CHARACTERIZATION OF INSULIN SECRETORY DEFECTS IN YOUNG TYPE 2 DIABETIC PATIENTS IN BANGLADESH

This dissertation is submitted as a requirement for the fulfillment of the degree of Doctor of Philosophy (PhD) in the Dept of Biochemistry and Cell Biology, BIRDEM, under the faculty of Postgraduate Medical Science & Research of the University of Dhaka

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STATEMENT OF ORIGINALITY

I hereby humbly declare that this Thesis titled as **'Characterization of insulin secretory defect in young type 2 diabetic patients in Bangladesh'** is based on work carried out by me in the Dept of Biochemistry and Cell Biology, BIRDEM, during the period of January 2003 to December 2010 and neither this Thesis as a whole nor any part of it has been submitted in any form for any other degrees or part there of.

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DECLARATION

The Thesis titled as **'Characterization of insulin secretory defect in young type 2 diabetic patients in Bangladesh'** has been submitted in fulfillment of the requirement for the degree of Doctor of Philosophy (PhD) in the Dept of Biochemistry and cell Biology, BIRDEM, under the faculty of Postgraduate Medical Science & Research of the University of Dhaka. This study has been carried out in the laboratory of Biomedical Research Group (BMRG), Bangladesh Institute of Research and Rehabilitation in Diabetes Endocrine and Metabolic disorders (BIRDEM), Dhaka. To the best of our knowledge no part of the work has been submitted for another degree or qualification in any other Institutes at home or in abroad.

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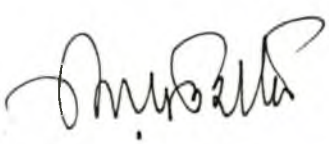

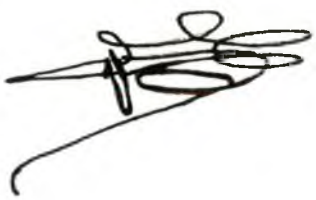

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LIST OF ABBREVIATIONS

%	Percent
µL	Microlitre
ADA	American Diabetes Association
ADP	Adenosine Di Phosphate
ATP	Adenosine Tri Phosphate
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders
BMI	Body Mass Index
BMRG	Biomedical Research Group
CI	Confidence interval
C-peptide	Connecting peptide
DBP	Diastolic Blood Pressure
DM	Diabetes Mellitus
EDTA	Ethylene Diamine Tetracetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FSG	Fasting Serum Glucose
FCPD	Fibrocalculous pancreatic diabetes
FFA	Free fatty acid
HDL-C	High Density Lipoprotein Cholesterol
IR	Insulin resistance
ISI	Insulin sensitivity index
IDDM	Insulin Dependent Diabetes Mellitus
IAA	Insulin auto antibodies
ICA	Islet cell antibodies
IFG	Impaired Fasting Glucose

IGT	Impaired glucose tolerance
LDL-C	Low Density Lipoprotein Cholesterol
KC	Kinetic calculation
Kg	Kilogram
L	Litre
m	Metre
mg	miligram
NIDDM	Non-insulin Dependent Diabetes Mellitus
OR	Odds Ratio
OPD	Out Patient Department
PDDM	Protein deficient diabetes mellitus
pmol	picomole
SBP	Systolic Blood Pressure
SD	Standard Deviation
SPSS	Statistical Package for Social Sciences
T Chol	Total Cholesterol
TIDM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglyceride
WHO	World Health Organization
TNF-α	Tumor necrosis factor-α
WHR	Waist-Hip Ratio

SUMMARY

Insulin secretory dysfunction and insulin resistance are well recognized as the two basic defects in type 2 diabetes mellitus (T2DM), but their relative contribution varies substantially among different populations as well as among different subgroups in the same population. An understanding of these defects in each group of diabetic population greatly help in designing a rational management and prevention policy. Bangladesh has one of the largest number of diabetic subjects in the world and, like many other population in the regional countries, a major proportion of these subjects are young and nonobese. The basic defects of diabetes have not yet been properly explored in these subjects. Preliminary data suggest a major role of the insulin secretory deficiency but its relative contribution as well as interaction with insulin resistance in the context of the more usual groups of adult diabetic and prediabetic subjects have not been investigated. In the above background the present work was undertaken to characterize the insulin secretory deficiency in young onset diabetic subjects in relation to insulin resistance and other determinants of insulin secretion and sensitivity in our population.

Under an observational group comparison design the following series of studies were conducted: a) Insulin deficiency and insulin resistance in young and nonobese Bangladeshi diabetic subjects (Study 1); b) Phase-wise insulin secretion in young and nonobese Bangladeshi diabetic subjects (Study 2): c) Insulin resistance and insulin secretory defect in young to middle aged Bangladeshi Type 2 diabetic subjects (Study 3): d) Phase-wise insulin secretion in middle aged Bangladeshi type 2 diabetic patients (Study 4); e) Relationship of serum proinsulin and glucagon levels in subjects with type 2 diabetes mellitus (Study 5); f) Association of serum resistin with hs-CRP and insulin sensitivity in type 2 diabetes mellitus subjects (Study 6); g) Insulin deficiency and insulin resistance in Bangladeshi IGT subjects (Study 7); h) Serum proinsulin status in IGT subjects (Study 8); and i) Insulin deficiency and insulin resistance and phase-wise insulin secretion in first degree relatives of young and nonobese Bangladeshi type 2 diabetic patients. The subjects were collected from the Outpatient Dept of BIRDEMD. Study 1 included 25 Diabetic and 10 Control subjects, Study 2 included 42 T2DM subjects and 43 Control subjects, Study 3 included 726 Diabetic and 317 Control subjects, Study 4 included 44 Diabetic and 30 Control subjects, Study 5 included 44 T2DM subjects and 44 Control subjects, Study 6 included 104 T2DM and 33 Control subjects, Study 7 included 46 IGT and 39 Control subjects, Study 8 included 50 IGT Diabetic and 44

Control subjects, and Study 9 included 29 first degree relatives of T2DM subjects and 33 Control subjects. Diabetes and IGT were diagnosed and classified as per WHO criteria. Serum glucose was measured by glucose-oxidase method, serum lipid profile was measured by enzymatic method. Serum C-peptide was measured by chemiluminescence-based ELISA technique. Serum insulin, proinsulin, glucagon, glucose dependent insulinotropic hormone (GIP) and resistin were measured by enzyme linked immunosorbent assay (ELISA). hs-CRP was measured by immunonephelometry. The maximum insulin secretory capacity was assessed by glucagon stimulation test and the usual capacity was assessed by homeostasis model assessment (HOMA%B). Insulin sensitivity (as a reverse of insulin resistance, was assayed by short insulin tolerance test and also by homeostasis model assessment (HOMA%S). 1st and 2nd phases of insulin secretion will be estimated by the following equations: 1st phase $_{est}$ = 1283+1.829.Ins_{30}-138.7.Glu_{30}+3.772.$Ins_{0;}$ 2nd $phase_{es}$ = 287+0.4164.Ins_{30}-26.07.Glu_{30}+0.9226.Ins_{0}. The data were analyzed by appropriate univariate, bivariate and multivariate statistical tests as appropriate using Statistical Package for Social Science (SPSS) for Windows version 11.5.

The young nonobese diabetic subjects showed only about 1/5th of maximum insulin secretory capacity compared to Control (Glucagon stimulated C peptide - glucose ratio, Mean ±SD, 0.19±0.20 in Diabetic vs 0.96±0.27 in Control, $p<0.001$). In contrast, there was no significant difference in insulin sensitivity between the two groups (kitt: Mean ±SD, 0.067±0.02 in Diabetic vs 0.068±0.01 in Control, $p=0.912$). The compromise in pancreatic β cell function was seen in both phases; however it was more dominant in the 1st phase [st phase insulin secretion: Median (Range), 593 (-2023-2347) in Diabetic vs 1700 (291-3936) in Control, $p<0.001$; 2nd phase insulin secretion: 203 (-287-626) in diabetic vs 444 (143-937) in Control, $p<0.001$)]. In contrast to the young nonobese subjects young to middle aged patients showed both insulin secretory defect and insulin resistance [HOMA%B: Median (Range), 78 (10-462) in Diabetic vs 120 (11-583) in Control, $p<0.0001$; HOMA%S: 47 (10-489) in Diabetic vs 68 (11-457)) in Control, $p<0.001$-0.0001]. Again the compromise was evident in both phases.

On multiple regression analysis, HOMA%B was found to have highly significant association ($p<0.0001$) consistently irrespective of age and BMI. On the other hand, the association of HOMA%S with diabetic status became marginal ($p=0.01$-0.03) or nonexistent when the effects of BMI and age were adjusted in the same regression model. On such analysis serum glucagons was found to be a major determinant of

HOMA%B and serum proinsulin, resistin and hs-CRP were important determinants of HOMA%S in these subjects.

In IGT subjects presence of insulin resistance was seen [HOMA%S: Median (Range) 39 (14-110) in IGT vs 54 (15-289) in Control, $p<0.023$), without any insulin secretory defect [HOMA%B: 162 (49-300) IGT vs 141 (18-386) in Control, $p<0.174$]. Proinsulin was found to be higher in both Diabetic (Proinsulin: Median (Range) 28.3 (2.1-268) in Diabetic vs 9.3 (1.8-156.7) in Control, $p<0.0001$) and IGT [Proinsulin: Median (Range) 17 (3-51) in IGT vs 9.2 (1.8-156) in Control, $p<0.0001$)] groups. On multiple regression analysis, on adjusting the effects of age and BMI, IGT was not found to be associated with HOMA%B, but it had strong tendency to be associated with HOMA%S, ($p=0.065$). With the same analysis proinsulin was significantly associated with HOMA%S ($p=0.037$), but not with HOMA%B. The first degree relatives of T2DM subjects did not show any difference in insulin sensitivity [HOMA%S Median(Range), 58 (15-258) in T2DMR vs 48 (21-620) in Control, $p=0.612$] but a strong tendency of hypersecretion was observed [HOMA%B, Median(Range), 131 (22-386) in T2DMR vs 90 (17-191) in Control, $p=0.095$]. The hypersecretory tendency was observed in both phases (T2DMR vs Control, $p=0.70$ in the first phase and $p=0.081$ in the 2nd phase). On multiple regression analysis, on adjustment of the effects of age and BMI, T2DM relative status did not show any significant association with HOMA%S but it showed a marginally significant positive association with HOMA%B.

The data from the series of studies lead to the following conclusions:

a) Insulin secretory deficiency seems to be the main basic defect in young onset T2DM among Bangladeshi population; b) The insulin secretory defect in young onset T2DM affects both the 1st and 2nd phases with 1st phase being affected more than the 2nd phase. c) DM in young to middle aged subjects are associated with both insulin deficiency and insulin resistance, but insulin resistance in these cases seem to be mainly a function of increased BMI (and to a lesser extent age) and insulin secretory deficiency is the consistently predominant basic defect in this disorder like that in young T2DM.; d) Irrespective of age and adiposity glucagon is a major determinant of insulin secretory deficiency in our population and, on the other hand proinsulin, resistin and hs- CRP are important determinants of insulin sensitivity; e) The predominant defect in impaired glucose tolerance is insulin resistance and it seems to have an association with hyperproinsulinemia; and f) Hyperinsulinemia may be an inherited feature in young onset type 2 diabetes mellitus.

INTRODUCTION

CHAPTER I

Introduction

Diabetes mellitus and its burden

Diabetes mellitus

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. When fully expressed, diabetes is characterized by fasting hyperglycemia, but the disease can also be recognized during less overt stages, most usually by the presence of glucose intolerance. The effects of DM include long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, heart and blood vessels. Diabetes mellitus confers a 2- to 4-fold increase in cardiovascular risk compared with the general population (American Heart Association, 2004). Although microvascular complications of diabetes result in increased rates of morbidity, macrovascular complications, including coronary artery disease, often cause death (Beckman et al., 2002).

Types of diabetes

The new classification system identifies four types of diabetes mellitus: type 1, type 2, 'other specific types' and gestational diabetes. Arabic numerals are specifically used in the new system to minimize the occasional confusion of type 'II' as the number '11.' Each of the types of diabetes mellitus identified extends across a clinical continuum of hyperglycemia and insulin requirements.

Type 1 diabetes mellitus (formerly called type I, IDDM or juvenile diabetes) is characterized by beta cell destruction caused by an autoimmune process, usually leading to absolute insulin deficiency. (WHO Expert Committee, 1997; National Diabetes Data Group, 1995). The onset is usually acute, developing over a period of a few days to weeks. Over 95 percent of persons with type 1 diabetes mellitus develop the disease before the age of 25, with an equal incidence in both sexes and an increased prevalence in the white population. A family history of type 1 diabetes

mellitus, gluten enteropathy (celiac disease) or other endocrine disease is often found. Most of these patients have the 'immune-mediated form' of type 1 diabetes mellitus with islet cell antibodies and often have other autoimmune disorders such as Hashimoto's thyroiditis, Addison's disease, vitiligo or pernicious anemia. A few patients, usually those of African or Asian origin, have no antibodies but have a similar clinical presentation; consequently, they are included in this classification and their disease is called the 'idiopathic form' of type 1 diabetes mellitus.

Etiological classification

The etiologic classifications of diabetes mellitus are listed in

Table 1: (Etiologic Classifications of Diabetes Mellitus) WHO Expert Committee, 1997)

Type 1 diabetes mellitus

Type 2 diabetes mellitus

Other specific types:
Genetic defects of beta-cell function
Genetic defects in insulin action
Diseases of the exocrine pancreas
- Pancreatitis
- Trauma/pancreatectomy
- Neoplasia
- Cystic fibrosis
- Hemochromatosis
- Others

Endocrinopathies
- Acromegaly
- Cushing's syndrome
- Glucagonoma
- Pheochromocytoma
- Hyperthyroidism

- Somatostatinoma
- Aldosteronoma
- Others

Drug- or chemical-induced

- Vacor†
- Pentamidine
- Nicotinic acid
- Glucocorticoids
- Thyroid hormone
- Diazoxide
- Beta-adrenergic agonists
- Thiazides
- Phenytoin
- Alfa-interferon
- Others

Infections

- Congenital rubella
- Cytomegalovirus
- Others

Uncommon forms of immune- mediated diabetes

Other genetic syndromes sometimes associated with diabetes

- Down syndrome
- Klinefelter's syndrome
- Turner's syndrome
- Wolfram syndrome
- Friedreich's ataxia
- Huntington's chorea
- Lawrence-Moon Beidel syndrome
- Myotonic dystrophy
- Porphyria
- Prader-Willi syndrome
- Others

Gestational diabetes mellitus

Type 2 diabetes mellitus (T2DM, formerly called NIDDM, type II or adult-onset) is characterized by insulin resistance in peripheral tissue, and an insulin secretory defect of the beta cell (WHO Expert Committee, 1995). This is the most common form of diabetes mellitus and is highly associated with a family history of diabetes, older age, obesity and lack of exercise. It is more common in women, especially women with a history of gestational diabetes, and in blacks, Hispanics and Native Americans. Insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance. Defective beta cells become exhausted, further fueling the cycle of glucose intolerance and hyperglycemia. The etiology of type 2 diabetes mellitus is multifactorial and probably genetically based, but it also has strong behavioral components.

Types of diabetes mellitus of various known etiologies are grouped together to form the classification called "other specific types." This group includes persons with genetic defects of beta-cell function (this type of diabetes was formerly called MODY or maturity-onset diabetes in youth) or with defects of insulin action; persons with diseases of the exocrine pancreas, such as pancreatitis or cystic fibrosis; persons with dysfunction associated with other endocrinopathies (e.g., acromegaly); and persons with pancreatic dysfunction caused by drugs, chemicals or infections.

Burden of diabetes

Global

By the end of the 20th century the worldwide diabetes pandemic had affected an estimated 151 million persons, distributed among both developed and developing countries (Zimmet et al., 2001; King et al., 1998). The global prevalence of T2 DM is expected to be double in the period 2000-2025 and may reach a level of almost 300 million people, the adult population will increase by 64%, prevalence of diabetes in adults will increase by 35%, and the number of people with diabetes will increase by 122% (King et al., 1998).

Diabetes imposes a large economic burden on the individual, national healthcare system and economy. Healthcare expenditures on diabetes are estimated to account

for 11.6% of the total healthcare expenditure in the world in 2010. Estimated global healthcare expenditures to treat and prevent diabetes and its complications are expected to total at least US Dollar 376 billion in 2010. By 2030, this number is projected to exceed some USD490 billion. Diabetes is a major cause of blindness, kidney failure, amputations, and cardiovascular disease, and its complications results in major reductions in both length and quality of life (Klein et al., 1984; Wetterhall et al., 1992; Geiss et al., 1997). In addition, the burden on individuals and on society extends past human suffering to include staggering economic costs, lost productivity, and social capital (Diabetes Health Economics Study Group: 1997; American Diabetes Association: 1998). The American Diabetes Association estimated that the US economy lost USD58 billion, equivalent to about half of the direct healthcare expenditure on diabetes in 2007, as a result of lost earnings due to lost work days, restricted activity days, lower productivity at work, mortality and permanent disability caused by diabetes. Such losses are perhaps relatively larger in poorer countries because premature death due to diabetes occurs at much younger ages. The World Health Organization (WHO) predicted net losses. The largest economic burden, therefore, is the monetary value associated with disability and loss of life as a result of the disease itself and its related complications (International Diabetes Federation, 2006.)

Regional

It has been estimated that there were 171 million people with diabetes in 2004 worldwide, with 83 million of these individuals living in countries of the World Health Organization's (WHO) South-East Asia and Western Pacific regions (Asia-Pacific region (Wild et al., 2004). Recent projections suggest that these figures will double by the year 2030 with the greatest burden of diabetes occurring in the Asia-Pacific region, where more than half of the world's population resides (Wild et al., 2004). In addition, the latest report from the WHO on chronic diseases predicts that deaths from diabetes in the WHO South-East Asia and Western Pacific regions will increase by 39% and 51%, respectively, over the next decade (World Health Organization, 2005).

India has a higher number of people with diabetes than any other country, with estimates ranging from 19.4 million in 1995 (King et al., 1998) to 32.7 million in 2000 (International Diabetes Federation, 2000). The International Diabetes Federation

gives an estimate of 12% prevalence in Pakistan, with a total of 8.8 million people with diabetes in 2000 (International Diabetes Federation, 2000). In Sri Lanka the 1999 census report records diabetes prevalence as 8% in rural areas and 12% in urban areas (Diabetes Association of Sri Lanka, 1999); equivalent current rates for Nepal have been reported as 3% and 15% respectively (Singh & Bhattarai, 2003). The number of people with diabetes is expected to rise by 195% in India during 1995-2025 to reach 57.2 million in 2025 (King et al., 1998).

Bangladesh

WHO listed 10 countries to have the highest numbers of people with diabetes in 2000 and 2030. According to this report, Bangladesh has 3.2 million of diabetic subjects in 2000 and the number is expected to increase to a staggering 11.1 million by 2030 placing her among the top 10 countries with diabetes (Wild et al., 2004). Several small-scale population based studies conducted in Bangladesh at different time points have revealed an increasing trend of diabetes prevalence in rural and urban communities (Hussain et al., 2007; Rahim et al., 2007; Hussain et al., 2005; Sayeed et al., 2003; Sayeed et al., 1997; Sayeed et al., 1997). A recent population based study (Rahim et al., 2007) showed a significant increase in the prevalence of DM in rural Bangladesh from 2.3% to 6.8% over 5 years. This prevalence was higher than found in the previous rural based studies on the same population (Sayeed et al., 2003; Sayeed et al., 1997).

Pathophysiology of type 2 diabetes

Basic defect of diabetes

Type 2 diabetes is characterized by defects in both insulin secretion and insulin action (DeFronzo et al., 1997). Both factors are important in the pathogenesis of the disease and both are influenced by environmental and genetic factors (DeFronzo et al., 1997; Bell & Polonsky, 2001). A related issue for individuals interested in the pathogenesis of type 2 diabetes is the debate as to whether insulin resistance or impaired β-cell function is the primary defect. By primary defect, the underlying genetic defect is meant. Type 2 diabetes has a strong genetic basis (DeFronzo et al., 19971; Bell & Polonsky, 2001; Ferrannini, 1998, Horikawa et al., 2000; Mahtani et al., 1996; Altshuler et al., 2000; Baier et al., 2000; Stumvoll et al., 2001; Fritsche et al., 2001; Jacob et al., 2000; Stumvoll et al., 2001). It seems pretty well established that type 2 diabetes is a polygenic disorder in which both hereditary and environmental or acquired factors are involved (Aitman et al., 1995), and both of these factors can affect β-cell function and insulin sensitivity (Iselius et al., 1985; Martin et al., 1992). The exact timing and relative importance with which these two factors appear and play a role in the natural history of the disease are still a matter of debate (Gerich, 1998; Ferrannini, 1998). The pathogenesis of diabetes the temporal sequence with which these metabolic abnormalities develop relative to one another during the different stages of the disease remains unknown, however. Several authors have proposed that a defect in insulin action is the predominant abnormality in the early stages of the development of type 2 diabetes, and that insulin secretory dysfunction occurs only at a later stage (DeFronzo, 1988, Reaven, 1988, DeFronzo, 1997, DeFronzo et al., 1992, Reaven et al., 1989, Lillioja et al., 1993, Martin et al., 1992, Warram et al., 1990). Others have suggested that a defect in insulin secretion may be the major early abnormality (Pimenta et al., 1996, Yoneda et al., 1992, O'Rahilly et al., 1986, Kosaka et al., 1996, Chen et al., 1995, Leahy, 1990, Gerich, 1998). The prospective studies provided evidence that insulin resistance and insulin secretory dysfunction predict the development of type 2 diabetes in various populations (Pratley et al., 2000; DeFronzo, 1997; Ferrannini, 1998; Gerich, 1998; Lillioja et al., 1993; Saad et al., 1991). Some researchers emphasize on the presence of insulin resistance in the first degree relatives of type 2 diabetes (Ericksson et al., 1989; Gulli et al., 1992; Perseghin et al., 1997), others strongly underscoring the early appearance of

defects in insulin secretion (Pimenta et al., 1995; Van Haeften et al., 1998; Zonderland et al., 2000). Some others have reported evidence for both abnormalities (Laakso et al., 1998; Nyholm et al., 1999). Various methods, different ethnic backgrounds, heterogeneity of the disease, and different criteria to select populations of control individuals may account for these seemingly disparate findings.

Insulin secretory defect in type 2 diabetes

Physiology of insulin secretion

The understanding of diabetes as a metabolic disease has evolved significantly since the discovery of insulin in the 1920s. Insulin was identified as a potent hormonal regulator of both glucose appearance and disappearance in the circulation. Until recently, insulin was the only pancreatic β-cell hormone known to lower blood glucose concentrations. Insulin, a small protein composed of two polypeptide chains containing 51 amino acids, is a key anabolic hormone that is secreted in response to increased blood glucose and amino acids following ingestion of a meal.

Insulin is produced and secreted by the β-cells of the islets of Langerhans, the endocrine part of the pancreas, so named after their discoverer, Paul Langerhans. It was to take another 20 years until Oscar Minkowski discovered their relationship to the pancreas and 50 years before the discovery of insulin was made.

Insulin secretion is controlled by β-cell electrical activity

Insulin is not secreted if the blood glucose concentration is $\leq$ 3.3 mmol/l, but is secreted in increasing amounts as glucose concentrations increase beyond this threshold (Gerich, 1993). Postprandially, the secretion of insulin occurs in two phases: an initial rapid release of preformed insulin, followed by increased insulin synthesis and release in response to blood glucose. Long-term release of insulin occurs if glucose concentrations remain high (Cryer, 1992; Gerich, 1993).

As in neurons and many other endocrine cells, electrical signals play a central role in the regulation of secretion. In 1968, Dean and Matthews demonstrated that the β-cell is electrically excitable (Ashcroft & Rorsman, 1989). When the extracellular glucose concentration is elevated from the basal level of 5 mM (at which little insulin

secretion is observed) to the insulin releasing concentration of 10 mM, the β-cell undergoes a slow depolarization from the resting potential (–70 mV) up to a threshold from which regenerative electrical activity is elicited. This electrical activity consists of oscillations in the membrane potential between depolarized plateaus, from which Ca2+-dependent action potentials originate, which are separated by electrically silent (repolarized) intervals. The induction of electrical activity is a central part of the cascade of events that leads to the initiation of insulin secretion. Very recently, it has been possible to demonstrate that the periods of electrical activity coincide with pulsatile release of insulin (Barbosa et al., 1998). Patch-clamp experiments during the last 15 years have revealed that two classes of ion channel are particularly important in the generation of β-cell electrical activity: ATP-regulated K+ channels (KATP channels) and voltage-gated L-type (dihydropyridine-sensitive) Ca2+ channels (Ashcroft & Rorsman, 1989). In the absence of glucose, the cytoplasmic ATP/ADP ratio is low and the KATP channels are open. The constant outflow of positively charged K+ through the open KATP channels accounts for the negative membrane potential of the b-cell in the absence of glucose. When the extracellular glucose concentration is elevated, the rapid uptake of glucose (via GLUT2 transporters) and subsequent metabolic degradation of the sugar result in an elevated cytoplasmic ATP/ADP ratio. This entrains the closure of the KATP channels, membrane depolarization, opening of voltage-gated Ca2+ channels, an increase in the cytoplasmic Ca2+ concentration, and, ultimately, the exocytosis of the insulin-containing secretory granules.

While glucose is the most potent stimulus of insulin, other factors stimulate insulin secretion. These additional stimuli include increased plasma concentrations of some amino acids, especially arginine, leucine, and lysine; GLP-1 and GIP released from the gut following a meal; and parasympathetic stimulation via the vagus nerve. (Holst, 1994; Drucker, 2001). Of crucial importance to make a clear distinction between the insulin response following an intravenous glucose challenge and that following glucose or food ingestion. The intravenous administration of glucose triggers a biphasic insulin response (featuring a rapid increase with a peak, an inter peak nadir, and a subsequent slower increasing phase) only when glucose concentration increases rapidly, as after a glucose bolus or a glucose infusion determining a square wave of hyperglycemia. A "slow-ramp" glucose input induces gradually larger secretion

without a well-defined first phase (Grodsky et al., 1972; Renson et al., 1980). More importantly, a well-defined first phase is lacking under physiological conditions, i.e., when glucose is given orally.

Before these issues are addressed, a precise definition of first-phase insulin secretion is required. Five different modes (or phases) of insulin secretion can be identified: 1) basal insulin secretion is the way insulin is released in the postabsorptive state; 2) the cephalic phase of insulin secretion is evoked by the sight, smell, and taste of food (before any nutrient is absorbed by the gut) and is mediated by pancreatic innervation (Ahren et al., 2000; Konturek et al., 2000); 3) first-phase insulin secretion is defined as the initial burst of insulin, which is released in the first 5–10 min after the β-cell is exposed to a rapid increase in glucose (or other secretagogues); 4) after the acute response, there is a second-phase insulin secretion, which rises more gradually and is directly related to the degree and duration of the stimulus; 5) finally, a third phase of insulin secretion has been described, albeit only in vitro (Grodsky, 1989). During all these stages, insulin is secreted, like many other hormones, in a pulsatile fashion, resulting in oscillatory concentrations in peripheral blood. Oscillations include rapid pulses (recurring every 8–15 min) superimposed on slower, ultradian oscillations (recurring every 80–120 min) that are closely related to fluctuations in glucose concentration (Lefebvre et al., 1987; Matthews et al., 1991; Polonsky et al., 1998; Pørksen et al., 2002).

Effects of first-phase insulin response on peripheral tissues. The time course of glucose uptake by peripheral tissues lags considerably following insulin response. It has been suggested that this delay is due to the time it takes insulin to cross the endothelial barrier and reach the interstitial medium bathing the insulin-sensitive cells (Bergman et al., 1990; Yang et al., 1989). In particular, it has been shown in dogs that changes in glucose uptake are closely predicted by changes in hindlimb lymph insulin (representing interstitial insulin bathing the skeletal muscle) (Poulin et al., 1994). Thus, to appreciate the effect of the biphasic insulin response on glucose uptake, one should make reference to the time course of insulin in the interstitial fluids, not in plasma. As insulin reaches the interstitial fluids, the biphasic pattern is no longer appreciable, because the first phase is considerably attenuated and merged with the second phase. Even though insulin loses its biphasic pattern when it reaches the

interstitial fluids, a large first-phase insulin secretion causes remote insulin to increase more quickly than it otherwise would. Getty et al., have shown in dogs that, when the contribution of the first-phase insulin response to the overall increase in insulin is of the order of ≥50%, this produces a rise in insulin lymph concentration that is much more rapid than the one observed when only the second phase is present. This, in turn, promotes a quick activation of glucose uptake by the tissues.

Because first-phase insulin secretion determines a time course of interstitial insulin concentration that has beneficial effects on glucose uptake, it is not unreasonable to hypothesize that it may also promote a quick activation of insulin's indirect effect on hepatic glucose production. It has been established that insulin's inhibitory effect on hepatic glucose production is in part direct, i.e., due to the interaction between hepatic sinusoidal insulin with the hepatocyte, and in part indirect, i.e., due to the action of insulin on extrahepatic tissues, the latter action consisting mainly of the antilipolytic effect of insulin on adipose tissue (Ader et al., 1990; Cherrington et al., 1998; Giacca et al., 1992; Lewis et al., 1996; Maheux et al., 1997; Prager et al., 1987; Rebrin et al., 1996.). The insulin-induced decrease of substrate flow to the liver and the subsequent inhibition of hepatic glucose production [i.e., the pillar of the so-called single-gateway hypothesis (Bergman et al., 1997)] is a rather slow process. It has been suggested that the delay in insulin suppression of lipolysis may be secondary to the delay in insulin reaching the interstitial fluids. Thus it is likely that insulin concentration in the interstitial fluids plays a crucial role also in regulating insulin's indirect inhibition of hepatic glucose production. Because the first-phase insulin response favors a more rapid elevation of insulin concentration in the interstitial fluids, as ascertained by Getty et al., it is plausible that insulin's indirect inhibition of hepatic glucose production is also activated more rapidly when the first-phase insulin response is present.

Effects of first-phase insulin response on the liver. Insulin has a direct inhibitory effect on hepatic glucose production. In dog studies, insulin's direct effect on hepatic glucose production has been found to be potent and more important that insulin's indirect effect, at least in nondiabetic animals (Cherrington et al., 1998). Lewis et al. (Lewis et al., 1996) have shown in humans that hepatic glucose production is inhibited to a greater extent with portal compared with peripheral insulin delivery, with

matched peripheral insulin levels. Maheux et al. (Maheux et al., 1997) have shown in humans that an increase in portal vein insulin concentration can rapidly inhibit hepatic glucose production independently of an increase in peripheral plasma insulin concentration. Thus, by acutely elevating liver sinusoidal insulin concentration, first-phase insulin secretion brings about a rapid inhibitory effect on hepatic glucose production. In addition, when the delayed version of the first phase reaches the interstitial fluids, the above-mentioned indirect effects of insulin will also become manifest and contribute to restrain hepatic glucose production. The relative importance and the precise timing of either the direct or indirect effects of first-phase insulin secretion on hepatic glucose production have not been fully elucidated yet, but experimental evidence has shown that the overall result on hepatic glucose production is remarkable and long lasting.

Insulin secretory defect

The reduced insulin secretion resulting from beta cell dysfunction is assumed to be the most direct and most important cause of clinical hyperglycemia (Weyer et al., 1999; Lillioja et al., 1988; Saad et al., 1989; Hansen et al., 1986; Reaven et al., 1989; Kahn et al., 2001; Bergman et al., 2002).

According to several recently conducted studies, the beta cell capacity is relatively low in Asian people compared to those of Western individuals (Yoon et al., 2003; Rhee et al., 2007; Rhee et al., 2007; Kim et al., 2001). It is therefore assumed that insulin secretory dysfunction may play a more important role in the progression of T2DM.

Studies of insulin responses in patients with type 2 diabetes and in those at high risk based on glucose challenge paradigms have documented various abnormalities in insulin secretion. For example, early (ie, 30 minutes) insulin responses during the oral glucose tolerance test (Pigon et al., 1996; Kahn, 2000; Porte, 1991) and first-phase insulin responses during intravenous infusions of glucose, (Pigon et al., 1996; Polonsky, 1999) or hyperglycemic clamp experiments, (Vaag et al., 1995) are reduced. In 1987, Nesher et al (Nesher et al., 1987) reported insulin secretion, as estimated by pancreatic responsiveness scores, was reduced by more than 80% in both lean and obese patients with type 2 diabetes. There is mounting evidence that insulin-secretory defects are the dominant problem among nonobese patients with diabetes. In a study of elderly men with diabetes, those who were lean exhibited only an insulin

secretory defect; in contrast, their obese counterparts displayed evidence of impaired insulin secretion and insulin resistance (Arner et al., 1991). In a recent study by Pigon et al, (Pigon et al., 1996) nonobese patients with type 2 diabetes had profound impairment in the secretion of insulin, a slight reduction in extrahepatic insulin sensitivity, and no evidence of impaired hepatic insulin sensitivity. To clarify further the relative contribution of these 2 abnormalities at different stages in the pathogenesis of type 2 diabetes, investigators have performed studies of the insulin-secretory capacity of glucose-tolerant individuals with a predisposing ethnicity or a family history of type 2 diabetes (Boden et al., 1999; Vaag et al., 1995; Byrne et al., 1996; Elbein et al.,1999; Elbein et al., 2000; Eriksson et al., 1989;Haffner et al., 1995; Henriksen et al., 1997; Pimenta et al., 1995; van Haeften et al.,1998; Weyer et al., 2001; Kahn et al., 2000). These studies showed that β-cell dysfunction, like insulin resistance, occurs in genetically predisposed individuals with normal glucose tolerance—well before the emergence of overt diabetes. (Boden et al., 1999; Vaag et al.,1995; Byrne et al., 1996; Elbein et al.. 1999; Elbein et al., 2000; Eriksson et al., 1989; Haffner et al., 1995; Henriksen et al., 1997; Pimenta, et al.,1995; Haeften et al., 1998; Weyer et al., 2001).

Though in the past three decades, the relevance of insulin secretion abnormalities in the pathogenesis of type 2 diabetes mellitus have been extensively debated (DeFronzo et al., 1988: Ferrannini et al., 1998; Polonsky et al., 1995; Porte et al., 1991: Pratley et al., 2001), a consensus has been reached that to fulfill its pivotal role in regulating glucose metabolism, insulin secretion must not only be quantitatively appropriate, but also possess qualitative, dynamic features that optimize insulin action on target tissues.

In particular, increasing emphasis has been placed on the importance of the so-called first-phase insulin secretion to glucose homeostasis (Cherrington et al., 2002; Del Prato et al., 2002; Henquin et al., 2002).

The earliest detectable defect in beta-cell function is commonly thought to be a reduction in first-phase insulin release (Cerasi, 1967; Luzi, 1989 and Lancet, 1994) while second-phase insulin release is also impaired, often by >50% (Van Haeften et al., 1991). Reductions in both phases of insulin release are equally early, that they precede insulin resistance other than that simply due to obesity and that they therefore

may represent the primary genetic risk factor predisposing individuals to type 2 diabetes (John and Gerich, 2002).

The diminution of the first phase can be detected in subjects with IGT and even in first degree relatives in type 2 diabetes mellitus, who are normoglycemic but at increased risk of developing the disease (Cerasi, 1972). For example, Pimenta et al (Pimenta et al., 1995) showed that both first- and second-phase insulin release, as assessed by hyperglycemic clamps, were significantly reduced among Caucasian first-degree relatives of patients with type 2 diabetes who had no insulin resistance compared with age-, sex-, and weight-matched controls. Interestingly, some of these individuals had impairments in both phases of insulin secretion, whereas others exhibited only first- or second-phase deficits. (Pimenta et al., 1995). These results were confirmed in a subsequent study by van Haeften et al, (van Haeften et al., 1998) who showed that second-phase insulin release (also assessed by hyperglycemic glucose clamps) was decreased among 21 Caucasian glucose-tolerant offspring of patients with type 2 diabetes compared with a well-matched control group. Of note, in both these studies, there were no significant differences with respect to insulin sensitivity between the predisposed subjects and the control groups (comprised of glucose tolerant subjects with no family history of type 2 diabetes), (Pimenta et al., 1995; van Haeften et al., 1998) suggesting that alterations in insulin secretion precede insulin resistance in patients at risk for developing type 2 diabetes.

In recent years, compelling evidence that impaired insulin secretion is the major genetic trait has been provided via the assessment of the degree of β-cell compensation to reduced insulin sensitivity among normal glucose-tolerant offspring or siblings of Caucasian familial type 2 diabetic kind reds (ie, families with at least 2 siblings diagnosed with type 2 diabetes before age 65 years). (Elbein et al., 1999; Elbein et al., 2000). For example, glucose-tolerant members of Caucasian familial type 2 diabetic kind reds (but not similarly obese control subjects) exhibit impairment in β-cell compensation for obesity related insulin resistance (Elbein et al., 1999).

Numerous animal and human studies had been done to elucidate the causes of β-cell dysfunction. Specific causes of β-cell dysfunction in patients with type 2 diabetes may include (Harris et al.,1998) an initial decrease in β-cell mass, (WHO Expert Committee, 2002) increased apoptosis/decreased regeneration (Kahn,1998), long-

standing insulin resistance leading to β-cell exhaustion (Rosenbloom et al.,1999), glucose toxicity-induced β-cell desensitization (Diabetes Control and Complications Trial Research Group, 1993), lipid toxicity to β cells, and [UK Prospective Diabetes Study (UKPDS) Group, 1998], amyloid deposition or other conditions with the ability to reduce β-cell mass (Kahn, 2000). Apoptosis of beta-cells is 3- to 10-fold more frequent in diabetic subjects than in weight-matched non-diabetic controls. Moreover, a 40% deficit in relative beta-cell volume has also been found in subjects with IFG (Butler et al., 2003), indicating that the loss of beta-cells is an early process in the pathogenesis of type 2 diabetes. The main mechanisms leading to increased beta-cell apoptosis include glucotoxicity, lipotoxicity, and deposits of islets amyloid polypeptide (IAPP). IAPP, which is co-secreted with insulin from beta-cells, exerts several physiological functions. Although IAPP is normally maintained in the form of soluble monomers, cytotoxic oligomers inducing apoptosis of beta-cells can also be formed. There are several possible mechanisms leading to the formation of oligomers (Haataja et al., 2008), including insulin resistance which disproportionately increases the expression of IAPP compared with insulin expression (Mulder et al., 1995; Bretherton-Watt et al., 1989). Gene variants may affect insulin secretion in type 2 diabetes through their effects on glucose-stimulated insulin release, incretin sensitivity or incretin secretion, proinsulin conversion, beta-cell proliferation, apoptosis etc.

Elevation of free fatty acids (FFAs) has been shown to promote proapoptotic effects on beta-cells (Unger et al., 1999), possibly as a result of endoplasmic reticulum stress (Cnop et al., 2005). Moreover, high levels of FFAs can also contribute to beta-cell dysfunction through the intracellular accumulation of triglycerides as a response to the activation of the sterol regulatory element binding proteins (SREBP) (Yamashita et al., 2004), or by increased expression of uncoupling protein 2 (UCP2), which regulates cellular ATP production (Joseph et al., 2004). The deleterious effects of FFAs are observed predominantly in the presence of high glucose.

Determinants of insulin secretory defect

Impaired proinsulin conversion as a marker of beta-cell dysfunction

The insulin gene is translated into proinsulin, a precursor of insulin. During insulin maturation, which takes place in the endoplasmic reticulum and Golgi apparatus of the beta-cells, proinsulin is cleaved by protein convertases 1 and 2 and

carboxypeptidase E into mature insulin molecule and C-peptide. Normally, only <10% of synthesised proinsulin escapes this process and gets into the circulation. Therefore, the increased proinsulin/insulin ratio reflects the efficiency of proinsulin conversion. Individuals with type 2 diabetes typically exhibit an elevated proinsulin to insulin ratio (Saad et al., 1990), which has been shown to correlate with decreased acute insulin response to glucose (Mykkanen et al., 1999). Moreover, hyperproinsulinemia has also been detected in individuals with pre-diabetes (Mykkannen et al., 1995; Hanley et al., 2002), suggesting that impaired conversion of proinsulin to insulin is an early event in the development of type 2 diabetes.

Hyperproinsulinemia is commonly present in type II diabetes (Porte, 1991; Leahy, 1990; Duckworth et al., 1972; Porte & Kahn, 1989; Temple et al., 1989; Mako & Rubenstein, 1977; Birkeland et al., 1994; Yoshioka et al., 1988; Saad et al., 1990; Reaven et al., 1993) and elevated proinsulin with proinsulin intermediates (collectively referred to as proinsulins) and proinsulin/immunoreactive insulin (P/IRI) ratios has been considered as a hallmarks of type 2 diabetes (Mako et al., 1977; et al., 1987; Yoshioka et al., 1988; Temple et al., 1989). Disproportionate hyperproinsulinemia has been advanced as an early marker for β cell dysfunction (Porte & Kahn, 1989). Literature provides support for the hypothesis that PI can be used as a sensitive marker of early β -cell dysfunction (Porte & Kahn et al., 1989). Although there is evidence that the plasma proinsulin to insulin ratio is higher in patients with noninsulin-dependent diabetes mellitus (NIDDM) and fasting hyperglycemia (Temple et al., 1989; Yoshioka et al., 1988; Saad et al., 1990; Reaven et al., 1993), the cause of this apparent abnormality in the processing of insulin within the ß-cell is not clear. A limited number of investigations have reported that elevated absolute and relative concentrations of PI and its split products are prospectively associated with risk of diabetes (Inoue et al., 1996; Nijpels et al., 1996; Mykkanen et al., 1995; Kahn et al., 1996; Haffner et al., 1997; Shin et al., 1997; Wareham et al., 1999). Recent studies have shown that PI and the PI-to-insulin ratio correlate significantly with acute insulin response (AIR) (Mykkanen et al., 1990–1995, 1997; Mykkanen et al., 1999). The causes of these secretory abnormalities are insufficiently clarified. In particular, it is unresolved whether conditions intrinsic to the β-cell are an important cause of elevated proinsulins and P/IRI ratios. The reasons for hyperproinsulinemia are, however, still unknown but several explanations have been

proposed (Hales et al., 1996). Hyperproinsulinemia could be due to a primary defect in the β cell, leading to increased secretion of incompletely processed insulin precursors, i.e. impaired proinsulin processing. However, the response of the normal β cell to increased demand has been hypothesized to result in enhanced processing of proinsulin into insulin (Porte & Kahn, 1989; Leahy et al., 1991). A positive relation has been demonstrated between the level of hyperglycemia and proinsulin in overt diabetes (Yoshioka et al., 1989; Davies et al., 1994; Clauson et al., 1997) but in some individuals, hyperproinsulinemia has been shown to be present without hyperglycemia (Haffner et al., 1995; Persson et al., 1991; Gelding et al., 1995; Kautzky-Willer et al., 1997).

Hyperproinsulinemia may be absolute, relative, or both. In simple obesity, there is a proportionate increase in proinsulin, and, therefore, only absolute hyperproinsulinemia is present (Shiraishi, et al., 991). In type II diabetes with fasting hyperglycemia, there is a disproportionate hyperproinsulinemia relative to insulinemia even in lean type II patients without absolute hyperinsulinemia or hyperproinsulinemia (Saad et al., 1990). Alternatively, other studies have suggested that hyperproinsulinemia is a secondary defect in subjects developing type II diabetes, and could be due to increased demand placed on the β cell by hyperglycemia and/or insulin resistance (Birkeland et al., 1994; Yoshioka et al., 1988; Haffner et al., 1994). Because insulin-resistant individuals often have hyperinsulinemia, it is not clear whether hyperproinsulinemia per se is a marker of β cell distress. Insulin resistance has, indeed, been associated with low proinsulin-to-insulin ratio in normoglycemic subjects (MykkaÈnen et al., 1990-1995), whereas the study by Wang et al. (Wang et al., 1997) could not found this association. One obvious explanation is that fasting hyperglycemia, or some metabolic defect associated with it, is responsible for this phenomenon. As such, the increase in the proinsulin to insulin ratio in plasma could be a manifestation of glucotoxicity and could contribute to the progressive decline in circulating insulin that is seen as glucose homeostasis deteriorates in patients with NIDDM (Reaven & Miller, 1968; Sicree et al., 1987; Saad et al., 1989; Haffner et al., 1990; Warram et al., 1990). Alternatively, the increased proinsulin to insulin ratio could simply be a function of an abnormal pancreatic ß-cell, stressed in its attempt to compensate for the resistance to insulin-mediated glucose disposal characteristic of patients with NIDDM (Ginsberg et al., 1975; Reaven et al., 1976; Reaven, 1983).

Although increased proinsulin concentrations are a characteristic feature of common type 2 diabetes (Duckworth & Kitabchi, 1972), there is convincing evidence based on studies in first degree relatives of patients with this disease that hyperproinsulinemia is not present before the deterioration of the glucose tolerance (Birkeland et al., 1994). Consequently, hyperproinsulinemia in 'common' type 2 diabetes has been considered as a secondary phenomenon due to hyperglycaemia-induced β-cell stress (Porte D Jr., 1991).

Previous studies aiming to investigate the role of hyperproinsulinemia as an early defect in type II diabetes have included individuals at high risk of developing type II diabetes, i.e. the first-degree relatives of patients with type II diabetes (Saad et al., 1990, Haffner et al., 1994; Gelding et al., 1995; Beer et al., 1990), had given contradictory results. These studies had shown that the first-degree relatives of type II diabetic patients have elevated proinsulin levels (Haffner et al., 1995; Gelding et al., 1995), whereas in one study it was observed that parental diabetes was related neither to fasting proinsulin levels nor to proinsulin-to-insulin ratio (Saad et al., 1990).
Therefore, disproportionately increased serum concentrations of proinsulin relative to insulin may be a surrogate marker of the failing pancreas (Porte & Khan, 1989). A phenomenon which has been reported in subjects with impaired glucose tolerance (Yoshioka et al., 1988).

Insulin resistance in type 2 diabetes mellitus

Physiology of insulin action

Insulin plays a central role in the control of the body's metabolism. It does so by accelerating glucose uptake into a number of tissues while simultaneously suppressing glucose production and lipolysis. Like many hormones, insulin exerts its actions through binding to specific receptors present on many cells of the body, including fat, liver, and muscle cells. The primary action of insulin is to stimulate glucose disappearance.

Plasma glucose concentration is a function of the rate of glucose entering the circulation (glucose appearance) balanced by the rate of glucose removal from the circulation (glucose disappearance). Circulating glucose is derived from three sources:

intestinal absorption during the fed state, glycogenolysis, and gluconeogenesis. The major determinant of how quickly glucose appears in the circulation during the fed state is the rate of gastric emptying. Other sources of circulating glucose are derived chiefly from hepatic processes: glycogenolysis, the breakdown of glycogen, the polymerized storage form of glucose; and gluconeogenesis, the formation of glucose primarily from lactate and amino acids during the fasting state. Insulin helps control postprandial glucose in three ways. Initially, insulin signals the cells of insulin-sensitive peripheral tissues, primarily skeletal muscle, to increase their uptake of glucose (Gerich et al., 1974). Secondly, insulin acts on the liver to promote glycogenesis. Finally, insulin simultaneously inhibits glucagon secretion from pancreatic α-cells, thus signalling the liver to stop producing glucose via glycogenolysis and gluconeogenesis. All of these actions reduce blood glucose (Cryer, 1992). Other actions of insulin include the stimulation of fat synthesis, promotion of triglyceride storage in fat cells, promotion of protein synthesis in the liver and muscle, and proliferation of cell growth (Cryer, 1992).

Mechanism of insulin resistance

Insulin exerts its biological functions by interacting with membrane-spanning insulin receptor (IR). Binding of insulin to IR elicits autophosphorylation of the IR, leading to binding of various scaffold proteins such as insulin receptor substrate (IRS) proteins. Phosphorylation of IRS proteins leads to their association with the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) and its activation. The subsequent steps, which involve PI3K-mediated actions of phopshoinositide-dependent protein kinase-1 (PDK1) and protein kinase B (PKB or Akt), lead to the facilitation of the translocation of glucose transporter-4 (GLUT4)-containing vesicles to the cell surface (Muoio et al., 2008). Finally, GLUT4 transports glucose into the cell. Other effects of insulin, such as its effects on glycogen synthesis, protein synthesis, lipogenesis, and supression of hepatic gluconeogenesis, are also mediated by PKB (Schinner et al., 2005). The main insulin-sensitive tissues are skeletal muscle (accounting for 60- 70% of whole-body glucose uptake), liver (30%) and adipose tissue (10%). Insulin resistance can be defined as the inability of these tissues to respond properly to normal circulating concentrations of insulin. To maintain euglycemia, the pancreas compensates by secreting increased amounts of insulin. However, elevated insulin levels can compensate for poor insulin action only for a

limited time. After a period of compensated insulin resistance, pre-diabetes or diabetes usually develops, especially in coexistence with impaired beta-cell function. An early appearance of insulin resistance in the natural history of diabetes was demonstrated by Vaag et al., (1992) who found that young healthy offspring of diabetic parents commonly exhibit insulin resistance and impaired skeletal muscle insulin signalling many decades before the onset of overt type 2 diabetes (Vaag et al., 1992). Although the mechanisms resulting in insulin resistance are largely unknown, multiple abnormalities in the insulin signalling pathway have been identified. The most important sites are IRS (-1 and -2), PI3K and PKB. Mutations in the IRS1 gene in humans are associated with insulin resistance (Whitehead et al., 1998), and IRS-2 knockout mice show insulin resistance in muscle, fat and liver, and develop diabetes resulting from beta-cell failure (Previs et al., 2000). Reduced activation of the PI3-kinase/Akt signalling pathway can lead to reduced glucose transport and utilisation in the skeletal muscle and adipocytes Giorgino et al., 2005). Several environmental and lifestyle factors also affect insulin sensitivity, such as nutritional factors in utero, diet, physical activity, smoking, drugs, and particularly obesity. Obesity, and especially visceral adiposity, is strongly associated with insulin resistance and type 2 diabetes.

Adipose tissue and insulin sensitivity

Adipose tissue can modulate whole body glucose metabolism by regulating the levels of circulating FFAs, and also by secreting adipokines, thereby acting as an endocrine organ (Schinner et al., 2005). Insulin-resistant visceral adipose tissue is resistant to the antilipolytic effects of insulin and releases excessive amounts of FFAs. Metabolic overload of the liver and muscle with FFAs causes mitochondrial dysfunction with impaired FFA oxidation. In the liver, impaired FFA oxidation leads to redirection of FFAs into lipid species localized in ER and cytoplasm, which promotes the development of hepatic steatosis and hepatic insulin resistance. In skeletal muscle, metabolic overload and physical inactivity lead to incomplete oxidation of FFAs, and lipid-derived intermediates accumulate in mitochondria, contributing to both mitochondrial stress and insulin resistance (Muoio et al., 2008). Moreover, elevated levels of plasma FFAs can increase insulin resistance also by affecting the insulin signalling cascade, particularly abolishing the insulin activation of IRS-1-associated PI3K-activity (Dresner et al., 1999). Adipokines secreted by the adipose tissue affect insulin sensitivity in either a positive (adiponectin, leptin, interleukin-10, etc.) or a

negative way (TNF α, resistin, interleukin-6, retinol binding protein 4, monocyte chemoattractant protein-1, plasminogen activator inhibitor-1 etc.). The best understood are the mechanisms whereby TNF α and adiponectin affect insulin sensitivity. TNF α is the main factor that triggers the secretion of FFAs from the adipose tissue into the circulation (Ruan et al., 2003). Furthermore, it mediates the repression of many genes responsible for glucose and FFA uptake and storage. The enhanced release of FFAs and cytokines as a result of TNF α action impairs insulin signalling in insulin responsive tissue, especially in skeletal muscle. TNF α has also been shown to downregulate the genes encoding adiponectin, GLUT4, IRS-1 etc. Adiponectin improves insulin sensitivity by various mechanisms, resulting in a decrease in plasma FFA and glucose levels. In the liver, adiponectin induces FFA oxidation, decreases lipid synthesis, decreases uptake of FFA and represses gluconeogenesis. In skeletal muscle, adiponectin increases glucose and FFA oxidation. Adiponectin also suppresses the secretion of TNF α (Mlinar et al., 2007).

Liver and insulin sensitivity

Although the liver accounts for only 30% of the whole-body glucose metabolism, hepatic insulin resistance plays an important role in the pathogenesis of type 2 diabetes. In the insulin-resistant liver, insulin does not inhibit sufficiently two key enzymes of gluconeogenesis (phosphoenolpyruvate carboxykinase and glucose-6-phosphatase catalytic subunit). Consequently, this leads to increased hepatic glucose production in the fasting state and to fasting hyperglycemia.

Hepatic glucose production

Increased hepatic glucose production resulting from unsuppressed gluconeogenesis and enhanced glycogenolysis in hepatocytes is an important mechanism contributing to hyperglycemia in type 2 diabetes. Hepatic glucose production is regulated by insulin and glucagon, which have opposite effects. Insulin suppresses both gluconeogenesis and glycogenolysis, and increased glucose hepatic output is therefore a consequence of insulin resistance in the liver. In contrast, glucagon stimulates gluconeogenesis and glycogenolysis, and increased glucagon secretion or enhanced hepatic glucagon sensitivity can contribute to the dysregulation of glucose production (Burcelin et al., 2008).

Insulin resistance is associated with a cluster of metabolic abnormalities known collectively as the insulin resistance syndrome, the metabolic syndrome, or syndrome X. (Bloomgarden, 1998; Groop, 1999; Lebovitz & Banerji, 2001; Reaven, 1999; Whitelaw & Gilbey 1998). Recently, the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) published criteria to identify the syndrome according to the following major components: abdominal obesity, hypertension, dyslipidemia, and abnormal glucose tolerance (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001). This syndrome has also been linked to a markedly increased risk for cardiovascular disease (Bloomgarden, 1998; Groop, 1999; Lebovitz & Banerji, 2001; Reaven, 1999; Whitelaw & Gilbey 1998). Insulin resistance impedes glucose disposal and disrupts lipid metabolism in insulin-sensitive tissues, particularly muscle, the liver, and adipose tissue (Gerich, 1988; American Diabetes Association, 1998; Kahn & Flier 2000; Reusch, 1998; Saltiel, 2001). In muscle, the condition manifests as inefficient glucose transport with subsequent impaired uptake, oxidation, and storage (as glycogen). (Gerich, 1988; American Diabetes Association, 1998; Kahn & Flier 2000; Saltiel, 2001). In the liver, insulin resistance reduces postprandial glucose storage and the suppression of glycogenolysis and gluconeogenesis in the fasting and postprandial states (Saltiel, 2001; Reusch, 1998). The ability of insulin to inhibit lipolysis in adipose tissue is also impaired (Gerich, 1988; 2002; Reusch, 1998; Saltiel, 2001).

Insulin Resistance in Patients with Type 2 Diabetes Mellitus and High-Risk Populations

Insulin resistance is a common condition. Data from the Insulin Resistance Atherosclerosis Study indicate that more than 90% of obese Caucasians, African Americans, and Hispanic Americans with type 2 diabetes could be classified as insulin insensitive (Haffner et al., 1997). Studies in populations at high risk for developing diabetes (eg, obese American Indians) have shown that insulin resistance is detectable in the earliest stages of deterioration of glucose tolerance and in obese glucose-tolerant individuals at high risk for developing diabetes later in life (Lillioja et al., 1988-1992; Martin et al., 1992; Gulli et al., 1992; Warram et al., 1990). The Pima Indians of Arizona, who are obese and have a disproportionately high incidence of type 2 diabetes, have served as subjects in several studies of insulin resistance and

insulin secretion during the prediabetic phase (Lillioja et al., 1988-1992). A prospective study that followed up 200 obese Pima Indians for a mean of 5.3 years found that insulin resistance was a strong predictor of type 2 diabetes (Lillioja et al., 1988-1992). Evidence of insulin resistance in conjunction with hyperinsulinemia also has been identified more than a decade before the onset of diabetes in the obese offspring of individuals with type 2 diabetes (Martin et al., 1992; Gulli et al., 1992; Warram et al., 1990). Insulin resistance in this setting, as in the Pima Indian population, (Lillioja et al., 1988-1992) significantly increased the risk of developing diabetes (Martin et al., 1992; Warram et al., 1990). In a study of 155 individuals whose parents both had type 2 diabetes, the 25-year incidence of type 2 diabetes was 76% among obese individuals with insulin resistance (Martin et al., 1992). Although these studies show the importance of insulin resistance as a risk factor for developing type 2 diabetes, they have been criticized for not properly assessing β-cell function and for not distinguishing between obesity and genetics as a cause of the insulin resistance (Gerich, 1998; Kahn, 2001).

Genetic and Environmental Mechanisms of Insulin Resistance

Genetic Influences.—The studies of high-risk populations imply that insulin resistance, like type 2 diabetes itself, has a genetic component and that heritable differences in insulin sensitivity may be one element of a "susceptibility genotype" predisposing members of high-risk populations to type 2 diabetes (Stern, 2000; Vaag, 1999). Additional evidence of genetic effects is derived from twin studies that provide estimates ranging from 47% to 66% for the heritability of insulin resistance (Stern, 2000). Whether this heritability depends on obesity is unclear. Insulin, like other hormones, exerts its effects by binding to specific cell surface receptors, initiating a cascade of intracellular reactions that ultimately lead to changes in glucose transport, glycogen and lipid synthesis, and gene expression (Alper, 2000). Genetic mutations in the insulin receptor have been identified, but these are rare causes of diabetes (Saltiel, 2001; Kahn et al., 2000). Because type 2 diabetes is a polygenic disorder, inheritable influences on type 2 diabetes are likely to involve alterations of several genes (Ferrannini 1998). To date, no genetic defect has been found in patients with typical type 2 diabetes that might cause their diabetes to be due solely to insulin resistance (McIntyre et al., 2002). Initial reports of polymorphisms involving calpain-10 and the peroxisome proliferator–activated receptor γ2 Pro12Ala had suggested that these may,

respectively, increase and decrease the risk for developing type 2 diabetes by modifying insulin sensitivity (Horikawa et al. 2000; Altshuler et al., 2000). However, subsequent studies suggested an overall small effect on the risk for type 2 diabetes, inconsistent results across different ethnic groups, and, more importantly, that alterations in β-cell function rather than insulin sensitivity may be involved (McIntyre et al., 2002; Stumvoll et al., 2002; Cox, 2001).

Environmental Factors: Focus on Obesity: Intraabdominal obesity, a consequence of a modern high-fat, high-energy diet and sedentary lifestyle, is strongly correlated with insulin resistance (Kahn, 2000; Bergman et al., 2000; Boyko et al., 2000; Fujimoto, 2000; Banerji et al., 1995). Central adiposity appears to be a major determinant of insulin resistance not only in obese individuals but also in apparently healthy nonobese individuals who have evidence of increased abdominal fat (Banerji et al., 1995; Carey et al., 1996). In obese patients with type 2 diabetes, weight loss has been shown to normalize insulin sensitivity completely, (Bak et al., 1992; Beck-Nielsen et al., 1979). increase insulin-stimulated glycogen synthase activity, (Bak et al., 1992) and reverse defective insulin receptor kinase activity (Freidenberg et al., 1988). Moreover, it can prevent the progression to type 2 diabetes in high-risk populations, including patients more in obese individuals with type 2 diabetes. Moreover, inverse correlations have been found with plasma triglycerides, postprandial plasma glucose levels, and insulin sensitivity (Hotta et al., 2000; Weyer et al., 2001). Weight loss and treatment with thiazolidinediones increased plasma adiponectin in animals (Berg et al., 2001) and humans (Maeda et al., 2001). Administration of adiponectin to obese or diabetic mice reduces food intake, tissue triglycerides, and plasma glucose levels, while it increases insulin sensitivity and muscle FFA oxidation (Berg et al., 2001; Maeda et al., 2001; Yamauchi et al., 2001). Furthermore, evidence shows that adiponectin may have antiatherogenic properties, (Tsao et al., 2002) thus providing a link between obesity, insulin resistance, and cardiovascular disease (Grundy, 2002).

Several acquired factors for the development of insulin resistance among nondiabetic, nonobese individuals have been identified, including decreased physical activity, (Nyholm et al., 1994) high-fat diets, (Swinburn, 1993) medications (eg, thiazide diuretics, protease inhibitors, corticosteroids, niacin), (Pandit et al., 1993) and glucose toxicity (Yki-Jarvinen, 1992).

Glucagon, resistin, and hs-CRP as determinants of insulin resistance

Glucagon

Glucagon is a key catabolic hormone consisting of 29 amino acids. It is secreted from pancreatic α-cells. Described by Roger Unger in the 1950s, glucagon was characterized as opposing the effects of insulin (Unger, 1971). Glucagon plays a major role in sustaining plasma glucose during fasting conditions by stimulating hepatic glucose production.

Insulin and glucagon are the key regulatory hormones for glucose homeostasis. Glucose homeostasis is regulated primarily by the opposing actions of insulin and glucagon, hormones that are secreted by pancreatic islets from β-cells and α-cells, respectively. Insulin secretion is increased in response to elevated blood glucose to maintain normoglycemia by stimulating glucose transport in muscle and adipocytes and reducing glucose production by inhibiting gluconeogenesis in the liver. Whereas glucagon secretion is suppressed by hyperglycemia, it is stimulated during hypoglycemia, promoting hepatic glucose production and ultimately raising blood glucose levels. The absolute levels and, even more so, the ratios of the two hormones are tightly regulated in vivo, depending on nutritional status.

Unger was the first to describe the diabetic state as a "bi-hormonal" disease characterized by insulin deficiency and glucagon excess. He further speculated that a therapy targeting the correction of glucagon excess would offer an important advancement in the treatment of diabetes (Unger, 1971). Hepatic glucose production, which is primarily regulated by glucagon, maintains basal blood glucose concentrations within a normal range during the fasting state. When plasma glucose falls below the normal range, glucagon secretion increases, resulting in hepatic glucose production and return of plasma glucose to the normal range (Orci et al., 1975; Gerich et al., 1979). This endogenous source of glucose is not needed during and immediately following a meal, and glucagon secretion is suppressed. When coupled with insulin's direct effect on the liver, glucagon suppression results in a near-total suppression of hepatic glucose output.

In the diabetic state, there is inadequate suppression of postprandial glucagon secretion (hyperglucagonemia) (Cryer, 1981; Dinneen et al., 1995) resulting in

elevated hepatic glucose production. Importantly, exogenously administered insulin is unable both to restore normal postprandial insulin concentrations in the portal vein and to suppress glucagon secretion through a paracrine effect. This results in an abnormally high glucagon-to-insulin ratio that favors the release of hepatic glucose (Baron et al., 1987). These limits of exogenously administered insulin therapy are well documented in individuals with type 1 or type 2 diabetes and are considered to be important contributors to the postprandial hyperglycemic state characteristic of diabetes. Diabetic hyperglycemia occurs as the result of insufficient insulin secretion from the β-cells and/or lack of insulin action due to peripheral insulin resistance. In normal animal and human subjects, the levels of insulin increase immediately after a meal, whereas the levels of glucagon decrease. Insulin is a physiological suppressor of glucagon secretion; however, at the cellular and molecular levels, how intra islet insulin exerts its suppressive effect on the α-cells is not very clear. In type 2 diabetic subjects, however, the postprandial secretion of insulin is delayed and depressed, whereas that of glucagon is not suppressed or is even elevated (Jiang and Zhang et al, 2003; Basu et al, 1996; Butler et al, 1991; Larsson et al, 2000 and Mitrakou et al, 1990).Such abnormality in insulin and glucagon secretion is associated with and predictive of glucose intolerance in type 2 diabetic human subjects (Ahren et al, 2001 and Larsson et al, 2000) and excessive secretion of glucagon from the α-cells is also a major contributor to the development of diabetic hyperglycemia. The cause-and-effect relationship between hyperglucagonemia and hyperglycemia is strongly implied in studies showing that suppression of postprandial hyperglucagonemia corrects postprandial hyperglycemia in type 2 diabetic subjects (Shah et al, 2000). Impaired suppression of glucagon release can be detected in individuals with impaired glucose tolerance (IGT) and type 2 diabetes following intravenous (Aronoff et al, 1997) or oral glucose administration (Mitrakou et al, 1992). Fasting glucagon concentrations exert a tonic stimulatory influence on hepatic glucose output, which in the dog has been estimated to account for one-third of the fasting rate of glucose release (Cherrington et al, 1997). Thus, under fasting conditions hyperglucagonemia sustains glucose overproduction (Cherrington et al, 1997) and impaired glucagon suppression after oral glucose or a mixed meal contributes to the postprandial hyperglycemia of type 2 diabetes (Mitrakou A et al, 1992).A lack of suppression of hyperglucagonemia has also been shown to contribute to postprandial glucose intolerance in type 1 diabetes (Dinneens et al, 1995) Although hyperglucagonemia results in glucose

intolerance in diabetic subjects with impaired insulin secretion or in normal subjects whose insulin secretion is experimentally blocked, it does not produce the same effects when insulin secretion is in tact (i.e., in normal healthy subjects) (Shah et al, 1999; Sherwin et al, 1976; Toft et al, 2002). Insulin appears to exert ongoing release inhibition upon glucagon secretion, probably via the intra-islet microvascular system that connects beta cells to alpha cells. Diabetic hyperglucagonemia in insulin deficient states appears to be secondary to lack of the restraining influence of insulin. The alpha cell response to glucopenia, by contrast, may be in large part mediated by release of noradrenaline from nerve endings in contact with alpha cells. Glucagon's action on glucose and ketone production by hepatocytes is mediated by increase in cyclic-AMP-dependent protein kinase. The opposing action of insulin upon glucagon-mediated events probably occurs largely at this level. Consequently, when glucagon secretion or action is blocked, cyclic-AMP-dependent protein kinase activity is low even in the absence of insulin, explaining why marked glucose and ketone production is absent in bi-hormonal deficiency states. Although the inhibitory effect of insulin on glucagon gene expression is an important means to regulate glucagon secretion, recent studies suggest that the underlying mechanisms of the intra islet insulin on suppression of glucagon secretion involve the modulation of K_{ATP} channel activity and the activation of the GABA-GABA receptor system. Nevertheless, regulation of glucagon secretion is multifactorial and yet to be fully understood. Not surprisingly, glucagon and glucagon receptor have been pursued extensively in recent years as potential targets for the therapeutic treatment of diabetes. But it is controversial whether the number of glucagon receptors is altered in diabetic states. Most studies, however, appear to suggest that the number of glucagon receptors is reduced in diabetic subjects. Interestingly, even in the presence of fewer glucagon receptors, the ability of glucagon to stimulate cAMP production may remain unchanged or even be elevated (Burcelin et al, 1996).This may be at least partially explained by the observation that the activation of adenylate cyclase by glucagon involves only 20% of glucagon receptors (Birnbaumer *et* al, 1972). In addition to the epigenetic effects of hyperglucagonemia on hyperglycemia, genetic polymorphism of the glucagon receptor has been reported to be associated with type 2 diabetes. A single heterozygous missense mutation in exon 2 of the glucagon receptor gene that changes a glycine to a serine ($Gly^{40}Ser$) has been found to be associated with type 2 diabetes in some French populations. The mutant receptor was shown to have a reduced affinity to bind to glucagon and to

produce cAMP in response to glucagon stimulation (Hansen et al, 1996).The significance of such a mutation in diabetes is likely to be limited, since it is not associated with diabetes in most other studies in various populations (Huang et al, 1999; Shiota et al, 2002).

Resistin

Inflammation is hypothesized to play a role in development in type 2 diabetes mellitus. Inflammation may play a crucial intermediary role in pathogenesis, thereby linking diabetes with a number of commonly coexisting conditions thought to originate through inflammatory mechanisms. Chronic subclinical inflammation is a component of the Insulin Resistance, or Metabolic Syndrome in which cardiovascular risk factors including dyslipidaemia, endothelial dysfunction, hypertension, obesity and Type 2 diabetes often occur in a cluster.

It has recently been identified that adipose tissue, in addition to its role as an energy reservoir, modulates energy metabolism via secretion of circulating adipocytokines. Thus adipose tissue plays an important role in insulin resistance through the dysregulated production and secretion of adipose-derived proteins, including TNF-α, Plasminogen activator inhibitor-1, leptin, angiotensinogen, adiponectin and resistin (Saltiel, 2001; Shuldiner et al., 2001; Goldfine and Kahn, 2002). Of adipocytokines, resistin appear to be important in regulating insulin sensitivity and insulin secretion and thus very important factor to type 2 diabetes.

Resistin has been proposed as an adipocyte-secreted factor that is thought to link obesity and type 2 diabetes, insulin resistance and hyperglycemia (Steppan et al., 2001). The relationship between these hormone and insulin sensitivity suggests that they may take part in the development of insulin resistance of type 2 diabetes (Hui-Ling et al., 2006).

There is more serum resistin protein in obese than lean individuals, with a significant positive correlation between resistin and BMI. BMI is a significant predictor of insulin resistance, but resistin adjusted for BMI is not. Resistin is present in human adipose tissue and in blood and that there is significantly more resistin in the serum of obese individuals. Serum resistin is not a significant predictor of insulin resistance in

humans (Youn et al., 2004; Rea and Donnelly, 2004). Steppan and his coinvestigators have subsequently proposed that resistin is increased in type 2 diabetes and suggested that it is a potential link between obesity and insulin resistance (Steppan et al, 2001). Nagaev suggest that resistin is not expressed in human primary adipocytes but it is present in immune cells which are found in subcutaneous WAT in obesity (Nagev et al, 2006). Resistin is expressed at very low levels, if at all, in human adipose cells, whereas high levels are expressed in mononuclear leukocytes, macrophages, spleen, and bone marrow cells (Patel et al., 2003, Nagaev et al., 2001, Lu et al., 2002). Low levels of resistin are also expressed in lung tissue, resting endothelial cells, and in placenta (Patel *et al,* 2003). However, no difference in resistin expression in adipocytes and myocytes was found between nondiabetic vs type 2 diabetic subjects (Nagaev, Smith. 2001, Lee et al., 2005), although circulating levels of resistin in these groups were different. The injection of recombinant resistin into mice reduces glucose tolerance and insulin action, whereas neutralization with anti-resistin antibodies improves insulin action (Mcternan et al., 2003). Obesity induced by a high-fat diet is associated with increased circulating resistin concentrations. Resistin increases blood glucose and insulin concentrations in mice and impairs hypoglycemic response to insulin infusion. In addition, anti-resistin antibodies decrease blood glucose and improve insulin sensitivity in obese mice (Ukkola et al., 2002). Resistin suppresses insulin-stimulated glucose uptake in cultured 3T3-L1 adipocytes, and this effect is prevented by anti-resistin antibodies. Thus resistin induces insulin resistance and that hyperresistinemia contributes to impaired insulin sensitivity in obese rodents (Shuldiner et al., 2001). However, other data do not confirm these results. (Way et al., 2001; Moore et al., 2001 and et al., 2001).

hs-CRP

Different molecules may be associated with inflammation. One such molecule is C-reactive protein (CRP) which is important for the regulation of the inflammatory process. Recently, different studies have confirmed that serum CRP concentration, other inflammation-sensitive plasma proteins and the acute-phase reaction have been significantly associated with clinical and biochemical indexes of insulin resistance, thus predict the development of diabetes mellitus and metabolic syndrome (Thorand *et al*, 2003; Han et al., 2002; Festa et al., 2002; Visser et al., 1999; Ford *et al*, 1999;

Leinonen et al., 2003; Chan et al., 2002; Laimer et al., 1992; Grimble et al., 2002). Previous research has established that CRP levels are higher in people with diabetes (Ford et al., 1999) and it is associated with higher HbA1c in people without diabetes (Wu et al., 2002). CRP is known to be higher also in people with impaired glucose tolerance (Ford et al., 1999; Wu et al., 2002). Furthermore, significantly higher serum concentration of CRP levels have been found to be a risk factor for later development of diabetes (McMillan et al., 1989; Pickup et al., 1997; Schmidt et al., 1999; Festa *et al*, 2000; Pradhan et al., 2001).

In these disorders, increased levels of inflammation mediators such as C-reactive protein and the cytokine series, mainly tumor necrosis factor a (TNF-a) and interleukin 6 (IL-6) (Mohamed-Ali et al., 1997; Mohamed-Ali et al., 1998; Basard et al., 1999 and Maachi et al., 2004), have been revealed.. The acute-phase protein including CRP, are mostly synthesized in the liver, and production is stimulated by cytokines of the innate immune response-mainly IL-6 and tumor necrosis factor (TNF)-α and other cytokines (Pickup et al., *2004*).

Although the detailed etiology of the Insulin Resistance Syndrome is unclear, prospective studies have found high CRP levels to be predictive of the development of insulin resistance, the Insulin Resistance Syndrome and Type 2 diabetes (Han et al., 2002; Festa et al., 2002). Furthermore, cardiovascular morbidity and mortality is increased in patients with elevated CRP levels (Ridker et al., 2003. This suggests that chronic inflammation may be an underlying cause of both atherosclerosis and insulin resistance. Consequently, treatments with anti-inflammatory properties may also reduce the incidence of macrovascular complications associated with insulin-resistant states such as Type 2 diabetes.

Development of insulin secretory defect & insulin resistance and role of inheritance

Studies on prediabetes

There have been numerous studies reporting baseline data on nomotolerant individuals who subsequently developed type 2 diabetes. Many of these have been epidemiological in that they compared, within a certain population, the baseline characteristics of individuals who did or did not subsequently become diabetic. These

studies have provided evidence that both impaired insulin secretion and insulin resistance are risk factors for development of type 2 diabetes (Lillioja et al., 1993; Haffner et al., 1995; Eriksson & Lindgarde, 1996; Charles et al., 1991; Sicree et al., 1987). However, they do not directly address the issue of which of these factors precedes the other and which is genetic because often those who subsequently developed diabetes were more obese or less physically active than those who did not develop diabetes (Lillioja et al., 1993; Eriksson & Lindgarde, 1996; Sicree et al., 1987).

Pre-diabetes is a common disorder in most populations. (Dunstan et al., 2004 Tapp et al., 2004; Ko et al., 1998; Cowie et al., 2006). The reported prevalence of pre-diabetes varies widely from study to study, in part because of evolving definitions of pre-diabetes but also because IFG and IGT appear to vary among populations with different ethnic backgrounds (Dunstan et al., 2004; Tapp et al., 2004; Ko et al., 1998; Cowie et al., 2006). Recent data from the USA indicate that the prevalence of IFG is ~26% and that of IGT is ~15% in the adult population (Cowie et al., 2006). Both IFG and IGT increase in prevalence with age (Shaw et al., 1999). The prevalence of IFG is similar in men and women, but IGT is more frequent in women (Shaw et al., 1999). Although there is some overlap between IFG and IGT, most studies have shown that these criteria define different populations at risk for type 2 diabetes and other complications (e.g. CVD) (Dunstan et al., 2004; Tapp et al., 2004; Ko et al., 1998; Cowie et al., 2006; Shaw et al., 1999). To clarify the pathophysiology of pre-diabetes, several studies have examined insulin resistance and insulin secretion in subjects with isolated IFG and isolated IGT (Weyer et al., 1999; Abdul et al., 2006). Both isolated IFG and isolated IGT are characterized by insulin resistance and impairment in insulin secretion. However, there are some differences in the nature of the defects between the two conditions. For example, individuals with isolated IFG manifest hepatic insulin resistance, but have relatively normal skeletal muscle insulin sensitivity. In contrast, those with isolated IGT are characterised by more severe muscle insulin resistance and less severe hepatic insulin resistance. Differences in insulin secretory abnormalities are also apparent between subjects with isolated IFG and isolated IGT. Whereas those with isolated IFG have defects in first-phase or early insulin secretion (in proportion to their fasting hyperglycaemia), individuals with isolated IGT have more severe defects in second-phase or late insulin secretion. As

might be expected, individuals with combined IFG/IGT manifest both hepatic and muscle insulin resistance as well as impairments in both first and second phase insulin secretion. Among subjects with pre-diabetes, those with combined IFG/IGT most closely resemble subjects with type 2 diabetes (Weyer et al., 1999; Abdul et al., 2006).

Many investigators have studied the role of insulin resistance and impaired insulin secretion in the pathogenesis of IFG and IGT. However, these studies have yielded contradictory results (Carnevale et al., 2003; Vaccaro et al., 2005; Qiau et al., 2002; Qiao et al., 2002; Haffner, 1998; Faerch et al., 2008; Laakso et al., 2008; Meyeret al., 2006; Abdul-Ghani et al., 2006; Weyer et al., 1999; Festa et al., 2004; Festa et al., 2008; Hong et al., 2008; van Haeften et al., 2002; Hanefeld et al., 2003; Kim et al., 2001; Davies et al., 2000; Melchionda et al., 2002; Snehalatha et al., 2003; Tripathy et al., 2000). Most of the studies using the clamp method to assess insulin sensitivity in different categories of glucose tolerance have shown impairment of peripheral insulin sensitivity in IGT, whereas subjects with IFG have been shown to have normal or impaired (similarly as in IGT) peripheral insulin sensitivity (Faerch et al., 2008; Laakso et al., 2008; Meyer et al., 2006; Abdul-Ghani et al., 2006; Weyer et al., 1999; Festa et al., 2004). When insulin resistance was assessed using HOMA-IR (describing mainly hepatic insulin resistance), several studies (Abdul-Ghani et al., 2006; Hanefeld et al., 2003; Melchionda et al., 2002; Tripathy et al., 2000) reported increased insulin resistance in subjects with IFG, whereas others did not (Faerch et al., 2008; Kim et al., 2001; Davies et al., 2000). Studies on insulin secretion disturbances in IFG and IGT have reported conflicting results. While some studies applying the intravenous glucose tolerance test (IVGTT) or clamp methods to assess insulin secretion have reported impaired first-phase insulin secretion only in IFG but not IGT (Faerch et al., 2008; Laakso et al., 2008; Weyer et al., 1999; Hong et al., 2008), other studies have reported impaired first- and also second-phase insulin secretion in isolated IGT (Meyer et al., 2006; van Haeften et al., 2002). Studies assessing insulin secretion on the basis of fasting glucose and insulin levels or OGTT have reported even more controversial results, reporting impaired insulin secretion in IFG (Davies et al., 2000; Snehalatha et al., 2003), in IGT (Hanefeld et al., 2003; Tripathy et al., 2000), or in both (Kim et al., 2001; Melchionda et al.,2002). Inconsistencies across these studies could be explained by differences in study populations, study designs and methods to assess insulin sensitivity and insulin secretion, and most importantly by a small

sample size. The categorization of glucose tolerance is based on arbitrary cut-off points of glucose levels, and therefore different subgroups cannot fully account for changes in beta-cell function and insulin action when glycemia increases. Only a few studies have examined insulin secretion and/or insulin sensitivity as a function of glucose concentrations (Abdul-Ghani et al., 2006; Weyer et al., 1999; Ferrannini et al., 2005; Gastaldelli et al., 2004; Abdul-Ghani et al., 2008; Ahrén, 2007; Godsland et al,, 2004).

In addition to insulin resistance and insulin secretory defects, various biomarkers may also be of value to better define which subjects will develop diabetes. For example, high adiponectin levels (an adipokine with antidiabetic, anti-inflammatory, and anti-atherogenic effects) which reflects better insulin sensitivity were associated with decreased risk for developing type 2 diabetes in subjects with IGT in the Hoorn Study (Snijder et al., 2006).

In a study of the Pima Indians (Lillioja et al., 1988), longitudinal data were given for 24 individuals who developed IGT. Although not analyzed, the baseline acute insulin response of these individuals (IVGTT) was less (190 *vs.* 220) than that of a control group while their glucose infusion rate (3.3 mg/kg/min) during a hyperinsulinemic clamp was comparable to that of the control group (3.8 mg/kg/min). Since the subjects that became diabetic were more obese (BMI 38 *vs.* 32 Kg/M^2), the small reduction in their glucose infusion rates could be attributable to their greater obesity. Moreover the fact that their acute insulin responses were not greater than that of the control group, despite their greater obesity, suggests that their β-cell function may have been impaired. These two studies (Lillioja et al., 1988; Savage et al., 1975) are widely cited as supporting genetically determined insulin resistance as the initial factor predisposing to type 2 diabetes but the evidence is equivocal.

The report of Martin et al. (Martin et al., 1992) represents the same data reported by Warram et al. (Warram et al., 1990), the observations of this study actually are consistent with the concept that superimposition of the insulin resistance of obesity upon a genetically impaired capacity to secret insulin is a common sequence leading to type 2 diabetes.

Chen et al. (Chen et al., 1995) reported baseline data on 23 individuals who developed type 2 diabetes and compared them to 144 individuals who remained nondiabetic. Those who developed type 2 diabetes initially had impaired early insulin release during an OGTT. Unfortunately, about half of each group had IGT at baseline. Thus, it is difficult to use these data to distinguish between a genetic cause and one due to glucose toxicity in explaining the reduced insulin secretion.

Taken together, these prospective studies indicate that both impaired insulin release and insulin resistance are risk factors for development of type 2 diabetes and that each can, and usually does, precede type 2 diabetes. However, they do not provide unassailable evidence that either the impaired insulin secretion or the insulin resistance necessarily has a genetic basis.

In recent years, several prospective studies, in which nondiabetic individuals are metabolically characterized on a single occasion and then followed for several years to determine who develops diabetes, have helped to identify metabolic abnormalities that predispose to diabetes. These studies have shown that obesity (Wilson et al., 1981, Knowler et al., 1990, Ohlson et al., 1985, Saad et al., 1991, Charles et al., 1991, Chen et al., 1995, Lundgren et al., 1990) and insulin resistance (Lillioja et al., 1993, Martin et al., 1992, Warram et al., 1990, Saad et al., 1991, Charles et al., 1991, Chen et al., 1995) predict the development of diabetes in many populations, whereas basal EGO was not predictive in the only study in which it was measured (Lillioja et al., 1993). A low early insulin response predicted diabetes in most (Lillioja et al., 1993, Chen et al., 1995, Nagi et al., 1995, Warram et al., 1996, Efendic et al., 1984, Lundgren et al., 1990) but not all (Martin et al., 1992, Warram et al., 1990) studies. According to several studies conducted with Caucasian and Hispanic subjects, reduced insulin secretion is assumed to begin in patients who can be categorized as having normal glucose tolerance (NGT) on the basis of current diagnostic criteria (Gastaldelli et al., 2004; Godsland et al., 2004; Tirosh et al., 2005). This indicates that beta cell dysfunction progresses, causing abnormal glucose metabolism, prior to the 'prediabetes' period of the current criteria.

In healthy young subjects, the absence of first-phase insulin release impairs glucose tolerance (Calles-Escandon & Robbins, 1987) and first-phase insulin appears to

regulate hepatic glucose production but not insulin-mediated glucose disposal (Luzi & DeFronzo, 1989). Although first-phase insulin release is absent in type 2 diabetes (DeFronzo et al., 1992), the significance of this metabolic defect has not been fully investigated (Bruce et al., 1988). It is recently demonstrated that elderly obese patients with type 2 diabetes have absent first-phase insulin release (Meneilly et al., 1996) but intact second-phase insulin release. These patients represent an ideal biological model in which to study the physiological role of first-phase insulin. The decrease in first-phase insulin secretion after intravenous glucose in patients with mild abnormalities of glucose tolerance was reported well before the WHO introduced the definition of IGT. (Cerasi et al., 1972). The concept received further support from studies of people with impaired glucose tolerance (IGT), a precursor of type 2 diabetes, showing that these individuals generally had reduced plasma insulin levels at 30 min after glucose ingestion and "normal or increased" plasma insulin levels at 120 min (Gerich et al., 1997). The assumption has been generally made that the 30-min response reflected first-phase insulin release, whereas the 120-min response reflected second-phase insulin release. Because insulin released early after glucose ingestion has been shown to be a key determinant of subsequent plasma glucose responses (Mitrakou et al., 1992; Calles-Escandon et al., 1987), it became widely accepted that reduced first-phase insulin release is responsible for the development of IGT. Long-term follow-up studies of patients with IGT have demonstrated conversion from IGT to type 2 diabetes in more than 50% of cases. Thus, IGT should be considered as an "at-high-risk" state for further development of type 2 diabetes. Most of the studies performed in patients with IGT disclosed abolition or a decrease in first-phase insulin secretion. (Ericksson et al., 1989; Yoneda et al., 1992; Davies et al., 1994). Conflicting results have also been published. Discrepancies are mostly related to an insufficient accounting for the degree of associated insulin resistance, though metabolic heterogeneity of the population of patients with IGT cannot be ruled out. However, most of the available data indicate that IGT shares the same pattern of alterations in insulin secretion than type 2 diabetes. The same holds true for IFG, as reported a long time ago by Brunzell et al., (1976) in a study of patients with differencing plasma glucose levels. First-phase insulin secretion was abolished as soon as fasting plasma glucose levels were in excess of 1.15 g/L (6.4 mmol/L). In a study with NGT and IGT subjects, hyperglycemic clamp experiments was done and it has been shown that both first- and second-phase insulin release were reduced in

people with IGT, as was insulin sensitivity. First-phase insulin release was reduced by ~35% and second-phase insulin release was reduced by ~28%, whereas insulin sensitivity was reduced by ~15%, it appears that the decrement in β-cell function was greater than that in insulin sensitivity. (Curry et al., 1968; Cerasi et al., 1967; Brunzell et al., 1976; Kahn et al., 1969). It should be noted, however, that the so-called "normal or increased" 120-min plasma insulin levels may not have been appropriate for the prevailing glycemic stimulus (Perley et al., 1967). Thus, if these late insulin responses reflect second-phase insulin release, this phase of insulin secretion may also have been reduced. Longitudinal studies have shown that the transition from normal glucose tolerance (NGT) to diabetes is associated with a progressive deterioration in early insulin secretion. In a study in Pima Indians progressing to IGT during a mean 5.1- year follow-up were compared with NGT subjects. Progression to IGT was accompanied by a 27% reduction in acute insulin response (AIR) and by a further 51% reduction in AIR during progression from IGT to diabetes. These data emphasize the pivotal role of early phase insulin secretion in preventing the development of type 2 diabetes in patients with deleterious environmental conditions. They also indicate that preservation of early phase insulin secretion is a rational target for this prevention. (Pratley & Weyer 2001)

Studies on first degree relatives of type 2 diabetes

First-degree relatives (FDRs) of diabetic patients should be quite helpful in reconstructing the natural history of the disease (Ericksson et al., 1989; Johnston et al., 1990; Gulli et al., 1992; Pimenta et al., 1995; Perseghin et al., 1997; Van Haeften et al., 1998; Laakso et al., 1998; Nyholm et al., 1999; Zonderland et al., 2000).

Over the past 30 yr, there have been more than 60 studies of insulin secretion and/or insulin sensitivity in normal glucose-tolerant individuals who were the first-degree relative of someone with type 2 diabetes (Pimenta et al., 1995; Cerasi, 1995; Warram et al., 1990; Byrne et al., 1996; O'Rahilly et al., 1988; Eriksson et al., 1989; Vaag et al., 1995; Kosaka et al., 1977; Laws et al., 1989; Ho et al., 1989; Ramachandran et al., 1990; Johnston et al., 1990; Osei et al., 1991; Elbein et al., 1991;. Henriksen et al., 1994; Lemieux et al., 1992; Gulli et al., 1992; Gelding et al., 1994; Armstrong et al., 1966; Taylor et al., 1967; Cerasi& Luft,1963; Clark et al., 1992; Vaag & Henriksen,

1992; Roder et al., 1993; Birkeland K et al., 1994; Nyholm et al., 1994; Schmitz et al., 1997; Migdalis et al., 1996; Fernandez-Castaner et al., 1996; Leslie et al., 1988; Handberg et al., 1993; Schalin-Jantti et al., 1992; Gelding et al., 1995; Eriksson et al., 1992; Vauhkonen et al., 1998; Henriksen et al., 1997; Van Haeften et al., 1998).

Normotolerant first-degree relatives of type 2 diabetic subjects (FDR) are an interesting model for the identification of early abnormalities of glucose homeostasis, which may be crucial to understanding the development of glucose intolerance. A high-risk state for type 2 diabetes is a history of type 2 diabetes in a first-degree relative. A risk of about 30% has been calculated in families of European origin, and similar, or even higher rates, have reported in other populations. Unfortunately, in most of these studies insulin secretion and insulin resistance were not simultaneously assessed. Furthermore, in most of the early studies, the methods used to evaluate insulin secretion or insulin sensitivity would not be considered to be state of the art by present standards. Finally and most importantly, in many studies subjects were usually not well matched for acquired factors such as age, gender, and obesity that are known to influence insulin secretion and insulin sensitivity. Although not all reports are in agreement, FDR subjects are frequently insulin resistant and exhibit β-cell function abnormalities (Ferrannini, 1998; Gerich, 1998; Van Haeften, 2002; Bonadonna et al., 2003.). During the past 10 years, as part of a multinational collaborative project involving centers in the U.S., Finland, Norway, Greece, and Italy, have been investigating β-cell function and insulin sensitivity in nondiabetic individuals with and without a first-degree relative with type 2 diabetes (Pimenta at el., 1995; Van Haeften at al., 2000; Van Haeften at el., 1998). These individuals were all Caucasian and had either normal or impaired glucose tolerance according to World Health Organization criteria.

In one of the earliest studies, Rojas et al. (Rojas et al., 1969) examined plasma insulin responses to intravenous glucose in control volunteers and NGT offspring of two diabetic parents who were carefully matched for age, gender, and obesity. It was found that glucose-stimulated insulin release was decreased in the NGT offspring. Warram et al., (1990) subsequently analyzed the data of these and additional offspring of two diabetic parents using the minimal model approach of Bergman et al. (Bergman et al., 1987; Bergman et al., 1981). Initial results of those who subsequently

either had or had not developed diabetes were compared. In this population, already demonstrated to have reduced β-cell function, presumably on a genetic basis, it was found that those who subsequently developed diabetes had been insulin resistant when they were still NGT, whereas those who did not develop diabetes had not been insulin resistant. It was concluded that insulin resistance was a risk factor for development of type 2 diabetes. This study is often cited in the literature as providing evidence that insulin resistance is the main genetic factor for type 2 diabetes. However, since the group who subsequently developed diabetes were markedly obese compared with the group that did not develop diabetes (*i.e.*, 140 *vs.* 106% ideal body weight), it is possible that the insulin resistance was simply the result of obesity. Indeed, comparison of the minimal model parameters of insulin secretion and insulin sensitivity in this group with those of similarly obese individuals having no family history of diabetes reported by Bergman et al. (Bergman et al., 1987; Bergman et al., 1981) provides evidence that the subjects studied by Warram et al., (1990) were no more insulin resistant than these individuals but had reduced first-phase insulin release. In subjects who are first-degree relatives of patients with type 2 diabetes, impairment of first-phase insulin secretion has been reported by various studies (O'Rahilly et al., 1986; Pimenta et al., 1995). Longitudinal studies, evaluating both early-phase insulin secretion and insulin sensitivity, have shown that both defects can predict the development of overt diabetes. (Lundgren et al., 1990; Weyer et al.,1999). The study of Warram et al. (Warram et al., 1990) showed that people with a genetic predisposition to impaired insulin secretion develop diabetes when acquired insulin resistance (due to obesity) is superimposed and exceeds the ability of the β-cell to compensate for it.

The study of Gulli et al., (1992), which examined plasma insulin responses during hyperglycemic clamp experiments in nondiabetic offspring of two type 2 diabetic parents with other nondiabetic Mexican-American subjects, is also often cited as finding insulin resistance without impaired insulin secretion.

It was found that people with a first-degree relative with type 2 diabetes had reduced B-cell function (but no insulin resistance) and that some had reductions in only first-phase insulin release; some had reductions only in second-phase insulin release,

whereas others had reductions in both phases of insulin release. The results are found to be consistent with genetic heterogeneity for impaired B-cell function.

Individuals with a first-degree relative with type 2 diabetes already have reductions in first- and second-phase insulin release while having no change in insulin sensitivity. This finding strongly suggests that impaired B-cell function precedes insulin resistance in those with a genetic predisposition to develop type 2 diabetes and thus that impaired B-cell function is the primary defect for type 2 diabetes.

To further assess the genetic influence on B-cell function and insulin sensitivity, the influence of family history of diabetes as well as other factors, such as age, sex, body weight, BMI, and waist-to-hip ratio, were examined using multiple linear regressions. It was found that first-phase and second-phase were correlated with family history and BMI, the latter probably reflecting the influence of insulin resistance on B-cell function. First- and second-phase insulin release only differed in that age was negatively correlated with second-phase release. In contrast, insulin sensitivity was not correlated with a family history of diabetes but was with BMI and waist-to-hip ratio. These observations did not provide evidence for a major genetic influence on the insulin resistance with type 2 diabetes but do suggest that a major factor is excess body weight and its distribution (Bouchard, 1995).

In an earlier study of subjects with normal glucose tolerance, differing only in whether they had a first-degree relative with type 2 diabetes (Pimenta at el., 1995), they also found that people with a first-degree relative with type 2 diabetes had reduced β-cell function (but no insulin resistance) and that some had reductions in only first-phase insulin release; some had reductions only in second-phase insulin release, whereas others had reductions in both phases of insulin release. They interpreted these results to be consistent with genetic heterogeneity for impaired β-cell function.

Special features of diabetes in Bangladesh and regional countries

Although the classical criteria like the age of diabetes onset, obesity, family history of diabetes, urinary ketosis and autoimmunity context allowed the classification of the major types of diabetes (type I and type II) in developing countries, a number of patients, based on these criteria, in under-developing tropical countries could not be

classified (Ahuja 1965; Mohan & Alberti 1992; West, 1980). Alberti (1993) has considered these parameters on the definition of type 2 diabetes relative to the exclusion of other types of diabetes. However, the problem in Africa and South Asia seems to be much more complex since intermediate forms of diabetes have been described in young and adult patients with normal weight and great variability in ketosis and insulin requirement (Ahren & Corrigan, 1985). Indeed, the term used for the classification of diabetes had considerably changed during the course of time. The first widely accepted classification of diabetes mellitus was published by WHO in 1980 (WHO Expert Committee on Diabetes Mellitus, 1980) and, in modified form, in 1985 (WHO Study Group, 1985). The 1980 and 1985 classifications of diabetes mellitus and allied categories of glucose intolerance included clinical classes and two statistical risk classes. The 1980 WHO Expert Committee proposed two major classes of diabetes mellitus and named them, IDDM or Type 1, and NIDDM or Type 2. In the 1985 WHO Study Group Report, the terms Type 1 and Type 2 were omitted, but the classes IDDM and NIDDM were retained, and a class of Malnutrition-Related Diabetes Mellitus (MRDM) was introduced. At that time, these particular forms of diabetes in tropical countries have been designated as 'tropical diabetes' though its characteristics remain largely unclear (Ahuja 1965; McMillan & Geevarghese, 1979; Nwokolo & Oli, 1980; Rao, 1983). Since the main pathogenic factor is malnutrition, this kind of diabetes has been then termed as Malnutrition Related Diabetes Mellitus (MRDM). The WHO Study Group proposed that MRDM should be classified as a separate sub-class of diabetes, as up to 80% of the cases of diabetes seen in some tropical countries belong to MRDM (Ekoe, 1985; Abu-Bakare et al., 1986). Various reports have previously tried to define the characteristics of this form of diabetes. It has been suggested that MRDM might be related to malnutrition alone or combination with cassava consumption and cyanide intoxication (Rao, 1983; WHO Study Group, 1985; Ekoe, 1985). However, it was unclear whether MRDM should be considered as a distinct clinical entity, though many epidemiological studies had failed to establish unequivocally the correlation ship between its prevalence and cassava consumption or protein malnutrition (Abu-Bakare et al., 1986; Teuscher et al., 1987; Akanji, 1990; Akanji et al., 1990).

An international workshop reviewed the evidence for, and characteristics of, diabetes mellitus seen in undernourished populations (Hoet et al., 1996; Tripathy & Samal,

1997). Whilst it appears that malnutrition may influence the expression of several types of diabetes, the evidence that diabetes can be caused by malnutrition or protein deficiency per se is not convincing. Therefore, it is recommended that the class 'Malnutrition-related diabetes' (MRDM) be deleted. The former subtype of MRDM, Protein-deficient Pancreatic Diabetes (PDPD or PDDM), may be considered as a malnutrition modulated or modified form of diabetes mellitus for which more studies are needed. The other former subtype of MRDM, Fibrocalculous Pancreatic Diabetes (FCPD), is now classified as a disease of the exocrine pancreas, fibrocalculous pancreatopathy, which may lead to diabetes mellitus.

In Bangladesh, there is a different group of diabetic patients, who are young, non obese, normotensive, hypo–to normoinsulinemic and generally normolipidemic. The clinical presentation of the patients is almost similar to that of Type 2 diabetes. They are usually within normal BMI range (taking 19 as the normal cut–point of normal BMI) and they do not present with ketoacidosis in spite of moderate to severe degree of hyperglycemia. These types of DM were more frequent in the 80's and 90's, but with improvement of nutritional situation and economic growth they are becoming rarer. Rather young diabetics with BMI <23 (non obese range) are becoming more common.

Rationale

The present study, designed to investigate the relative roles of insulin deficiency vs insulin resistance in the pathogenesis of type 2 diabetes mellitus, and characterization of the insulin secretory defect was based on the availability of a considerable number of young diabetic patients in our country. Many of these young patients have a number of characteristics, which make them especially suitable for investigating the relative roles of insulin secretion and insulin resistance in the natural history of type 2 diabetes. Many of these young diabetic rarely present with obesity and their body weight falls within normal to subnormal ranges. Most of these young diabetes subjects are found to be normotensive and normolipidemic. Preliminary results of antibody (ICA, IAA and GAD) measurements point against autoimmune involvement in most of these patients, however possibility of a modified Type I like diabetes mellitus in some of the patients can not be ruled out. As per present ADA or WHO

Criteria. These patients can be grouped as Type 2 diabetes mellitus patients about the real grouping of these subjects require many more studies on characterization.

In the context of the availability of such a group we have undertaken the present study to characterize the insulin secretory defect in these patients and to explore the coexistence and interrelationship of these defects with insulin resistance. This will help to better understand the etiopathogenesis of Type 2 diabetes mellitus in this population. Apart from the basic scientific point of view, the measurement of insulin secretory capacity and insulin resistance in various groups of patients in our country has got practical relevance in designing appropriate management and prevention policies. In the light of the considerable degree of heterogeneity regarding both insulin secretion and insulin sensitivity, it is important to measures those two physiological parameters in different racial and environmental situations. Although, the present investigation covers only a section diabetic subjects in our population it may give important methodological and technical tools for further studies in this direction.

Research Hypothesis

***The following hypotheses were made*:**

1) Insulin secretory deficiency is the predominant basic defect for young onset DM in Bangladeshi population and this defect is coexistent with insulin resistance.
2) First phase of insulin secretion is more effected in young onset DM compared to 2nd phase secretion;
3) Age, BMI, proinsulin, glucagon, and hs-CRP (as a marker of chronic subclinical inflammation) are equally strong determinants of insulin secretory dysfunction and insulin resistance in Bangladeshi T2DM subjects;
4) IGT in Bangladeshi population does not differ from young onset DM regarding insulin secretion, sensitivity, phases of insulin secretion and their determinants.
5) Both insulin secretory defect and insulin resistance in young onset DM have hereditary component.

OBJECTIVES

CHAPTER II

General Objective:

The general objective of the study was to characterize the insulin secretory deficiency in young onset T2DM in relation to insulin resistance and various determinants of insulin secretion and sensitivity.

Specific Objectives:

The series of studies were undertaken with the following objectives;

1. To investigate the insulin secretory deficiency and insulin resistance in young T2DM subjects;
2. To investigate which phase of insulin secretion is more affected in young onset DM;
3. To explore whether age, BMI, proinsulin, glucagon and hs-CRP are associated with insulin secretory dysfunction and in insulin resistance in young onset T2DM;
4. To compare IGT and young onset T2DM regarding the contribution of basic defects of diabetes and the determinants of those defects; and
5. To find out whether insulin secretory defect and insulin resistance in young onset T2DM have any hereditary component;

SUBJECTS AND METHODS

CHAPTER III

SUBJECTS AND METHODS

Place of the studies

The studies were conducted in the Department of Biochemistry & Cell Biology of the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Dhaka.

Study period

This study was done during the period of January 2003 to December 2010.

Study design

All studies were conducted under an observational group comparison design.

Sampling technique

A purposive sampling technique was followed in all the studies.

Study Series

To test the 6 hypotheses the following series of studies were conducted.

For Hypothesis 1

Study 1: Insulin deficiency and insulin resistance in young and nonobese Bangladeshi diabetic subjects

Study 3: Insulin resistance and insulin secretory defect in young to middle aged Bangladeshi Type 2 diabetic subjects

For Hypothesis 2

Study 2: Phase-wise insulin secretion in young and nonobese Bangladeshi diabetic subjects

Study 4: Phase-wise insulin secretion in middle aged Bangladeshi type 2 diabetic patients

For Hypothesis 3

Study 5: Relationship of serum proinsulin and glucagon levels in subjects with type 2 diabetes mellitus

Study 6: Association of serum resistin with hs-CRP and insulin sensitivity in type 2 diabetes mellitus subjects.

For Hypothesis 4

Study 7: Insulin deficiency and insulin resistance in Bangladeshi IGT subjects

Study 8: Serum proinsulin status in IGT subjects

For Hypothesis 5

Study 9: Insulin deficiency and insulin resistance and phase-wise insulin secretion in first degree relatives of young and nonobese Bangladeshi type 2 diabetic patients

Study subjects

Study 1

25 Diabetic and 10 Control subjects (aged between 15-30 years, BMI< 23) were included.

Study 2

42 T2DM subjects and 43 Control subjects (aged between 15-30 years, BMI< 23) were included.

Study 3

726 Diabetic and 317 Control subjects (aged between 30-55 years, BMI> 18) were included.

Study 4

44 Diabetic and 30 Control subjects (aged between 30-55 years, BMI>18) were included.

Study 5

44 T2DM subjects and 44 Control subjects (aged between 30-55 years, BMI>18) were included.

Study 6

104 T2DM subjects and 33 Control subjects (aged between 30-55 years, BMI>18) were included.

Study 7

46 IGT and 39 Control subjects (aged between 30-55 years, BMI>18) were included.

For Study 8

50 IGT Diabetic and 44 Control subjects (aged between 30-55 years, BMI>18) were included.

For Study 9

29 first degree relatives of T2DM subjects and 33 Control subjects (aged between 30-55 years, BMI>18) were included.

Exclusion Criteria

In all studies, the following subjects were excluded:

- Patients with serious co-morbid diseases and complications of DM
- Pregnant women

Recruitment of subjects

All patients were recruited from the Outpatient Department of BIRDEM.

Subjects were diagnosed as T2DM and IGT using WHO Expert Committee Guidelines(R

Preparation of the subjects

After selection, the purpose of the study was explained in details to each subject and informed consent was taken from each of them. Subjects were requested to fast overnight (12 hours) and were advised not to take any kind of medicine or smoke on

the previous day. The patients were then requested to attend the Biomedical Research Group laboratory of BIRDEM on the next morning.

History and clinical examination

Detailed socio-demographic data were recorded in a pre-designed data collection form. (Appendix XII)

METHODS

Anthropometric measurements

Anthropometric parameters including height (m), body weight (kg), waist circumference (cm), hip circumference (cm), mid arm circumference were measured using standardized techniques. Body mass indexes (BMI) of the subjects were calculated using standard formula. BMI= Weight (kg)/ [Height (m)] ^{2}

Recording of blood pressure

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

Collection of blood samples

Fasting blood was collected between 8.00-9.00 am. Venous blood (05 ml) was taken with the subject sitting comfortably in a chair. Then the patient was given 75 g of glucose in 300 ml of water. Patient was advised not to smoke, not to take any food and to take rest. Then the blood sample was collected 30 min and 120min after glucose to determine blood glucose level and also insulin level in fasting and 30 min blood sample. Blood samples were kept in glass test tubes sealed with parafilm. After 10-15 minutes blood sample was centrifuged for 10 minutes at 3000 rpm to obtain serum. All serum was frozen at – 70^{0}C until analysis. Fasting serum glucose, triglyceride, total cholesterol, HDL cholesterol, were measured in the same day.

Analytical techniques

- Glucose (fasting) was measured by Glucose Oxidase (GOD-PAP) method (Randox Laboratories Ltd., UK) (Appendix I).
- Total cholesterol was measured by enzymatic endpoint method (Cholesterol Oxidase/ Peroxidase) method (Randox Laboratories Ltd., UK) (Appendix II).
- Triglyceride was measured enzymatic colorimetric (GPO-PAP) method (Randox Laboratories Ltd., UK) (Appendix III).
- HDL cholesterol was measured enzymatic colorimetric (cholesterol CHOD-PAP) method (Randox Laboratories Ltd., UK) (Appendix IV).
- Low Density Lipoprotein (LDL) Cholesterol was calculated by using Friedewald's formula (Appendix V).
- Serum insulin was estimated by the Enzyme Linked Immunusorbant Assay (ELISA) (LINCO Research Kit, USA). (Appendix VI)
- Fasting serum C-peptide was assessed by Chemiluminescence Technique using Chemiluminescence enzyme immunoassay (Immulite, UK) (Appendix VII)
- Serum proinsulin was estimated by the Enzyme Linked Immunosorbent Assay (ELISA) method (LINCO Research Kit, USA) (Appendix VIII)
- Serum glucagon was estimated by the Enzyme Immuno Assay (EIA) method (Peninsula Laboratories, LLC-A Member of the Bachem Group, USA) (Appendix IX)
- Serum Resistin measured by enzyme linked immunosorbent assay (ELISA) method (Linco Research Inc., USA). (Appendix X)
- Serum hs-CRP measured by immunonephelometry (BN System; USA) (Appendix XI)

Assessment of insulin secretory capacity

Insulin secretory capacity were assessed by Glucagon Stimulation Test and Homeostasis Model Assessment

Glucagon Stimulation Test

Insulin secretory capacity of the patient was measured by Glucagon Stimulation Test (Faber & Binder 1977). Glucagon was given intravenously at a bolus dose of 1g/subject and blood sample for glucose, lipids and C-peptide were drawn at 0 and 6 minutes.

Calculation of HOMA%B

HOMA B % was calculated from fasting blood glucose (mmol/l) and fasting Serum insulin (pmol/l) values by homeostasis model assessment (HOMA) using HOMA-CIGMA software (Levy, Mathews & Hermans, 1998).

Estimation of 1st and 2nd Phases of insulin secretion

After a 10-h overnight fast, subjects ingested a solution containing 75g of dextrose, and venous blood samples were obtained at 0, 30, 60, 90 and 120 min. for determination of serum glucose and serum insulin.

1st and 2nd phases of insulin secretion will be estimated by the following equations:

1st $phase_{est}$ $=1283+1.829.Ins_{30}-138.7.Glu_{30}+3.772.Ins_0$

2nd $phase_{est}$ $= 287+0.4164.Ins_{30}-26.07.Glu_{30}+0.9226.Ins_0$ [22]

Assessment of insulin sensitivity

Insulin sensitivity was assessed by Short insulin tolerance test and Homeostasis Model Assessment.

Short Insulin Tolerance Test

Insulin sensitivity was measured by Short Insulin Tolerance Test (Akinmokun et al., 1992). Arterial serum glucose level was measured during 3 to 15 minutes after a bolus injection of insulin (0.05 U/kg of body weight). Insulin sensitivity was derived from the linear slope of the blood glucose concentration and expressed as mole/l glucose fall per minute.

Calculation of HOMA%S

HOMA S % was calculated from fasting blood glucose (mmol/l) and fasting Serum insulin (pmol/l) values by homeostasis model assessment (HOMA) using HOMA-CIGMA software (Levy, Mathews & Hermans, 1998).

Statistical Analysis

Data were expressed as mean ±SD and/or median (range) where appropriate. Comparison between two groups was done using Students't' test (unpaired) or Mann-Whitney 'U' test as apropriate. Bivariatte correlation analysis was done by using Pearson's or Spearman's Correlation analysis. Multivariate analysis (multiple regressions) was done with appropriate outcome and independent variables. Data were managed and statistical analyses were performed using Statistical Package for Social Science (SPSS) for Windows version 11.5. A p value <0.05 was taken as level of significance

RESULTS

CHAPTER IV

RESULTS

Study1: Insulin deficiency and insulin resistance in young and non-obese Bangladeshi diabetic subjects

Clinical & biochemical features of young and nonobese diabetic subjects (Table 1)

Age (years)

Mean (±SD) value of age of Control and T2DM subjects was 25± 3 and 25± 3 respectively. The value did not show any statistically significant difference (p=0.380) (Table 1).

Table 1: Clinical features of the young and nonobese diabetic subjects

Variable	**Control** (n=10)	**T2DM** (n=25)	**t/p values**
Age (years)	25± 3	25± 3	0.36/0.72
BMI (kg/m^2)	21±2	19±3	1.44/0.15
WHR	0.88±0.05	0.86±0.07	0.69/0.49
SBP (mm Hg)	111±8	109±11	0.14/0.88
DBP (mm Hg)	71±5	71±8	0.39/0.69
T Chol (mg/dl)	123±24	142±34	4.48/0.042
TG (mg/dl)	131±94	114±46	2.74/0.107
HDL- C (mg/dl)	29±6	26.58±7	0.54/0.58

Results are expressed as mean±SD. Significance of difference was calculated by unpaired student's t- tests. n = numbers of subjects; BMI, Body mass index; WHR, Waist hip ratio; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; T chol, Total cholesterol; TG, Triglycerides; HDL chol, high density lipoprotien cholesterol; LDL chol, low density lipoprotien cholesterol . $p<0.05$

BMI (kg/m^2)

Mean (±SD) value of BMI of Control and T2DM subjects was 21±2 and 19±3 respectively. The value did not show any statistically significant difference (p=0.72) (Table 1).

WHR

Mean (±SD) value of WHR of Control and T2DM subjects was 0.88±0.05 and 0.86±0.07 respectively. The value did not show any statistically significant difference (p=0.49) (Table 1).

Systolic blood pressure (mm Hg)

Mean (±SD) value of SBP (mm Hg) of Control and T2DM subjects was 111±8 and 109±11 respectively. The value did not show any statistically significant difference (p=0.88) (Table 1).

Diastolic blood pressure (mm Hg)

Mean (±SD) value of DBP (mm Hg) of Control and T2DM subjects was 71±5 and 71±8 respectively. The value did not show any statistically significant difference (p=0.69) (Table 1).

Total Cholesterol (mg/dl)

Mean (±SD) value of T cholesterol (mg/dl) of Control and T2DM subjects was 123±24 and respectively 142±34. The value showed statistically significant difference (p=0.042) (Table 1).

Triglyceride (mg/dl)

Mean (±SD) value of TG (mg/dl) of Control and T2DM subjects was 131±94 and 114±46 respectively. The value did not show any statistically significant difference (p=0.107) (Table 1).

HDL cholesterol (mg/dl)

Mean (±SD) value of HDL-C (mg/dl) of Control and T2DM subjects was 29±6 and 26.58±7 respectively. The value did not show any statistically significant difference (p=0.58) (Table 1).

Fasting and Glucagon stimulated blood glucose and C-peptide levels young and non-obese diabetic subjects (Table 2)

Fasting glucose (mmol /l)

Mean (±SD) value of fasting serum glucose of Control and T2DM subjects were 4.67±0.73 and 13.45±4.25 respectively. The value showed statistically significant difference (p=0.001) (Table 2).

Glucagon stimulated glucose (mmol /l)

Mean (±SD) value of glucagon stimulated glucose of Control and T2DM subjects were 5.82±1.16 and 14.64±5.60 respectively. The value showed statistically significant difference (p=0.001) (Table 2.

Fasting C-peptide (ng/ml)

Mean (±SD) value of fasting C peptide of Control and T2DM subjects were 2.01±0.60 and 1.02±0.85 respectively. The value showed statistically significant difference (p=0.001) (Table 2).

Glucagon stimulated C-peptide (ng/ml)

Mean (±SD) value of Glucagon stimulated C peptide of Control and T2DM subjects were 5.4±1.61 and 2.07±1.86 respectively. The value showed statistically significant difference (p=0.001) (Table 2).

Fasting C peptide and fasting glucose ratio

Mean (±SD) value of FC-pep: FGl of Control and T2DM subjects were 0.46±0.23 and 0.09±0.09 respectively. The value showed statistically significant difference (p=0.001) (Table 2).

Glucagon stimulated C peptide and Glucagon stimulated glucose ratio (Table2)

Mean (±SD) value of GSC-pep: GSGl of Control and T2DM subjects were 0.96±0.27and 0.19±0.20 respectively. The value showed statistically significant difference (p=0.001)

Table 2: Fasting and Glucagon stimulated blood glucose and C-peptide levels in the young and nonobese diabetic subjects during glucagon stimulation test

Variable	**Control** (n=10)	**T2DM** (n=25)	**t/p values**
F Glucose (mmol/l)	4.67±0.73	13.45±4.25	9.94/0.001
GS Glucose (mmol/l)	5.82±1.16	14.64±5.60	7.43/0.001
F C-pep (ng/ml)	2.01±0.60	1.02±0.85	23.66/0.001
GS C-pep (ng/ml)	5.4±1.61	2.07±1.86	19.16/0.001
FC-pep: FGl	0.46±0.23	0.09±0.09	10.18/0.001
GSC-pep: GSGl	0.96±0.27	0.19±0.20	12.97/0.001

Results are expressed as mean±SD. Significance of difference was calculated by unpaired student's t- tests. n= numbers of subjects; F Glucose, Fasting glucose; F C-pep, Fasting C-peptide; GS Glucose, Glucagon stimulated glucose; GS C-pep, Glucagon stimulated C-peptide.

Fasting insulin and insulin sensitivity of the study subjects by short insulin tolerance test (Table 3)

Fasting insulin (mU/l)

Mean (±SD) value of fasting insulin of Control and T2DM subjects were 17.29±14.81 and 9.26±7.91 respectively. The value showed statistically significant difference (p=0.044) (Table 3).

Fasting insulin and fasting glucose ratio

Mean (±SD) value of F Ins: FGl of Control and T2DM subjects were 4.13±3.49 and 0.82±.080 respectively. The value showed statistically significant difference (p=0.015) (Table 3).

Kitt

Mean (±SD) value of Kitt of Control and T2DM subjects were 0.068±0.01 and 0.067±0.02 respectively. The value did not show statistically significant difference (p=0.912) (Table 3).

Table 3: Fasting insulin and insulin sensitivity of young and nonobese diabetic subjects by short insulin tolerance test

Variable	**Control** (n=10)	**T2DM** (n=25)	**t/p values**
F Insulin (mU/l)	17.29±14.81	9.26±7.91	11.11/0.044
FGl (mmol/l)	4.26±0.60	13.52±5.7	7.98/0.0001
FIns: FGl	4.13±3.49	0.82±.080	9.38/0.015
Kitt	0.068±0.01	0.067±0.02	0.33/0.912

Results are expressed as mean±SD. Unpaired student's t-tests was performed n= numbers of subjects; F Insulin, Fasting insulin

Study 2: Phase-wise insulin secretion in young and nonobese Bangladeshi diabetic subjects

Clinical & biochemical features of the non obese diabetic subjects (Table 4)

Age, BMI, Waist hip ratio, Diastolic Blood pressure, Systolic Blood pressure, Triglyceride, Total Cholesterol and LDL cholesterol level of Control and T2DM subjects were not significantly different. (Table 4)

HDL cholesterol (mg/dl)

Median (range) value of HDL cholesterol of Control and T2DM subjects were 26 (20-39) and 31 (12-39) respectively. The value showed statistically significant difference (p=0.009) (Table 4).

Fasting glucose (mmol/l)

Median (range) value of fasting glucose of Control and T2DM subjects were 4.7 (3.5-6.5) and 7 (3.8-20) respectively. The value showed statistically significant difference (p=0.001) (Table 4).

30 min glucose (mmol/l)

Median (range) value of 30 min glucose of Control and T2DM subjects were 8.8 (3.7-15.4) and 13 (4-30) respectively. The value showed statistically significant difference (p=0.001) (Table 4).

120 min glucose (mmol/l)

Median (range) value of 120 min glucose of Control and T2DM subjects were 5.5 (3.9-11.5) and 16 (4-28) respectively. The value showed statistically significant difference (p=0.001) (Table 4).

Table 4: Clinical & biochemical features of nonobese young diabetic subjects

Variable	Control (n=43)	T2DM (n=42)	t/p values
Age (years)	26±2	27±2	1.15/0.251
BMI (kg/m^2)	19±3	18±3	1.15/0.252
WHR	0.8±0.07	0.9±0.06	1.88/0.063
DBP (mm Hg)	74±7	75±7	1.88/0.063
SBP (mm Hg)	114±7	111±11	1.30/0.194
TG (mg/dl)	139 (60-399)	146 (55-540)	0.65/0.510
T Chol (mg/dl)	166 (74-400)	179 (135-270)	1.58/0.113
HDL-C (mg/dl)	26 (20-39)	31 (12-39)	2.60/.009
LDL-C (mg/dl)	107 (12-334)	107 (53-223)	0.02/0.979
F glucose (mmol/l)	4.7 (3.5-6.5)	7 (3.8-20)	5.47/0.001
30 min glucose (mmol/l)	8.8 (3.7-15.4)	13 (4-30)	3.94/0.001
120 min glucose (mmol/l)	5.5 (3.9-11.5)	16 (4-28)	6.48/0.001

Results are expressed as mean±SD & median (range). Significance of difference was calculated by unpaired student's t- tests & Mann –Whitney U test as the test of significance at 5% significance level. n= numbers of subjects; BMI, Body mass index; WHR, Waist hip ratio; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; T chol, Total cholesterol; TG, Triglycerides; HDL-C, high density lipoprotien cholesterol; LDL-C, low density lipoprotien cholesterol; F glucose, fasting glucose; $p<0.05$

Insulinemic status, phase-wise insulin secretion of non obese diabetic subjects (Table 5)

Fasting insulin (pmol/l)

Median (range) value of fasting insulin of Control and T2DM subjects were 88 (33-275) and 96 (14-414) respectively. The value did not show any statistically significant difference (p=0.507) (Table 5).

30 min insulin (pmol/l)

Median (range) value of 30 min insulin of Control and T2DM subjects were 612 (151-1791) and 297 (55-945) respectively. The value showed statistically significant difference (p=0.001) (Table 5).

Table 5: Insulinemic status and phase-wise insulin secretion nonobese diabetic subjects

Variable	Control (n=43)	T2DM (n=42)	t/p values
F Insulin (pmol/l)	88 (33-275)	96 (14-414)	0.66/0.507
30 min Insulin (pmol/l)	612(151-1791)	297 (55-945)	4.50/0.001
1st Phase insulin secretion	1700 (291-3936)	593 (-2023-2347)	4.54/0.001
2nd Phase insulin secretion	444 (143-937)	203 (-287-626)	4.49/0.001

Results are expressed as median (range). Significance of difference was calculated by Mann –Whitney U test as the test of significance at 5% significance level. n= numbers of subjects; F Insulin, fasting insulin. $p<0.05$

1st Phase insulin secretion

Median (range) value of 1st Phase insulin secretion of Control and T2DM subjects were 1700 (291-3936) and 593 (-2023-2347) respectively. The value showed statistically significant difference (p=0.001) (Table 5).

2nd Phase insulin secretion

Median (range) value of 2nd Phase insulin secretion of Control and T2DM subjects were 444 (143-937) and 203 (-287-626) respectively. The value showed statistically significant difference (p=0.001) (Table 5).

Fasting Proinsulin level of nonobese diabetic subjects (Table 6)

Median (range) value of fasting serum proinsulin of Control and T2DM subjects were 7.1 (2.4-60) and 11.2 (1.7-39) respectively. The value did not show statistically significant difference (p=0.275) (Table 6).

Table 6: Fasting proinsulin level in nonobese diabetic subjects

Variable	Control (n=43)	T2DM (n=42)	t/p values
F S Proinsulin (pmol/l)	7.1 (2.4-60)	11.2 (1.7-39)	1.09/0.275

Results were expressed as Median (range). Difference between groups was calculated using ManWhitney U test. n= number of subjects; F S Proinsulin, Fasting serum proinsulin. p<0.05

Study 3: Insulin resistance and insulin secretory defect in young to middle-aged Bangladeshi Type 2 diabetic subjects

Clinical & biochemical characteristics of the young to middle-aged T2DM subjects (Table 7)

Age (years)

Mean value of age of the Control and T2DM subjects were not significantly different.

BMI (kg/m^2)

Median value of BMI of the Control and T2DM subjects was 23 (19-34) and 24 (19-34) respectively. The value showed statistically significant difference (p=0.001) (Table 7).

Triglyceride (mg/dl)

Median value of TG (mg/dl) of the Control and T2DM subjects were 139 (5-600)and 170 (50-998) respectively. The value showed statistically significant difference (p=0.0001) (Table 7).

HDL-Cholesterol (mg/dl)

Median value of HDL-C (mg/dl) of the Control and T2DM subjects was 31 (15-59) and 30 (11-108) respectively. The value did not show any statistically significant difference (p=0.948) (Table 7).

LDL-Cholesterol (mg/dl)

Median value of LDL-C (mg/dl) of the Control and T2DM subjects were 125 (29-302) and 125 (11-380) respectively. The value did not show any statistically significant difference (p=0.861) (Table 7).

Total Cholesterol (mg/dl)

Median value of T Chol (mg/dl) of the Control and T2DM subjects were 186 (65-450) and 125 194 (50-441) respectively. The value showed statistically significant difference (p=0.034) (Table 7).

Fasting glucose (mmol/l)

Median value of Fasting glucose (mmol/l) of Control and T2DM subjects were 5.1 (3.1-9.6) and 7.2 (3.50-23) respectively. The value showed statistically significant difference (p=0.001) (Table 7).

120 min glucose (mmol/l)

Median value of 120 min glucose (mmol/l) of Control and T2DM subjects were 5.9 (3.3-12) and 15.7 (3.70-34) respectively. The value showed statistically significant difference (p=0.001) (Table 7).

Fasting insulin (pmol/l)

Median value of fasting insulin (pmol/l) of Control and T2DM subjects were 77 (12-420) and 94 (3.8-634) respectively. The value showed statistically significant difference (p=0.001) (Table 7).

Fasting insulin and fasting glucose ratio

Median value of fasting insulin and fasting glucose ratio of Control and T2DM subjects were 15 (2.2-149) and 13 (0.20-100) respectively. The value showed statistically significant difference (p=0.012) (Table 7).

Insulin secretory capacity (HOMA%B)

Median (range) value of HOMA%B of Control and T2DM subjects were 120 (11-583) and 78 (10-462) respectively. The value showed statistically significant difference (p=0.0001) (Table 7).

Table 7: Clinical & biochemical features of young to middle-aged T2DM subjects

Variables	Control (n=317)	T2DM (n=726)	t/p value
Age (years)	40 (25-58)	41 (30-60)	1.434/0.152
BMI (kg/m^2)	23 (19-34)	24 (19-34)	4.327/0.0001
TG (mg/dl)	139 (5-600)	170 (50-998)	5.101/0.0001
T Chol (mg/dl)	186 (65-450)	194 (50-441)	2.120/0.034
HDL-C (mg/dl)	31 (15-59)	31 (11-108)	0.065/0.948
LDL-C (mg/dl)	125 (29-302)	125 (11-380)	0.175/0.861
F glucose (mmol/l)	5.1 (3.1-9.6)	7.2 (3.5-23)	18.636/0.0001
12o min glucose (mmol/l)	5.9 (3.3-12)	15 (3.7-34)	11.854/0.0001
Fasting Insulin (pmol)	77 (12-420)	94 (3.8-634)	3.518/0.0001
Insulin : Glucose	15 (2.2-149)	13 (0.20-100)	2.512/0.012
HOMA%B	120 (11-583)	78 (10-462)	10.580/0.0001
HOMA %S	68 (11-457)	47 (10-489)	6.656/0.0001

Results are expressed as mean±SD & median (range). Significance of difference was calculated by unpaired student's t- tests & Mann –Whitney U test as the test of significance at 5% significance level. n= numbers of subjects; BMI, Body mass index; WHR, Waist hip ratio; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; T chol, Total cholesterol; TG, Triglycerides; HDL-C, high density lipoprotien cholesterol; LDL-C, low density lipoprotien cholesterol; F glucose, fasting glucose; HOMA%B, ß-cell function assessed by homeostasis model assessment; HOMA%S, insulin sensitivity assessed by homeostasis model assessed $p<0.05$

Insulin sensitivity (HOMA %S)

Median (range) value of HOMA%S of Control and T2DM subjects were 68 (11-457) and 47 (10-489) respectively. The value showed statistically significant difference (p=0.0001) (Table 7).

Multiple regression analysis taking HOMA%B as a dependent variable in young to middle-aged DM subjects (Table 8)

Multiple regression analysis was done taking HOMA%B as a dependent variable and Group (Control/T2DM), Age, BMI as independent co variables. HOMA%B had significant positive association (p=0.0001) with Group (Control/T2DM) and BMI (p=0.001).

Table 8: Multiple regression analysis taking HOMA%B as a dependent variable in young to middle-aged DM subjects

Variable	β value	P value
Age	0.007	0.813
BMI	0.096	0.001
Group (Control/T2DM)	0.289	0.0001

Multiple regression analysis taking HOMA%S as a dependent variable in young to middle-aged DM subjects

Multiple regression analysis was done taking HOMA%S as a dependent variable and Group (Control/T2DM), Age, BMI, TG as independent co variables. HOMA%S had significant positive association (p=0.011) with Group (Control/T2DM). HOMA%S had significant negative association with Age (p=0.045), BMI (p= 0.0001) and TG (p=0.006).

Table 9: Multiple regression analysis taking HOMA%S as a dependent variable in young to middle-aged DM subjects

Variable	β value	P value
Age	-0.060	0.045
BMI	-0.186	0.0001
Group (Control/T2DM)	0.078	0.011
TG	-0.084	0.006

Clinical & biochemical characteristics of the young to middle-aged DM subjects (BMI= 19- 23) (Table 10)

Age (years)

Mean value of age of the Control and T2DM subjects were not significantly different.

BMI (kg/m^2)

Median value of BMI of the Control and T2DM subjects were 21(19-23) and 22 (19-23) respectively. The value showed statistically significant difference (p=0.0001) (Table 10).

Triglyceride (mg/dl)

Median value of TG (mg/dl) of the Control and T2DM subjects were 121 (5.2-1200) and 161 (50-800) respectively. The value showed statistically significant difference (p=0.0001) (Table 10).

HDL-Cholesterol (mg/dl)

Median value of HDL-C (mg/dl) of the Control and T2DM subjects were 30 (15-59) and 32 (11-108) respectively. The value did not show any statistically significant difference (p=0.098) (Table 10).

Table 10: Insulin resistance and insulin secretory defect in young to middle-aged Bangladeshi Type 2 diabetic subjects of different ranges of BMI

	Age (years)	**BMI** (kg/m^2)	**TG** (mg/dl)	**T Chol** (mg/dl)	**HDL-C** (mg/dl)	**LDL-C** (mg/dl)	**F glucose** (mmol/l	**120 min glucose** (mmol/l)
BMI= 19-23								
Control (n=187)	40 (20-58)	21(19-23)	121 (5.2-1200)	179 (67-450)	32 (15-58)	118 (52-302)	5 (3.1-19)	5.4(3.3-9)
T2DM (n=275)	41(30-60)	22 (19-23)	161 (50-800)	191 (50-380)	31(16-65)	122. (11-271)	7.4 (3.7-23)	17 (3.7-34)
t/p values	1.546/0.122	5.761/0.0001	4.815/0.0001	3.385/0.001	1.092/0.275	1.744/0.081	13.30/0.001	7.462/0.0001
BMI= 23-27								
Control (n=150)	40 (28-58)	24 (23-27)	155 (52-570)	195 (65-372)	30 (15-59)	133 (29-278)	5.1 (3.1-10)	5.7 (3.3-9.3)
T2DM (n=462)	41(30-55)	24 (23-27)	171 (50-800)	198 (50-441)	32 (11-108)	129 (17-380)	7.2 (3.5-23)	14 (4.1-33)
t/p values	0.673/0.501	2.026/0.053	2.065/0.039	0.105/0.916	1.655/0.098	0.778/0.436	12.917/0.0001	8.810/0.0001
BMI> 27								
Control (n=48)	40 (30-55)	28 (28-34)	146 (51-600)	210 (100-303)	30 (16-45)	140 (49-237)	5.2 (3.6-6.2)	6.5 (4-12)
T2DM (n=102)	41 (30-55)	28 (28-34)	195 (56-998)	193 (112-332)	30 (18-73)	120 (27-242)	7.2 (4.3-19)	15 (5.1-30)
t/p values	0.947/0.344	0.236/0.814	2.121/0.034	1.563/0.118	0.151/0.880	2.560/0.010	8.007/0.0001	5.687/0.0001

Results are expressed as mean±SD & median (range). Significance of difference was calculated by unpaired student's t- tests & Mann –Whitney U test as the test of significance at 5% significance level. n= numbers of subjects; BMI, Body mass index; WHR, Waist hip ratio; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; T chol, Total cholesterol; TG, Triglycerides; HDL-C, high density lipoprotien cholesterol; LDL-C, low density lipoprotien cholesterol; F glucose, fasting glucose; $p<0.05$

LDL-Cholesterol (mg/dl)

Median value of LDL-C (mg/dl) of the Control and T2DM subjects were 133 (29-278) and 129 (17-380) respectively. The value did not show any statistically significant difference (p=0.436) (Table 10).

Total Cholesterol (mg/dl)

Median value of T Chol (mg/dl) of the Control and T2DM subjects were 195 (65-372) and 198 (50-441) respectively. The value did not show any statistically significant difference (p=0.916) (Table 10).

Fasting glucose (mmol/l)

Median value of Fasting glucose (mmol/l) of Control and T2DM subjects were 5 (3.1-19) and 7.4 (3.7-23) respectively. The value showed statistically significant difference (p=0.0001) (Table 10).

120 min glucose (mmol/l)

Median value of 120 min glucose (mmol/l) of Control and T2DM subjects were 5.4(3.3-9) and 17 (3.7-34) respectively. The value showed statistically significant difference (p=0.001) (Table 10).

Fasting insulin (pmol/l)

Median value of fasting insulin (pmol/l) of Control and T2DM subjects were 56 (13-420) and 87 (6.6-634) respectively. The value showed statistically significant difference (p=0.0001) (Table 10).

Fasting insulin and fasting glucose ratio

Median value of fasting insulin and fasting glucose ratio of Control and T2DM subjects were 15 (2.2-149) and 13 (0.20-100) respectively. The value did not show any statistically significant difference (p=0.901) (Table 10).

Clinical & biochemical characteristics of the young to middle-aged DM subjects (BMI= 23-27) (Table 10)

Age (years)

Mean value of age of the Control and T2DM subjects were not significantly different.

BMI (kg/m^2)

Median value of BMI of the Control and T2DM subjects were 24 (23-27) and 24 (23-27) respectively. The value showed statistically significant difference (p=0.053) (Table 10).

Triglyceride (mg/dl)

Median value of TG (mg/dl) of the Control and T2DM subjects were 155 (52-570) and 171 (50-800) respectively. The value showed statistically significant difference (p=0.039) (Table 10).

HDL-Cholesterol (mg/dl)

Median value of HDL-C (mg/dl) of the Control and T2DM subjects were 32 (15-58) and 31(16-65) respectively. The value did not show any statistically significant difference (p=0.275) (Table 10).

LDL-Cholesterol (mg/dl)

Median value of LDL-C (mg/dl) of the Control and T2DM subjects were 118 (52-302) and 122 (11-271) respectively. The value did not show any statistically significant difference (p=0.081) (Table 10).

Total Cholesterol (mg/dl)

Median value of T Chol (mg/dl) of the Control and T2DM subjects were 179 (67-450) and 191 (50-380) respectively. The value showed statistically significant difference (p=0.001) (Table 10).

Fasting glucose (mmol/l)

Median value of Fasting glucose (mmol/l) of Control and T2DM subjects were 5.1 (3.1-10) and 7.2 (3.5-23) respectively. The value showed statistically significant difference (p=0.0001) (Table 10).

120 min glucose (mmol/l)

Median value of 120 min glucose (mmol/l) of Control and T2DM subjects were 5.7 (3.3-9.3) and 14 (4.1-33) respectively. The value showed statistically significant difference (p=0.0001) (Table 10).

Fasting insulin (pmol/l)

Median value of fasting insulin (pmol/l) of Control and T2DM subjects were 89 (20-295) and 93 (8-639) respectively. The value did not show any statistically significant difference (p=0.194) (Table 10).

Fasting insulin and fasting glucose ratio

Median value of fasting insulin and fasting glucose ratio of Control and T2DM subjects were 17 (4-59) and 13 (0.58-100) respectively. The value showed statistically significant difference (p=0.0001) (Table 10).

Clinical & biochemical characteristics the young to middle-aged DM subjects (BMI= >27) (Table 10)

Age (years)

Mean value of age of the Control and T2DM subjects were not significantly different.

BMI (kg/m^2)

Median value of BMI of the Control and T2DM subjects were 28 (28-34) and 28 (28-34) respectively. The value did not show any statistically significant difference (p=0.814) (Table 10).

Triglyceride (mg/dl)

Median value of TG (mg/dl) of the Control and T2DM subjects were 146 (51-600) and 195 (56-998) respectively. The value showed statistically significant difference (p=0.034) (Table 10).

HDL-Cholesterol (mg/dl)

Median value of HDL-C (mg/dl) of the Control and T2DM subjects were 30 (16-45) and 30 (18-73) respectively. The value did not show any statistically significant difference (p=0.880) (Table 10).

LDL-Cholesterol (mg/dl)

Median value of LDL-C (mg/dl) of the Control and T2DM subjects were 140 (49-237) and 120 (27-242) respectively. The value showed statistically significant difference (p=0.010) (Table 10).

Total Cholesterol (mg/dl)

Median value of T Chol (mg/dl) of the Control and T2DM subjects were 210 (100-303) and 193 (112-332) respectively. The value did not show any statistically significant difference (p=0.118) (Table 10).

Fasting glucose (mmol/l)

Median value of Fasting glucose (mmol/l) of Control and T2DM subjects were 5.2 (3.6-6.2) and 7.2 (4.3-19) respectively. The value showed statistically significant difference (p=0.0001) (Table 10).

120 min glucose (mmol/l)

Median value of 120 min glucose (mmol/l) of Control and T2DM subjects were 6.5 (4-12) and 15 (5.1-30) respectively. The value showed statistically significant difference (p=0.0001) (Table 10).

Fasting insulin (pmol/l)

Median value of fasting insulin (pmol/l) of Control and T2DM subjects were 91(12-643) and 114 (3-698) respectively. The value did not show any statistically significant difference (p=0.182) (Table 10).

Fasting insulin and fasting glucose ratio

Median value of fasting insulin and fasting glucose ratio of Control and T2DM subjects were 17 (2-149) and 15 (0.20-84) respectively. The value did not show any statistically significant difference (p=0.164) (Table 10).

Insulin secretory capacity and insulin sensitivity of the young to middle-aged DM subjects (BMI= 19-23) (Table 11)

Insulin secretory capacity (HOMA%B)

Median (range) value of HOMA%B of Control and T2DM subjects were 108 (11-383) and 71 (10-451) respectively. The value showed statistically significant difference (p=0.0001) (Table 11).

Insulin sensitivity (HOMA %S)

Median (range) value of HOMA%S of Control and T2DM subjects were 83 (11-473) and 49 (10-382) respectively. The value showed statistically significant difference (p=0.0001) (Table 11).

Insulin secretory capacity and insulin sensitivity of the young to middle-aged DM subjects (BMI=23-27)

Insulin secretory capacity (HOMA%B)

Median (range) value of HOMA%B of Control and T2DM subjects were 132 (11-498) and 80 (10-462) respectively. The value showed statistically significant difference (p=0.0001) (Table 11).

Insulin sensitivity (HOMA %S)

Median (range) value of HOMA%S of Control and T2DM subjects were 57 (15-209) and 47 (10-489) respectively. The value showed statistically significant difference (p=0.002) (Table 11).

Insulin secretory capacity and insulin sensitivity of young to middle-aged subjects (BMI> 27)

Insulin secretory capacity (HOMA%B)

Median (range) value of HOMA%B of Control and T2DM subjects were 121 (54-583) and 81 (11-241) respectively. The value showed statistically significant difference (p=0.0001) (Table 11).

Insulin sensitivity (HOMA %S)

Median (range) value of HOMA%S of Control and T2DM subjects were 55 (14-246) and 38 (12-531) respectively. The value showed statistically significant difference (p=0.039) (Table 11).

Table 11: Insulin secretory capacity and insulin sensitivity the young to middle-aged DM subjects

BMI 19-23		
Control (n=187)	108 (11-383)	83 (11-473)
T2DM (n=275)	71 (10-451)	49 (10-382)
t/p values	6.392/0.0001	6.379/0.0001
BMI 23-27		
Control (n=150)	132 (11-498)	57 (15-209)
T2DM (n=462)	80 (10-462)	47 (10-489)
t/p values	8.159/0.0001	3.142/0.002
BMI> 27		
Control (n=48)	121 (54-583)	55 (14-246)
T2DM (n=102)	81 (11-241)	38 (12-531)
t/p values	4.504/0.0001	2.069/0.039

Results were expressed as Median (range). Difference between groups was calculated using Man-Whitney U test. n= number of subjects; HOMA%B, ß-cell function assessed by homeostasis model assessment; HOMA%S, insulin sensitivity assessed by homeostasis model assessed. p<05.

Multiple regression analysis taking HOMA%B as a dependent variable (Table 12)

BMI= 19-23

Multiple regression analysis was done taking HOMA%B as a dependent variable and Group (Control/T2DM), Age, BMI as independent co variables. HOMA%B had significant positive association (p=0.0001) with Group (Control/T2DM) and BMI (p=0.004).

BMI= 23-27

Multiple regression analysis was done taking HOMA%B as a dependent variable and Group (Control/T2DM), Age, BMI as independent co variables. HOMA%B had significant positive association (p=0.0001) with Group (Control/T2DM).

BMI>27

Multiple regression analysis was done taking HOMA%B as a dependent variable and Group (Control/T2DM), Age, BMI as independent co variables. HOMA%B had significant positive association (p=0.0001) with Group (Control/T2DM).

Table 12: Multiple regression analysis taking HOMA%B as a dependent variable

Variable	β value	P value
BMI= 19-23		
Age	0.032	0.479
BMI	0.137	0.004
Group (Control/T2DM)	0.278	0.0001
BMI= 23-25		
Age	0.006	0.884
BMI	0.007	0.864
Group (Control/T2DM)	0.273	0.0001
BMI>27		
Age	0.127	0.100
BMI	0.027	0.722
Group (Control/T2DM)	0.394	0.0001

Multiple regression analysis taking HOMA%S as a dependent variable (Table 13)

BMI 19-23

Multiple regression analysis was done taking HOMA%S as a dependent variable and Group (Control/T2DM), Age, BMI, TG as independent co variables. HOMA%S had significant positive association (p=0.036) with Group (Control/T2DM). HOMA%S had significant negative association with BMI (p=0.0001) and TG (p=0.043).

BMI 23-27

Multiple regression analysis was done taking HOMA%S as a dependent variable and Group (Control/T2DM), Age, BMI, TG as independent co variables. HOMA%S had significant negative association with Age (p=0.029).

BMI>27

Multiple regression analysis was done taking HOMA%S as a dependent variable and Group (Control/T2DM), Age, BMI, TG as independent co variables. HOMA%S had significant negative association with TG (p=0.05).

Table 13: Multiple regression analysis taking HOMA%S as a dependent variable

Variable	β value	P value
BMI= 19-23		
Age	-0.080	0.069
BMI	-0.293	0.0001
TG	-0.090	0.043
Group (Control/T2DM)	0.096	0.036
BMI= 23-27		
Age	-0.090	0.029
BMI	-0.028	0.498
TG	-0.032	0.433
Group (Control/T2DM)	0.019	0.649
BMI>27		
Age	-0.144	0.079
BMI	-0.108	0.187
TG	-0.160	0.051
Group (Control/T2DM)	0.030	0.708

Study 4: Phase-wise insulin secretion in middle aged Bangladeshi type 2 diabetic patients

Clinical & biochemical features of the study subjects (Table 14)

Mean (±SD) value of Age, BMI, Waist hip ratio, Diastolic Blood pressure, Systolic Blood pressure of Control and T2DM subjects were not significantly different. (Table 4A)

Fasting glucose (mmol/l)

Mean (±SD) value of Fasting glucose (mmol/l) of the Control and T2DM subjects were 4.38±0.6 and 7.3±2.4 respectively. The value showed statistically significant difference (p=0.0001) (Table 14).

30 min glucose (mmol/l)

Mean (±SD) value of 30 min glucose of Control and T2DM subjects were 7.33±1.8 and 11.3±3.2 respectively. The value showed statistically significant difference (p=0.0001) (Table 14).

120 min glucose (mmol/l)

Mean (SD) value of 120 min glucose (mmol/l) of Control and T2DM subjects was 5.95±2.0 and 13.9±3.7 respectively. The value showed statistically significant difference (p=0.0001) (Table 14).

Table 14: Clinical and Anthropometric characteristics of T2DM subjects (n=74)

Variables	Control (n=30)	T2DM (n=44)	t/p values
Age (years)	41±7	44±7	1.804/0.075
BMI (kg/m^2)	25.31±3.61	25.46±3.26	0.183/0.856
WHR	0.92±0.013	0.91±0.013	1.202/0.233
SBP (mm Hg)	124±10	127±13	0.970/0.335
DBP (mm Hg)	84±8	82±9	1.000/0.321
F Glucose (mmol/l)	4.38±0.6	7.3±2.4	7.710/0.0001
30 min glucose (mmol/l)	7.33±1.8	11.3±3.2	6.772/0.0001
120 min glucose (mmol/l)	5.95±2.06	13.9±3.7	11.809/0.0001

Results were expressed as mean ±SD. Difference between groups was calculated using unpaired student's t-test. n= number of subjects; S Glucose, Serum glucose. Results were expressed as mean ±SD. Difference between groups was calculated using unpaired student's t-test. n= numbers of subjects; BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; WHR, Waist to hip ratio; F Glucose, fasting glucose. $p<0.05$

Insulinemic status, phase-wise insulin secretion, insulin secretory capacity and insulin Sensitivity of T2DM subjects (Table 15)

Fasting insulin (pmol/l)

Median (range) value of Fasting insulin (pmol/l) of Control and T2DM subjects were 74 (6.25-216.89) and 53 (6.67-162.58) respectively. The value showed statistically significant difference (p=0.0001) (Table 15).

30 min insulin (pmol/l)

Median (range) value of 30 min insulin of Control and T2DM subjects were 544 (33.27-1168.64) and 132 (17.78-422.19) respectively. The value showed statistically significant difference (p=0.013) (Table 15).

1st Phase insulin secretion

Median (range) value of 1st Phase insulin secretion of Control and T2DM subjects were 1655 (-109-2831) and 208 (-1412-1230) respectively. The value showed statistically significant difference (p=0.0001) (Table 15).

2nd Phase insulin secretion

Median (range) value of 2nd Phase insulin secretion of Control and T2DM subjects were 418 (51-679) and 101 (-217-341) respectively. The value showed statistically significant difference (p=0.0001) (Table 15).

Insulin secretory capacity (HOMA%B)

Median (range) value of HOMA%B of Control and T2DM subjects were 163 (48-381) and 53(10-162) respectively. The value showed statistically significant difference (p=0.0001) (Table 15).

Insulin sensitivity (HOMA %S)

Median (range) value of HOMA%S of Control and T2DM subjects were 65 (21-259) and 79 (26-226) respectively. The value did not show any statistically significant difference (p= 0.088) (Table 15).

Table 15: Insulinemic status, phase-wise insulin secretion, insulin secretory capacity and insulin Sensitivity of T2DM subjects

Variables	Control (n=30)	T2DM (n=44)	t/p value
F Insulin (pmol/l)	74 (6.25-216.89)	53 (6.67-162.58)	129/0.0001
30 min Insulin (pmol/l)	544 (33.27-1168.64)	132 (17.78-422.19)	433.50/0.013
1st Phase insulin secretion	1655 (-109-2831)	208 (-1412-1230)	87/0.0001
2nd Phase insulin secretion	418 (51-679)	101 (-217-341)	94/0.0001
HOMA B%	163 (48-381)	53(10-162)	115/0.0001
HOMA S%	65 (21-259)	79 (26-226)	505/0.088

Results were expressed as Median (range). Difference between groups was calculated using Man-Whitney U test. n=number of subjects, F Insulin, fasting insulin. 1st Phase insulin secretion, first phase insulin secretion; 2nd Phase insulin secretion, second phase insulin secretion. HOMA B%, B-cell function assessed by homeostasis model assessment, HOMA S%, Insulin sensitivity assessed by homeostasis model assessment.$p<0.05$

Study 5: Relationship of Serum Proinsulin and Glucagon Levels in Subjects with Type 2 Diabetes Mellitus

Clinical and biochemical characteristics of T2DM subjects (Table 16)

Age (years) Mean (±SD) value of age of control and T2DM subjects were 36.0±6.0 and 42.4±7.0 respectively. The value showed statistically significant difference (p=0.0001) (Table 16).

Mean (±SD) value of BMI, Waist hip ratio, Diastolic Blood pressure, Systolic Blood pressure, Triglyceride, HDL-Cholesterol level of the Control and T2DM subjects were not significantly different (Table 16).

Total Cholesterol (mg/dl)

Mean (±SD) value of T Chol (mg/dl) of the Control and T2DM subjects were 145.6±86.8 and 191.5±96.2 respectively. The value showed statistically significant difference (p=0.021) (Table 16).

LDL Cholesterol (mg/dL)

Mean (±SD) value of LDL-C (mg/dl) of the Control and T2DM subjects were 178.7±41.2 and 196.4±33.2 respectively. The value showed statistically significant difference (p=0.030) (Table 16).

Fasting glucose (mmol/l)

Mean (±SD) value of Fasting glucose (mmol/l) of Control and T2DM subjects were 5.3±0.4 and 10.0±3 respectively. The value showed statistically significant difference (p=0.0001) (Table 16).

Table 16: Clinical and biochemical characteristics of T2DM subjects

Variables	Control (n=44)	T2DM (n=44)	p values
Age (yrs)	36.0±6.0	42.4±7.0	0.0001
BMI (kg/m^2)	24.1±3.0	25.5±3.0	0.070
Diastolic BP (mmHg)	108.8±8.0	142.6±14.0	0.139
Systolic BP (mmHg)	71.1±12.0	78.4±8.0	0.003
TG (mg/dL)	119.3±38.9	128.2±37.9	0.279
T Chol (mg/dL)	145.6±86.8	191.5±96.2	0.021
LDL-C (mg/dL)	178.7±41.2	196.4±33.2	0.030
HDL-C (mg/dL)	30.2±7	29.9±7.3	0.826
F glucose (mmol/L)	5.3±0.4	10.0±3	0.0001
120 min glucose (mmol/L)	5.9±1.1	17.7±5.9	0.0001
HOMA %B	96(46-498)	42(9-164)	4.711/.0001
HOMA %S	68(19-270)	38 (9-259)	2.253/.024

Results were expressed as mean ±SD. Difference between groups was calculated using unpaired student's t-test. n= number of subjects; BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; WHR, Waist hip ratio; T chol, Total cholesterol; TG, Triglycerides; HDL-C, high density lipoprotien cholesterol; LDL-C, low density lipoprotien cholesterol; F Glucose, fasting glucose. HOMA%B. ß-cell function assessed by homeostasis model assessment; HOMA%S, insulin sensitivity assessed by homeostasis model assessed $p<0.05$

120 min glucose (mmol/l)

Mean (±SD) value of 120 min glucose (mmol/l) of the Control and T2DM subjects was 5.9±1.1 and 17.7±5.9 respectively. The value showed statistically significant difference (p=0.0001) (Table 16).

Insulin secretory capacity (HOMA%B)

Median (Range) value of HOMA%B of Control and T2DM subjects 96 (46-498) were and 42 (9-164) respectively. The value showed statistically significant difference (p=0.0001) (Table 16).

Insulin sensitivity (HOMA %S)

Median (Range) value of HOMA%S of Control and T2DM subjects were 68(19-270) and 38(9-259) respectively. The value showed statistically significant difference (p= 0.024) (Table 16)

C-peptide, Proinsulin & glucagon level of the T2DM subjects (Table 17)

Fasting C-peptide (ng/ml)

Median (Range) value of Fasting C-pep (ng/ml) of Control and T2DM subjects were 1.9 (0.5-7.0) and 2.6 (0.4-6.8) respectively. The value showed statistically significant difference (p= 0.024) (Table 17).

Fasting serum proinsulin (pmol/L)

Median (Range) value of Fasting serum Proinsulin (pmol/L) of Control and T2DM subjects were 9.3 (1.8-156.7) and 28.3 (2.1-268) respectively. The value showed statistically significant difference (p= 0.0001) (Table 17).

Fasting serum glucagon ((ng/ml))

Median (Range) value of Fasting serum Glucagon (ng/ml) of Control and T2DM subjects were 20.1 (13.8-36.5) and 12.3 (2.39-19.7) respectively. The value showed statistically significant difference (p= 0.0001) (Table 17).

Table 17: C-peptide, Proinsulin & glucagon level of the Study Subjects

Variables	Controls (n=44)	T2DM (n=44)	t/p values
F C-pep (ng/ml)	1.9 (0.5-7.0)	2.6 (0.4-6.8)	1.24/.214
F S Proinsulin (pmol/L)	9.3 (1.8-156.7)	28.3 (2.1-268)	4.63/.0001
F S Glucagon (ng/ml)	20.1 (13.8-36.5)	12.3 (2.3-19.7)	7.20/0.0001

Results were expressed as Median (range). Difference between groups was calculated using Man-Whitney U test. n= number of subjects; F C-pep. Fasting C-peptide; F S Proinsulin, Fasting Serum proinsulin; F S glucagon, Fasting serum glucagon. $p<0.05$

Multiple Regression analysis taking proinsulin as a dependent variable (Table 18)

Multiple regression analysis was done taking proinsulin as a dependent variable and Group (Control/T2 DM), glucagon, HOMA %B, HOMA %S as independent co-variables. Proinsulin had significant negative association (p=0.05) with glucagon. Proinsulin had significant negative association with HOMA %S (p=0.005).

Table 18: Multiple regression analysis taking proinsulin as a dependent variable

Variable	β value	P value
Glucagon	- 0.281	0.050
HOMA %B	0.175	0.170
HOMA %S	- 0.325	0.005
Group (Control/T2DM)	0.253	0.139

Multiple regression analysis taking glucagon as a dependent variable (Table 19)

Multiple regression analysis was done taking glucagon as a dependent variable and Group (Control/T2DM), proinsulin, HOMA %B, HOMA %S as independent co variables. Glucagon had significant negative association (p=0.0001) with Group (Control/T2DM). Glucagon had significant negative association with proinsulin (p=0.05) and HOMA %B (p=0.05).

Table 19: Multiple regression analysis taking glucagon as a dependent variable

Variable	β value	P value
F S proinsulin (pmol/l)	- 0.157	0.0500
HOMA %B	- 0.229	0.0500
HOMA %S	- 0.049	0.6700
Group (Control/T2DM)	- 0.822	0.0001

Study 6: Association of serum resistin with hs-CRP and insulin sensitivity in Type 2 diabetes mellitus subjects

Clinical and biochemical characteristics of the T2DM subjects (Table 20)

Mean (±SD) value of age, BMI, Waist hip ratio, Diastolic Blood pressure, and Systolic Blood pressure, median value of Total cholesterol, HDL-Cholesterol, LDL-Cholesterol level of Control and T2DM subjects were not significantly different. (Table 20)

Triglyceride (mg/dl)

Median value of TG (mg/dl) of the Control and T2DM subjects were 136 (67-600) and 177 (50-536) respectively. The value showed statistically significant difference (p=0.001) (Table 20).

Table 20: Clinical and biochemical characteristics of T2DM subjects

Variable	Control (n=33)	T2DM (n= 104)	t/p value
Age (yrs)	40 ± 4	40 ± 6	0.24/0.807
BMI (kg/m^2)	25 ± 3	24 ±2	1.55/0.123
WHR	0.89± 0.05	1.02± 0.89	0.84/0.399
TG (mg/dL)	136 (67-600)	177 (50-536)	2.16/0.03
T Chol (mg/dL)	194 (152-270)	200 (115-326)	0.48/0.629
HDL- C (mg/dL)	36 (30-59)	35 (18-50)	1.22/0.214
LDL-C (mg/dL)	124(61-201)	122(48-223)	0.556/0.578
F glucose (mmoL/L)	5.3(4.5-6.0)	9.1(5.20-19.5)	8.34/0.0001
120 min glucose (mmol/L)	6.1(3.6-7.8)	18.6(9.8-34.3)	2.35/0.019
HOMA %B	83 (52-309)	25 (2-119)	7 /0.001
HOMA %S	106 (14-217)	114 (20-531)	2/0.112

Results are expressed as mean±SD & median (range). Significance of difference was calculated by unpaired student's t- tests & Mann –Whitney U test as the test of significance at 5% significance level. n= numbers of subjects; BMI, Body mass index; WHR, Waist hip ratio; T chol, Total cholesterol; TG, Triglycerides; HDL-C, high density lipoprotien cholesterol; LDL-C, low density lipoprotien cholesterol; F glucose, fasting glucose; HOMA%B, ß-cell function assessed by homeostasis model assessment; HOMA%S, insulin sensitivity assessed by homeostasis model assessed. $p<0.05$

Fasting glucose (mmol/l)

Median (Range) value of Fasting glucose (mmol/l) of Control and T2DM subjects were 5.3(4.5-6.0) and 9.1(5.20-19.5) respectively. The value showed statistically significant difference (p=0.0001) (Table 20).

120 min glucose (mmol/l)

Median (Range) value of 120 min glucose (mmol/l) of Control and T2DM subjects was 6.1(3.6-7.8) and 18.6(9.8-34.3) respectively. The value showed statistically significant difference (p=0.019) (Table 20).

Insulin secretory capacity (HOMA%B)

Median (Range) value of HOMA%B of Control and T2DM subjects were 83 (52-309) and 25 (2-119) respectively. The value showed statistically significant difference (p=0.001) (Table 7B).

Insulin sensitivity (HOMA %S)

Median (Range) value of HOMA%S of Control and T2DM subjects were 106 (14-217) and 114 (20-531) respectively. The value showed statistically significant difference (p= 0. 0.112) (Table 7B).

Serum hs-CRP and serum resistin level of T2DM subjects (Table 21)
Fasting serum Resistin (ng/ml)

Median (Range) value of Fasting serum Resistin (ng/ml) of Control and T2DM subjects were 20.8 (7.2-47.7) and 26.1 (11.0-80.3) respectively. The value showed statistically significant difference (p=0.006) (Table 21).

High sensitive C Reactive Protein (mg/L)

Median (Range) value of hs-CRP (mg/L) of Control and T2DM subjects were 1.5 (0.2-9.9) and 2.8 (0.2-26.2) respectively. The value showed statistically significant difference (p=0.004) (Table 21).

Table 21: Serum hs-CRP and serum resistin levels of T2DM subjects

Variable	Control (n=33)	T2DM (n= 104)	t/p value
F S Resistin (ng/mL)	20.8 (7.2-47.7)	26.1 (11.0-80.3)	2.758/ 0.006
hs-CRP (mg/L)	1.5 (0.2-9.9)	2.8 (0.2-26.2)	2.897/ 0.004

Results were expressed as Median (range). Difference between groups was calculated using Man-Whitney U test. n= number of subjects; F S Resistin, fasting serum resistin; hs-CRP, high sensitive C reactive protein. $p<0.05$

Multiple Regression analysis taking resistin as dependent variables (Table 22)

Multiple regression analysis was done taking resistin as dependent variable and others as independent co variables. Resistin was significantly associated with Hs-CRP (p=<0.001) and HOMA %S (p=0.004), but no significant association was found with WHR and BMI.

Table 22: Multiple regression taking resistin as dependent variable in the study subjects

Variables	β value	P value
CRP	0.499	<0.001
HOMA %S	0.267	0.004
WHR	0.145	0.097
BMI	-0.096	0.284

Multiple regression analysis taking CRP as a dependent variable in the study subjects (Table 23)

Multiple regression analysis was done taking hs-CRP as dependent variable and others as independent co variables. Hs-CRP had positive significant association with Resistin (p=<0.001) and strong negative association HOMA %S (p=0.004).

Table 23: Multiple Regression analysis taking CRP as a dependent variable

Variables	β value	P value
Resistin	0.484	<0.001
HOMA %S	-0.276	0.002
WHR	-0.141	0.100
BMI	0.143	0.104

Study 7: Insulin deficiency and insulin resistance in Bangladeshi IGT subjects

Clinical and biochemical characteristics IGT subjects (Table 24)

Mean (±SD) value of age, BMI, Waist hip ratio, Systolic Blood pressure, median (Range) value of Total cholesterol, LDL-Cholesterol level of the Control and IGT subjects were not significantly different. (Table 8A)

Diastolic blood pressure (mm Hg)

Mean (±SD) value of DBP (mm Hg) of control and IGT subjects was 77±10 and 72±9 respectively. The value showed statistically significant difference (p=0.0169) (Table 24).

Triglyceride (mg/dl)

Median (Range) value of TG (mg/dl) of the Control and IGT subjects were 152 (52-386) and 78 (82-540) respectively. The value showed statistically significant difference (p=0.008) (Table 24).

HDL-Cholesterol (mg/dl)

Median (Range) value of HDL-C (mg/dl) of the Control and IGT subjects were 27 (19-47) and 30 (15-78) respectively. The value showed statistically significant difference (p=0.008) (Table 24).

Fasting glucose (mmol/l)

Median (Range) value of Fasting glucose (mmol/l) of the Control and IGT subjects were 5.2 (2.8-7.9) and 5.2 (4-8) respectively. The value did not any statistically significant difference (p=0.3650) (Table 24).

Table 24: Clinical & biochemical features of IGT subjects

Variable	Control (n=39)	IGT (n=46)	t/p value
Age (years)	43±13	43±7	0.029/0.977
BMI (kg/m^2)	20±5	20±3	0.582/0.562
WHR	0.92±0.06	0.92±0.06	0.339/0.735
DBP (mm Hg)	77±10	72±9	0.457/0.016
SBP (mm Hg)	118±13	117±15	0.327/0.745
TG (mg/dl)	152 (52-386)	78 (82-540)	2.646/0.008
T Chol (mg/dl)	189 (82-400)	183 (140-400)	0.044/0.965
HDL-C (mg/dl)	27 (19-47)	30 (15-78)	2.668/0.008
LDL-C (mg/dl)	135 (12-334)	111 (41-320)	1.821/0.069
F glucose (mmol/l)	5.2 (2.8-7.9)	5.2 (4-8)	0.905/0.365
30 min glucose (mmol/l)	7.9 (3.7-12.2)	8.6 (5.9-11.5)	2.016/0.044
120 min glucose (mmol/l)	5.5 (3.5-17.8)	8 (5.1-13.8)	4.239/0.001

Results are expressed as mean±SD & median (range). Significance of difference was calculated by unpaired student's t- tests & Mann –Whitney U test as the test of significance at 5% significance level. n= numbers of subjects; IGT, impaired glucose tolerance; BMI, Body mass index; WHR, Waist hip ratio; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; T chol, Total cholesterol; TG, Triglycerides; HDL-C, high density lipoprotien cholesterol; LDL-C, low density lipoprotien cholesterol; F glucose, fasting glucose; p<0.05

30 min glucose (mmol/l)

Median (Range) value of 30 min glucose of Control and IGT subjects were 7.9 (3.7-12.2) and 8.6 (5.9-11.5 respectively. The value showed statistically significant difference (p=0.044) (Table 24).

120 min glucose (mmol/l)

Mean (±SD) value of 120 min glucose (mmol/l) of the Control and IGT subjects were 5.5 (3.5-17.8) and 8 (5.1-13.8) respectively. The value showed statistically significant difference (p=0.001) (Table 24).

Insulinemic status, phase-wise insulin secretion, insulin secretory capacity and insulin sensitivity of the IGT subjects (Table 25)

Fasting insulin (pmol/l)

Median (range) value of fasting insulin of the Control and IGT subjects were 87 (13-327) and 116 (42-325) respectively. The value showed statistically significant difference (p=0.029) (Table 25).

30 min insulin (pmol/l)

Median (range) value of 30 min insulin of Control and IGT subjects were 547 (6.53-1353) and 479 (127-1020) respectively. The value did not show any statistically significant difference (p=0.148) (Table 25).

1st Phase insulin secretion

Median (range) value of 1st Phase insulin secretion of Control and IGT subjects were 1700 (844-3301) and 1462 (347-2627) respectively. The value did not show any statistically significant difference (p=0.101) (Table 25).

2nd Phase insulin secretion

Median (range) value of 2nd Phase insulin secretion of Control and IGT subjects were 443 (221-792) and 380 (142-631) respectively. The value did not show any statistically significant difference (p=0.148) (Table 25).

Insulin secretory capacity (HOMA%B)

Median (Range) value of HOMA%B of Control and IGT subjects were 141 (18-386) and 162 (49-300) respectively. The value did not show any statistically significant difference (p=0.174) (Table 25).

Table 25: **Insulinemic status, phase-wise insulin secretion, insulin secretory capacity and insulin sensitivity of IGT subjects**

Variable	Control (n=39)	IGT (n=46)	t/p
F Insulin (pmol/l)	87 (13-327)	116 (42-325)	2.183/0.029
30 min Insulin (pmol/l)	547 (6.53-1353)	479 (127-1020)	1.446/0.148
1st Phase insulin secretion	1700 (844-3301)	1462 (347-2627)	1.640/0.101
2nd Phase insulin secretion	443 (221-792)	380 (142-631)	1.446/0.148
HOMA%B	141 (18-386)	162 (49-300)	1.358/0.174
HOMA%S	54 (15-289)	39 (14-110)	2.328/0.020

Results were expressed as Median (range). Difference between groups was calculated using Man-Whitney U test. n=number of subjects, IGT, impaired glucose tolerance; F Insulin, fasting insulin. 1st Phase insulin secretion, first phase insulin secretion; 2nd Phase insulin secretion, second phase insulin secretion. HOMA%B, ß-cell function assessed by homeostasis model assessment; HOMA%S, insulin sensitivity assessed by homeostasis model assessed $p<0.05$

Insulin sensitivity (HOMA %S)

Median (Range) value of HOMA%S of Control and IGT subjects were 54 (15-289) and 39 (14-110) respectively. The value showed statistically significant difference (p= 0.020) (Table 25).

Study 8: Serum Proinsulin status in IGT subjects (Table 26)

Clinical and biochemical characteristics of IGT subjects

Age, BMI, Waist hip ratio, Systolic Blood pressure, Diastolic Blood Pressure, Total cholesterol, LDL-Cholesterol, HDL-Cholesterol level of the Control and IGT subjects were not significantly different. (Table 26)

Triglyceride (mg/dl)

Median (Range) value of TG (mg/dl) of the Control and IGT subjects were 119 (51-474) and 178 (82-540) respectively. The value showed statistically significant difference (p=0.001) (Table 26).

Fasting glucose (mmol/l)

Median (Range) value of Fasting glucose (mmol/l) of the Control and IGT subjects were 5.3 (3.8-6) and 5.2 (4-12) respectively. The value did not any statistically significant difference (p=0.297) (Table 26).

120 min glucose (mmol/l)

Mean (±SD) value of 120 min glucose (mmol/l) of the Control and IGT subjects were 6.1 (3-7.8) and 7.9 (5-21) respectively. The value showed statistically significant difference (p=0.01) (Table 26).

Insulin secretory capacity (HOMA%B)

Median (Range) value of HOMA%B of Control and IGT subjects were 97 (46-498) and 164 (17-300) respectively. The value showed statistically significant difference (p=0.01) (Table 26).

Insulin sensitivity (HOMA %S)

Median (Range) value of HOMA%S of Control and IGT subjects were 68 (19-270) and 39 (15-110) respectively. The value showed statistically significant difference (p= 0.001) (Table 26).

Fasting serum proinsulin (pmol/L) (Table 9C)

Median (Range) value of F S Proinsulin (pmol/L) of Control and IGT subjects were 9.2 (1.8-156) and 17 (3-51) respectively. The value showed statistically significant difference (p= 0.001) (Table 26).

Table 26: Clinical biochemical characteristics IGT subjects

Variable	Control (n=44)	IGT (n=50)	t/p values
Age (years)	40±6	40±5	0.186/0.853
BMI (kg/m^2)	23±3	22±2	1.55/0.123
SBP (mm Hg)	108±8	118±15	1.680/0.096
DBP (mm Hg)	71±12	72±9	1.053/0.397
TG (mg/dl)	119 (51-474)	178 (82-540)	4.107/0.001
T Chol (mg/dl)	180 (65-272)	186 (140-400)	1.308/0.191
HDL-C (mg/dl)	29 (19-45)	31 (15-78)	1.043/0.297
LDL-C (mg/dl)	117 (29-201)	111 (41-320)	0.572/0.567
F glucose (mmol/l)	5.3 (3.8-6)	5.2 (4-12)	1.043/0.297
120 min glucose (mmol/l)	6.1 (3-7.8)	7.9 (5-21)	5.912/0.001
HOMA%B	97 (46-498)	164 (17-300)	4.463/0.001
HOMA%S	68 (19-270)	39 (15-110)	4.512/0.001
F S Proinsulin (pmol/L)	9.2 (1.8-156)	17 (3-51)	3.470/0.001

Results are expressed as mean±SD & median (range). Significance of difference was calculated by unpaired student's t- tests & Mann –Whitney U test as the test of significance at 5% significance level. n= numbers of subjects; IGT, impaired glucose tolerance; BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; T chol, Total cholesterol; TG, Triglycerides; HDL-C, high density lipoprotien cholesterol; LDL-C, low density lipoprotien cholesterol; F glucose, fasting glucose; HOMA%B, ß-cell function assessed by homeostasis model assessment; HOMA%S, insulin sensitivity assessed by homeostasis model assessed. F S Proinsulin, Fasting serum insulin $p<0.05$

Multiple Regression Analysis Taking HOMA%B as a Dependent Variable in IGT subjects (Table 27)

Multiple regression analysis was done taking HOMA%B as a dependent variable and Group (Control/T2DM), Age, BMI as independent co variables.

Table 27: Multiple regression analysis taking HOMA%B as a dependent variable in IGT subjects

Age	0.076	0.505
BMI	0.024	0.836
Proinsulin	0.037	0.755
Group (Control/T2DM)	-0.095	0.406

Multiple regression analysis taking HOMA%S as a dependent variable in IGT subjects (Table 28)

Multiple regression analysis was done taking HOMA%S as a dependent variable and Group (Control/T2DM), BMI, as independent co variables. HOMA%S had significant negetive association (p=0.037) with proinsulin.

Table 28: Multiple regression analysis taking HOMA%S as a dependent variable in IGT subjects

Variable	β value	P value
BMI	-0.141	0.183
Proinsulin	-.0228	0.037
Group (Control/T2DM)	0.197	0.065

Study 9: Insulin deficiency and insulin resistance and phase-wise insulin secretion in first degree relatives of young and nonobese Bangladeshi type 2 diabetic patients

Clinical and biochemical parameters first degree relatives of T2DM subjects

(Table 29)

Age (years)

Mean (±SD) value of age BMI, Waist hip ratio, Diastolic Blood pressure, Systolic Blood pressure, Triglyceride, Total cholesterol, HDL-Cholesterol, LDL- cholesterol level of the Control and the first degree relatives of T2DM subjects (FDR) were not significantly different (Table 30).

Fasting glucose (mmol/l)

Median (range) value of Fasting glucose (mmol/l) of the Control and the first degree relatives of T2DM subjects (FDR) were 5.2 (2.8-15.7) and 5.7 (4.4-8.9) respectively. The value did not show any statistically significant difference (p=0.162) (Table 29).

30 min glucose (mmol/l)

Median (range) value of 30 min glucose (mmol/l) of the Control and the first degree relatives of T2DM subjects (FDR) were 8 (3.3-17.6) and 8.6 (5.6-18.9) respectively. The value showed statistically significant difference (p=0.041 (Table 29).

120 min glucose (mmol/l)

Median (range) value of 120 min glucose (mmol/l) of the Control and the first degree relatives of T2DM subjects (FDR) were 6.6 (3.5-24.8) and 7.4 (3.5-22.5) respectively. The value did not show any statistically significant difference (p=0.220) (Table 29).

Table 29: Clinical and anthropometric measurements of first degree relatives of T2DM subjects

Variable	Control (n=33)	T2DMR (n=29)	*t/p value*
Age	49±11	45±9	1.533/0.131
WHR	0.90±0.02	0.9±0.05	0.226/0.823
DBP (mm Hg)	77±11	74±7	1.454/0.151
SBP (mm Hg)	119±16	114±15	1.197/0.236
TG (mg/dl)	154 (21-386)	151 (80-451)	0.663/0.507
T Chol (mg/dl)	200 (95-325)	189 (140-277)	0.953/0.341
HDL-C (mg/dl)	26 (20-51)	29 (15-54)	0.391/0.696
LDL-C (mg/dl)	145 (15-263)	124 (77-210)	1.661/0.097
F glucose (mmol/l)	5.2 (2.8-15.7)	5.7 (4.4-8.9)	1.398/0.162
30 min glucose (mmol/l)	8 (3.3-17.6)	8.6 (5.6-18.9)	2.046/0.041
120 min glucose (mmol/l)	6.6 (3.5-24.8)	7.4 (3.5-22.5)	1.228/0.220

Results are expressed as mean±SD & median (range). Significance of difference was calculated by unpaired student's t- tests & Mann –Whitney U test as the test of significance at 5% significance level. n= numbers of subjects; T2DMR, T2DM relatives;; BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; T chol, Total cholesterol; TG, Triglycerides; HDL-C, high density lipoprotien cholesterol; LDL-C, low density lipoprotien cholesterol; F glucose, fasting glucose; $p<0.05$

Insulinemic status, phase-wise insulin secretion, insulin secretory capacity and insulin sensitivity in first degree relatives of T2DM subjects (Table 30)

Fasting insulin (pmol/l)

Median (range) value of F Insulin (pmol/l) of Control and the first degree relatives of T2DM subjects (FDR) were 76 (17-327) and 90 (13-2190 respectively. The value did not show any statistically significant difference (p=0.910) (Table 30)

30 min insulin (pmol/l)

Median (range) value of 30 min Insulin (pmol/l) of Control and the first degree relatives of T2DM subjects (FDR) were 342 (25-1330) and 312 (93-786) respectively. The value did not show any statistically significant difference (p=0.287) (Table 30)

1st Phase insulin secretion

Median (range) value of 1st Phase insulin secretion of Control and the first degree relatives of T2DM subjects (T2DMR) were 1086 (-417-2259) and 1175 (-568-3301) respectively. The value did not show any statistically significant difference (p=0.070) (Table 30)

2nd Phase insulin secretion

Median (range) value of 2nd Phase insulin secretion of Control and the first degree relatives of T2DM subjects (T2DMR) were 284 (7-568) and 307 (-31-792) respectively. The value did not show any statistically significant difference (p=0.081) (Table 3)

Insulin secretory capacity (HOMA%B)

Median (Range) value of HOMA%B of Control and the first degree relatives of T2DM subjects (FDR) were 90 (17-191) and 131(22-386) respectively. The value did not show any statistically significant difference (p=0.095) (Table 30).

Insulin sensitivity (HOMA %S)

Median (Range) value of HOMA%S of Control and the first degree relatives of T2DM subjects (FDR) were 48 (21-2620 and 58 (15-252) respectively. The value showed statistically significant difference (p= 0.612) (Table 30).

Table 30: Insulinemic status, phase-wise insulin secretion, and insulin secretory capacity insulin and sensitivity first degree relatives of T2DM subjects

Variable	Control (n=33)	FDR (n=29)	t/p value
F Insulin (pmol/l)	76 (17-327)	90 (13-2190	0.113/0.910
30 min Insulin (pmol/l)	342 (25-1330)	312 (93-786)	1.065/0.287
1st Phase insulin secretion	1086 (-417-2259)	1175 (-568-3301)	1.813/ 0.070
2nd Phase insulin secretion	284 (7-568)	307 (-31-792)	1.742/0.081
HOMA%B	90 (17-191)	131(22-386)	1.672/0.095
HOMA%S	48 (21-2620	58 (15-252)	0.508/0.612

Results were expressed as Median (range). Difference between groups was calculated using Man-Whitney U test. n=number of subjects, T2DMR, T2DM relatives; F Insulin, fasting insulin. 1st Phase insulin secretion, first phase insulin secretion; 2nd Phase insulin secretion, second phase insulin secretion. HOMA%B, β cell secretory capacity; HOMA%S, insulin sensitivity by homeostasis model assessment; $p<0.05$

Multiple Regression Analysis Taking HOMA%B as a Dependent Variable (Table 31)

Multiple regression analysis was done taking HOMA%B as a dependent variable and Group (Control/T2DM), Age, BMI as independent co variables. HOMA%B had significant positive association (p=0.05) with Group (Control/T2DM).

Table 31: Multiple regression analysis taking HOMA%B as a dependent variable in T2DMR subjects

Variable	β value	P value
Age	0.141	0.305
BMI	-0.032	0.812
Group (Control/T2DM)	0.251	0.051

Multiple regression analysis taking HOMA%S as a dependent variable in T2DMR subjects (Table 32)

Multiple regression analysis was done taking HOMA%S as a dependent variable and Group (Control/T2DM), Age, BMI, TG as independent co variables.

Table 32: Multiple regression analysis taking HOMA%S as a dependent variable in T2DMR subjects

Variable	β value	P value
Age	0-.061	0.674
BMI	-0.206	0.144
TG	-0.027	0.840
Group (Control/T2DM)	0.061	0.648

DISCUSSION

CHAPTER V
DISCUSSION

Exploration of the relative role of insulin secretory dysfunction and insulin resistance in T2DM is of practical clinical importance as it is central in designing the management as well as preventive policies in this disorder. It is now evident that heterogeneity exists in the patahophysiology of T2DM and thus the relative role of the basic defects need to be explored in various population as well as in subsets of the same population. The present series of studies were under taken to investigate these basic defects primarily in young lean Bangladeshi subjects, but other groups with higher age and body weight were also explored to compare the data and thus have greatly insight on the understanding the mechanism of the disorder.

Insulin secretory defect and insulin resistance in young diabetic subjects

The present data indicate that the young lean Bangladeshi diabetic subjects have both insulin secretory dysfunction and insulin resistance (Table 2C). However, analysis of the data reveals that the pancreatic β-cell dysfunction is markedly predominant in these subjects compared to insulin resistance. This applies both for the fasting and stimulated states, and it can be seen from even the absolute values of serum C-peptide (the best marker for endogenous insulin secretion). The mean value of fasting C-peptide in diabetic is only about 50% of the Control subjects and almost the same ratio is found on stimulation by glucagon (one of the most potent insulin secretogagues). The secretory defect, however, becomes more predominant when the C-peptide-glucose ratio is analyzed. From this analysis of the mean values the diabetic subjects were found to have only 20% of the C-peptide compared to control both at the fasting and glucagon stimulated states.

In contrast to the insulin secretory defects the insulin sensitivity in the diabetic subjects did not show any significant difference from the control as explored by the shot insulin tolerance test (kitt, 0.068±0.01 in the control vs 0.067±0.02 in the diabetic, Table 1C). However, presence of hyperinsulinemia and higher insulin glucose ratio in the fasting state indicate some degree of insulin resistance in these subjects. From the over all analysis of the data a markedly predominant presence of insulin secretory defect may be inferred in these populations.

The present finding is in contrast with the observations of (Das, 2009) who reported that insulin resistance is the major defect in young lean T2DM populations. However, insulin secretory defect has also been reported in Indian population by other Authors. It may suggest that even Indian lean young subjects may not contribute a homogenous population and the defects should be explored in different ethnic groups and may be in different subsets of the same ethnic groups.

To have a clearer idea about the uniqueness of the lean young subjects some other subgroups of diabetic subjects were studied in the present series. It is interesting to observe that, in middle aged subjects with BMI in the normal to obese range, the contribution of pancreatic β-cell dysfunction and insulin resistance became almost equal [deduction in Median values for both HOMA%B {Control, 119 (11-583); T2DM, 78 (10-462); (p=0.0001)] and HOMA%S [Control, 71 (11-489); T2DM, 47 (10-531) respectively, p=0.0001) Table 3B], when the defects were explored in subjects with BMI only the range of over weight to obesity , the difference in HOMA%B between Control and Diabetic groups was highly significant (p<0.0001), whereas the difference in HOMA%S was marginally significant (p=0.023) (Table 5B). This may indicate that the predominance in the secretory defect in T2DM subjects is not only an exclusive phenomenon for young lean subjects; rather it seems to be common for all subjects of diabetic subjects in our population. The finding is in contrast with the usual notion that the insulin resistance is the earliest and predominant defect in T2DM (Defronzo & Tripathy, 2009) and insulin secretory defect is secondary to the primary defect of insulin resistance.

The insulin secretory defect in the diabetic subjects were further characterized in terms of the phases of secretion. Loss of secretion in the 1st phase has been described as the characteristic feature of T2DM (John and Gerich, 2002). In the young lean subjects, the compromise in 1st phase response was evident even by the absolute insulin values where there was about 50% compromise in diabetic subjects compared to Control at the 30 minute. The compromise, however, was more evident with proper analysis where less than 1/2 of the secretory capacity was seen in the diabetics as compared to Control [Median (range) value of HOMA%B of Control, 140 (19-366); T2DM, 66 (11-271); respectively, p=0.001, (Table 2C)].

In contrast to the 1st phase the compromise in the 2nd phase insulin secretion was proportionately less (about 50%). The data show that the defects in insulin secretion in

these subjects correspond to the usual trend reported for type 2 diabetes (Van Haeften et al., 1991)

The experiment on the nonobese subjects was replicated on young to middle aged subjects with higher BMI. Mirroring the findings in the young, Diabetic to Control Ratio for Phase 1 insulin secretion was only 0.34 in contrast to 0.45 for Phase 2 secretion. The compromise was also evident form the ratio, Phase 1 was 4 times higher than Phase 2 in Controls, but it was only 3 times in case of T2DM. John and Gerich (2002) has reported a loss of 1st phase insulin secretion in Caucasian population. So far no published report was found on the phase wise defect of insulin secretion in any Indian population.

Determinants of insulin secretory defect and insulin resistance in young diabetic subjects

Age is known to be a major determinant for insulin secretion and sensitivity (Akehi et al., 2007; Jackson et al., 1988). Particularly insulin sensitivity is known to detoriate over time and it is related to increasing incidence of diabetes after middle age. It is recognized that people of Indian subcontinent develop diabetes in a relatively earlier age compared to the Caucasian population. In the present study specific attention was given to analyze the interaction of age with BMI and the basic defects of diabetes. On multivariate regression insulin sensitivity was inversely associated with age.

Compared to age BMI had a much stronger association with insulin secretory dysfunction and insulin resistance both on bivariate and multivariate analysis. It was especially interesting to note that insulin resistance had a clear stepwise reduction in the control subjects when the BMI ranges were categorized in 19-23 (normal weight) to 23-27 (overweight) and >27 (obese) groups. The association remained significant in Pearson's correlation analysis both with total subjects as well as with various subgroups. The association was maintained on multiple regression, both in insulin resistance on adjusting the effects of age and BMI. For the diabetic group the stepwise reduction as per BMI subgroups was not evident as the lowest BMI group already showed about 50% fall in HOMA%S and it further fell by only 30% in the >27 BMI group. Thus it seems that insulin resistance produces a BMI related background environment, but the other triggering factor β cell dysfunction is a must for the development of DM as proposed by others (Pimenta et al., 1996, Yoneda et al., 1992, O'Rahilly et al., 1986, Kosaka et al., 1996, Chen et al., 1995, Leahy, 1990, Gerich, 1998).

Association of proinsulin and glucagon with the basic defects of diabetes

Increased proportion of proinsulin is implicated with T2DM (Mako et al., 1977; et al., 1987; Yoshioka et al., 1988; Temple et al., 1989). It may be considered as a type of insulin secretory defect, but at the same time, it may also lead to insulin resistance (MykkaEnen et al., 1995). In the present study, proinsulin was found to be substantially elevated in diabetic subjects as compared to control both in the normal weight as well as overweight and obese groups. On multivariate analysis proinsulin did not show any significant association with insulin secretory defect, but it was associated with insulin resistance in the T2DM subjects. Elevated proinsulin and its association with insulin resistance have been claimed by Lele et al (2006) on Indian population.

Glucagon is the major insulin antagonist and its rise in T2DM has been reported to be associated with insulin resistance (Cryer, 1981; Dinneen et al., 1995; Ivan, 2008). Lower level of serum glucagon has been observed in the present subjects, on multivariate analysis it was found to be associated with HOMA%B but not with HOMA%S. It also showed a highly significant association with the diabetic status. Thus glucagon status seems to be a major determinant of insulin secretion in this population.

Association of adipocytokines and chronic inflammatory markers with insulin secretory defect and insulin resistance has been postulated before. Resistin is an important cytokine which has derived its name from its association with insulin resistance (Steppan et al., 2001). It was explored whether this adipocytokines are associated with the basic defects of T2DM in our population. Serum resistin was found to be significantly raised in the diabetic group and it was associated with insulin resistance on bivariate as well as multivariate analysis. The data is compatible with the finding of Valsamakis (Valsamakis et al, 2004).

Subclinical chronic inflammation, with hsCRP as a marker, has been implicated as a meeting point for the genesis of a large number of NCDs and it is thought that insulin resistance is associated with hsCRP. The present data suggest the presence of subclinical chronic inflammation in diabetic subjects. It supports the findings of Joshua et al (2003). On further analysis, subclinical chronic inflammation seem to be associated with insulin secretory defect and insulin resistance (Pickup et al, 1997; Mohamed-Ali et al., 1998) even after adjustment of age and BMI as confounding variables.

Difference between young prediabetic and diabetic subjects regarding the basic defects of diabetes

Impaired glucose tolerance (IGT) is considered to be an intermediate stage in the pathogenesis of T2DM (Genuth et al., 2003) and it is one of the major types of prediabetes along with impaired fasting glucose (IFG) and the combination of the two (IFG IGT). This condition has a sort of parallelism with young onset T2DM as it precedes the development of T2DM in the older adults. It was felt important to explore the basic defect(s) of IGT in this population and compare it with that of the young onset T2DM for gaining more insight into the causal links of the underlying mechanisms. The IGT and their control subjects were recruited with BMI in the nonobese range to make it comparable to the nonobese young T2DM subjects. Although there was a mild degree of hyperinsulinemia in the IGT groups (Table 25), neither the 1st phase or nor the 2nd phase insulin secretion showed any significant difference with the control. However, a clear tendency for the compromise was evident in both the phases ($p<0.101$ and $p<0.148$ respectively). Calculation of HOMA%B rather showed hypersecretion, but a loss of insulin sensitivity was evident on calculation of HOMA%S. Presence of insulin resistance without significant insulin secretory defect has previously been reported in this population (Roy et al., 2007), but they were from a higher BMI range. The present data confirms that the basic phenomenon is the same in the nonobese IGT subjects. Comparison of the IGT data with those young and T2DM subjects indicate that these two disorders may constitute distinct pathophysiological entities with insulin secretory dysfunction as predominant feature in T2DM in contrast to insulin resistance in IGT. The conversion of IGT to adult T2DM seems to be triggered by a failure of the pancreatic β cells on an inherited background of insulin resistance proposed by Lillioja et al (1993and); Weyer et al (2001).

The association of IGT with proinsulin was also explored and it was found to be almost double compared to the Control. Thus proinsulin seems to be an important determinant of insulin resistance in IGT (Haffner et al., 1994). Haffner et al have reported a raised proinsulin level in IGT subjects and it corresponds to the present findings.

Inherited defects in young onset T2DM

DM is known to have a genetic and environmental component and it was felt important to explore the inheritance of the basic defects in young onset T2DM. A family based study

was designed with the parents of the young patients. A tendency of insulin hypersecretion was revealed both in 1st phase and 2nd phases ($p< 0.07$ and 0.08 respectively) and it was also reflected in HOMA%B values ($p=0.095$). However, no significant defect in insulin secretion or sensitivity was found in these relatives. Thus, it still remains unsettled whether insulin secretory defect or insulin resistance or both are genetically inherited in these young subjects.

The following conclusions may be made from the series of the studies:

a) Insulin secretory deficiency seems to be the main basic defect in young onset T2DM among Bangladeshi population;

b) The insulin secretory defect in young onset T2DM affects both the 1st and 2nd phases with 1st phase being affected more than the 2nd phase.

c) DM in young to middle aged subjects are associated with both insulin deficiency and insulin resistance, but insulin resistance in these cases seem to be mainly a function of increased BMI (and to a lesser extent age) and insulin secretory deficiency is the consistently predominant basic defect in this disorder like that in young T2DM.;

d) Irrespective of age and adiposity glucagon is a major determinant of insulin secretory deficiency in our population and, on the other hand proinsulin, resistin and hs- CRP are important determinants of insulin sensitivity;

e) The predominant defect in impaired glucose tolerance is insulin resistance and it seems to have an association with hyperproinsulinemia;

f) Hyperinsulinemia may be an inherited feature in young onset type 2 diabetes mellitus

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CHAPTER VI

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APPENDICES

CHAPTER VII
APPENDICES

Appendix I

Estimation of serum glucose

Serum glucose was estimated by enzymatic colorimetric (GOD-PAP) method (Trinder, 1969) using reagents from Randox Laboratories, UK. The estimation was carried out in an autoanalyzer, Hitachi 704.

Principle

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

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

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

Glucose estimation reagents

Contents	Initial concentration of solution
Buffers	
Phosphate buffer	0.1 mol/l, pH 7.0
Phenol	11 mol/l
GOD-PAP Reagent	
4-aminophenazone	0.77 mmol/l
Glucose oxidase	>1.5 kU/l
Peroxidase	>1.5 kU/l
Standard	
Glucose	5.55 mmol/l (100 mg/dl)

Additional reagent: Uranyl Acetate 0.16%

Materials required

Microcentrifuge tube, micropipettes, pipette tips

Procedure

Procedure for glucose GOD-PAP assay without deproteinisation. The instrument was calibrated before estimation.

Serum and reagent was taken in specific cup and arranged serially into the analyzer (Hitachi 704). The autoanalyzer was programmed for the estimation of glucose and allowed to run with following procedure:

For reaction proper 5 l serum sample was mixed with 500 l reagent and incubated at 37 o C for 10 minutes and absorbance was taken against the reagent blank at 500 nm.

Calculation of result

The unit or auto analyzer was calibrated every day. Values for unknown samples were obtained extrapolating control sera.

Validity of the results of unknown samples

After each run check was carried out for values of QC1 and QC2 and internal control. Once satisfied with the levels of QC1 and QC2 and the internal control, the results of unknown samples was accepted and subsequently recorded into the database.

Appendix II

Estimation of Serum Total cholesterol

Total cholesterol was measured by enzymatic cilorimatric (cholesterol oxidase /peroxidase) method using reagents from Randox Laboratories, UK. The estimation was carried out in an autoanalyzer Hitachi 704.

Principle

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

$$\text{Cholesterol} + H_2O \xrightarrow[\text{eesestrase}]{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + \text{Cholestene} \xrightarrow[\text{eesestrase}]{\text{Cholesterol oxidase}} \text{Cholestene-3-one} + H_2O_2$$

$2H_2O_2$ +4-aminoantiphyrine + Phenol $\xrightarrow{\text{Peroxidase}}$ Quinoneimine + H2O

Reagent composition

Contents	Initial Concentration of Solution
Reagents	
4- aminoantipyrine	0.30 mmol/l
Phenol	6 mmol/l
Peroxidase ≥0.5 U/ml	
Cholesterol esterase	≥0.15 U/ml
Cholesteol oxidase	≥0.1 U/ml
Pipes Buffer	80 mmol/l; p^H 6.8
Standard	5.17 mmol/l (200 mg/dl)

Materials

Microcentrifuge tube, micropipettes and pipettes tips

Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the autoanalyzer .5 µl sample and 500 µl reagent were mixed and incubated at 37° C for 5 minutes within the autoanalyzer (Hitachi 704). The reaction occurred in reaction cup or cell. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

The unit or auto analyzer was calibrated every day. Values for unknown samples were obtained extrapolating control sera.

Validity of the results of unknown samples

After each run check was carried out for values of QC1 and QC2 and internal control. Once satisfied with the levels of QC1 and QC2 and the internal control,

the results of unknown samples was accepted and subsequently recorded into the database.

Appendix III

Estimation of Serum Triglyceride (TG)

Serum triglyceride was estimated by enzymatic colorimetric (GOD-PAP) method using reagents from Randox Laboratories, UK. The estimation was carried out in an autoanalyzer, Hitachi 704.

Principal

The triglyceride is determined after enzymatic hydrolysis with lipases. The indicator is a quinoemine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase (Fossati and Prencipe, 1982).

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

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + 0_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{8-dihydroxyacetone phosphate} + H_2O_2$$

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

Reagents

Contents	Concentration in the Test
1. Buffer	
Pipes Buffer	40 mmol/l
4-chlorophenol	5.5 mmol/l
Magnesium ions	17.5 mmol/l
2. Enzyme Reagents	
4-aminophenazone	0.5 mmol/l
ATP	1.0 mmol/l
Lipases	>150 U/ml
Glycerol 3-phosphate oxidase	1.5 U/ml
Peroxidase	0.5 U/ml
3. Standard	2.29 mmol/l (200 mg/dl)

Materials, micropipettes and pipettes tips

Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the autoanalyzer. 5 µl of serum sample and 500 µl of reagent were mixed and incubated at 37° C for 5 minutes within the autoanalyzer.

The reaction occurred in reaction cell. The absorbance of the sample and the standard against the reagent blank were measured at within 60 minutes.

Calculation of result

The unit or auto analyzer was calibrated every day. Values for unknown samples were obtained extrapolating control sera.

Validity of the results of unknown samples

After each run check was carried out for values of QC1 and QC2 and internal control. Once satisfied with the levels of QC1 and QC2 and the internal control, the results of unknown samples was accepted and subsequently recorded into the database.

Appendix 1V

Estimation of Serum High Density Lipoprotein cholesterol (HDL-c)

Serum high density lipoprotein (HDL) was measured by enzymatic colorimetric (cholesterol CHOD-PAP) method (RANDOX Laboratories, UK)

Principle

High density lipoproteins (HDL) were separated from chylomicrons, VLDL (Very low density lipoproteins) and LDL (Low density lipoproteins) by the addition of a precipitating reagent (phosphotungstic acid-magnesium chloride) to serum or plasma. After centrifugation, the cholesterol contents of HDL fraction which remained in the supernatant was determined by the enzymatic colorimetric method (CHOD-PAP) using an autoanalyzer, Hotachi 704 (Friedwald, 1972).

Reagent composition

Buffer, Enzymes and Standard 50 mg/dl (1.29 mmol/l)

Materials

Microcentrifuge tube, micropipettes and pipettes, disposable tips

Procedure

Samples (200 ml) and precipitating reagents (500µl) 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 µreagent were mixed and incubated at 37°C for 5 minutes within the autoanalyzer. The reaction occurred in reaction cell. The absorbance and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of result

The unit or auto analyzer was calibrated every day. Values for unknown samples were obtained extrapolating control sera.

Validity of the results of unknown samples

After each run check was carried out for values of QC1 and QC2 and internal control. Once satisfied with the levels of QC1 and QC2 and the internal control, the results of unknown samples was accepted and subsequently recorded into the database.

Appendix V

Estimation of Low Density Lipoprotein (LDL) Cholesterol

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

The Formula is as follows:

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

Appendix VI

Estimation of serum insulin

Serum insulin was estimated by the Enzyme Linked Immunusorbant Assay (ELISA) (LINCO Research Kit, USA).

Principles of procedure

This assay is a Sandwich ELISA based, sequentially, on: 1) capture of human insulin molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of monoclonal mouse anti-human insulin antibodies and the binding of a second biotinylated monoclonal mouse anti-human antibody to the captured insulin, 2) wash away of unbound materials from samples, 3) conjugation of horseradish peroxidase to the immobilized biotinylated antibodies, 4) wash away of free enzyme conjugates, and 5)

quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3`,5,5`-tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured human insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human insulin.

A. **REAGENTS SUPPLIED**

Each kit is sufficient to run one 96-well plate and contains the following reagents:

1. **Microtiter plate**

Coated with Mouse Monoclonal anti-Human insulin Antibodies, ready to use

2. **Adhesive Plate Sealer**

1 sheet, ready to use

3. **10X HRP Wash Buffer Concentrate**

10X concentrate of 50 mM Tris Buffered Saline containing Tween-20, 50ml

Preparation: Dilute 1:10 with deionized water

4. **Human Insulin Standards**

Human Insulin in Buffer: 2, 5, 10, 20, 50, 100 and 200 μU/ml, 0.5ml/bottle,ready to use.

5. **Quality Controls 1 and 2**

Purified Recombinant Human Insulin in Assay Buffer, 0.5ml/bottle, ready to use.

6. **Matrix Solution**

Heat-treated Charcoal stripped Off the Clot Human Serum, 1ml/vial, ready to use.

7. **Assay Buffer**

0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% Sodium Azide, and 1% BSA, 20ml/vial, ready for use.

8. **Human Insulin Detection Antibody**

Pre-titered Biotinylated Monoclonal Mouse anti-Human Insulin Antibody, 3ml/vial, ready to use.

9. **Enzyme Solution**

Pre-titered Streptavidin-Horseradish Peroxidase Conjugate in Buffer, 12ml/vial, ready to use.

10. **Substrate**

3,3',5,5'-tetramethylbenzidine in Buffer 12 ml/vial, ready to use. Minimize the exposure to light.

11. **Stop Solution**

0.3 M HCl 12 ml/vial, ready to use.

Caution: Corrosive Solution

Materials Required

1. Pipettes and Pipette Tips: 10 µl –20 µl or 20 µl-100 µl
2. Multi-Channel Pipettes and Pipette Tips: 5~50 µl and 50~300 µl
3. Buffer and Reagent Reservoirs
4. Vortex Mixture
5. Deionized Water
6. Microtiter Plate Reader capable of reading absorbency at 450 nm
7. Orbital Microtiter Plate Shaker
8. Absorbant Paper or Cloth

Reagent Preparation

1. HRP Wash Buffer: Dilute the 10X concentrated HRP wash buffer 10 fold by mixing the entire content of buffer with 450 ml deionized water or glass distilled water.

Assay Procedure

1. Remove the microtiter assay plate from the foil pouch and fill each well with 300µl of diluted HRP wash buffer. Incubate at room temperature for 5 minutes. Decant wash buffer and remove the residual amount from all wells by inverting the plate an tapping it smartly onto absorbent towels several times. Do not let wells dry before proceeding to the next step. If an automated machine is used for the assay, follow the manufacturer's instructions for all washing steps described in this protocol.

2. Transferred detection antibody solution to a reagent reservoir and add 20 µl of this solution to each well with a multi-channel pipette.

3. Added 20µl matrix solution to the NSB, Standard, and Control wells.

4. Added 20 µl assay buffer to each of the sample wells.

5. Added 20µl assay butter to the NSB wells. Add in duplicate 20 µl human insulin standards in the order of ascending concentration to the appropriate wells.

6. Added 20 µl QC1 and 20 µl QC2 to the appropriate wells.

7. Added sequentially 20 µl of the unknown samples in duplicate to the remaining wells. For best result all additions should be completed within one hour. Cover the plate with plate sealer and incubate at room temperature for 1 hour on a orbital microtiter plate shaker set to rotate at moderate speed (approximately 400 to 500 rpm)

8. Removed plate sealer and decant solutions from the plate. Tapped as before to remove residual solutions in the wells.

9. Washed wells 3 times with diluted HRP wash buffer, 300 µl per well per wash. Decant and tap after each wash to remover residual buffer.

10. Added 100µl enzyme solution to each well. Cover the plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.

11. Removed sealer, decant solutions from the plate, and tapped plate to remove the residual fluid.

12. Washed wells 5 times with diluted HRP buffer, 300µl per well per wash. Decant and tapped after each wash to remover residual buffer.

13. Added 100µl of substrate solution to each well, covered plate with sealer and shake on the plate shaker for 8 to 10 minutes. Blue color should be formed in wells of insulin

standards with intensity proportional to increasing concentrations of insulin. Removed sealer and added 100µl stop solution and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Read absorbance at 450 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well. The absorbance of highest insulin standard should be approximately 1.8- 2.6

Calculations

The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function.

Appendix VII

Determination of Serum C-peptide

Determination of Serum C-peptide

Serum C-peptide was estimated by the Chemiluminescence enzyme immunoassay method (LINCO Research Kit, USA).

Principle for the estimation of C-peptide

Circulating fasting serum C-peptide was assessed by Chemiluminescence Technique using IMMULITE/IMMULITE 1000 C-peptide in a solid-phase, competitive Chemiluminescence enzyme immunoassay.

Incubation Cycles: 1X60 minutes.

Specimen Collection

The patient should be fasting. Blood was collected by venipuncture, avoiding hemolysis, into plain tubes (without anticoagulant) and separated the serum or plasma from the cell. The use of an ultracentrifuge is recommended to clear lipemic samples.

Volume required

10 µL serum (Sample cup must contain at least 100µL more than the volume required).

Storage

Assay within 2-3 hours, or store frozen at -20^{0}C for a week.

Materials Supplied for C-peptide determination

Components are a matched set. The barcode labels are needed for the assay.

C-PEPTIDE Test Units (LPEP1)

Each barcode labeled unit contains one bead coated with a polyclonal rabbit anti- C-peptide. Stable at 2-8°C until expiration date.

C-peptide Reagent Wedges (LPEP2) with barcodes

7.5 ml alkaline phosphatase (bovine calf intestine) conjugated to C-peptide in buffer, with preservative.

C-peptide Adjustors (L PEPL, LPEPH)

Two vials (Low and High) of lyophilized C-peptide in buffered human albumin with preservative.

Kit Components

Supplied Separately

C-peptide Sample Diluent (LPEZ)

For the manual dilution of patients samples.

One vial containing 25 ml of C-peptide free buffered human albumin, with preservatives. Stable at 2-8°C for 30 days after opening.

LSUBX : Chemiluminescent Substrate.

LPWS2 : Probe Wash Module.

LKPM : Probe cleaning kit.

LCHx-y : Sample Cup holders (Barcoded).

LSCP : Sample Cups (Disposable)

LSCC : Sample cup caps (Optional)

Also required

Sample transfer pipettes, distilled or deionized water, controls.

Assay procedure for the estimation of Serum C-peptide

Note that for optimal performance, it is important to perform all routine maintenance procedure as defined in the IMMULE/IMMULITE 1000 operator's manual for: preparation, setup, dilutions, adjustment, assay and quality control procedures.

Recommended adjustment interval: 2 weeks.

Quality control samples: At least two levels (low and high) of C-peptide are used as quality control.

Expected Values: Samples were collected from a total of 136 fasting healthy individuals and are analyzed by the IMMULITE C-peptide procedure yielding a median of 2.2 ng/mL (728 pmol/L) and non-parametric central 95% reference range of: 0.9 – 7.1 ng/mL (298 – 2,350 pmol/L).

Appendix VIII
Determination of serum proinsulin

Serum proinsulin was estimated by the Enzyme Linked Immunosorbent Assay (ELISA) method (LINCO Research Kit, USA).

2.6.6.1. Principle for the estimation of serum proinsulin

Measurement of total in human is a Sandwich ELISA method in which two monoclonal antibodies are directed against antigenic determinants on the proinsulin molecule. During incubation proinsulin in the sample reacts with the horseradish peroxidase-conjugate to the immobilized biotinylated antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine.The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured human proinsulin in the unknown sample, the later can be derived by interpolation from a reference curve generated in the assay with reference standards of known concentrations of human proinsulin.

2.6.6.2. Reagents for proinsulin determination

Each kit is sufficient to run one 96 –well plate and contains the following reagents:

1. Microtiter plate

Coated with pretreated guinea pig anti-human insulin antibodies.

2. Adhesive Plate Sealer
3. 10XConcentrate HRP Wash Buffer

Washing solution (buffer with preservatives) 2x50mL.

4. Standards

Human proinsulin in buffer: 2, 5,10,20,50,100 and 200 pM, ready to use.

5. Quality Control 1 and 2

Purified recombinant human proinsulin in assay buffer, ready to use.

6. Matrix Solution

Charcoal stripped depleted human serum 2x1 mL, ready to use.

7. Assay Buffer

0.025 M Phosphosaline, pH 6.8, containing 0.025M EDTA, 0.08% Sodium Azide, 1 % BSA, 2x9 mL, ready to use.

8. Detection Antibody

Pretreated biotinylated monoclonal mouse anti- human C-peptide antibody (2x12), ready for use.

9. Enzyme Solution

Pretreated streptovidin-horseradish peroxidase.

Conjugate buffer (2x12), ready to use.

10. Substrate

3, 3', 5, 5'-tetramethylbenzidine in Buffer (2x12mL), ready for use. The reagent was stored in dark.

11. Stop Solution

0.3 M HCL (2x12), ready to use.

Reagent preparation for proinsulin determination

The concentrated HRP wash buffer was diluted 10 times by the entire content of each bottle with 450 mL deionized water.

Assay procedure for the estimation of proinsulin

Each well of the microplate, which was coated with polyclonal guinea pig anti-human insulin antibodies, was washed with 300µl of diluted wash buffer and the plate was sharply strike on absorbent paper to remove the residual droplets. Sixty (60) µL assay buffer was added to each standard and control wells and 80 µL assay buffer to blank and sample wells .Again, 20 µL matrix solution was added to the blank, standard and quality sample wells. Then, 20 µL of each standard, control an sample was dispensed into the appropriate wells in duplicate, the plate was covered with plate sealer and incubated for 1 hour at room temperature on orbital microtiter plate shaker set at 400 rpm .After the incubation the content of the wells was briskly shacked out and the wells were rinsed 3 times by dispensing 300 µL diluted HRP wash buffer into each well. Then, 100 µL of antibody solution was dispensed into each well and incubated at room temperature for 1 hour on orbital microtiter plate shaker at 400 rpm .After the incubation, the solution was then decanted from the plate and again washed with HRP wash buffer for 3 times. After washing, 100 µL enzyme solutions was dispensed into each well and the plate was incubated with moderate shaking at room temperature for 30 minutes .The plate was then washed for 6 times with the same buffer .Finally, 100 µL substrate solution was added to each solution, the plate was covered and kept on the shaker for incubation. After 15-20 minutes stop solution was added to each well to stop the reaction. Absorbance of the colored solution was taken at 450 nm.

Calculation for the amount of proinsulin

The proinsulin value of each sample was obtained as follows:

- Plotted the net absorbance value for each level, obtained by subtracting the value for the assay buffer from the value of individual.
- The smooth curve was drawn that fits 5 plotted points

The results of unknown samples calculated using logistic function.

Appendix IX

Determination of Serum Glucagon:

Serum glucagon was estimated by the Enzyme Immuno Assay (EIA) method (PENINSULA LABORATORIES, LLC-A Member of the Bachem Group).

Principle for the estimation of serum glucagon:

Circulating fasting serum was assessed by Competitive Immunoassay Technique using Peptide Enzyme Immunoassay (EIA).

Coated plate

↓

Specific Antiserum ↔ Standard or Sample Peptide

↓

Bt-Tracer

↓

SA-HRP

↓

TMB↔Developing Blue 650 nm Color

↓

2 N HCL

↓

Stopped Yellow 450 nm Color

The **antiserum** is immobilized on a 96-well plate. A constant concentration of **Bt-tracer** (biotinylated tracer) and varying concentrations of unlabeled standard or sample peptide compete for binding specifically to the antiserum. Captured Bt- tracer is subsequently boundly by **SA-HRP** (streptavidin-conjugated horseradish peroxidase), which produces a soluble colored product after **TMB** (3, 3, 5, 5"-tetramethyl benzidine dihydrochloride)is added.

Reagent for glucagon determination:

- EIA buffer concentrate (50 ml 20 x concentrate)
- One 96-well immunoplate with acetate plate sealer.
- One vial of peptde antibody (lypholized powder)
- One vial of peptide standard (1 µg lypholized).
- One vial of biotinylated peptide (lypholized powder)
- Streptavidin-HRP concentrates (100 µl 200X concentrate).
- Substrate solution (11ml of TMB and H_2O_2).
- 2 N HCL (15 ml)
- One vial standard diluent (peptide-free rat or human serum) for extraction-free kits only (8 ml).

Assay Procedure for the estimation of glucagon

1- Into each well of the immunoplate add
   50 µl standard or sample
   25 µl antiserum
   25 µl Bt-tracer

2- Incubate at room temperature for 2 hours

3- Wash immunoplate 5 times with 300 µl per well of EIA buffer

4- Add 100 µl / well of streptavidin-HRP

5- Incubate at room temperature for 1 hour

6- Wash immunoplate 5 times

7- Add 100 µl / well of TMB solution

8- Incubate at room temperature for usually 30-60 minutes

9- Terminate reactions by adding 100 µl 2N HCL per well

10- Read absorbance at 450 nm within 10 minutes

Calculation for the concentration of glucagon:

Glucagon was estimated by using SPSS curve-fitting software analysis. The concentration of unknown samples was calculated using a computer program known as Kinetic Calculation (KC).

Appendix X

Estimation of serum resistin

Serum Resistin was estimated by Enzyme Linked Immunosorbent Assay (ELISA).

Principle

This assay is based, sequentially, on: 1) capture of human resistin from sample by a monoclonal antibody, immobilized in the wells of a microwell plate, 2) washing off unbound materials including free materials from samples, 3) binding of the biotinylated monoclonal human resistin antibody to the other side of captured human resistin molecules, 4) conjugation of SA-HRP (Poly-HRP-labeled streptavidin) enzyme to the biotinylated antibodies, and 5) quantification of bound detection conjugate by monitoring SA-HRP enzyme activity in the presence of TMB (tetramethylbenzidine) substrates. The enzyme activity is measured spectrophotometrically by the absorbency at 450 nm due to production of the photometric product. Since the amount of photometric product is directly proportional to the concentration of human resistin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human resistin.

Reagents

This Human Resistin ELISA kit of LINCO RESEARCH, USA used for the non-radioactive quantification of Human Resistin in serum, plasma, and adipocyte extracts or cell culture media samples. The kit specifically measures native Human Resistin and has no cross reactivity to Mouse Resistin. Each kit is sufficient to run one 96-well plate and contains the following reagents:

A. Human Resistin ELISA Plate

Coated with anti- Human Resistin Monoclonal Antibody

Quantity: 1 plate

Preparation: Ready to use

B. Adhesive Plate Sealer

Quantity: 2 Sheets

Preparation: Ready to use

C. 10X HRP Wash Buffer Concentrate

10X concentrate of 50 mM TBS Buffer containing 0.05%Tween 20

Quantity: 2 bottles containing 50 ml each

Preparation: Dilute 1:10 with distilled or deionized water

D. Human Resistin Standards

Human Resistin, lyophilized.

Quantity: 2.5 ng/0.25 ml (10 ng/ml) upon hydration.

Preparation: Contents Lyophilized. Reconstitute with 250 µL distilled or deionized water to obtain 10.0 ng/ml.

E. Human Resistin Quality Controls 1 and 2

One vial each, lypholized, containing Human Resistin in Assay Buffer.

Quantity: 0.25 ml/vial upon hydration.

Preparation: Contents Lyophilized. Reconstitute each vial with 250 µL distilled or deionized water.

F. Assay Buffer

0.05M PBS, pH 7.4, containing 0.025 M EDTA, 0.08% Sodium Azide and 1% BSA.

Quantity: 12 ml

Preparation: Ready to use

G. Human Resistin Detection Antibody

Biotinylated anti-human resistin monoclonal antibody

Quantity: 9 ml

Preparation: Ready to use

H. Enzyme Solution

Pre-titered Streptavidin-Horseradish Peroxidase Conjugate (SA-HRP)

Quantity: 12 ml

Preparation: Ready to use

I. Substrate

3, 3', 5, 5'-tetramethylbenzidine (TMB)

Quantity: 12 ml

Preparation: Ready to use

J. Stop Solution

Quantity: 12 ml

Preparation: Ready to use

Sample Preparation

1. All the reagents were allowed to come to room temperature.
1. Serum samples were diluted 1:5 in Assay Buffer.

Human Resistin Standard Preparation

1. Using a pipette, Human Resistin Standard was reconstituted with 250 µL distilled or deionized water into the glass vial to give a 10 ng/mL concentration of Standard. Inverted and mixed gently, waiting for 5 minutes then vortexed gently.
2. Six tubes were labelled 5, 2.5, 1.25, 0.625, 0.312, and 0.16 ng/ml. 100 µL Assay Buffer were added to each of the six tubes. Serial dilutions prepared by adding 100 µL of the 10 ng/ml reconstituted standard to the 5 ng/ml tube, mixed well and 100 µL of the 5 ng/ml reconstituted standard was transfered to the 2.5 ng/ml tube, mixed well and 100 µL of the 2.5 ng/ml Standard was transfered to the 1.25 ng/ml tube, mixed well and 100 µL of the 1.25 ng/ml Standard was transfered to the 0.625 ng/ml tube, mixed well and 100 µL of the 0.625 ng/ml Standard was transfered to the 0.312 ng/ml tube, mixed well and 100 µL of the 0.312 ng/ml Standard was transfered to the 0.16 ng/ml tube and mixed well.

Quality Control 1 and 2 Preparation

Using a pipette, each of the Human Resistin Quality Control 1 and Quality Control 2 were reconstituted with 250 µL distilled or deionized water into the glass vials. Invert and mixed gently, waiting for 5 minutes then mixed well.

Assay Procedure

All reagents to room temperature.

1. 10X concentrated Wash Buffer was diluted 10 fold by mixing the entire content of each bottle of Wash Buffer with 450 ml deionized or distilled water
2. Microtiter Assay Plate was removed from the foil pouch and 300 µl of diluted Wash Buffer was added to each well. Incubated at room temperature for 5 minutes. Decant Wash Buffer and the residual amount was removed from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. 60 µl Assay Buffer added into all wells.
4. 20 µl of Assay Buffer was added to blank wells.
5. 20 µl of Human Resistin Standards added in order of ascending concentration to the appropriate wells.
6. 20 µl QC1 and 20 µl QC2 added to the appropriate wells
7. 20 µl of diluted serum or plasma samples were sequentially added in duplicate to the remaining wells.
8. The plate was covered with plate sealer and incubate at room temperature for 1.5 hours on an orbital microtiter plate shaker at moderate speed, approximately 400 to 500 rpm.
9. Plate sealer was removed and decant solutions from the plate. Tapped as before to remove residual solutions in the wells.
10. Each wells were washed 3 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tapped firmly after each wash to remove residual buffer.
11. 80 µl Detection Antibody added to all wells. The plate was covered with plate sealer and incubate at room temperature for 1 hour on an orbital microtiter plate shaker at moderate speed, approximately 400 to 500 rpm.
12. Plate sealer was removed and decant solutions from the plate. Tapped as before to remove residual solutions in the wells.
13. Each wells were washed 3 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tapped firmly after each wash to remove residual buffer.
14. 80 µl Enzyme Solution added to each well. Plate covered with sealer and incubate with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.
15. Sealer was removed, decant solutions from the plate, and tapped plate to remove the residual fluid.

16. All the wells washed 3 times with diluted Wash Buffer, 300 µl per well per wash. Decant and tapped firmly after each wash to remove residual buffer.
17. 80 µl of Substrate Solution added to each well, plate covered with sealer and shaked on the plate shaker for approximately 8 to 15 minutes. Blue color was formed in wells of Resistin standards with intensity proportional to increasing concentrations of Resistin.
18. Sealer was removed and 80 µl Stop Solution was added and plate was shaked by hand to ensure complete mixing of solution in all wells. The blue color was turned to yellow after acidification.
19. Absorbance was read at 450 nm and 590nm in a plate reader within 5 minutes

Calculations

The dose-response curve of the assay fits best to a sigmoidal 5-parameter logistic equation. The results of unknown samples were calculated with the computer program having a 5-parameter logistic function. Final results should be multiplied by a 5 dilution factor

Sensitivity

The lowest level of human resistin that can be detected by this assay is 0.16 ng/mL.

Appendix XI

Estimation of serum high-sensitive C-reactive protein level

Principle

Polystyrene particles coated with monoclonal antibodies specific to human CRP are aggregated when mixed with samples containing CRP. These aggregates scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The result is evaluated by comparison with a standard of known concentration. The assigned value of CRP in N Rheumatology Standard SL is standardized against the international reference preparation BCR-CRM 470

Cardio phase* *hs*CRP is an in vitro diagnostic reagent for the quantitative determination of C-reactive protein (CRP) in human serum, and heparin and EDTA serum by means of particle enhanced immunonephelometry using BN* Systems. In acute phase response, increased levels of a number of plasma proteins, including C-reactive protein, is observed. Measurement of CRP is useful for the detection and evaluation of infection, tissue injury, inflammatory disorders and associated diseases. High sensitivity CRP (*hs*CRP) measurements may be used as an independent risk marker for the identification of individuals at risk for future cardiovascular disease. Measurements of *hs*CRP, when used in conjunction with traditional clinical laboratory evaluation of acute coronary syndromes, may be useful as an independent marker of prognosis for recurrent events, in patients with stable coronary disease or acute coronary syndromes.

Reagents

Materials provided

Cardio Phase* *hs*CRP Reagent, Code No. OQIY

Five vials containing 5ml each or three vials containing 2ml each.

Composition

Cardio Phase* *Hs*-CRP Reagent consists of a suspension of polystyrene particles coated with mouse monoclonal antibodies to CRP.

Procedure

1. Consult your BN* System instruction Manual for details regarding operation of the instrument.
2. Allow reagents and samples to equilibrate to room temperature (+15 to +25^{0}C) before use on the BN* 100 System. With a BN* II or BN Pro Spec System reagents and samples stored at +2 to +8^{0}C can be used immediately.
3. On the BN* 100 System, samples should be run at approximately the same ambient temperature (maximum 3^{0}C deviation) as the measurements used for recording the reference curve.

Results

The results are evaluated automatically by the analyzer and are represented in mg/L or in a unit selected by the BN* System user.

Interpretation of Results

Recent medical events resulting in tissue injury, infection or inflammation, which may cause elevated CRP levels should also be considered when interpreting results.

The AHA/CDC Scientific Statement5 provides the following risk assessment guidelines:

Risk	hsCRP (mg/L)
Low	<1.0
Average	1.0-3.0
High	>3.0

Appendix XII

Data Collection Sheet

1. Identification No:

2. Particulars of the Subjects:

i) Name:_____

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

iii) Age:_____ years

iv) Gender: Male / Female

v) Address:

Present: _____

Permanent: _____

vi) Phone/Cell:_____

vii) Date of 1st Examination:_____

3. Past Medical history:

Renal disease	**Y / N**	Hypertension	**Y / N**
Cardiac disease	**Y / N**		

4. Drug history:

Anti-HTN:	**Y / N**	Anti-obesity:	**Y / N**
Lipid Lowering:	**Y / N**	Anti-ischemic:	**Y / N**

5. Family history:

Family history of Diabetes

Family history of HTN

6. Social history:

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

ii) **Scio-economics Status:**

Total members of the family:

Income of the family (Monthly / Yearly):

iii) **Educational Status:**

Class I-VIII / SSC-HSC / Graduate / Others (_____)

iv) **Occupation:**

Current Designation Duration:

Type of Work

Professional / Housewife / Sales Person / Domestic help /

Laborer / Others (_____)

v) **Habit:**

Exercise:

Present (Type_____, Duration)

Past (Type _____, Duration)

Not

Smoking:

Present (Duration_____, Sticks/day_____)

Past (Duration_____, Sticks/day_____)

Not

Alcohol: Yes / No

7. Physical Examination:

(a) Anthropometry:

vi) Height in meter:

vii) Weight in Kg:

viii) Waist circumference:

ix) Hip circumference:

x) WHR:

xi) Mid-upper arm circumference (MUAC):

xii) Skin fold thickness:

xiii) Triceps:

xiv) Sub-scapular skin folds thickness:

xv) Body fat mass:

(b) Blood pressure:

	1st reading	2nd reading	Average reading
Systolic			
Diastolic			