., 2000; Pfutzer et al., 2000) proving that these mutations are clearly associated with pancreatitis. Interestingly, chronic pancreatitis occurred with heterozygous, compound heterozygous or nomozygous genotypes (Witt *et al*., 2000; Pfutzer *et al*., 2000), and the severity

of pancreatitis or age of disease onset among genotypes is similar (Pfutzer *et al*., 2000). Furthermore, because SPINK1 N34S and P55S mutations are common in the western general population (2%) and idiopathic chronic pancreatitis is rare, the risk of an asymptomatic SPINK1 mutation carrier developing chronic pancreatitis is low (1% given the observed frequency for N34S mutations and a population prevalence for idiopathic chrome pancreatitis of 1/16,000 100). Thus, the disease mechanism is more complex than a simple autosomal recessive one. SPINK1 mutations appear to act as disease modifiers (Pfutzer *et al*., 2000), lowering the threshold for initiating pancreatitis or possibly worsening the severity of pancreatitis caused by other genetic and/or environmental factors. SPINK1 represents the first line of defense against prematurely activated trypsinogen within the pancreas (Whitcomb *et al*., 1996; Witt *et al*., 2000; Pfutzer *et al*., 2000; Rossi *et al*., 1998). If the SPINK1 N34S and other mutations cause SPINK1 loss of function (Pfutzer *et al*., 2000), then the model would predict that the levels of active trypsin within the pancreas would increase above normal basal levels. However, if the trypsin R122 side-chain autolysis mechanism remains intact (above), the pathophysiologic activation process would typically fail to progress beyond the fail-safe trypsin autolysis phase. If so, only patients with inherited or acquired deficiencies or impairments of other pancreatic protective mechanisms would develop pancreatitis (Whitcomb *et al*., 2001).

In a recent study from India, it was noted that both TCP and FCPD were highly associated with the SPINK 1 N34S mutation (Schneider *et al*., 2002; Suman *et al.*, 2001). The high prevalence of the N34S mutation in patients with (FCPD) and without (TCP) diabetes in the Indian study suggests that these two

subtypes have a similar genetic predisposition (Schneider *et al*., 2002; Suman *et al*., 2001).

Recently several studies have been carried out in Bangladeshi with TCP and FCPD patients The most initial study (Rossi et al 2001) was carried out with 12 TP (TCP and FCPD) patients and 4 healthy controls. SPINK 1 mutation was found in 6 of 8 (75%) FCPD patients but none in 3 cases of TCP or 4 controls. This was just confirmed and extended to TCP in an article by Chandak et al in 2002. In another study in 2002 Hassan et al. used a family- based and case-control approach in two separate ethnic groups from the Indian subcontinent to determine whether N34S was associated with susceptibility to FCPD. Clear excess transmission of SPINK 1 N34S with FCPD in 69 Bangladeshi families was observed (P <.0001; 20 transmissions and 2 no transmissions). In the total study group (Bangladeshi and southern Indian) the N34S variant was present in 33% of 180 subjects with FCPD. 44% of 861 non diabetic subjects (odds ratio 10.8:P <.0001 compared with FCPD). 3.7% of 219 subjects with type 2 diabetes, and 10.6% of 354 subjects with early-onset diabetes (aged <30 years P=.02 compared with the ethnically matched control group). These results suggest that the N34S variant of SPINK1 is a susceptible gene for FCPD in the Indian subcontinent, although by itself, it is not sufficient to cause the disease. Scheinder et al in 2002 conducted another study where Bangladeshi patients with a variety of pancreas-associated diseases including TCP, FCPD and type 2 diabetes were included to determine the role of SPINK' mutations in this study, SPINK1 N34S mutations appeared in 1.3% of controls, 55% of FCPD, 20% of TCP and 14% of type 2 diabetic subjects which concludes that in

Bangladesh, the SPINK1 N34S mutation increases the several forms of pancreatic diseases, including TCP, FCPD and type2 diabetes mellitus.

In another study carried out by Hassan et al. in 2002, SPINK1 N34S mutation in Bangladeshi controls have been found to be 5.7%. The frequency of the mutation in the control subjects appears to be relatively higher compared to the observation of Schneider et al 2002. It may be noted that the age of the two groups of controls are different at the time of recruitment: in the Hassan et al. study they are of younger age group (22.5±4.9. yrs) who may have undiagnosed sub-clinical disease, compared to relatively older (mean age, 28 yrs) cohort of only 76 controls which left a chance of underestimation of the frequency. In this study unrelated Bangladeshi FCPD patients showed 39% 5'HA/K7 gene variant 'G allele leading to N34S mutations compared to the controls ($p<0.0001$). SPINK1 gene N34S mutation has also been analyzed in idiopathic and alcoholic pancreatitis patients. Frequency of the mutation was found to be 9-20% in idiopathic pancreatitis. Among alcoholic pancreatitis patients the mutation was found in 6% cases (Threadgold et al., 2002) whereas in different studies frequency of positivity for the variant genotype was 50-75% in FCPD patients. The high frequency of SPINK1 gene N34S mutation in FCPD compared to idiopathic pancreatitis clearly suggests that mutant ‘G’ allele, possibly, confer an increased risk for development of pancreatitis in the tropical pancreatitis patients.

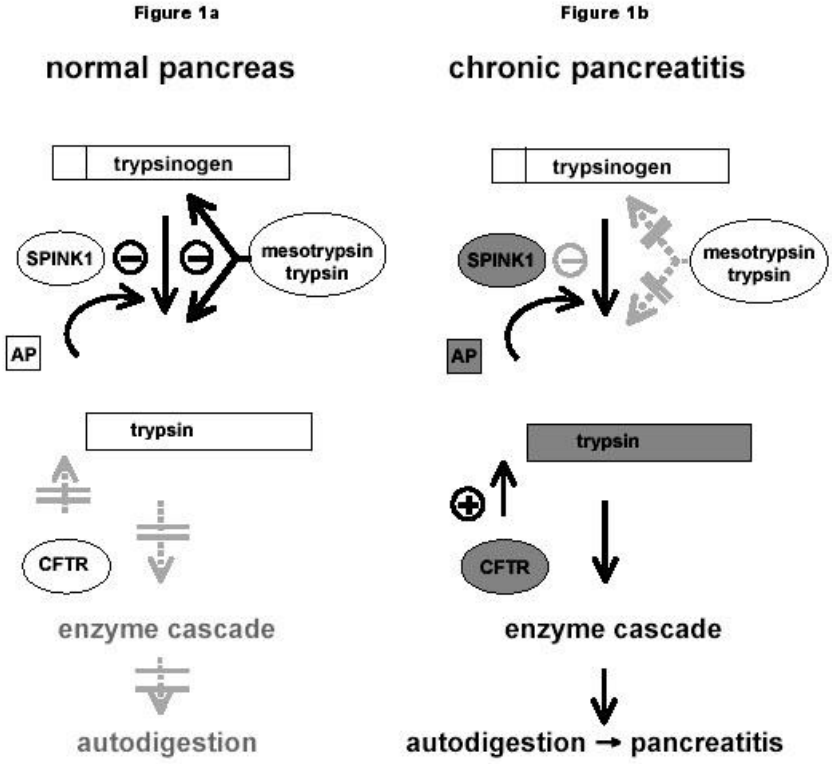

Figure 3: Model of chronic pancreatitis. [a]Condition in the normal pancreas: Natural defense mechanism prevents activation of pancreatic enzyme cascade within the pancreas and autodigestion. [b]Condition in chronic pancreatitis: Disruption of defense system leading to unopposed intrapancreatic activation of digestive enzyme. Dark boxes represent product of mutated genes AP, Activated peptide [Truninger *et al.,* 2001].

2.2.5.3 CFTR mutations – An autosomal Rrcessive/ModiÞer Gene

Cystic fibrosis (CF) is a common autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan *et al*., 1989). Major mutations in both alleles result in the commonly recognized CF clinical features of abnormal sweat chloride concentrations, neonatal hypertrypsinogenemia, pancreatic pseudocysts formation, and fibrosis (i.e. “cystic fibrosis”) with clinical chronic pancreatitis, and progressive pulmonary disease. Among CF patients, 66% have a 3–base pair deletion of

the phenylalanine-coding codon 508 (DF508), although approximately 900 other mutations have been reported (Duri, 1998; Tsui and Duri, 1997). Most CFTR mutations can be classified into 1 of 5 severity categories based on the demonstrated or presumed molecular consequences (Zielenski and Tsui, 1995; Durie, 200). Typical CF patients with pancreatic insufficiency tend to have two severe mutations (i.e., class I, II, or III), whereas CF patients with pancreatic sufficiency from birth tend to have at least o ne CF "mild allele" (i.e., class IV or V) (Durie, 2000). In 1998, 2 groups reported that a significant association between patients with idiopathic chronic pancreatitis and various CFTR mutations (Sharer *et al*., 1998; Cohn *et al*., 1998). Indeed, several mild, "pancreas-sufficient" mutations (e.g., CFTR R117H and the intron 8 "5T allele," which results 80% reduction of exon 9 expression (Chillon *et al*., 1995; Strong *et al*., 1993) seem to be associated with idiopathic chronic pancreatitis (Sharer *et al*., 1998; Cohn *et al*., 1998) as well as another feature of CF, congenital bilateral absence of the vas deferens (CBAVD) (Chillon *et al*., 1995; Costes *et al*., 1995). Other mild CFTR mutations e.g., L997F (Gomez *et al*., 2000) may also be associated with neo-natal hypertrypsinemia and/or idiopathic pancreatitis, but not lung disease or an abnormal sweat chloride. Although initial reports suggested that idiopathic chronic pancreatitis was associated with a single allelic mutation of CFTR, more recent evidence suggest that patients with chronic pancreatitis may actually have compound heterozygous mutations of CFTR and mild CF because they also have abnormal nasal bioelectrical responses that accurately identifies abnormal CFTR function (Cohn et al., 2000). Thus, a subset of patients with idiopathic chronic pancreatitis have a variety of CFTR mutations without other features of CF.

2.2.5.4 Interleukin 18 (IL-18) gene mutation in TCP and FCPD

Regarding the causation of TCP and FCPD some data suggest that cofactors of tropical pancreatitis are related to rural tropical environment and low socioeconomic status (Sarles *et al*., 1987; Rajasuriya *et al*., 1997). Helmith infestation is a common problem in children of poor families in Bangladesh and

other developing counties (Northrop-Clewes *et al*., 2001) with the prevalence of ascaris infections in poor urban communities ranging from 64% to 95% (Hall *et al.*, 1999). The nematode ascaris lumbricoides is frequently found in these children and is also a common cause of acute pancreatitis. Subsequently recurrent acute attack may lead to chronic pancreatitis.

IL-18 is a pleomorphic cytokine involved in the regulation of the immune response. In rodents IL-18 over expression promotes the persistence of helminthic infections (Helmby *et al*., 2001; Pfaff *et al*., 2003).

2.2.6.5 TCP and FCPD subjects in Bangladesh

The relationship between tropical calcific pancreatitis (TCP) and fibrocalculus pancreatic diabetes (FCPD) is still unclear.(khan and ali, 1997) A substantial number of isolated national and international collaborative works have been initiated at BIRDEM to investigate the etiopathogenesis of TCP and FCPD subjects.

2.2.6.1 Epidemiology

No population-based survey, as yet, exists on the incidence or prevalence of TCP or FCPD in Bangladesh. TCP is not a common disease; however, it is found not uncommonly in gastroenterological practices in Bangladesh. Due to an organized system of diabetes care provided by the Diabetic Association of Bangladesh (DAB), a relatively large number of FCPD patients have registered with the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), the central institute of DAB. The outpatient department of BIRDEM now takes care of the highest number of diabetic patients *(*> 150000 registered by the end of 1995) in any one centre in the world, and it also has a special clinic for the care of young diabetic patients under 30 years of age. Among 1449 diabetic patients under 30 years old registered at BIRDEM between January 1990

and May 1992, 309 (55%) were diagnosed as suffering from MRDM and 179 (13%) had calcific pancreatitis (Khan *et al*., 1994)

2.2.6.2 Aetiology

It has been mentioned previously that alcohol may be excluded as a causative agent of TCP or FCPD in Bangladesh. Cyanogen containing foods such as cassava have been implicated in the aetiology of this type of pancreatitis (McMillan DE and Geevarghese PJ, 1979). However, in Bangladesh cassava is neither grown nor imported. A search for cyanogen was made in some common foods of Bangladesh and no significant amount could be found (Khan *et al*., 1991). However, this does not exclude the possibility of cyanogen existing in some relatively uncommon types of food consumed in Bangladesh.

Although Braganza in 1988 suggested the possible role of free radicals in the aetiopathogenesis of FCPD, no substantial data have been produced to test this hypothesis. Our recent findings provide evidence regarding the involvement of oxidative damage in FCPD patients (Khan and Ali, 1997). A significantly higher number of patients showed single stranded DNA (a marker of free radical mediated damage of double stranded DNA) compared with control and age-matched NIDDM subjects. Various agents (chemicals, fertilizers, food adjuncts, etc.) which may possibly lead to free radical production are now under investigation.

2.2.6.3 Clinical characteristics

2.2.6.3.1 Age

In contrast to what is expected, non-diabetic TCP subjects usually present at an older age FCPD patients (Khan and Ali, 1997; Rossi *et al*.,2004) (Table 2).

2.2.6.3.2 Sex

There is a male preponderance among both the TCP and FCPD patients. However, this may not reflect the true picture in the general population as there may be a bias for males to present to a hospital, for different socio-economic and cultural reasons, in a country like Bangladesh (Khan and Ali, 1997; Rossi *et al*., 2004) (Table 2).

2.2.6.3.3 Body mass index (BMI))

FCPD patients present with a significantly lower value of BMI compared to TCP patients (Khan and Ali, 1997; Rossi *et al*., 2004). The mean (±SD) of TCP subjects was 17.53±2.94), showing that TCP subjects fall both above and below the mean BMI of the normal, Western, adult population (i.e. 19). The mean BMI of the normal Bangladeshi population has not yet been worked out. It is, therefore, difficult to ascertain whether TCP patients are of low weight or normal weight. All of the FCPD patients presented low body weights (BMI mean±SD 15. 02 ±3. 04) (Khan and Ali, 1997) (Table 2).

2.2.6.3.4 Demographic and socio-economic background

Both TCP and FCPD patients came predominantly from a rural background (Rahman, 1996). However, economic conditions varied greatly between the two groups. The mean annual income of the FCPD group was one-third of the annual income of the TCP group. According to the socio-economic index followed in Bangladesh FCPD subjects belonged to the poorest class, whereas the TCP subjects belonged to the middle-class income group. However, it must be mentioned that the TCP patients were collected from the private practices of the gastroenterologists where a bias towards affluent people may be found (Khan and Ali, 1997) (Table 2).

Table 2: Socio-demographic and clinical characteristics of the TCP and FCPD subjects

Parameter	TCP (n=24)	FCPD (n=15)
Age-(years)	21.83 ± 5.91	21.33 ± 5.61
Male: Female	24:7	17:7
Rural: Urban	19:8	16:3
Annual income in	300-5000	250-1800
Family history of diabetes(up to second degree relatives)	2 (8%)	3 (20%)
Family history of TCP	1	0
BMI, mean ± SD	17.53 ± 2.94	15.02 ± 3.04

Results are expressed as mean ± SD.

2.2.6.3.5 Presenting clinical features

TCP subjects were selected from gastroenterologists in Dhaka, and all of the patients presented with abdominal pain suggestive of relapsing pancreatitis. In the FCPD subjects, only 25% presented with similar pain. The FCPD patients showed the typical symptoms of diabetes; however, they did not develop ketoacidosis despite high fasting blood glucose levels. Almost one hundred percent of the FCPD patients showed signs of malnutrition and many of them had various complications of diabetes. In contrast, only 6% of the TCP patients had any sign of malnutrition (Khan and Ali, 1997).

2.2.6.3.6 Biochemical characteristics at presentation

2.2.6.3.6.1 Glycaemic status

Obviously FCPD differed clearly TCP with regard to glycemic control, as observed by the significantly elevated plasma glucose and HBA1c in FCPD subjects (Khan and Ali, 1997; Rossi *et al*., 1998). Both Fasting blood glucose and 2h postprandial values of the FCPD subjects was about 4 times higher than that of the TCP subjects. In another study of 11 FCPD and seven TCP subjects (Saha *et al*., 2000), fructosamine values were found to be 3.5 times higher in the FCPD group compared with the TCP group (Table 3).

In another study (Ali *et al*., 2001), regarding glycemic status compared to TCP, early FCPD patients had about 1.33 times and late FCPD had about 3.01 times higher fasting serum glucose, early FCPD had 3.07 times and late FCPD had 4.26 times higher postprandial serum glucose.

2.2.6.3.6.2 Lipid profile

The values of lipids were within normal limits for both the groups. Serum TG and HDL cholesterol were similar in the two groups. However, serum total cholesterol and LDL cholesterol were significantly higher in the TCP group (Khan and Ali, 1997) (Table 3).

Table 3: Biochemical Characteristics of the TCP and FCPD subjects

Parameter	TCP (n=24)	FCPD (n=15)	t/P values
Serum glucose, fasting (mmol/L)	4. 42 ±0.99	14. S9± 10.15	-3.99/0.001
Serum glucose, 2h after BF (mmol/L)	7, 13 ±2. 19	23. 09± 10.53	-5.80/0.000
S C-peptide, fasting (ng/dL)	1.27±0.60	0.69 ±0.50	3.10/0.004
S Triglyceride (mg/dL)	142.54±51.72	125.47±57.77	0.093/0.358
S Cholesterol (mg/dL)	169.42±51.36	139.73 ±27.48	2.34/0.025
S HDL-cholesterol (mg/dL)	32.38±11.86	32.33±7.81	0.01/0.990
S LDL cholesterol (mg/dL)	108.10±41.71	86.47 ±20.26	2.16/0.037
S Total protein (g/dL)	8.15±1.34	7.57±L19	1.38/0.176
S Albumin (g/dL)	4.26±0.82	3.86 ±0.73	1.55/0.129
S Creatinine (mg/dL)	0.76±0.43	1.33±0.27	-3.47/0.003

Results are expressed as mean ± SD. Signification of differences between the groups were compared by unpaired t-test.

2.2.6.3.6.3 Renal function

In the series of seven TCP and 11 FCPD patients (Saha et al., 2000) both serum urea and creatinine were found to be higher in the FCPD group compared with the TCP group (scrum urea, mg/dL, mean±SEM: 21.7 ±2.0 in TCP vs 27.7 ± 1.8 in FCPD, P <0.06; serum creatinine, mg/dL, mean±SEM: 0.77 ±0.16 in TCP vs 1.33 ±0.08 in FCPD, P<0.05). Although the values are still within normal limits, they may indicate some nephropathic changes in the FCPD group.

Early renal haemodynamic and microvascular changes have been assessed in FCPD patients along with age-matched non-diabetic controls (Alam, 1994). The percentage of patients with increased GFR (> 143mL/1.73m2 body surface) was 0 in the control group and was 58% in the FCPD group. Kidney sizes of the FCPD patients were similar to those of the controls. Albumin-creatinine ratios (ACR) and transferrin-creatinine ratios (TCR) were raised in the FCPD when compared to the control group (1.6 times for ACR and 3.24 times for TCR, respectively). These findings also indicate an early renal involvement in FCPD patients (Alam, 1994).

2.2.6.3.6.4 Trace elements

Serum and urinary levels of Zn, Cu and Mg have been measured by atomic absorption spectrophotometry in six TCP and 13 FCPD subjects (Liauqe et al., 1999). Although the small number of TCP subjects precludes any conclusion, the data are presented as preliminary information (Table 4)

Table 4: Serum levels of Mg, Zn, Cu and Zn-Cu ratio in TCP and FCPD subjects Parameter

Parameter	**TCP (n=6)**	**FCPD (n=13)**	**t/P values**
SMg (mmol/L)	0.74 + 0.04	0.66 + 0.02	2.31/<0.05
SZn (umol/L)	23.60 + 2.66	31.68 + 3.00	1.66/Ns
SCu (umol/L)	17.08 + 1.14	19.50 + 0.87	1.22/Ns
Zn: Cu	1.38 + 0.20	1.66 + 0.18	0.96/Ns

Results are expressed as mean ± SEM. Significance of differences between the groups were calculated by unpaired 't' test.

2.2.6.3.6.5 Other biochemical features

Serum total protein and serum albumin are similar in the two groups. There was, also, no difference with regard to serum calcium (Saha *et al*., 2000).

2.2.6.3.6.6 Endocrine pancreatic function

Fasting serum C-peptide in TCP subjects was around two times higher than that in FCPD subjects. The fasting C-peptide/glucose ratio also showed a marked difference between the two groups (Khan and Ali, 1997) (Table 3). Compared to TCP, early FCPD had 1.40 times less and late FCPD had 2.48 times less fasting C-Peptide and early FCPD had 1.83 times and late FCPD had 3.85 times less postprandial C-peptide (Ali *et al*., 2001) (Table 5a).
Compared to TCP, early FCPD patients had about 1.83 times less and late FCPD patients had about 3.85 times less serum insulin level. Early FCPD had 2.24 times less and late FCPD had 3.38 times less post-prandial serum insulin (Table 5a).

Table 5a: Glycemic status, serum C-peptide and insulin values of the study subjects

Group	Serum glucose(mmol/L)		SerumC-peptide(nmol/L)		Serum Insulin(pmol/L)	
	0 min	120 min	0 min	120 min	0 min	120 min
TCP (n=14)	5.00± 0.78	5.42± 2.24	0.52± 0.15	1.50± 0.74	40.17± 14.56	258.80± 100.00
Early FCPD (n=10)	6.66± 3.09	16.66± 3.48	0.37± 0.14	0.82± 0.27	24.28± 8.66	115.59± 45.94
FCPD (n=7)	15.09± 3.28	23.11± 6.48	0.21± 0.07	0.39± 0.08	9.49± 3.11	76.52± 8.81

Results are expressed as mean ± SD. Signification of differences between the groups were compared by unpaired t-test.

Fasting and postrandial C-peptide/glucose ratio were 0.11 ± -.04, 0.06 ± 0.03, 0.01 ± 0.005 and 0.32 ± 0.20, 0.05 ± 0.02, 0.02 ± 0.007 in TCP, early FCPD and late FCPD. Fasting and postprandial insulin/glucose ratios were found as 8.07 ± 2.29, 4.27 ± 2.02, 0.67 ± 0.31 and 60.32 ± 45.61, 7.19 ± 3.17 and 3.62 ± 1.43 in TCP, early FCPD and late FCPD groups (Ali *et al*., 2001) (Table 5b).

Table 5b: C-peptide-glucose, insulin-glucose and C-peptide-insulin ratios of the study subjects.

Group	C-peptide glucose		Insulin/Glucose		C-peptide/Insulin	
	0 min	120 min	0 min	120 min	0 min	120 min
TCP (n=14)	0.11± 0.04	0.32± 0.02	8.07± 2.99	60.32± 45.61	0.01± 0.009	0.006± 0.005
Early FCPD (n=10)	0.06± 0.03	0.05± 0.02	4.27± 2.02	7.19± 3.17	0.02± 0.007	0.007± 0.002
FCPD (n=7)	0.01±0.005	0.02± 0.007	0.67± 0.31	3.62± 1.43	0.02± 0.014	0.005± 0.001

Results are expressed as mean ± SD. Signification of differences between the groups were compared by unpaired t-test.

Endocrine and exocrine pancreatic functions after Arginine stimulation test

Arginine stimulation test (Rossi *et al*., 2004) revealed plasma C-peptide levels were stimulated more than twofold from baseline to 30 min in healthy controls and in TCP compared to a reduced response in FCPD subjects. Basal plasma C-peptide levels clearly differed between TCP, FCPD and TCP groups.

As expected, FCPD showed a significant reduced β-cell response to arginine which was expressed as a diminished incremental response compared to controls. The same arginen infusion also led to a more than twofold increase of plasma glucagon from baseline to 30 min in controls. As expected, basal as well as arginine-stimulated values for glucagon were slightly higher in FCPD compared to TCP and control subjects. When incremental responses were considered, FCPD subjects showed preserved glucagon response to arginine stimulation, did not differ from healthy control subjects. Plasma levels of pancreatic polypeptide were higher in FCPD and controls compared to TCP. They were not altered significantly by arginine infusion in the control group and a similar pattern could be seen in FCPD and TCP as shown by the incremental responses.

2.2.6.3.6.7 Exocrine pancreatic function

Exocrine pancreatic function was studied in three studies. In one study exocrine function was assessed by measuring urinary p-aminobenzoic acid (PABA) after ingestion of NBT-PABA (Khan *et al*., 1991). The values of the 15 healthy controls were similar to those reported for healthy West European subjects. Patients in both groups had substantially lower mean values of urinary PABA excretion (with FCPD subjects having the lowest mean values); however, the wide scatter of values across the FCPD and TCP groups made any meaningful comparison difficult. In another study (Rossi *et al.*, 2004), secretine test was done to assess the exocrine function of TCP and FCPD. Secretine-stimulated bicarbonate output in duodenal juice increased rapidly and significantly higher in controls subjects than in TCP and FCPD subjects. When secretory data were expressed as mean bicarbonate output during the last 45 min of secretine stimulation corrected for body weight, the cut-off value of 70 μmol.15 min^{-1} kg^{-1} discriminated every patients with TCP or FCPD from healthy control subjects. No difference of exocrine pancreatic deficiency was thereby found between TCP and FCPD subjects.

In one study, severe exocrine pancreatic insufficiency were found in 85.72% of TCP, 90% of early FCPD and 100% of late FCPD subjects, as per results of estimation of fecal pancreatic elastase-1 in study subjects (Ali *et al*., 2001) (Table 6a).

Table 6a: Fecal pancreatic elastase-1 [Median (range)] in different groups

Group	**Fecal pancreatic elastase-1 (μg/ stool)**
TCP(n=14)	13.97(4.30 - 175.69)
Early FCPD(n=10)	3.69(1.58 – 109/81)
Late FCPD(n=7)	8.76(5.44 – 24.36)

Results are expressed as median (minimum-maximum). Signification of differences between the groups were compared by unpaired t-test.

2.2.6.3.6.8 ERCP findings

ERCP was performed in 19 TCP and eight FCPD cases (Rahman et al., 2000). Ductal changes in ERCP were scored using the criteria of Axon *et al*., 1984. The number (percentage) of subjects showing mild, moderate and severe changes of chronic pancreatitis were 1 (5.3%), 2 (10.5%) and 16 (84.2%), respectively, in the TCP group and 0, 0 and 8 (100%), respectively, in the FCPD group. No significant difference was found between the two groups regarding the degree of ductal change.

In another study, there were marked or severe change in ERCP in 100% of early FCPD and 100% of late FCPD cases, in TCP, 85.72% (12 out of 14) patients showed marked changes and 7.14% (1 out of 14) cases showed mild and moderate changes in ERCP. Mild to moderate pancreatic exocrine insufficiency were found in 14.28% of TCP, 10% of early FCPD and none in late FCPD (Ali *et al*., 2001) (Table 6b).

From ERCP findings and fecal pancreatic elastase values, it has been observed that there were seven pancreatic exocrine damage (marked changes of ERCP -85.72% cases) and seven pancreatic exocrine insufficiencies in about 85.72% cases in TCP patients (Table 6b). But the endocrine status still remained intact in TCP patients.

Both the study results suggest that FCPD should not be considered as a form of secondary diabetes and consequent to generalized pancreatic damage in TCP subjects only.

Table 6b: ERCP finding *vs* fecal pancreatic elastase-1 in different groups.

		TCP (n=14)		**Early FCPD (n=10)**				**Later CPD(n=7)**	
	Mild	**Moderate**	**Severe**	**Mild**	**Moderate**	**Severe**	**Mild**	**Moderate**	**Severe**
	No.(%)	No.(%)	No.(%)	(%)	No.(%)	No.(%)	(%)	No.(%)	No.(%)
ERCP	1	1	12	0	0	10	0	0	7
	(7.14)	(7.14)	(85.72)			(100.0)			(100.0)
	Mild-Moderate		Severe	Mild-Moderate		Severe	Mild-Moderate		Severe
	No.(%)		No.(%)	No.(%)		No.(%)	No.(%)		No.(%)
Fceal		3	12		1	9		0	7
Pancreatic		(14.28)	(85.72)		(10.0)	(90.0)			(100.0)
elastase									

Gradation of ERCP changes as per Axon *et al*., 1984. Arbitrary grading of fecal pancreatic elastase-I level as per Western studies.

a) Severe pancreatic exocrine insufficiency < 100ug/g of stool

b) Pancreatic exocrine insufficiency < 200ug/g of stool.

Correlation-coefficient of fecal pancreatic elastase-I with C-peptide levels in the fasting states, which reflects their insulin secretory capacity, showed that there is a significant positive correlation between fecal pancreatic elastase and fasting C-peptide only in TCP group (Table 6c). However, in the early FCPD and late FCPD groups, there is no significant positive correlation of fecal pancreatic elastase-I with C-peptide (Table 6c).

Table 6c: Coefficient-correlation of fecal pancreatic elastase-I value with glucose, C-peptide and insulin in the study groups.

		Serum glucose		**C-peptide**		**Serum insulin**	
Group	r/P	0 min	120 min	0 min	120 min	0 min	120 min
TCP	R	-0.4609	0.2205	0.6513	0.1916	-0.3258	-0.6341
(n=14)	P	0.097	0.449	0.012	0.512	0.256	0.015
Early	R	-0.0835	-0.2812	-0.3093	-0.0497	0.4963	0.2367
FCPD	P	0.819	0.431	0.385	0.892	0.145	0.510
(n=10)							
Late	R	-0.7844	0.0208	-0.4904	-0.1645	0.3019	-0.2684
FCPD	P	0.037	0.965	0.264	0.725	0.510	0.561

2.2.6.3.6.9 Ketosis resistance in FCPD subjects

A conspicuous clinical feature among the FCPD subjects is the absence of ketosis in spite of high serum glucose levels (fasting values usually > 16 mmol/L). The absence of ketosis in FCPD is probably due to a defect in the keton body synthesis pathway and/or in the regulation of the counterbalancing hormones (Khan and Ali, 1997)

2.2.6.3.6.10 Genetic aspects of TCP and FCPD

The idea that a certain genetic predisposition together with one or several environmental factors, including pancreatitis, is responsible for diabetes mellitus in FCPD patients, has led to initiate a series of studies on genetics both related to pancreatitis and diabetes.

Recently several studies have been carried out with Bangladeshi TCP and FCPD patients The most initial study (Rossi *et al*., 2001)) was carried out with 12 TP (TCP and FCPD) patients and 4 healthy controls. SPINK 1 mutation was found in 6 of 8 (75%) FCPD patients but none in 3 cases of TCP or 4 controls. This was just confirmed and extended to TCP in an article by Chandak *et al* in 2002. In another study in 2002, Hassan *et al*. used a family- based and case-control approach in two separate ethnic groups from the Indian subcontinent to determine whether N34S was associated with susceptibility to FCPD. Clear excess transmission of SPINK 1 N34S with FCPD in 69 Bangladeshi families was observed (P <.0001; 20 transmissions and 2 no transmissions). In the total study group (Bangladeshi and southern Indian) the N34S variant was present in 33% of 180 subjects with FCPD. 44% of 861 non diabetic subjects (odds ratio 10.8:P <.0001 compared with FCPD). 3.7% of 219 subjects with type 2 diabetes, and 10.6% of 354 subjects with early-onset diabetes (aged <30 years P=.02 compared with the ethnically matched control group). These results suggest that the N34S variant of SPINK1 is a susceptible gene for FCPD in the Indian subcontinent, although by itself, it is not sufficient to cause disease. Scheinder *et al*., in 2002 conducted another study where Bangladeshi patients with a variety of pancreas-associated diseases including TCP, FCPD and type 2 diabetes were included to determine the role of SPINK' mutations in this study, SPINK1 N34S mutations appeared in 1.3% of controls. 55% of FCPD. 20% of TCP and 14% of type 2 diabetic subjects which concludes that in Bangladesh, the SPINK1 N34S mutation increases the several forms of pancreatic diseases, including TCP, FCPD. and type2 diabetes mellitus.

In another study (Hassan, 2006) *SPINK1* N34S mutation in Bangladeshi controls have been found to be 5.7%. The frequency of the mutation in the

control subjects appears to be relatively higher compared to the observation of Schneider *et al..* 2002. It may be noted that the age of the two groups of controls are different at the time of recruitment: in the Hassan et al. study they are of younger age group (22.5±4.9. yrs) who may have undiagnosed sub-clinical disease, compared to relatively older (mean age, 28 yrs) cohort of only 76 controls which left a chance of underestimation of the frequency. In this study unrelated Bangladeshi FCPD patients showed 39% 5'HA/K7 gene variant 'G allele leading to N34S mutations compared to the controls ($p<0.0001$). *SPINK1 gene* N34S mutation has also been analyzed in idiopathic and alcoholic pancreatitis patients. Frequency of the mutation was found to be 9-20% in idiopathic pancreatitis. Among alcoholic pancreatitis patients the mutation was found in 6% cases (Threadgold *et al.,* 2002) whereas in different studies. frequency of positively for the variant genotype was 50-75% in FCPD patients. The high frequency of *SPINK1* gene N34S mutation in FCPD compared to idiopathic pancreatitis dearly suggests that mutant ‘G’ allele, possibly, confer an increased risk for development of pancreatitis in the tropics pancreatitis patients.

General Objective:

To explore the genetic and biochemical characteristics of TCP and FCPD Bangladeshi population.

Specific objectives:

1. To study the etiopathogenesis of TCP and FCPD subjects.
2. To investigate TCP and FCPD subjects for their exocrine and endocrine pancreatic functions and their interrelationships.
3. To investigate a group of Bangladeshi TCP and FCPD patients regarding their SPINK1 gene mutation.
4. To characterize the SPINK1 mutation positive and negative cases of TCP and FCPD subjects for the insulin secretary capacity, insulin sensitivity, exocrine and endocrine pancreatic functions and autoimmune status both for diabetes and pancreatitis.
5. To explore the role of IL 18 and CFTR gene mutations of theTCP and FCPD subjects and to investigate their relations with the environmental factors.

4. Subjects and Methods

4.1 Subjects

The study was performed at the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), a large WHO collaborating center for Research and Rehabilitation on Diabetes, Endocrine and Metabolic Disorders, Dhaka, Bangladesh, University of Basel, Switzerland and University of Pittsburg, USA between 2001 and 2008. The study protocol was approved by the local Ethics Committee. Type 2 Diabetes mellitus (T2DM) and Fibrocalculus pancreatic diabetes (FCPD) patients were prospectively selected from the outpatient department of BIRDEM. Tropical calcific pancreatitis (TCP) was chosen from the gastroenterology units at BIRDEM, Bangobandhu Sheik Mujib medical university (BSMMU) in Dhaka and from local gastroenterologists. All the patients were selected within an age band of 30-55 years.

4.1.1 Selection criteria

Both TCP and FCPD are not among the commonest diseases in Bangladesh and no epidemiological data are so far available for these disorders. So, there are practical difficulties to follow strict statistical procedure for sampling purposes in this study. Efforts have been taken, however, to include maximum number of patients in the planned study period. We were aiming to get patients based on an earlier experience and which made us think that it would be possible to collect about 100 TCP and 100 FCPD in the study period. Accordingly, 100 non-pancreatitis diabetic controls would be included. As

alcoholic pancreatitis, which would have been served as the controls for TCP, are uncommon in Bangladeshand as expected, had not been found during the study period.

The study had been started in April 2002 and during the period up to the first week of October 2008 the following numbers of patients had been collected and are grouped as:

Group 1: TCP – 34

Group 2: FCPD - 82

Group 3: T2DM - 48

4.2 Methods

4.2.1 Study design

After routine examinations (patient history, physical examination, oral glucose tolerance test, Glycosolyteted hemoglobin (HbA1c), as defined by WHO, blood screening, plain abdominal X-ray) patients with diabetes mellitus and pancreatic calcifications on abdominal X-ray were classified as FCPD and T2DM according to the 1985 WHO classification, which was valid at the time of this investigation. After providing oral informed consent, additional screening investigation was done (anthropometric measurements). The same screening procedures were used for TCP.

A modified set of Case Record Form was filled out for each patients and detailed history was taken. Subjects were given appointment for the tests in two sucsecive days.

Patients, who were under oral hypoglycemic agents or insulin were asked to refrain from taking the last evening dose and the morning dose prior to the experiments. As most of the patients in the OPD are of lower socioeconomic status, it was not possible to follow thw standard protocol of patient preparation set for the measurement of total stool fat like, having normal fat diet for consecutive 3 days and collection of stool (whole day fraction for the next consecutive 3 days). Insteed, subjects were asked to bring spot stool sample in a provided container for the purpose of measuring stool fat and fecal elastase 1.

Day 1:

Glycemic status was assessed by fasting glucose and HbA1c.From the fasting blood samples for lipid profile, serum urea, serum creatinine, SGPT, SGOT were taken to assess general clinical conditions. Sample for ICA, IAA, IA2, GAD antibody and CA-II antibody were preserved to assess the immunological status, both for diabetes and pancreatitis. Samples for DNA analysis were preserved. All samples were immediately frozen at -20^{o}c until further analysis.

Routine and microscopic examinations of the stool were done to exclude fat, parasites and blood. Samples for fecal elastase 1 were preserved at -20^{o}c until further analysis.

Day 2:

Pancreatic endocrine functions were measured by Arginine Stimulation Test (C-peptide, insulin, pancreatic and glucagon) [16].

Arginine stimulation test

Arginine stimulation test was performed in 12 hr over night fasting patient [16]. Cannulae were inserted into veins of both forearms, one for blood sampling and the other for arginine infusion.Blood samples were drawn in 15 min intervals during 90 minute time span (basal, arginine infusion and post infusion period each 30 min). Arginine-HCL (Arginen Hydrochloride 21% Braun©, Vifor, St. Gallen, Switzerland), 1.4 mmol/kg body weight diluted in 300 ml of sterile pyrozen-free water was infused during 30 min using an infusion pump (Diginfusa©, Arcomed AG, Regensdorf, Switzerland). Blood samples were drawn into pre cooled tubes containing EDTA (Monovette©, Sarstedt, Germany). Tubes were centrifuged at 4oc and 3000 rpm for 10 minutes. Plasma was then pipette into pre cooled eppendorf c tubes for glucose and hormone determinations. Samples for glucagon analysis contained the protease inhibitor aprotinin (Trasylol c, Bayer, Germany). All samples were immediately frozen at -20oc until further analysis.

4.2.2 Technique

Plasma glucose, total cholesterol. Triglyceride, HDL, creatinine, SGPT and SGOT were estimated in Autoanalyzer (AutoLab, Analyzer Medical System, Rome, Italy).

The plasma LDL-Cholesterol was calculated by using Friedewald formula [Friedwald 1972].

HbA1c was measured by HPLC (Bio-Rad).

Insuline sensitivity was assessed by HOMA Model [27] using the software HOMA-CIGMA (version 2.0).

Extraction of DNA was done and preserved at -20^{o}c for further analysis. All these analysis were done at BIRDEM.

Preserved plasma and stool samples were transported to Basel, Switzerland strictly following the criteria for preservation of the samples.

Plasma C-peptide, insulin and Pancreatic glucagon were measured by RIA.

ICA, IA2 and GAD antibody are also measured by RIA.

Fecal elastase I in stool was detected with a sandwich ELISA based on two monoclonal antibodies (ScheBo®. Tech GmbH)

Extracted DNA samples were transported to Pittsburgh, USA for screening of the pancreatitis related genes: SPINK1/PSTI, CFTR and IL-18.

4.3 Statistical Method

Statistical analysis was performed using Statistical Package for SocialScience (SPSS) for Windows Version 11.0 and $p<0.05$ was taken as the level of significance throughout. Unpaired‘t’ test. Proportion test, non-parametric tests (eg, Mann-Whitney test, Chi square test and Odds ratio) were applied where applicable.

5. RESULTS

5.1 Groups and gender distribution of the study subjects

Among the 165 subjects, 34 (21.8%) were TCP, 82 (49.7%) were FCPD and 48 (29.1%) were T2DM. Male and female distribution among the subjects were 92 (54.8%) and 74 (44%). In case of TCP 21 (61.8%) were male and 13 (38.2%) were female. In case of FCPD subjects, male and female distribution was 46 (56.1%) and 36 (43.9%) respectively. and in case of T2DM subjects the corresponding proportion was 24 (51%) and 23 (49%). There appeared to be male preponderance in all the groups (Table 7).

5.2 Age (years) of the study subjects

Mean (±SD) age of TCP, FCPD and T2DM were 28.5±8.8, 27.9±8.8 and 29.9±8.4 respectively. Mean (±SD) age of onset of diabetes in FCPD (62) is 33.59±13.30 and that in T2DM (37) is 29.46±7.17. Duration (Mean±SD) of diabetes in FCPD (65) and T2DM (37) are 5.32±.5.24 and 0.58±0.90 respectively which are statistically significant (p<.000) (Table 7).

5.3 BMI of the study subjects

BMI was calculated for the subjects with age 18 years and above. Mean (±SD) BMI of the TCP subjects (n=31) was 20.36±4.32, that of FCPD (n=80) 18.59±3.14 and that of T2DM (n=48) 23.82±4.65.Significantly higher BMI had been found in TCP vs FCPD (p=0.000) and FCPD vs T2DM (p=.000) subjects (Table 7).

Table 7: Clinico-biochemical variables of the study subjects

Variables	**TCP** (n=34)	**FCPD** (n=82)	**T2DM** (n=49)
Gender			
Male [N (%)]	21 (61.8)	46 (56.1)	24 (51)
Female [N (%)]	13 (38.2)	36 (43.9)	23 (49)
Age (yrs)	28.5±8.8	27.9±8.8	29.9±8.4
BMI (Kg/m^2)	20.36±4.32 [a]	18.59±3.14[a*]	23.82±4.65[*]

N= no of subjects. Results are expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus; BMI, body mass index

Unpaired Student's-'t' test is performed to calculate statistical differences.

*[a]Significantly different between TCP and FCPD (p=0.000); *SignificanIty different between FCPD and T2DM (p=0.000).*

5.4 Demographic distribution of the study subjects

Among the 34 TCP 61.8%, 11.8% and 17.6% individuals came from rural, semi-urban and urban areas respectively. In case of 82 FCPD subjects the proportions were 60.2%, 18.1% and 19.3%, and in the 48 T2DM 37.5%, 16.7% and 43.8% respectively (Table 3). High proportions of TCP and FCPD subjects were from the rural compared to more of the T2DM from urban area (X^2=10.842, p= 0.028) (Table 8).

Table 8: Demographic distribution of the study subjects

Area	**TCP** (N=34)	**FCPD** (N=82)	**T2DM** (N=49)
Rural (%)	61.8	60.2	37.5
Semi urban (%)	11.8	18.1	16.7
Urban (%)	17.6	19.3	43.8
	X^2= 10.842, p= 0.028		

N= no of subjects. Results are expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus.
Chi square test is done to show statistical difference.

5.5 Anthropometric measurements of the study subjects

Mean (±SD) of mid-arm circumference (MAC, cm) in the TCP (n=25) is 22.90±2.29, that in FCPD (n=72) is 20.81±3.62 and that in T2DM (n=44) is (25.04±3.41). No significant difference has been found among the subjects regarding MAC (Table 10). Mean (±SD) biceps skin-fold thickness (BSF, mm) is 6.11±2.7 in TCP (n=28), 6.03±2.15 in the FCPD (n=73) and 10.11±5.11in T2DM (n=40). BSF values between FCPD and T2DM shows statistical significant difference (p=.000) (Table 10). Mean (±SD) of waist hip ratio (WHR) is 1.10±.161 in TCP (n=28), 1.20±0.11 in FCPD (n=73) and 1.20±0.06 in T2DM (n=41) subjects. Significant difference (p=.945) had been found in WHR value between FCPD and T2DM subjects (Table 9).

Table 9: Anthopometric measurement of the study subjects

Variables	**TCP (N)**	**FCPD (N)**	T2DM (N)
MAC (cm)	20.90±2.29 (25)	20.81±3.62 (72)	25.05±3.41 (44)
BSF (mm)	6.11±2.7 (28)	6.03±2.51* (73)	10.11±2.71* (40)
WHR	1.10±0.161 (28)	0.85±0.07* (73)	0.84±0.05* (40)

N=number of subjects. Results are expressed as mean±SD;
Statistical difference calculated by unpaired student's 't' test;
**Significantly different between FCPD and T2DM*
MAC, mid arm circumference in centimeter; BSF, biceps skin fold thickness in millimeter; WHR, waist hip ratio; cm, centimeter.

5.7 Blood glucose level of the study subjects

In TCP subjects fasting blood glucose (mmol/l, mean±SD) level is 5.36±0.75 as expected. FCPD subjects show higher fasting blood glucose level 10.56±5.64 than the T2DM 9.91±4.24 which is statistically insignificant (p=0.374) (Table 11). After 2 hours after breakfast blood glucose level in TCP was 6.36±1.63 as expected also. FCPD subjects show higher blood glucose level (17.65±7.03) than that of T2DM (15.32±6.41) which is also statistically insignificant (p= 0.05) (Table 11). HBA1c (%) in TCP is 5.42±0.350 which is also expected. HBA1c (%) FCPD and T2DM are 9.20±2.94 and 9.58±3.61 respectively showing no significant difference (p= 0.639) (Table 11). Age of inset of diabetes (yr) in FCPD and T2DM were 32.59±13.30 and 29.46±7.71 showing significant difference statistically (p=0.142) (Table 12). Duration of diabetes (yr) was 5.32±5.24 in FCPD and 0.575±0.903 in T2DM which was also statistically significant (p=0.000) (Table 11).

Table 11: Glucose level (mmol/l), age of onset and duration of diabetes in the study groups of the study subjects

Parameters	**TCP** (N=34)	**FCPD** (N=82)	**T2DM** (N=49)
FG (mmol/l)	5.36±0.75	10.56±5.64	9.91±4.24
2AB (mmol/l)	6.36±1.63	17.65±7.03	15.32±6.41
HBA1c (%)	5.42±0.350	9.20±2.94	9.58±3.61
Age of inset of diabetes (yr)		32.59±13.30[a]	29.46±7.71[a]
Duration of diabetes (yr)		5.32±5.24*	.575±0.903*

N= no of subjects.Results were expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus; FG, fasting glucose; 2AB, blood glucose 2 hour after breakfast; Age of onset of diabetes includes FCPD n=62 and T2DM n=37; Duration of diabetes includes FCPD n=65 and T2DM n=37.

Unpaired Student's-'t' test was performed to calculate statistical differences.

*[a]significantly different in age of onset of diabetes between FCPD and T2DM (p=.0.009);;*Significanly different in duration of diabetes between FCPD and T2DM (p=0.000).*

5.8 Lipid profiles (Mean±SD) of the study subjects

Plasma total Cholesterol level (mg/dl) in TCP, FCPD and T2DM subjects were 166±56, 165±37 and 180±51 respectively. Significant difference had been found in TCP vs FCPD (t/p value=-0.004/0.997). Triglyceride level (mg/dl) in TCP, FCPD and T2DM were 161±110, 150.71±100.10 and 189±92 respectively showing no significant differences within the groups. High density lipoprotein cholesterol (HDL-c) level (mg/dl) in TCP was 33.7±10.31, in FCPD 38.3±11.61 and in T2DM 37.1±9.6 showing no significant differences within the groups. In TCP, FCPD and T2DM subjects low density lipoprotein (LDL-c) level (mg/dl)

were 104±45, 90±29 and 112±38 respectively which shows significant difference between TCP and FCPD subjects, t/p value=1.588/0.120 (Table 12).

Table 12: Lipid profile of the study subjects

Parameters	**TCP** (n=34)	**FCPD** (n=82)	**T2DM** (n=49)
Total Cholesterol (mg/dl)	166±56*	165±37*	180±51
Triglyceride (mg/dl)	161±110	150.71±100.10	189±92
HDL-c (mg/dl)	33.7±10.31	38.3±11.61	37.1±9.6
LDL-c (mg/dl)	104±45*	90±29*	112±38

Results are expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus; LDL-c low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol.

*Unpaired Student's-'t' test was performed to calculate statistical differences. *Significanly different in levels of total cholesterol (p= 0.997) and LDL-c (p=0.120) between TCP and FCPD.*

5.9 Plasma creatinine and ALT status in the study subjects

Serum level (mg/dl) of creatinine in TCP, FCPD and T2DM are 0.971±0.208, 1.319±1.841 and 0.806±0.183 respectively. Regarding s creatinine level TCP shows significant difference with T2DM (p= 0.001). FCPD and T2DM subjects also show significant difference among the groups (p=.018). The results clearly show the TCP having normal S creatinine while FCPD and T2DM having higher s creatinine levels than normal range. FCPD shows higher value than T2DM subjects. Serum ALT level (U/l) in TCP, FCPD and T2DM are 26.10±9.91, 30.96±19.61 and 36.02±23.81 respectively. Regarding ALT levels TCP differs significantly with T2DM (p=.019) (Table 13).

Table 13: Plasma creatinine and ALT status in the study subjects

Parameters	TCP (n=30)	FCPD (n=77)	T2DM (n=42)
S creatinine (mg/dl)	0.971±0.208*	1.319±1.841[a]	0.806±0.183*[a]
ALT (U/l)	26.10±9.91*	30.96±19.61	36.02±23.81*

Results are expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus; s creatitine, serum creatinine.

*Unpaired Student's-'t' test was performed to calculate statistical differences. *Significanly different in s creatinine value (p=0.001) and ALT value (p=0.019) between TCP and T2DM; [a]significantly different in s creatinine value between FCPD and T2DM subjects.*

5.10 Antibody status of the study subjects

GAD antibody and IA-2 antibody are absent in all the three groups. ICA anibody has been detected in all the groups (X^2=0.150, p=0.928). 2 out of 32 TCP subjects showed presence of ICA antibody which is 6.3%. In 76 FCPD subjects 5 (6.6%) showed presence of ICA antibody. 4 (8.2%) out of 49 T2DM subjects came out with presence of ICA antibody (Table 14).

Table 14: Antibody status of the study subjects

Group	ICA Antibody		GAD Antibody		IA-2 Antibody	
	Presence, n (%)	Absence n (%)	Presence n (%)	Absence n (%)	Presence, n (%)	Absence n (%)
TCP	2 (6.3)	30 (93.8)	0 (0%)	32 (100%)	0 (0%)	32 (100%)
FCPD	5 (6.6)	71 (93.4)	0 (0%)	77 (100%)	0 (0%)	77 (100%)
T2DM	4 (8.2)	45 (91.8)	0 (100%)	49 (100%)	0 (100%)	49 (100%)
X^2=0.150, p=0.928						

Results are expressed as number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus; ICA, islet cell antibody; GAD, glutamic acid anhydrase decarboxylate; IA-2,insulin antigen.
Chi square test is done to show statistical difference.

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5.11 Exocrine function of the study subjects as measured by Fecal pancreatice elastase) 1(μg/g stool)

Exocrine function of the study subjects is evaluated by measuring Fecal elastase 1 ((μg/g stool) in the stool. All the groups show severe pancreatic exocrine insufficiency according to the arbitrary grading of fecal elastase 1 level as per western studies, where <100 (μg/g stool) indicates severe exocrine insufficiency. The result sows significant difference redarding fecal ealastase 1 between FCPD and T2DM groups (p= .004) explaining more exocrine insufficiency in FCPD than T2DM (Table 15).

Table 15: Exocrine function of the study subjects

Parameter	TCP (N=20)	FCPD (N=41)	T2DM (N=18)
Fecal elastage 1 ((μg/g stool)	1.35±0.75	1.07±0.36*	1.78±0.88*

Results are expressed as mean±SD. N, number of subjects; TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus. Unpaired Student's-'t' test was performed to calculate statistical differences. Superscript in the column indicated statistical significant difference between two groups

5.12 Pancreatic endocrine functions as evaluated by performing Arginine stimulation test in the study subjects

5.12.1: Glucose (mmol/l) level at different time points of Arginine infusion test of the study subjects

Arginine infusion has not altered plasma glucose levels in any of the groups (Table 16).

Table 16: Glucose (mmol/l) level at different time points of Arginine infusion test of the study subjects

Time points	**TCP** (n=34)	**FCPD** (n=82)	**T2DM** (n=49)
-15 min	5.97±2.75	9.51±5.45	8.88±4.02
0 min	5.91±2.71	9.31±5.41	9.05±4.08
15 min	6.22±2.61	9.61±5.22	9.56±4.17
30 min	6.24±2.53	9.74±5.21	9.88±4.19
45 min	5.84±2.61	9.62±5.27	9.52±3.93
60 min	5.71±2.61	9.35±5.32	9.29±3.95

Results were expressed as mean±SD. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus; min, minute. Unpaired Student's-'t' test was performed to calculate statistical differences.

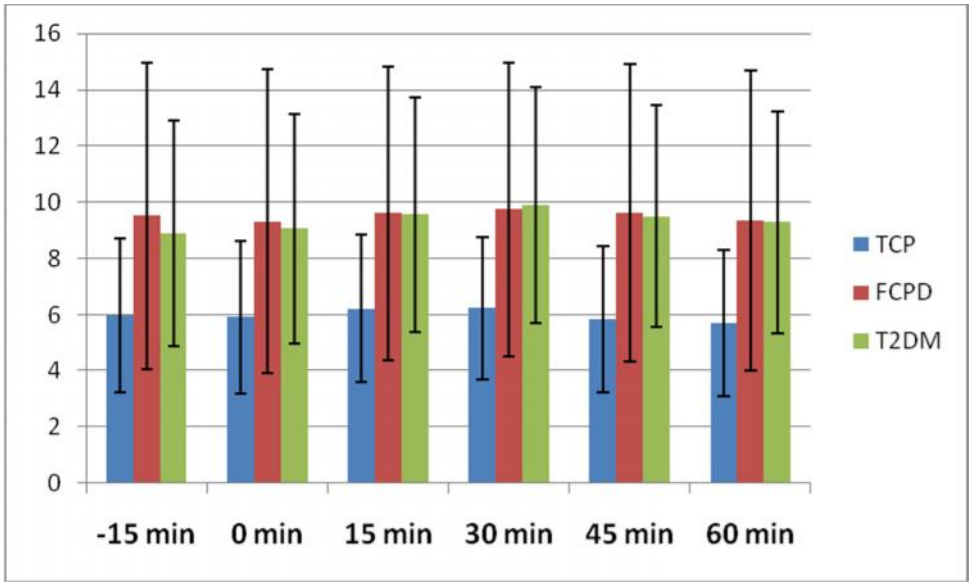

Figure 4: Glucose (mmol/l) level at different time points of Arginine infusion test of the study subjects

Results were expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus; min, minute.
Unpaired Student's-'t' test was performed to calculate statistical differences.

5.12.2: C-peptide (nmol/ml) level at different time points of Arginine infusion test of the study subjects (Table 14)

Arginine stimulation test revealed almost 2 fold increase in serum C-peptide level (nmol/ml) in TCP from base line to 30 min (0.264±0.206 and 0.455±0.389 respectively) and FCPD subjects showed near 1.5 fold increase of the same (0.255±0.208 and 0.360±0.405 respectively) compared to that of T2DM which remained un changed (1.045±0.824 and 1.026±0.734 respectively). In TCP and FCPD, basal serum level of C-peptide did not differ significantly while that of T2DM showed almost 5 fold higher levels (Table 16).

Table 17: C-peptide (nmol/ml) level at different time points of Arginine infusion test of the study subjects

Time points	**TCP** (n=34)	**FCPD** (n=82)	**T2DM** (n=49)
-15 min	0.264±0.206	0.255±0.208	1.045±0.824
0 min	0.335±0.253	0.248±0.215	1.015±0.628
15 min	0.403±0.387	0.249±0.218	0.866±0.761
30 min	0.455±0.389	0.360±0.405	1.026±0.734
45 min	0.388±0.332	0.333±0.309	1.202±0.831
60 min	0.301±0.252	0.268±0.230	0.705±0.490

Results were expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus; min, minute. Unpaired Student's-'t' test was performed to calculate statistical differences. Different superscript in the column indicated statistical significant difference between two groups.

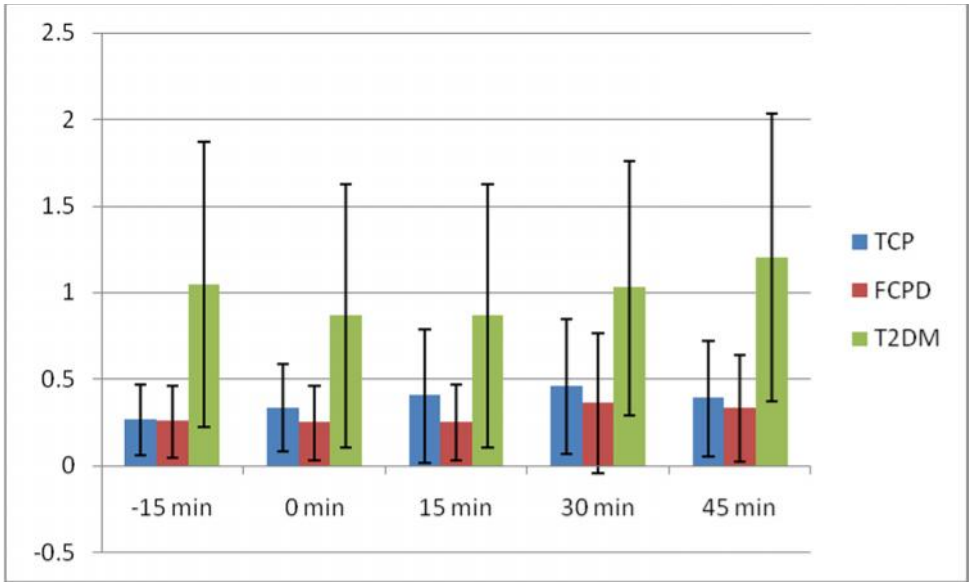

Fig 5: C-peptide (nmol/ml) level at different time points of Arginine infusion test of the study subjects

5.11.3: Glucagon (pg/ml) level at different time points of Arginine infusion test of the study subjects

The basal value of serum glucagon (pg/ml) revealed same in TCP, FCPD and T2DM during arginine stimulation test (41.84±14.66, 48.86±20.33 and 52.36±34.04 respectively) After 30 minutes of arginine infusion, serum glucagon level showed almost 2 fold increase in both TCP and FCPD with out any significant difference in between (86.09±42.71 and 88.76±48.06 respectively) while T2DM showed almost 3 fold increase of serum glucagon level after 30 minutes of arginin infusion (151.90±62.04) (Table 17).

Table 18: Glucagon (pg/ml) level at different time points of Arginine infusion test of the study subjects

Time pints	**TCP** (n=34)	**FCPD** (n=82)	**T2DM** (n=49)
-15 min	41.84±14.66	48.86±20.33	52.36±34.04
0 min	51.91±18.2	54.50±26.87	69.34±31.44
15 min	91.15±65.20	82.34±48.68	133.56±48.70
30 min	86.09±42.71	88.76±48.06	151.90±62.04
45 min	64.61±34.02	60.99±28.21	76.16±34.02
60 min	46.04±16.02	52.82±28.74	85.31±50.02

Results were expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus. Unpaired Student's-'t' test was performed to calculate statistical differences.

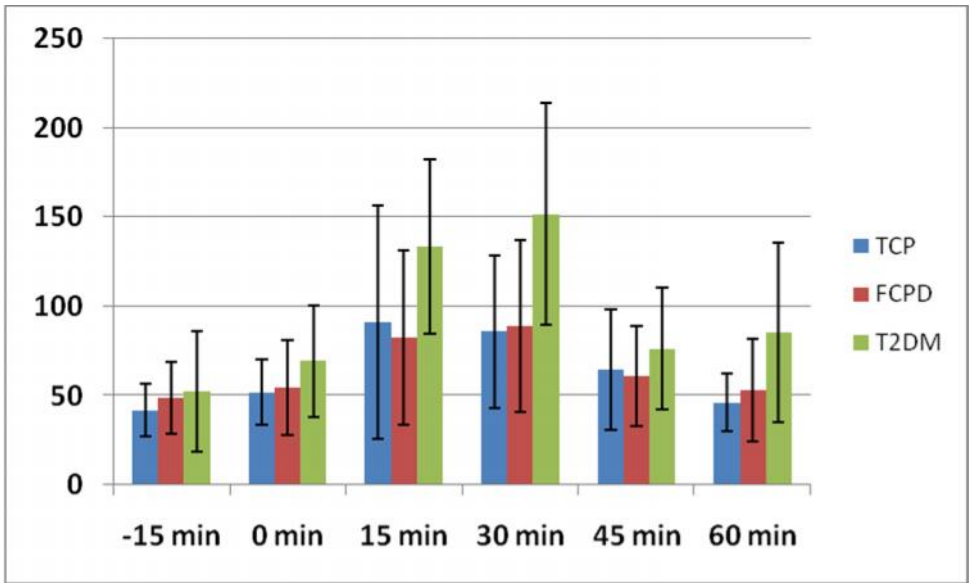

Fig 6: Glucagon (pg/ml) level at different time points of Arginine infusion test of the study subjects

Table 19: Incremental response of glucose, cpeptide and glucagon of study subjects in Arginine test

	TCP	FCPD	T2DM
Glucose (mmol/l)	.573±.944	.533±1.257	1.50±2.61
C-peptide (nmol/ml)	.172±.336	.0737±.195	-.0170±.379
Glucagon (pg/ml)	.172±.336	.0737±.195	-.0710±.379

Results were expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus. Unpaired Student's-'t' test was performed to calculate statistical differences.

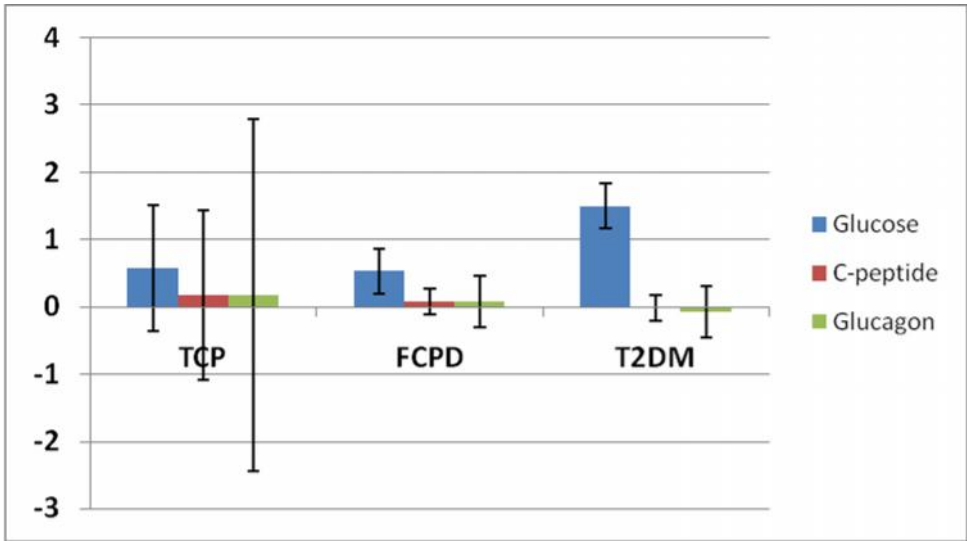

Fig 7: Incremental response of glucose, cpeptide and glucagon of study subjects in Arginine test

5.10: Genetic analysis of the study subjects

5.10.1: SPINK1 geno typing

Genotype frequencies of the SPINK gene (N34S genotype) were 0.583, 0.688 and 0.879 for homozygous wild type in TCP, FCPD and T2DM subjects respectively, 0.375, 0.266 and 0.121 for heterozygous variant and 0.042, 0.046 and 0 for homozygous variants among the groups respectively. The genotype frequency distribution in TCP, FCPD and T2DM subjects have not shown statistically significant in χ^2 test. When the hetero and homozygous variants have been considered together the frequency distribution of variants were 0.417, 0.312 and 0.121 in TCP, FCPD and T2DM respectively and the distribution have been shown statistically significant in χ^2 test (=6.67; p=0.036) (Table 16).

Frequencies of the SPINK N34S allele were 0.770 and 0.230 for A and G alleles in TCP group, 0.821 and 0.179 in FCPD group, and 0.9395 and 0.0605 in T2DM group respectively.

Table 20: Distribution of SPINK1 Genotype among the study subjects

Genotype	TCP [% (n)]	FCPD [% (n)]	T2DM [% (n)]
Wild Type	0.583 (14)	0.688 (44)	0.879 (29)
Variant, Hetero	0.375 (9)	0.266 (17)	0.121 (4)
Variant, Homo	0.042 (1)	0.046 (3)	0
	X^2=7.17, p=0.127		
Wild Type	0.583 (14)	0.688 (44)	0.879 (29)
Variant	0.417 (10)	0.312 (20)	0.121 (4)
	X^2=6.67, p=0.036		

Allele frequency	TCP [% (n)]	FCPD [% (n)]	T2DM [% (n)]
A	0.770	0.821	0.9395
G	0.230	0.179	0.0605

Results were expressed as mean±SD and number (percentage) as appropriate.

TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus

Unpaired Student's-'t' test was performed to calculate statistical differences. Odds ratio (OR), confidence interval (CI) and corresponding p values are calculated by Fisher's Exact Test as appropriate. Binominal tests of proportions are used to compare the allele frequencies in different groups. .

5.10.2: CFTR geno typing

Genotype frequencies of the CFTR Ex22 gene 1.0, 0.983 and 1.0 for homozygous wild type in TCP, FCPD and T2DM subjects respectively, 0, 0.017 and 0 for heterozygous variant and 0, 0 and 0 for homozygous variants among

the groups respectively. The genotype frequency distribution in TCP, FCPD and T2DM subjects have not shown statistically significant (Table 17).

Frequencies of the CFTR (G ➔T) allele were 1.0 and 0 for G and T alleles in TCP group, 0.991 and 0.009 in FCPD group, and 1.0 and 0 in T2DM group respectively.

Table 21: Distribution of CFTR ex22 Genotype among TCP, FCPD and T2DM patients in Bangladeshi population

Genotype	**TCP [% (n)]**	**FCPD [% (n)]**	**T2DM [% (n)]**
Wild Type	1.00 (24)	0.983 (58)	1.00 (31)
Variant, Hetero	0(0)	0.017 (1)	0 (0)
Variant, Homo	0	0	0
	Fisher's Exact value, X^2=0.94, p=0.62		

Allele frequency	**TCP [% (n)]**	**FCPD [% (n)]**	**T2DM [% (n)]**
G	1.00	0.991	1.00
T	0	0.009	0

Results were expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus

Unpaired Student's-'t' test was performed to calculate statistical differences. Odds ratio (OR), confidence interval (CI) and corresponding p values are calculated by Fisher's Exact Test as appropriate. Binominal tests of proportions are used to compare the allele frequencies in different groups.

5.10.3: IL-18 geno typing of the study subjects

Genotype frequencies of the IL-18 -607 gene are 0.083, 0.156 and 0.031 for homozygous wild type in TCP, FCPD and T2DM subjects respectively, 0.500,

0.391 and 0.438 for heterozygous variant and 0.417, 0.453 and 0.531 for homozygous variants among the groups respectively. The genotype frequency distribution in TCP, FCPD and T2DM subjects have not shown statistically significant. When the hetero and homozygous variants have been considered together the frequency distribution of variants were 0.917, 0.844 and 0.969 in TCP, FCPD and T2DM respectively and the distribution have not shown statistically significant (Table 19a).

Frequencies of the IL-18 (A → C) allele were 0.333 and 0.667 for A and C alleles in TCP group, 0.351 and 0.649 in FCPD group, and 0.250 and 0.750 in T2DM group respectively.

The IL-18 -607CC genotype is more frequent in patients with TCP (15) and FCPD (22) compared to T2DM (43) (Armitage trend test $p<0.005$, OR 3.15) while the AC and AA genotypes are less frequent in patients with TCP and FCPD (Table 19b). A significant increase in the non-functional polymorphism at position -656 in patients with PCP and FCPD, a polymorphism wich is in complete linkage with the polymorphism at position -607.

A significant negative correlation is detected between IL-18 -607 CC genotype and the SPINK1 (N34S) haplotype in the subgroup of patients with FCPD (12/22 N34S positive ; r=.49 ; p=0.02). 15 TCP and 22 PCPD have either the IL-18 CC genotype and/or the SPINK1 n34S haplotype (Table 19c).

Table 22a: Distribution of IL-18 -607 Genotypes among TCP, FCPD and T2DM patients in Bangladeshi population

Genotype	TCP [% (n)]	FCPD [% (n)]	T2DM [% (n)]
Wild Type	0.083 (1)	0.156 (7)	0.031 (1)
Variant, Hetero	0.500 (13)	0.391 (4)	0.438 (14)
Variant, Homo	0.417 (12)	0.453 (80)	0.531 (17)
	X^2=4.197, p=0.380		
Wild Type	0.083 (1)	0.156 (7)	0.031 (1)
Variant	0.917 (22)	0.844 (54)	0.969 (31)
	X^2=3.64, p=0.162		

Allele frequency	TCP [% (n)]	FCPD [% (n)]	T2DM [% (n)]
A	0.330	0.351	0.250
C	0.667	0.649	0.750

Results were expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus

Unpaired Student's-'t' test was performed to calculate statistical differences. Odds ratio (OR), confidence interval (CI) and corresponding p values are calculated by Armitge trend test and Fisher's Exact Test as appropriate. Binominal tests of proportions are used to compare the allele frequencies in different groups.

Table 22b: Distribution of IL-18 -607 Genotypes among TCP, FCPD and T2DM patients in Bangladeshi population

Group (no)	A/A	A/C	C/C
TCP (15)	1 (7%)	2(13%)	12(80%)
FCPD (22)	1 (5%)	4 (18%)	17 (77%)
T2DM (43)	4 (9%)	17 (40%)	22 (51%)

Results were expressed as number (percentage) as appropriate.
TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus
Unpaired Student's-'t' test was performed to calculate statistical differences. Odds ratio (OR), confidence interval (CI) and corresponding p values are calculated by Armitge trend test and Fisher's Exact Test as appropriate. Binominal tests of proportions are used to compare the allele frequencies in different groups. Spearman rank analysis is used for comparisions between genes.

Table 22c : IL-18 -607 A/C genotypes and SPINK1 N34S status in TCP and FCPD subjects

Group	IL-18 -607 SPINK1 N34S	A/A Neg	 Pos	A/C Neg	 Pos	C/C Neg	 Pos
TCP	15	0	1	0	2	10	2
FCPD	22	0	1	0	4	8	9

Results were expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus
Unpaired Student's-'t' test was performed to calculate statistical differences. Odds ratio (OR), confidence interval (CI) and corresponding p values are calculated by Armitge trend test and Fisher's Exact Test as appropriate. Binominal tests of proportions are used to compare the allele frequencies in different groups. .

5.10.4 : Biochemical characteristics of the study subjects according to SPINK genotype

When the data were reanalyzed on the basis of SPINK1 Genotype, it has been found that glycemic and lipidemic status in variant genotype were not significantly different compared to the wild genotype. Fecal elastase 1 concentration in the variant genotype was significantly lower (p=0.006) compared to the wild type genotype.

Table 23: Biochemical characteristics of the study subjects according to SPINK genotype

Parameters	SPINK		*t/p-value*
	Wild type	**Variant**	**Wild vs Variant**
FG	9.3±5.2	9.9±5.5	-0.599/0.55
AG	14.5±6.9	16.4±7.9	-1.266/0.208
HbA1c	8.7±3.2	8.4±3.1	0.490/0.625
TG	171±105	161±108	0.474/0.636
Chol	173±45	158±33	1.686/0.095
HDL	36±10	39±13	-1.078/0.284
LDL	104±37	88±29	1.997/0.033
Creat	1.01±0.34	0.94±0.23	1.017/0.312
SGPT	32±19	29±14	0.767/0.445
HOMA B	59±85	58±75	0.11/0.991
HOMA S	530±995	220±286	0.967/0.339
Elastase	62±110	11±25	2.890/0.006

Results were expressed as mean±SD and number (percentage) as appropriate. TCP, tropical calcific pancreatitis; FCPD, fibrocalculus pancreatic diabetes; T2DM, type 2 diabetes mellitus
Unpaired Student's-'t' test was performed to calculate statistical differences.

5.10.5 Increment of plasma C-peptide, glucagon and glucose in TCP, FCPD and DM subjects according to SPINK genotype

SPINK 1 N34S positive FCPD subjects show significantly higher C-peptide incremental value than the wild type (p=0.022). Incremetal value of glucagon do not differ in both wild and mutant subtype of SPINK1 in TCP and FCPD subjects.

Table 24: Increment of plasma C-peptide, glucagon and glucose in TCP, CPD and DM subjects according to SPINK genotype

		SPINK genotype		
Variables	**Groups**	**Wild type**	**Variants**	**t /p value**
Increment of C-peptide	**TCP**	0.50±0.75	0.03±0.22	2.07/0.058
	FCPD	0.167±0.343	-0.069±0.204	2.38/0.022
	DM	-0.086±0.575	-0.315±0.381	0.761±0.451
Increment of glucagon	**TCP**	0.364±0.854	0.322±0.653	0.126/0.901
	FCPD	0.539±1.219	0.610±1.114	-0.219/0.827
	DM	1.508±2.259	2.047±2.710	-0.437/0.665
Increment of glucose	**TCP**	0.364±0.854	0.322±0.653	0.126/0.901
	FCPD	0.539±1.219	0.610±1.114	-0.219/0.827
	DM	1.508±2.259	2.047±2.710	-0.437/0.665

5.10.6 : Distribution of ICA antibody among the study subjects according to SPINK1 genotype

ICA antibody is found in 1 SPINK 1 positive TCP subjects. ICA antibody is found in 1 SPINK 1 positive FCPD subjects also. T2DM SPINK 1 variants show no presence of ICA antibody.

Table 25: Distribution of ICA antibody according to SPINK1 genotype

Group	Genotype		ICA Antibody	
			Presence, n(%)	**Absence,** n (%)
TCP	**SPINK Genotype**	**Wild type**	1 (50)	12 (57.1)
		Variants	1 (50)	9 (42.9)
FCPD	**SPINK Genotype**	**Wild type**	2 (66.7)	38 (67.9)
		Variants	1 (33.3)	18 (32.1)
T2DM	**SPINK Genotype**	**Wild type**	2 (100)	27 (87.1)
		Variants	0 (0)	4 (12.9)

5. Discussion

The results indicate that

5.1 Sociodemographic status

5.1.1 Sex

There is male preponderance in all the groups. Male preponderance in TCP (Male 61.8% and female 38.2%) and FCPD tally with the other studies (Lin et al., 2000; Khan and Ali, 1997; Rahman *et al*., 2000; Zahid *et al.*, 2000; Rossi *et al.*, 2003) done in Bangladesh and abroad. The smaller proportion of females do not reflect the true prevalence of the disease that is approximately equally distributed between both genders, but rather can probably be explained by the greater reluctance of females to participate in such a study.

5.1.2 Age

There is no significant difference in age of the study subjects which has been also shown in other studies (Lin et al., 2000; Khan and Ali, 1997; Rahman *et al*., 2000; Zahid *et al.*, 2000; Rossi *et al.*, 2003). Although Mohan *et al.,* 2003 defined TCP as a juvenile form of chronic pancreatitis, onset of TCP and FCPD in infancy (Premalatha and Mohan, 1990), childhood (Mohan *et al*., 1990) and elderly is not uncommon (Mohan *et al.*, 1999).

5.1.3 BMI

FCPD subjects showed low BMI compared to TCP subjects though not statistically significant which also have been found in other studies (Khan and

Ali 1997, Zahid *et al.*, 2000, Schneider *et al.*, 2002, Rossi *et al.*, 2003). Rahman *et al.*, 2000 showed FCPD with a higher BMI than TCP which is not statistically significant.

5.1.4 Sociodemographic status

Both TCP and FCPD subjects are from lower socioeconomic strata of the society which was also observed by Zuidema in 1959, although Khan and Ali 1997 explained that according to the socioeconomic index followed in Bangladesh FCPD subjects belonged to the poorest class, whereas the TCP subjects belonged to the middle class income group.

5.1.5 Anthropometry

The study groups do not differ in respect of anthropometric parameters MAC, BSF and WHR as also shown by Rossi *et al.*, 2004.

5.2 Routine microscopic examination of stool

5.2 Glycemic status

Obviously, TCP subjects differed clearly from FCPD subjects with regard to Glycemic control, as observed by significant elevated fasting plasma glucose and HbA1c. Fasting plasma glucose in FCPD subjects is more than 2 times higher than that of TCP subjects. Study carried out by Khan and Ali 1997 and Rossi *et al.*, 2004 showed 4 times higher plasma glucose in FCPD than TCP subjects. The different higher level of Plasma glucose might reflect the difference in study population, age of onset and detection of diabetes. HbA1c

also showed more than 2 times higher value in FCPD subjects than TCP subjects.

5.3 Lipid profile of the study subjects

Significant difference in total cholesterol and LDL-c has been found in TCP vs FCPD (t/p value=-.004/.997 and 1.588/.120). Triglyceride and HDL-c level shows no significant differences within the groups. These results tally with the results observed by Khan and Ali in 1997.

5.5 S creatinine levels in the study subjects.

Serum creatinine level in FCPD is higher than TCP and T2DM subjects. Same result had been shown by Khan and Ali in 1997. Although the levels are within normal limit, higher value in FCPD may indicate early nephropathic changes in FCPD.

5.6 Antibody status of the study subjects

This study reports for the first time the presence of ICA antibody in TCP. 2 out of 32 TCP subjects showed presence of ICA antibody which is 6.3%. In 76 FCPD subjects 5 (6.6%) showed presence of ICA antibody. 4 (8.2%) out of 49 T2DM subjects came with presence of ICA antibody. GAD antibody and IA-2 antibody are absent in TCP, as expected and also in FCPD. Mohan *et al.*, 1998a reported presence of GAD 7% of FCPD and ICA in 4,3% of FCPD and GAD antibody and ICA in 5.6% and 53.8% of T2DM. Dabadghao *et al.*, *in* 1996 reported absences of ICA in both FCPD and T2DM. Sanjeevi *et al. in* 1999 found GAD antibody in 7% of FCPD. Singh *et al.*, *in* 2000 reported absence of

ICA and IA-2 antibody in both FCPD and T2DM. Goswami *et al.*, in 2001 reported presence of GAD antibody in 7.5% of FCPD.

5.7 Pancreatic exocrine function of the study subjects

All the 3 groups show severe exocrine insufficiency as assessed by measuring fecal elestase 1. Keller *et al.*, in1984 carried out a study where pancreatic exocrine function was assessed by using determination of urinary excretion of p-aminobenzoic acid after ingestion of n-benzoyl-l-tyrosyl-p-aminobenzoic acid (NBT-PABA test). The study revealed impaired exocrine function both in TCP and FCPD. Rossi *et al.*, in 2004 examined the pancreatic exocrine function by Secretin stimulation test where both TCP and FCPD subjects showed impaired function as well.

5.8 Pancreatic endocrine function of the study subjects as assessed by arginine stimulation test

5.8.1 Pancreatic $\mathcal{B}$ cell function

Arginine infusion has not altered plasma glucose levels in any of the groups. This result perfectly tally with the study carried out by Keller *et al.*, in 1984 and Rossi *et al*., in 2004. In TCP and FCPD, basal serum level of C-peptide did not differ significantly. Arginine stimulation test revealed almost 2 fold increases in

serum C-peptide level in TCP from base line to 30 min and FCPD subjects showed near 1.5 fold increase of the same. Almost similar result had been shown in the studies of Keller *et al.*, in 1984 and Rossi *et al.*, in 2004 although both the study showed decreased basal c-peptide level in FCPD than that of TCP subjects.

5.8.2 Pancreatic α cell function

The basal value of serum glucagon reveal the same in both the TCP, FCPD subjects. After 30 minutes of arginine infusion, serum glucagon level showed almost 2 fold increase in both TCP and FCPD without any significant difference in between. This result shows that pancreatic α cell function is preserved in both TCP and FCPD subjects. Similar results had been shown in the study of Rossi *et al.*, in 2004 where there were slightly higher levels of basal as well as arginine stimulated values of plasma glucagon in FCPD subjects compared to that of TCP subjects although insignificant, also suggesting preserved α cell function. Keller *et al.*, in 1984 showed lower value of basal glucagon in FCPD than that of TCP subjects though insignificant. In contrast, pancreatic glucagon level failed to increase during arginine stimulation in FCPD subjects than that of TCP subjects showing impaired α cell functions in FCPD.

5.9 Genetic analysis of the study subjects

5.9.1 SPINK1 genotyping

SPINK1 genotyping has been carried out in 24 TCP, 64 FCPD and 33 T2DM subjects. Among the TCP subjects, 14 are of wild homozygous N34S genotype,

9 are of heterozygous N34S mutation and 1 is of homozygous N34S mutation. Among the FCPD subjects, 44 wild homozygous N34S genotype, 17 heterozygous N34S mutant and 3 homozygous N34S mutant have been detected. In T2DM, 29 are of wild type and 4 are of heterozygous mutant. Thus, SPINK 1 N34S mutation has been found in 42%% of TCP, 31% of FCPD and 12% of T2DM subjects. In another study carried out in Bangladesh (Schneider *et al*., 2002), SPINK 1 N34S mutations were present in 55% of FCPD, 20% of TCP and 14% of T2DM. In another study carried out in India (Bhatia *et al*., 2002) showed that among TCP and FCPD subjects the frequency of N34S carriers (47% vs. 43%) and N34S homozygotes (12% vs. 14%) was similar. The association between the SPINK 1N34S gene and TCP and FCPD subjects has also been reported by other groups (Etemad and Whitcomb, 2001; Balakrishnan *et al*., 2006; Kazal *et al*., 1948; Laskowski and Wu, 1953; Whitt *et al*., 2000).

5.9.2 CFTR genotyping of the study subjects

The study reports for the first time, the association of CFTR gene in TCP and FCPD subjects. Out of 26 TCP subjects, 24 (92%) are of wild homozygous CFTR ex22 genotype. CFTR ex22 gene mutation is not found in any of the TCP subjects. Out of 60 FCPD subjects, 58 (96%) are of wild homozygous CFTR ex22 genotype and 1 (1.7%) heterozygous mutant has been detected and no homozygous mutant have been detected. Among the 33 T2DM subjects, 31 (94%) are of wild homozygous type and no CFTR ex22 mutant have been detected. A number of groups reported the association of CFTR gene mutation in idiopathic chronic pancreatitis but not in TCP and FCPD subjects in particular

(Chon et al., 1998; Sharer et al., 1998; Audrezet et al., 2002; Noone et al., 2001; Cohn et al., 2005).

5.9.3 IL-18 genotyping of the study subjects

The study also reports for the first time, the association of IL-18 gene in TCP, FCPD and T2DM subjects. Among 15 TCP, 22 FCPD and 43 T2DM subjects, IL-18 -607 homozygous variant has been found in 12 TCP (80%), 17 FCPD (77%) and 22 T2DM (51%) subjects. Heterozygous variant has been detected in 13% of TCP, 18% of FCPD and 40% of T2DM subjects. Wild type Il-18 -607 has been found in 7% of TCP, 5% of FCPD and 9% of T2DM subjects. Helminthes infestation is a common problem in children of poor families in Bangladesh and other developing countries (Northrop-Clewes *et al*., 2001) with prevalence of ascaris infections in poor urban communities ranging from 64% to 95% (Hall *et al*., 1999). Although, a significant percentage of IL-18 -607 gene mutation have been found, none of the subjects showed presence of helminth infestation (in regards to the absence of any larva in the stool). This scenario might be due to a wide line of practice of prescribing anthelminthic drug in a regular interval to the poor of rural as well as urban population.

5.9.4 Biochemical and genetic characteristics of the study subjects according to SPINK 1 N34S genotype

The study reports for the first time the correlation of the biochemical parameters and other genetic status of TCP and FCPD subjects with SPINK 1 N34S mutant. Among the biochemical parameters only fecal elastase 1 concentration is significantly lower (p=0.006) compared to the wild genotype. A significant

negative correlation is detected between IL-18 -607 CC genotype and the SPINK1 (N34S) haplotype in the subgroup of patients with FCPD (12/22 N34S positive ; r=.49 ; p=0.02). 15 TCP and 22 FCPD has either the IL-18 CC genotype and/or the SPINK1 n34S haplotype

Conclusions

The study concludes that:

1. There is a male preponderance among both the TCP and FCPD subjects

2. Both TCP and FCPD subjects are from lower socioeconomic strata of the society, however FCPD subjects show low BMI compared to TCP subjects

3. TCP subjects show significantly higher value of total cholesterol and LDL cholesterol than that of FCPD subjects.

4. Significantly higher value of serum creatinine in FCPD may indicate early nephropathic changes in FCPD.

5. ICA antibody is present in 6.1% of the TCP subjects.

6. Severe pancreatic exocrine insufficiency marked by significant decreased level of fecal elatase 1in T2DM subjects indicates presence of a common gene mutation in T2DM, TCP and FCPD subjects.

7. Pancreatic α cell function is preserved in both TCP and FCPD subjects as revealed by increased serum glucagon level after arginine stimulation test which supports the concept of diabetes in FCPD not being straight-forward consequence of TCP.

8. SPINK 1 N34S gene mutation is present both in TCP and FCPD subjects

9. Although no CFTR gene mutations have been detected in the TCP subjects but in 1 out of 59 FCPD subjects, heterozygous CFTR ex22 gene mutation has been found.

10. IL-18 -607 homozygous variant has been found in 12 of TCP, 17 of FCPD and 22 of T2DM subjects. Heterozygous variant has been detected in 2 of TCP, 4 of FCPD and 17 of T2DM subjects.

11. Pancreatic exocrine function is more compromised in SPINK 1 N34S positive TCP and FCPD subjects than the wild type.

POINTS TO CONSIDER:

Alcoholic pancreatitis subjects are not included in the study due to non availability. Inclusion of this group of subjects may clarify more precisely the mechanism of presence of diabetes in FCPD subjects which also may support the concept of two different pathogenic mechanisms of occurrence od diabetes in alcoholic pancreatitis, T2DM and FCPD groups.

SUGGESTED FURTHER STUDY

This study, for the first time detects the presence of ICA antibody in TCP, presence of CFTR ex22 gene mutation in FCPD and presence of IL-18 -607 gene mutation in TCP, FCPD and T2DM subjects. More extensive studies with large no of TCP, FCPD and T2DM cohorts should be carried out to understand the actual status regarding these issues. Determination of carbonic anhydrase II antibody is a good indicator of pancreatic exocrine function which should be included in the future studies.

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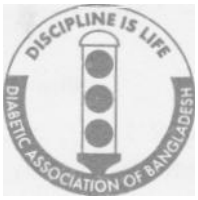
Appendix I

Case Record Form



Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM)
122 Kazi Nazrul Islam Avenue, Dhaka- 1000, Bangladesh Phone: 8616641-49. 9661551-60/ext 2233, 2282; Fax 880-2-8613004

CASE RECORD FORM

Registration no: Date:

Name Age Sex

Present address

Permanent address

Reference

Urban Rural Semiurban

Socioeconomic status

1. No of family members:
   -
2. Yearly family income :
   P

Visit Date

CLNICAL PROFILE
A Medical History (Y, Yes; N, No, D, Doubtful)

1. Measles: Y N

2. Mumps: Y N

3. Recurrent abdominal pain suggesting panceratitis : Y N D

4. Steatonrrhea suggesting exocrine pancreatic dysfunction : Y N D

5. History of protein deprivation before diabetes mellitus (peripheral edema, ascitis or autritonal suppliments) : Y N D

6. History suggestive of hepatic or gall bladder disease (Jaundics, Bilary colic etc) Y N D

7. History of alcohol intake Y N D

8. History of excess panta & shutki intake Y N D

B. Family History (ND. Nondiabetic; D, Diabetic, 1', Total)

1. Father :	N	D	
2. Mother:	N	D	
3. Brothers:	T	N	D
4. Sisters:	T	N	D
5. Cousins, maternal:	T	N	D
6. Cousins, paternal:	T	N	D
7. Uncle/ aunt, maternal:	T	N	D
8. Uncle/ aunt, paternal :	T	N	D
9. Grand father, maternal:	T	N	D
10. Grand father, paternal :	T	N	D
11. Grand mother, maternal :	T	N	D
12. Grand mother, paternal :	T	N	D
13. Sons:	T	N	D
14. Daughters:	T	N	D

C. Presentation of Diabetes Mellitus

a. Typical

Polyuria

Polydipsia

F. Anthropometry

t. Height (m)r 5 `c,-2. Weight (kg) 3. BMI
. Mid arm circumference 6. Others (MAC), cm

5. Skin fold thickness (mm)

c. Others

l 11. **STOOL EXAMINATION REPORT**

Name of Laboratory

Serial No

Date of examination :

Consultant .

A. Physical examination
1. Quantity:
2. Colour
3. Odor
4. Consistensy
5. Mucus
6. Blood

B. Chemical examination
1. Reaction:
2.Occult blood:
3. Reducing substance:

C. Microscopic examination
1 Protozoa of:
2.Cyst of:
3.Ova of:
4.Larva of:
5.RBC:
6.Pus cell:
7. Macrophages:
8.Vegetable cell
9. Starch granules:
10. Fat granules:

11. Muscle fibre'
12. Charcot-leyden crystal:
13. Bacteria:
14. Others:

VI. SPECIAL INVESTIGATIONS

A. Oral Glucose Tolerance Test (O(i'I'T)

Date: Starting time:

Amount of glucose (g):

Supervising scientist:

Results

Parameter	Time in minutes
	0 30 60 90 120
S glucose (mmol/1):	
S insulin (lu/ml)	
S C-peptide (ng/ml)	

H. Arginine Infusion Test (AIT)

Date : Starting time:

Superivisiting scientist:

Method in brief:

Results

Parameters	Time in minutes
	-15 0 15 30 45 60
S Glucose (mmol/L)	
S Glucagon (u/1)	
S insulin (ng/ml)	

Appendix II

Estimation of fasting Plasma Glucose

Serum glucose was estimated by enzymatic Glucose-Oxidase (GOD-PAP) method in Autoanalyzer (AutoLab, Analyzer Medical System, Rome, Italy) using reagents of Randox Laboratories, UK [Barham & Trinder 1972].

Principle

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

Reaction Principle

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{quinoneimine} + 4H_2O$$

Reagents composition

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

2. **GOD-PAP Reagent:** 4-aminophenazone (0.77 mmol/l), Glucose oxidase (≥1.5 kU/l) and Peroxidase (≥1.5 kU/l).
3. **Standard:** Glucose (5.55 mmol/l)

Procedure

The AutoLab Unit was calibrated before the assay. Serum was taken in the sample cup and GOD-PAP reagent was taken in the reagent container. Then the sample cups and reagent containers were placed in the sample and reagent holder. The Auto lab was programmed for the estimation of glucose and allowed to run with the following procedure:

5 μl sample and 500 μl reagent were taken to the reaction cell and mixed .The mixture was then incubated for 10 minutes at 37°C within the AutoLab. Reading was taken 500 nm.

Calculation of result for unknown sample is as follows:

Result of unknown sample = [(Standard Concentration / OD for Standard) × OD of unknown sample]

Appendix III

Estimation of Fasting Plasma Triglycerides

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in Autoanalyzer (Analyzer Medical System, Rome, Italy) using reagents of Randox Laboratories, UK [Trinder 1969].

Principle

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

Reaction Principle

$$\text{Triglyceride} + H_2O \xrightarrow[GK]{\text{Lipases}} \text{glycerol + fatty acids}$$

$$\text{Glycerol + ATP} \longrightarrow \text{glycerol-3-phosphate + ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{GPO} \text{dihydroxy acetone phosphate} + H_2O_2$$

$$2H_2O_2 + \text{4 aminophenazone} + \text{4 chlorophenol} \xrightarrow{POD} \text{quinoneimine} + HCl + 4H_2O$$

Reagents:

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

Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for test was entered in the AUTOLAB. Five (5) μl sample and 500 μl reagent were mixed and incubated at 37^0C for 5 minutes within the AUTOLAB. Reading was taken at 500 nm wavelngth.

Calculation of result for unknown sample is as follows:

Concentration of unknown sample = [(Standard Concentration / OD for Standard) × OD of unknown sample]

Appendix IV

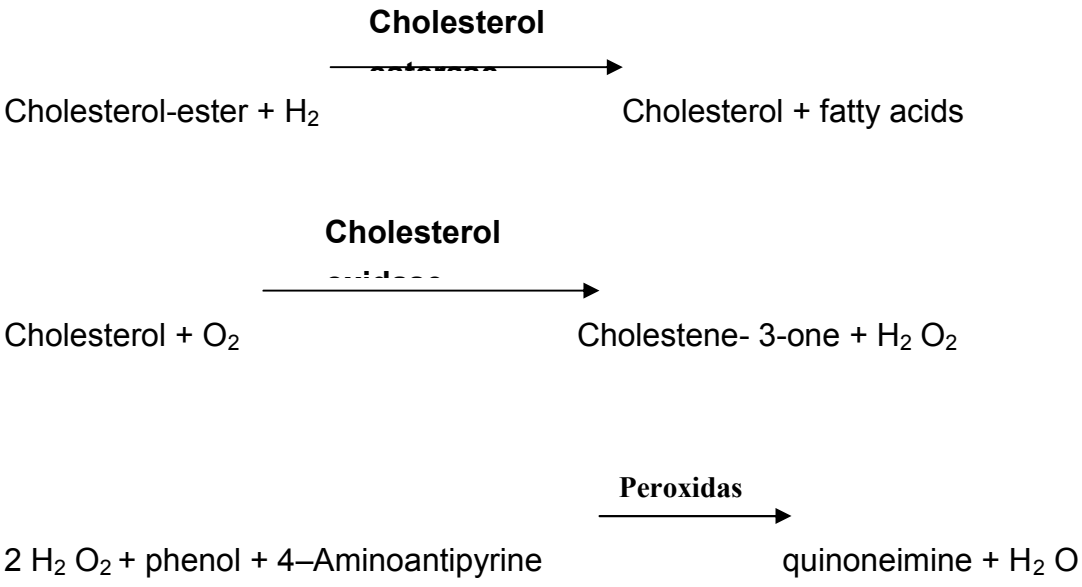
Estimation of Plasma Total Cholesterol

Total cholesterol was measured by enzymatic endpoint method (cholesterol Oxidase/ Peroxidase) method in Autoanalyzer (Analyzer Medical System, Rome, Italy) using reagents of Randox Laboratories, UK [Trinder 1988].

Principle

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantiphyrine in the presence of phenol and peroxidase.

Reaction Principle:



Reagent composition

1. Enzyme Reagent: Cholesterol oxides (≥ 0.1 U/ml), Cholesterol esterase (> 0.15 U/ml), Peroxidase **(>** 0.5 U/ml**),** 4-Aminoantipyrine (0.30 mmol/l), Phenol (6 mmol/l) and Pipes Buffer (80 mmol/l; pH 6.8).

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

Procedure

The equipment was calibrated before assay. Serum was taken in the sample cup and enzyme reagent was taken in the reagent container. Then the sample cups and reagent containers were placed in the Autolab analyzer (Analyzer medical system, Rome, Italy). The Autolab was programmed for the estimation serum cholesterol and allowed to run with the following steps: 5 μl sample and 500 μl reagent were taken to the reaction cell and mixed .The mixture was then incubated for 10 minutes at 37°C within the unit. Reading was taken at 500 nm wavelngth.

Calculation of result for unknown sample is as follows:

Concentration of unknown sample = [(Standard Concentration / OD for Standard) × OD of unknown sample]

Appendix V

Estimation of Plasma High Density Lipoproteion (HDL) Cholesterol

Serum High density Lipoprotein (HDL) was measured by enzymatic colorimetric (cholesterol CHOD-PAP) method in Autoanalyzer (Analyzer Medical System, Rome, Italy) using reagents of Randox Laboratories, UK [Assmann 1979].

Principle

HDL (High Density Lipoproteins) was separated from chylomicrons, VLDL (very low density lipoproteins) and LDL (Low density lipoproteins) by the addition of a precipitating reagent phosphotungstic acid in the presence of magnesium ions to serum or plasma. After centrifugation, the cholesterol concentration in the HDL fraction, which remains in the supernatant, was determined by the enzymatic colorimetric method using CHOD- PAP.

Procedure

Samples (200 ml) and precipitating reagent (500 μl) were taken in a microcentrifuge tube. Then it was mixed and allowed to sit for 10 minutes at room temperature. Then it was centrifuged at 4000 rpm for 10 minutes.

The supernatant was used as sample for determination of cholesterol content by the CHOD-PAP method. The sample and reagents were taken in specific cup or cell. They were arranged serially then ID number for test was entered in the AUTOLAB. Then 5 μl sample and 500 μl reagent were mixed and incubated

at 37^0C for 5 minutes within the AUTOLAB. The reaction occurred in reaction cell. Reading was taken at 500 nm.

Calculation of result for unknown sample is as follows:

Concentration of unknown sample = [(Standard Concentration / OD for Standard) $\times$ OD of unknown sample]

Appendix VI

Estimation of Low Density Lipoprotein (LDL) Cholesterol

The LDL-Cholesterol level in serum was calculated by using Friedewald formula [Friedwald 1972].

The Formula is as follows:

LDL-Cholesterol = Total cholesterol – [1/5 (Triglycerides) + HDL cholesterol].

Appendix VII

ESTIMATION OF HbA_{1c}

Percentage of HbA_{1c} was estimated in whole blood by a Variant hemoglobin testing system (Bio-Rad mode) using a modified HPLC method[93]

Principle

The variant Hemoglobin Testing System utilizes the principle of ion exchange high performance liquid chromatography (HPLC) for the automatic and accurate separation of hemoglobin A_{1c} by its Variant HbA_{1c} Program. The principle of ion exchange is that a charged substance is separated on the basis of its relative adsorption to the oppositely charged ion exchanger and to the oppositely charged substances of the solvent. For separation of HbA_1 or HbA_{1c}, which are negatively charged at neutral pH, a cation exchange column was used.

The mobile phase (buffers land 2) pumps through the apparatus by two dual piston pump system. Buffer 1 (sodium phosphate, pH 5.9) washes off HbA_{1c}. Thus decrease in pH increases the charge of HbA_{1c} and helps in its elution the separated hemoglobin is detected by a filter photometer (absorption maximum 415 nm) and further background variations are corrected by an additional filter at 690 nm. A built-in intergrator computes all chromatograms, retention times and peak areas.

Assay Conditions

Flow Rate:	2.50 ml/min
Low Pressure Limit:	142 psi
High Pressure Limit:	1428 psi
Column Temp:	40^0C
Temp of the Sample Tray:	8^0C

Reconstituting the Primer

The lyophilyzed primer was reconstituted by adding 1 ml of deionzed water and allowed to stand for 10 minutes at 15-30^{0}C. The reconstituted primer amy stored at 2-8^{0}C Reconstituting the Calibrator.

The lyphilyzed calibrator was reconstituted by adding 10 ml of cold calibrator diluent and allowed to stand for 10 minutes at 15-30^{0}C. The reconstituted calibrator (9.4% HbA1c) was stored at 2-8^{0}C.

A Simple pretreatment of the sample (the EDTA-treated whole blood pool) was required to provide a freshly prepared hemolysate before each assay.

Preparation of sample

Primer was reconstituted following recommended procedure.

Samples were made homogenous gentle inversion of contained for few times. the Then 5μl of blood was removed and added 100o μl haemolysis buffer and mxed thoroughly. The vials were then placed in position of the sample tray. Reconstituted samples may stand at 18-24^{0}C for at least 10 minutes.

Test procedure

Before starting analysis, the system would follow a 5-minute WARM-UP program. The sample size was mentioned and the Unit was set run.

At the end of the run the system followed a 3 min wash program.

Interpretation of Results

Analyte	**Retention time (mins)**	**Band (mins)**	**Limits (mins)**	**Observed (mins)**
Injection peak	0.09	0.08	0.01-0.17	N.A
A_{1a}	0.26	0.09	0.17-0.35	0.26-0.35
A_{1b}	0.48	0.13	0.35-0.61	0.36-0.61
F	0.73	0.12	0.61.-0.85	0.66-1.11
A_{1c}	1.1	0.08	1.02-1.18	1.06-1.11
S	1.8	0.08	1.72.1.88	1.78-1.82
C	2.02	0.14	1.88-2.16	1.98-2.08

Analyte identification Window for Interpretation of HbA_{1c} Test Results.

The reportable range of HbA_{1c} is 3-17%. When HbA_{1c} values exceeded above this value. The samples were diluted with 2 ml hemolysing agent (1:400) and then the assay was performed.

Everyday after entire run, the piston seal port was flushed with 10 ml of deionized water.

Appendix VIII

Estimation of serum creatinine

Creatinine in alkaline solution reacts with picric acid to form a colored complex.The amount of the complex formed directly proportional to the creatinine concentration.

SAMPLE COLLECTION AND PREPARATION

Serum, Heparinized or EDTA plasma, Stable for 7 days at +2 to +8^{o}C.

REAGENT COMPOSITION

Contents	Initial Concentration of Solutions
Standard	177 □mol/l (2mg/dl)
Picric acid	35 mmol/l
Sodium hydroxide	0.32 mol/l

MATERIALS PROVIDED

Standard

Picric acid

Sodium hydroxide

MATRIALS REQUIRED BUT NOT PROVIDED

Pipetting devices for the delivery of 100□l, 200□l, 1 ml and 2 ml.

Timing device and water bath or heating block to maintain temperature at 25, 30 or 37^{o}C.

Spectrophotometer with wavelength capability of 490 to 510nm.

Randox Assayed Multisera Level 2 (Cat. No. HN 1530) and level 3 (Cat. No. HE 1532)

PROCEDURE NOTES

Reaction rate and absorbance of the reaction product are very sensitive to temperature. The specified temperature must therefore be maintained

PROCEDURE

Wavelength: Cuvette:	340 run (I lg 334 nm **or 149** 365 nm) 1 cm light path
Temperature: Measurement:	30/37°C against air

Pipette into cuvette:

	Macro	Micro
Sample	0.2 ml	0.1 ml
R1. Enzyme/Coenzyme/a-oxoglutarate	2.0 ml	1.0 ml

Mix, read initial absorbance after 1 mm.

Read again after 1, 2, and 3 min. Note lithe absorbance change per minute is between

0.11 and 0.16 at 340 nm/Hg 334 nm

0.06 and 0.08 at Hg 365 am

use only the values for the first 2 minutes for the calculation.

Appendix IX

ESTIMATION OF SERUM SGPT

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

Principle:

$\alpha-$ Oxoglutarate + L-alanine ALT L-glutamate + pyruvate

Pyruvate + NADH + H^+ LD L-lactate + NAD^+

SAMPLE

Serum or EDTA plasma

Reagent Composition

Content	Concentration in the test tube
Buffer/Substrate Tris buffer L-alanine	 100mmol/l, pH 7.5 0.6 mol/l
Enzyme/Coenzyme/α-oxoglutarate α-oxoglutarate LD NADH	 15 mmol/l ≤ 1,2 U/ml 0.18 mmol/l

Preparation of Solutions:

1. Buffer/Substrate: Buffer/Substrate supplied in the kit was used as it is. 2. Enzyme/Coenzyme/α-oxoglutarate: One vial of Enzyme/Coenzyme/α-oxoglutarate 2 was reconstituted with the appropriate volume of Buffer/Substrate 1: 2 ml for the 20 x 2 ml kit (AL 1200) 10 ml for the 20 x 10 ml kit (AL 1205) 20 ml for the 5 x 20 ml kit (AL 1268) One vial of

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

PROCEDURE

Wavelength	340 nm (Hg 334 nm or Hg 365 nm
Cuvette	1 cm light path
Temperature	30/37°C
Measurement	against air
Pipette into cuvette	
	Macro Micro
Sample R1.Enzyme/Coenzyme/α-oxoglutarate	0.2 ml 0.1 ml 2.0 ml 1.0 ml

Mixed and initial absorbance was read after 1 minute. Again after 1, 2 and 3 minutes the absorbance was read. The absorbance change per minute was noted and if the value is between 0.11 and 0.16 at 340 nm/Hg 340 nm 0.06 and 0.08 at Hg 365 nm Only then the values for the first 2 minutes were used for the calculation.

Calculation: To calculate the ALT activity the following formulae was used: U/l = 1746 x ΔA 340 nm/min U/l = 1780 x ΔA Hg 334 nm/min U/l = 3235 x ΔA Hg 365 nm/min

Appendix X

Estimation of fecal elastase 1

1 Introduction

Human pancreatic elastase 1 (E1) remains undegraded during intestinal transit. Therefore its concentration in feces exocrine pancreatic function. During an inflammation of the pancreas, E 1 is released into the blood circulation. Thus the quantification of pancreatic elastase 1 in serum allows diagnosis or exclusion of acute pancreatitis.

1.2 Advantages

In contrast to other laboratory parameters for the diagnosis of pancreatic disease (amylase and lipase activity in serum for the diagnosis of acute pancreatitis and fecal chymotrypsin activity for the diagnosis of exocrine pancreatic insufficiency), the determination of pancreatic elastase 1 has the following advantages:

- E1 is absolutely pancreas-specific.
- Since E1 is stable during intestinal transit, the fecal elastase 1 /^{f} concentration reflects the secretory capacity of the pancreas (diagnosis or exclusion of pancreatic exocrine insufficiency).
- E1 determination correlates with the gold standard invasive secretinpancreozymin test and the secretin-caerulein test,
- Intra-individual variation of fecal E 1 concentration is low.
- A substitution therapy has no influence on the determination of E1. The monoclonal antibodies used in the test do not cross-react with elastases of animal origin, which are contained in enzyme substitution preparations.
- Like other pancreatic enzymes, E1 is released into the blood circulation during an inflammation. Due to its longer half-life,

compared to amylase and lipase, its concentration remains elevated longer, and an acute pancreatitis is detectable even three or four days after onset of the disease.

Two ELISA test kits (based on monoclonal antibodies) are available for the determination of pancreatic elastase 1. The serum test quantifies E1 in serum, allowing the diagnosis or exclusion of an acute pancreatitis or an inflammatory episode of chronic pancreatitis or ERCP- or gallstone-induced pancreatitis. The stool test quantifies El in stool (or duodenal juice), allow' e diagnosis or exclusion of pancreatic exocrine insufficiency, ic can be c chronic pancreatitff~

cystic fibrosis, pancreatic tumor, cholelithiasis or diabetes mellitus r example.

E1 serum test: cat. no.: 06

E1 stool test: cat. no.: 07

1.3 Sensitivity and specificity

The diagnostic efficiency of pancreatic elastase 1 determination in stool has been evaluated in several clinical studies. Stein et al. (1993, 1996 & 1997) and Loser et al. (1995 & 1996) compared the El determination with invasive intubation tests, the secretin-pancreozymin test and the secretin-caerulein test, respectively. Both authors report a sensitivity and specificity greater than 90 % for the diagnosis of exocrine pancreatic insufficiency. In contrast to the fecal chymotrypsin assay,

n a mo erate pancreatic insufficiency can be detected by El determination (Loser et al., 1995 & 1996, Gullo et al., 1999).

In a study by Dominguez-Munoz et al. (1995) the elastase 1 determination was compared to the pancreolauryl test. According to this study El determination is more specific than the pancreolauryt test at a comparable sensitivity.

In addition, studies by Terbrack et al. (1996), Soldan et al. (1996), Gullo et al. (1997), Wallis et al. (1997), Walkowiak et al. (1999) and Code et al. (2000)

showed an excellent sensitivity and specificity for the diagnosis of cystic fibrosis with pancreatic involvement.

1.4 Basic principle of the assay

The ELISA plate is coated with a monoclonal antibody which only recognizes human pancreatic elastase 1 (El). El from samples and standards binds to the antibody and is immobilized on the plate. A complex of monoclonal anti-Elastase 1-Biotin and Peroxidase (POD)-Streptavidin binds to El during the next incubation. The peroxidase oxidizes ABTS (2, 2'-Azino-bis-(3-ethylbenzothiazolin-bsulfonic acid) diammonium salt), which turns dark green. Finally, the concentration of oxidized ABTS is determined photometrically.

1.5 Detection limit

Pancreatic elastase 1 is determined within the range of 15 to 500 pg El/g stool. Concentrations below the lowest standard should be stated as < 15 pg El/g stool. Values above the highest standard should be indicated as > 500 pg E 1 /g stool.

2 Reagents

1. 12 ELISA-strips with 8 wells each coated with a monoclonal antibody to human pancreatic Elasfase 1 (E1) 96 wells

2. Sample-/ washing buffer concentrate (5x) (black cap) 100 ml phosphate buffered saline, pH 7.2, with detergent

3. Extraction buffer concentrate (5x) for stool specimen (square flask, green cap) 100 ml (not included if E1 Quick-Prep (cat. no. 07-9uick)) is ordered phosphate buffered saline, pH 7.2, with detergent and sodium azide

4. E 1 standards 1 to 4, ready-to-use (blue cap) 700 NI each human pancreatic elastase 1 in aqueous solution with sodium azide

5. Control, ready-to-use (violet cap) 700 NI human pancreatic elastase 1 in aqueous solution with sodium azide

6. Complex of monoclonal anti-El-biotin and POD-Streptavidin (=anti-El-bio-POD-Streptavidin-Comp.), ready-to-use, light sensitive (black plastic vial with black cap) 8 ml in aqueous solution with preservative

7. Substrate solution, ready-to-use, light sensitive (red cap) 12 ml ABTS in aqueous solution

8. Stop solution, ready-to-use (white cap) 12 ml alkaline aqueous solution

9. Plastic bag containing a desiccant for unused ELISA-strips

3. Sample material and sample stability

Sample material: A single random stool sample

Sample stability: Samples may be stored in the laboratory for up to three days at 4 - 8 °C or up to a year at -20 °C. Undiluted stool extracts may be stored at 4 - 8 °C for one day or up to a year at -20 °C,

4 Storage and stability of the test kit

All components of the test kit are stable at 4 - 8 °C until the expiry date shown on the kit labels. Unused ELISA-strips must be stored in the well-sealed plastic bag containing a desiccant.

5 Additional utensils required

- Polystyrene test tubes (5 m1,10 ml and 12 ml) with caps
- 500 ml graduated cylinder
- vortex mixer
- adjustable precision pipettes: 0-50 NI, 50-200 NI, and 200-1000 pi
- 5 ml and 10 ml pipettes

• adjustable 8 channel pipette 50-250 pi

• ELISA reader capable of reading absorbance at 405 nm.

Precautions

For in vitro diagnostic use only. Extraction buffer, standards, control and the complex of anti-El-bio and POD-Streptavidin contain a preservative. Do not pipette by mouth. Wear disposable gloves while performing the tests. A materials safety data sheet is available on request. Do not mix materials from different master lots.

8 Test procedure

8.1 **Preparation**

8.1.1 Preparation of sample-/washing buffer

100 ml sample-/washing buffer 5x (black cap) + 400 ml bidistilled water.

solution).

The diluted sample-/washing buffer is stable for 6 months at 4 - 8 °C.

8.1.2 Preparation of ELISA plate

Bring ELISA plate to room temperature before opening. Desired number of ELISA strips are left in the microplate frame. Unused ELISA strips must be stored in the well-sealed plastic bag containing the desiccant.

8.1.3 Preparation of stool specimen

Weighing method: stool specimen can be weighed (see 8.1.3.1) or alternatively

The E 1Quick-PrepT"^ dosing device can be used: for speed and convenience it is recommended to use the El Quick-PrepT"' dosing device (cat. no. 07-Quick, see 8.1.3.2)

8.1.3.1 Performance with the weighing method

Preparation of extraction buffer:

100 ml extraction buffer 5x (square flask, green cap) + 400 ml bidistilled water.

The diluted extraction buffer is stable for 6 months at 4 - 8 °C

Weighing of stool specimen

Tare tube and inoculating loop on a sensitive digital laboratory balance. Take a small sample (approximately 100 mg) from the stool specimen with the inoculating loop and replace the loop into the tube to weigh the sample. A wooden applicator or tooth pick may be used instead of the inoculating loop.

Add extraction buffer to the stool sample according to the mass of the sample, (e.g. 100 mg stool + 10 ml extraction buffer or 75 mg stool + 7,5 ml extraction buffer). The final concentration must be 10 mg stool/ml extraction buffer.

Homogenisation and extraction of stool samples

The stool suspension is mixed thoroughly at room temperature (please use a vortex mixer). Stool suspensions must be homogenized to ensure a complete extraction of pancreatic elastase I. After an extraction period of at least five minutes the suspension is mixed once more. Then, after any particles have settled, the dilutions are performed. Because pancreatic elastase 1 is very stable, the extraction period may be extended for up to 24h at 4 - 8 °C and the dilutions can be performed the next day.

Dilution of stool extracts (1:250)

Preparation of 1:250 dilution:

10 NI extracted stool sample + 2.5 ml sample-/washing buffer.

8.2 Assay procedure

8.2.1 Incubation of samples and standards

	1	2	3	4	5	6	7	8	9	10	11	'12
A	Blank	Blank	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
B	STD I	STD I	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
C	STD2	STD2	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
D	STD3	STD3	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
E	STD4	STD4	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
F	CON	CON	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
G	S1	Sl	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
H	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42

<---2 strips --->

<-------- 4 test-strips -------->

<---------- whole ELISA plate, 12 test-strips ---------->

Possible plate layout

STD: standards

CON: control

Sl-542: samples

Blank = wells A1 and A2; pipette 50 NI of sample-/washing buffer into each well.

Standards (blue cap) are ready-to-use; pipette 50 uI of each standard (undiluted) into columns 1 and 2 as duplicate,

Stool Standard 1 corresponds to 15 pg/g

Standard 2 corresponds to 50 pg/g

Standard 3 corresponds to 150 pg/g

Standard 4 corresponds to 500 pg/g

Control, ready-to-use (violet cap); pipette 50 NI into wells F1 and F2.

Stool Control corresponds to 200 pg/g ± 15 %

Pipette 50 pl of extracted diluted stool specimen (see 8.1.3.1 und 8.1.3.2) from each sample into each of two adjacent wells.

Incubate for 30 minutes at room temperature.

Pancreatic Diastase 1 in Stool

Pancreatic Elastase 1 in Stool

Washing: Empty the wells and wash each well 3 times with sample-/washing buffer (8 channel pipette, 250 NI/well). Invert the plate and tap it hard on a clean paper towel to completely remove any remaining liquid.

8.2.2 Incubation with complex of anti- El-bio and POD-Streptavidin

Add 50 NI/well ready-to-use complex of anti-El-biotin and POD-Streptavidin anti-El-bio-POD-Streptavidin-Comp. (black plastic vial with black cap)

Incubate for 15 minutes in the dark at room temperature.

Washing: Empty the wells and wash each well 3 times with sample-/washing buffer (8 channel pipette, 250 ul/well). Invert the plate and tap it hard on a clean paper towel to completely remove any remaining liquid.

8.2.3 Color Reaction

Add 100 NI of ready-to-use substrate solution (red cap) to each well.

Incubate for 15 minutes in the dark at room temperature. (Please shorten this time when using an ELISA reader which reads extinctions only up to 1.5 or 2).

8.2.4 Stopping the color reaction

Stop the substrate reaction by adding 100 uI of stop solution per well (ready-touse, white cap). Mix contents well by agitating the plate.

8.2,5 Measurement

Read the optical density at 405 nm with a microtiter plate reader between 5 and 30 minutes after addition of the stop solution. Mix contents well before measuring. 492 nm can be used as a reference wavelength.

8.3 Quantification of results

Evaluation by ELISA - software

Define blank, standards and samples according to the plate layout (figure 2). Use the curve - fit method (linear regression) with log - log scale.

8.3.3 Reference concentrations for pancreatic elastase 1

In stool:

normal: 200 to >500 Ng El /g stool

moderate to mild exocrine pancreatic insufficiency: 100-200 Ng E 1 /g stool

severe exocrine pancreatic insufficiency: < 100 pg E 1 /g stool

These pancreatic elastase 1 concentrations refer to formed stool samples. In case of pathological elastase 1 concentrations (< 200 pg El/ g stool) in watery stool samples a second formed stool sample should be requested (see section 1.7).

Appendix XI

Estimation of Plasma Glucagon

Double Antibody Glucagon

Principle of the Procedure

Double Antibody Glucagon procedure is a sequential radioinimunoassay. After preincubation of the patients sample with anti-glucagon antibody, $^{l\cdot5}$14abeled glucagon competes with glucagon in the sample for antibody sites. After Incubation for a fixed time, separation of bound from free is achieved by the PEG-accelerated double-antibody method, followed by centrifugation. The precipitate containing the antibody-bound fraction is then counted, and patient concentrations are read from a calibration curve.

Reagents to Pipet: 3

Total Incubation Time: Two 24-hour Incubations

Total Counts at Iodination:

approximately 25,000 cm

Separation:

The ready-to-use Precipitating Solution combines second antibody and dilute PEG.

Materials Supplied

Glucagon Antiserum (GND1)

Lyophilized glucagon antiserum, raised in rabbits, with preservative. Reconstitute by adding 10 mL distilled water. Mix by gentle inversion. Stable at 2-13"C for 30 days after reconstitution. Color: blue. KGNI31: 1 vial

"^{2}1 Glucagon (GND2)

Lyophilized, iodinated synthetic human glucagon, with preservative. Just before assay, reconstitute by adding 10 ml distilled water. Mix by *gentle* inversion. Stable at 24°C for 30 days after reconstitution. KGNI31: 1 vial

Glucagon Zero Calibrator (GND3)

Lyophilized zero calibrator in a protein-based matrix, with preservative. ***At least 30 minutes before use,*** reconstitute each vial with **10 ml.** distilled water. Mix by *gentle* inversion. Stable at 2-8"C for 30 days after reconstitution, or for 2 months (aliquotted) at -20°C. KGN01: 2 vials

Glucagon Calibrator F (GND8)

Lyophilized calibrator containing synthetic human glucagon in a protein-based matrix, with preservative. *At least 30 minutes before use,* reconstitute each vial with **2.0 mL** distilled or deianized water. Use a volumetric pipet and mix by *gentle* Inversion. *Discard alter use.* -

KGN131: 3 vials

The calibrator F has a *lot-specific* value of approximately 500 picograms of synthetic human glucagon per milliliter (pgiml); equivalently 144 picoinoles per liter (email.). Refer to the vial labels for exact values in pa/m1 . The assay is standardized in terms of the World I lealth Organization's International Standard for Glucagon. number 69/194.

Precipitating Solution (GNRG)

110 mt. of Precipitating Solution consisting of goat anti-rabblt gamma globulin (GARGG) and dilute polyethylene glycol (PEG) in saline, with gentainicin as a preservative. The Precipitating Solution is supplied In liquid form, ready to use. Remove the aluminum foil seal completely and store refrigerated: stable at 2-8°C for 30 days alter opening Since a fine (but visible) precipitate may form after refrigeration, the Precipitating Solution should be thoroughly mixed before use, without foaming. Color; red.

KGN131: 1 vial

Glucagon Controls (GNC01, GNCO2) Two vials, labeled Glucagon Control **1** and 2, containing synthetic human glucagon in a protein-based matrix, with

preservative. The controls are supplied lyophilized *At least 30 minutes before use,* reconstitute each vial with 1,0 **mL** distilled water. Use a volumetric pipet and mix by *gentle* inversion. *Discard after Use.* Refer In the control insert for Glucagon values in pg/mL:

KGN01: 3 sets.

Required But Not Provided

Gamma counter

Centrifuge - Refrigerator and capable of at least 1500xg

Vortex mixer

Reagent Preparation

Distilled of deionized wale;

Pipets for measuring 0 sel and 10 ml. Volumetric pipet: 1.0 nil.

RadloImmunoassay

Plain 12x75 mm *glass* tubes Tubes should be made of high-quality borosilicate glass. Because of Glucagon tendency to adsorb to plastic surfaces, glass tubes (rather than plastic) should be employed for the assay.

Micropipets -50 mL, 100 pL, 2013 lit, SOO **pt.** and 1.0 mt.. For the 1130 pL additions, a reliable repeating dispenser is recommended. A syringe-style dispenser accurate to within 10.05 mL is recommended for the 1.0 mL addition of

Precipitating Solution.

Parafilm

Foam decanting rack - available from Siemens Healthcare Diagnostics (catalog number: FOR).

Logil-log graph paper

Radioimmunoassay Procedure

All components except the Precipitating Solution must be at room temperature (15-28°C) before use.

Caution: Because of the tendency of glucagon to adsorb to plastic surfaces. *glass* tubes rather than plastic should be employed for the assay. (The use of plastic tubes could result in the loss of all detectable glucagon)

1. Just before assay, dilute the

500 pg/inL Glucagon Calibrator Fin the Glucagon Zero Calibrator, using *glass* tubes or *gloss* vials, as described in the table below. Vortex each dilution thoroughly.

Add this ..	**To this._ ML Calib.**			**Yielding this Calltnutur**	
50 Colib F	950	Zero	B	26	7
100 Colib. F	900	Zero	C	50	14
203 Colib. F	800	Zero	D	100	29
SO) Cali). F	500	Zero	E	293	72

2 Label sixteen glass tubes in duplicate: T (total counts), NSB (nonspecific binding), A (maximum binding) and B through F. Label additional tubes, also in duplicate, for plasma samples *and* controls.

Approkmate pg/mL	Approomate prnolit
0	0
25	7
50	14
100	29
250	72
500	144

Mete: The value el 'he wlibrators we *kg-spertic.*

Refer to the US labels for exact values in pgint.

3 Pipet 200 ml of the zero calibrator A into the NSF3 and A tubes, and 200

mL of each of the remaining calibrators **B** through F into correspondingly labeled tubes. Pipet **200 mL** of each patient plasma sample and each control into the tubes prepared.

It is good practice to use a disposable tip micropipet, changing the tip between samples, in order to avoid carryover contamination.

Samples expected to contain

glucagon concentrations greater than the highest calibrator (calibrator F. 500 pg/mL) should be diluted in the • zero calibrator boron) assay.

4 Add 100 *pl.* of Glucagon Antiserum (sLuE) to all tubes except the NSB and T tubes. Vortex.

A repeating dispenser is recommended for this step and for the addition of tracer at step 6.

5 Cover the rack and incubate for 24 hours at 2-13°C.

Cover the entire rack with Parafitm *cc* place the racks in plastic bags to avoid evaporation.

Add 100 pl. of ^{125}1 Glucagon (CLEAR) to all tubes. Vortex.

Remove the T tubes for counting at

step 11; they require no further processing.

7 Cover the rack and incubate for 24 hours at 2-8°C.

Cover the entire rack with Paratitin or place the racks in plastic bags to avoid evaporation.

8 Add 1.0 mt. of *cold* Precipitating Solution (Rai) to all tubes. Vortex.

For the 1.0 mL reagent addition, a repeating dispenser may be employed.

9 Centrifuge for 15 minutes at 1.500xg.

10 Using a foam decanting rack, decant (or aspirate) the supernatant. retaining the precipitate for counting.

Let the tubes stand inverted on absorbant paper for 10 minutes. Then lap the tubes gently and blot the rims, to remove residual droplets.

11 Count each tube foci minute.

Calculation of Results

To obtain results in terms of concentration from a logit-log representation ol the calibration curve, first calculate for each pair of tubes the average NS13-corrected counts per minute.

Net Counts = (Average CPM) minus (Average NSI3 (WM)

Then determine the binding of each pair of tubes as a percent of maximum binding *(MB),* with the NS13-corrected counts of the A tubes taken as 100%:

Appendix XII

Estimation of serum C-peptide

Principles of Procedure

In radiniaanuneassay. a axed concentration of faceted foxau antigen Is incubated with a (mistime dilution of °Mean= such flue the concentration of antgen bird ng sites on the antibody is limited, for CX:11111110. *only* 50% of the total trance corownkation may be bound by an6tody. If unlabeled antigen is added to lb* system. trete is competition between labeled tracer and urea beled antigen for tne limited and constant number of ending silos on the antibody. Thus. the amout of tacor bound to antibody will decrease as the concentration of unlabeled antigen increases. This con lie measured after separating antibody-bound trona free tracer and counting ore or the other, or both *fractions.* A coLotatina Cr standard curve * sot up with increasing concentrators o'slandaid unlabeled antgen and from ths mewl the amount ot antigen in unknown samples can be calculated Thus. the kim th15·4 necessities tor a radiemmunnassay system aro. a specific antiserum to lee antoen to be measured, the avail:SI ty ol a radioactive labeled karts id The antgen, a method wite. way antibody-bound tracer caa be soprimityl from the unbound tracer, and loath', an instrument to count radioactively.

The Malcom Human C-Peptide assay utilize tt Hlanaued Human C- Peptide a Human C-Peptide antiserum to determine the level of C-Peptide in serum, Marsala of tissue cuhure media by the double antibo PEG technique.

H. Reagent Supplied

Each kit is stiff dent to nit 250 tubes and contains the following reagents

A Assay *Suffer*

0.05M Pnosphosalino pH 74 containing ft 025M EDTA, 0.08% Sodium Arida. 1% RIA Grade BSA Quantity. 40 nit:va

Preparation: Ready to use

B Human C-Peptide Antibody

Guinea Pig anti Iluman 0-Peofide Antibody in Assay Butter
Quantity. 26 mLevial

Preparation: Ready to use.

C ^{125}: I-Human C4^{7}eptide

^{125} I-Human C-Pepbde Label, HPLC purdim..ml (saetalic activity 658 pCi/pg)

LYOPM:74X1inl slalidity. rteStly icy:Mated label contains 43 LC*, (111 kBo), calibrated to the 1st Monday of each month

Quality, 27 ml/ vial under hydration

Preparation:: Contents Lyophilized. Hydrate with entire contents of level Hydrating buffer. Allow to set at room temp for 30 minutes, with occasional gentle mixing

REAGENTS SUPPLIED (continued)

a. Label Hydrating Butter

Assay Buffer ooMantng No-mal Guinea lag Serum as a camer. Used to hydrate ^{In}t-Human C. Peulide.

Quality 27 ml/vial

Preparation: Ready to use

b. Human Ca:Peptide Standards

Purifed Human C-Peptide in Assay Fiona al the following concentrations: 0.1.0.2, 0.5. 1.2, 5 nett

Quality 28 ml/vial

Preparation: Ready to use

c. Quality Controls 1 82

Punted Human areptide In Assay Buffer

Quantity: 1 mL/vial

Preparation: Ready to use

d. Precipitating Reagent

Coat anti-Guinea Pig IgG Scrum. 3,4 PEG and 0.05% Triton X-103 in 0.0511Phosphssatire. 0.025M ED A.

041% Sodom Aide

Quantity 260 minted

Preparation Ready to use. Chill to 4^0 C

Rr. STORAGE AND STABILITY

Refrigerate all reagents *between* 2 and St fur shod term storage. For prolonged storage (..-2 weeks), freeze at

s-20"C Avoid multiple (55) freeze /thaw cycles. Refer to date on battle Re expicalinn Mum slow: ;It C Nit Do not mix reagents from different kits unless hey have he same lot number.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

12 x 75 mm. (NOTE Polypropylene or poilystyrene lobes may be used. If the investigator finds dial Ins pellet formation is acceptably stable their system

2 100 ml pipet wit, disposable tire

3 100 ml & 1.0 ml. Repeating Dispenser

4 Rehigmaled svAng micket centrifuge capable of develeping 2.000 - 3.300 xg. (Use 011.xed-argle buckets are rOt rocaliMended)

5 Absorbent paper

6 Vortex mixer

7 Refrigerator

8 Gamma Counter

VII. SPECIMEN COLLECTION AND STORAGE

1. A maximum of 100 pi par assay tube cd Sotum or plasma can be used, telhDogll. DO pl per assay kibn iff adequate rev 1111ni applicatines. l.ssuo dilute and other mislia may also be used.
2. Cate must be taken ohs" using heparin as an anticoagulant since an excess vAll provide falsely hip values [2] *Use* no mom than 10 heoann per ml.. ol blood coleded
3. Human C•peptide must 're protected horn prohnlysis dunce assay pte4edures anti sample Storage Tlaflytel (Avg air) at a conevidtahmi of 560 KIU *net* mL of ant um or plasma should be added to samples to protect nine protcolysis

   For ImprOleded sastpfea hero is a kiss of a Ogre% italeY [25]%[1]CP row [3] cortsgts storage [at=] -

   Xrc. No loss was observed when unnimeciod hampiew Wert: MIMS *at* s .70°C let up to 12 manger.
4. Specimens can be deed at A'C:f they will be tested wibir 24 bows e: deaden re longer see age, specimens mid4d be she ed at s -20"C Avoid mull isle (P5) freezen haw cycles.
5. Avoid using samples with gross hemolysis or Lipemia

ASSAY PROCEDURE

For optimal results, accurate pipetting and adherence to the protocol are recommended

A. Day One

Pipet 200 pt. of Assay butte - to tile Non Specific(NSB) tubes (3-4) and. 100 pl. in

Rey:fence (Bo) tubes ib-6).

2 ***Poet***pl. of Sin ^dards and Quality Controls in duchcate (see flow churl)

3Pm ICO p101 eau sample in duplicate NOTE: Smaller trctuanes OF sacople may be used when

Human C.Peplitle tres:entraiions are anticipated to be etevated or when sample *size* as tmited. Adotional Assay Buffer should *es ace.]* to compensate or the dievence so that the volume Is egtirealent to IUU p1 (e g.. vt eta usrg SO pL of sample. add SO pL of Assay Buller). Refer to Souion 0(tor calrailation mudaltation.

Pipet 100 pL of ^{125}144urnsn C Peptide to all tubes. Impolant: For precaution, *sae* Sootier, 111. Part

C. Pace! 100 pl. of Human C Peptide antibody Ita all tubes except Total Count tubes (1 2) and NSB tu Nee (3 4)

8.Vona; cover. aul incubate oveinighl (2C.24 hours) al 4'C

B. Day Two

7Add 1.0 mi./gonad PCC)Paccipitatrag Reagent to all tubes (tomcat Total Count tubes).

B.Voiles and incubate 20 manotes at CC.

9.Centufuge. 4°C. all tubes (except Tot's Cowl tubes (1.2)) for 20 minutes at 2,000-3 OM xg NOTE:

If less than 2.000 xg is used or if sipped pellets have been ohamved in previous rurs, tile tune of centatugotkri must be increased to obtain a lion pellet 10 g , 40 minutes). Multiple oantrituge runs veillta an assay must be consistent

Conversion of rpm to am

lag a $^{(1}$ 12x 10)(r) $(rpm)^2$

r Indiol distant° in cm (from axis of rotation to the bottom
01 the tube) rpm = revolutions ;ma mute

10 reviled altdy decant the sub.:mate of all tubea except tat CI **"Ini** lacx;s (1-21, dean Lubin tor at **10:134115-60** sectods (be consistent bloaveen oaks). and blot excess equid from Up at tubes NOTE, invert lutes only

ono time Pellets ore fragile rad Waco may occur.

11. Count all tubesgamma counter for I minute. Calculate Me I maril of Homan C.Poblide in

unknown sompaes owe automated data reduction pocedircs

CALCULATIONS

JL' Explanation

The calculations **10**, **11110100** C Peptide cull be automatically performed by most gamma counters

paessiny data teclueliret enpahilitta re by irderentSent Inietment of the raw data using a commeteciolly nvnilabie software packaujeCaoctse weighted **4-parameter** Of oeighled lnolugit for the mathematic-II treatment of the *data* OTE: Be tartan the procedure used strident the NSB counts from each average count except Total Courts. boor to limit data recta:lion j

B. Manual Calculation

1.Avelage duplicate counts for Total Court tubes (1-2). NSB tubes **tad).** Total Binding tubes

(reference, Bo) (b.6). and all duracnie lobes for standards and samples to the *end* of the assay 2 Subtract the average NSB countsfrom eacn average count (except tar Total Crawls). These counts are used in the katenving caltulations

4Calculate the parentage of tracer tound : (lota Sinew° Ciontsfic **bin! Clltlf.(s) X** 100.

It's Should he 3550%

4.Calculate the cemented° of total Diftdi^c **(i.liEn)** tot mat standard .1or1 sample

tobfBe = (Sample Of Standard:Toed xling) X inn

Pkal the % Brno for each standard cn they-an and re known ooncenfration el the stondrird on tea x-axis using log-log graph Paper.

6. Construct *the reference* curve by Oiling the pants wth a emOeth Min)

7. I)etatnamine the aghnl of Iturnon C.PePtide

interpralatkm of the frt. crence Curve.in the *unknown* samples Nnknoors

and Merit) by

NOTC When sample volumes assayed differ from IUU pl, an appropriate malhem;:tinal adjustment must toe made to acconunodale tor the dilation factor (e.g. if 50 pl- of sample is Used 'hen calmtaled data must be multiplied by 2).

Calculation

U/l = 174 x AA 340 nm/min

U/l = 178 x AA Hg 334 nmtmin

U/l = 323 x AA Hg 365 nm/min

INTERFERENCE

Avoid haemolysis as it interferes with the a ssay,

NORMAL VALUES IN SERUM

25°C	30DC	37°C
Men	up to 22 Ufiup to 29 11/1	up to 40 WI
Women up toll U/1	up to 22 U/l*up tb* 31 U/l	

It is recommended that each laboratory established its own reference range to reflect the age, sex, diet ard geographical location of the population.

LINEARITY

If the absorbance change per minute exceeds

0.16 at 340 nm/Hg 334 nm

0.08 at Hg 365 nm

dilute 0.1 ml of sample with 0.9 ml of 0.9% *NaCI* soi Wien and reassay. Multiply the result by 10.

Appendix XIII

High through-put DNA Extraction using QIAGEN Kit

Principle

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

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

Procedure

1. 25 µl of Qiagen Protease stock solution was added to each of the 96 wells of round-well block.
2. 200 µl aliquot of peripheral blood sample was added to each well.
3. 200 µl of chaotropic solution AL (QIAGEN) was added to each well. Wells were capped and the block shaken vigorously for 15 seconds.
4. The 96 well block was then spun briefly at 3000 rpm.
5. The block was incubated at 65°C for 10 minutes in an oven and spun briefly at 3000 rpm.
6. 210 µl of 100% ethanol was added to each well, the block was shaken vigorously for 15 seconds and spun briefly at 3000 rpm. Proper attention was paid to avoid any accidental swap of strip caps for wells.
7. QIAamp 96 column plate was placed on a plate receptacle and reaction mix (635 µl/well) transferred into the column. Plate was sealed with an adhesive strip and centrifuged for 6000 rpm for 4 minutes.
8. The 96 well column plate was washed with 500 µl of Qiagen buffer AW1. The plate was centrifuged at 6000 rpm for 2 minutes.
9. The second wash was done with 500 µl of Qiagen buffer AW2. The plate was centrifuged at 6000 rpm for 4 minutes. In each step of washing fresh sealing film was used to avoid any outside chance of cross contamination.
10. The QIAamp 96 column plate was removed from the waste receptacle block and placed on a 96 microtube collecting rack and incubated in an oven at 70^{o}C for 10 minutes to dry the silica membrane.

11. Preheated (at 70^{o}C) Qiagen AE Buffer 200 µl was added to 96 column membranes, sealed and incubated at room temperature for 5 minutes.
12. DNA elution into collecting tubules was done by centrifuging at 6000 rpm for 4 minutes. This step was repeated using 50 µl of preheated AE Buffer.
13. To maximise the DNA extraction a third elute was done using 100 µl Buffer AE in a separate collection block.
14. Random samples from the eluted DNA were electrophoresed on 1% agarose gel to assess the integrity, uniformity of yield. Approximate concentration of yield was assessed comparing with known quantity of molecular marker. DNA yield was approximately 30-40 ng/µl in the first elute.
15. Aliquot of DNA was diluted to 10 ng/µl in 96 deep well plates and preserved at 4^{o}C.

    Quantification of extracted DNA has been carried following standard procedure

Appendix XIV

Mutational screenibg of IL-18 gene promoter region

Screening for mutations in the promoter region of IL-18 gene is accomplished by direct sequencing of all subjects using PCR primers developed using GenBank sequences (Accession NT_009151) and encompassing the single nucleotide polymorphism at position -656, -607 of the IL-18 gene promoter region. All results are confirmed by double sequencing.

The following oligonucleotide primer sequence is designed and used for mutational screening for the positions -656 and -607: 5' CTC TGC TCT TCA AAC GTT AC 3', down 5' CCA AGC TCA ATA TGG TGT C 3', resulting in a 340 bp amplification product.

PCR is performed using 25 ng of genomic DNA template, 0,625 U Taq DNA polymerase (5U/μl) (Gene choice Inc / Kemp Biotechnologies Inc, Frederick, MD, USA, Cat No 62-6086-02). 250 nmol of each primer (IDT Inc, CoralVille. IA, USA) in 25 μl reaction volume. The cycle conditions are 3 minutes of denaturation at 96^{o}C followed by 35 cycles of 40 seconds at 96^{o}C, 40 seconds of annealing at 54^{o}Cand 50 seconds of extension at 72^{o}C, and final extention time of 10 minutes at 72^{o}C.

The PCR products are purified with Exosap purification kits (usb, Cleveland, OH, USA). Direct DNA sequencibg is performed by using the ABI Prism Big Dye Terminator Cycle Sequencing kit (AB, Foster City, CA, USA). 4.0 ml of Big Dye Terminator 5x Sequencing Buffer (AB, Foster City, CA, USA) and 2.5ml DEPH treated water (Ambion Inc. Austine, TX, USA) in a 10ml reaction. The amplified products are separated by electrophoresis on an 2% agarose gel and

visualized by UV light illumination using ethidium bromide staining. Sequence analysis is performed using Sequencher 3.1. (GeneCodes Corp., Ann Arbor MI).

Appendix XV

Mutational screening of SPINK 1 N34S promoter region

Screening for mutations in the SPINK 1 gene is accomplished by direct sequencing of exon 3 in all the subjects and of all exons and adjacent intronic nucleotides. All sequencing is performed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis is performed with sequencer 3.1 (Gen Codes Corp., Ann Arbor, MI).

For verification of any base changes or in case of ambiguity noted, sequencing of the opposite strand is performed with the appropriate polymerase chain reaction primer.