ROLE OF LEPTIN IN THE PATHOGENESIS OF TYPE 2 DIABETES MELLITUS

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DECLARATION

The thesis titled 'Role of leptin in the pathogenesis of type 2 diabetes mellitus' is submitted in partial fulfillment of the requirement for the degree of Master of Philosophy (MPhil) degree in the Department of Biochemistry and Molecular Biology, University of Dhaka. This work was carried out in the Biomedical Research Group of BIRDEM, Dhaka and Department of Biochemistry and Molecular Biology, University of Dhaka. To the best of my knowledge no pat of the work has been submitted for another degree or qualification in any other Institutes at home or in abroad.

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DECLARATION

I hereby humbly declare that this Thesis, entitled **"Role of leptin in the pathogenesis of type 2 diabetes mellitus"**, is based on works carried out by me and no part of it has been presented previously for any higher degree.

The research work was carried out in:

(a) The Department of Biochemistry and Molecular Biology &

(b) Biomedical Research Group of BIRDEM

Under the guidance of Honorable Prof, Professor and Head of the Department of Biochemistry and Molecular Biology, University of Dhaka and Prof Liaquat Ali, Professor, Department of Biochemistry and Cell Biology, BIRDEM.

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Dedicated
To
My Beloved Parents

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Abstract

Leptin is a multifactorial hormone, which may be involved in the pathogenesis of type 2 diabetes by influencing insulin production or sensitivity and insulin resistance. The present study was designed to analyze insulin secretory defect in relation to leptin level in type 2 diabetic subjects and also to see whether blood fatty acid composition has any association with these abnormalities.

A cross-sectional analytical study included 59 newly diagnosed type 2 diabetic subjects whose age ranges between 30 to 45 years. Sixty two age-matched healthy non-diabetic subjects were taken as control. Anthropometric measurements of all subjects were done following standard procedures. Circulating fasting insulin and leptin were assayed by enzyme-linked immunoassay (ELISA). Fasting serum NEFA was measured by enzymatic-colorimetric method. Plasma fatty acid composition was characterized gas liquid chromatography using modified Dole Extraction method. Beta-cell function and insulin sensitivity were calculated by the homeostasis model assessment (HOMA) using HOMA-CIGMA software.

The diabetic subjects showed highly significant β-cell dysfunction and also insulin resistance as evident from HOMA B [20.20(4.20-89.60) in control vs. 78.35(35.50-365.7) in diabetic, $p<0.001$] and HOMA S [84.6(39.1-226.4) vs. 118.8(22.0-3573.7), $p<0.004$]. Serum body fat normalized leptin level was found to be lower in diabetic subjects [5.44 (0.65-34.7)] compared to control [8.35 (1.36-55), $p=0.012$]. The serum leptin level inversely correlated with insulin secretory dysfunction. The total NEFA level in the diabetic subjects was higher than control (0.652±0.196 vs. 0.42±0.15, $p<0.001$). NEFA was positively correlated with fasting blood glucose level. Leptin level is positively correlated with palmitoleic (C16:1) acid, linolenic (C18:3) acid and inversely correlated with palmitic (C16:0) acid, linoleic (C18:2) acid, behenic acid as well as of linoleic/ linolenic (C18:2/C18:3) acid, palmitic/ palmitoleic (C16:0/C16:1) acid ratios.

The conclusions of the study are : a) Serum leptin level in normal to mildly overweight Bangladeshi type 2 diabetic subjects is lower compared to age and BMI-matched healthy population; b) Lower leptin in type 2 diabetes mellitus subjects is associated with insulin secretory dysfunction; c) Modulation of serum leptin in type 2 diabetes mellitus is not related to total NEFA, but few individual fatty acids are associated with leptin both on higher and lower sides; and d) Few individual NEFAs are associated with increasing or decreasing insulin secretion or sensitivity.

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LIST OF ABBREVIATIONS

μL	Microlitre
AA	Arachidonic acid
ACC	Acetyl CoA Carboxylase
ACO	Acyl CoA Oxidase
ACS	Acyl CoA Synthetase
ADA	American Diabetic Association
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders
BMI	Body Mass Index
BMRG	Biomedical Research Group
CHD	Coronary Heart Disease
C-peptide	Connecting peptide
CPT-1	Carnitine palmitoyl Transferase 1
DBP	Diastolic Blood Pressure
DHA	Docosahexaenoic acid
DM	Diabetes Mellitus
EASD	European Association for the Study of Diabetes
EDTA	Ethylene Diamine Tetra Acetic Acid
EFA	Essential Fatty Acid
ELISA	Enzyme Linked Immuno sorbant Assay
EPA	Ecosapentaenoic acid
eq	Equivalent
FAME	Fatty Acid Methyl Ester
FAS	Fatty Acid Synthase
FFA	Free Fatty Acid
GAPT	Glycerol Phosphate Acyl Transferase

GLC	Gas Liquid Chromatography
GDM	Gestational Diabetes Mellitus
HbA_{1c}	Glycosylated Hemogloboin
HDL-C	High Density Lipoprotein- Cholesterol
HOMA B	Homeostasis Model Assessment β-cell Function
HOMA S	Homeostasis Model Assessment Insulin Sensitivity
HPLC	High Performance Liquid Chromatography
IR	Insulin Resistance
LDL-C	Low Density Lipoprotein- Cholesterol
M	Mean
MBP	Mean Blood Pressure
MC	Melancortin
mg	Milligram
min	Minute (s)
mm Hg	Millimeter of Mercury
mmol	Millimole
mmol/l	Millimole per Liter
MNT	Medical Nutrition Therapy
mRNA	Messenger Ribonucleic Acid
MSH	Melanocyte Stimulating Hormone
MUFA	Monounsaturated Fatty Acid
NEFA	Non- Esterified Fatty Acid
NGT	Normal Glucose Tolerance
NIDDM	Non-Insulin Dependent Diabetes Mellitus
NO	Nitric Oxide
NPY	Neuropeptide Y
Ob gene	Obesity Gene

OGTT	Oral Glucose Tolerance Test
OPD	Out Patient Department
p	Probability
%	Percent
PKC	Protein Kinase C
pmol/l	Picomol per Liter
POMC	Proopiomelanocortine
PPAR	Peroxisome Proliferator Activated Receptor
PPRE	PPAR Response Element
P-STAT	Phosphorylated STAT
PUFA	Polyunsaturated Fatty Acid
SBP	Systolic Blood Pressure
SD	Standard Deviation
SFA	Saturated Fatty Acid
SPSS	Statistical Package for the Social Science
SPT	Serine Palmitoyl Transferase
STAT	Signal Transducers and Activators of Transcription
STR	Subscapular Triceps Skinfold Ratio
TG	Triglyceride
TNF-α	Tumor Necrosis Factor Alfa
UCP	Uncoupling Protein
UFA	Unsaturated Fatty Acid
VLDL	Very Low Density Lipoprotein
WAT	White Adipose Tissue
WHO	World Health Organization
WHR	Waist Hip Ratio

INTRODUCTION

Introduction

Diabetes Mellitus is a metabolic disorder characterized by chronic hyperglycemia, resulting from defects in insulin secretion, insulin action or both (Anon, 1999; Stumvoll et al, 2005).

According to WHO, Diabetes Mellitus is classified as Type 1 (β-cell destruction, usually leading to absolute insulin deficiency), Type 2 (insulin resistance or insulin secretory defect) and other specific types (genetic defects of β-cell function, genetic defect in insulin action, disease of the exocrine pancreas, endocrinopathies, uncommon forms of immune-mediated diabetes) (WHO, 1999). Type 2 diabetes affects large numbers of people from a wide range of ethnic groups and at all social and economic levels throughout the world.

Diabetes mellitus (DM) is becoming a modern epidemic due to the high prevalence of obesity and physical inactivity. Currently DM has reached epidemic proportions, affecting more than 170 million individuals worldwide, with an estimated increase of at least 50% by 2010, especially in developing countries, where the prevalence will rise from 4.2 to 5.6% of which > 80% of the persons will be found in the developing countries by the year 2003 to 2025 (Sicree *et al.* 2003) and type 2 diabetes constitutes about 90-95% of all diabetic cases. The prevalence of DM varies widely among populations, due to differences in environmental influences and genetic susceptibility (Amot *et al.* 1997) at all social and economic levels throughout the world.

The pathophysiology of type 2 diabetes mellitus is a field of rigorous investigations. Type 2 diabetes is well characterized by defects in insulin secretion, insulin action, free fatty acids (FFA) and fat distribution (Boden, 1997; Kraegen *et al.* 2001). The progressive deterioration of pancreatic insulin secretion has been implicated as the proximate cause of the progressive increase in plasma glucose level (Taylor *et al.* 1994). The patients with type 2 diabetes display a marked decline in insulin secretion,

although the etiology of this impairment in β-cell function is not known. It may result from pre-programmed genetic abnormalities or from acquired insults such as that due to the chronic effect of mild hyperglycemia, commonly referred to as glucotoxicity. Regardless of this etiology, this decrease in insulin secretion is a major contributor to the development of the overt Type 2 DM state.

The insulin secretory dysfunction has been studied in different phases of secretion. The first and second phase of insulin secretion has been recognized for many years, the particular importance of the first phase in the dynamic signaling of insulin action had only recently been acknowledged (Luzi, 1989; O'Rahilly *et al*, 1994). Under physiological conditions, the most important determinant of insulin secretion is the blood glucose concentration, although numerous other endocrine, metabolic, or neural factors play physiological roles in the fine-tuning of insulin secretion. Abrupt glucose stimulated insulin release is biphasic, comprising a rapid first phase lasting 5-10 min, followed by a prolonged second phase, which continues for the duration of the stimulus. However, sustained (>24 hours *in vivo*) levels high glucose stimulation results in a reversible desensitization of the B cell response to glucose but not to other stimuli (Masharani, Karam and German, 2004). The first phase of insulin release may be due to release of insulin secretory granules that are directly adjacent or 'marginated' to the B cell membrane. Second-phase insulin release involves synthesis of new insulin molecules as well as ATP-dependent mobilization of granules from a storage pool into the rapidly releasable pool. Moreover, insulin release is pulsatile, undergoing short and long oscillations. B cell abnormalities in type 2 diabetes include: diminution in first and second phase insulin release, decreased early insulin responses during oral glucose tests, decreased pulsatile and oscillatory insulin release, increase release of proinsulin-like molecules, and impaired ability to compensate for superimposed tissue insulin resistance. The first phase is more efficient signal than the second, both in enhancing

glucose clearance and in 'priming' the liver to shut down glucose production. (Luzi, 1989; O'Rahilly *et al.* 1994).

In the early stages of type 2 diabetes or in patients with IGT, first-phase insulin release is almost invariably lost, despite the common enhancement of second phase insulin secretion. Although the physiological and metabolic implications of the biphasic beta-cell response are not yet fully understood, available evidence suggest a physiological role for the dynamics of insulin secretion and the acute surge of insulin release into the portal circulation in regulating glucose homeostasis. The first phase is blunted or lost early type 2 diabetes mellitus (Luzi, 1989; Lancet, 1994). It was found that individuals with a first-degree relative with type 2 diabetes already have reductions both 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 primarily defect for type 2 diabetes (Van Haeften *et al.* 2000).

Insulin resistance also plays a significant role in the pathophysiology of type 2 diabetes mellitus. Insulin resistance is a state in which a given concentration of insulin produces a less than normal biologic response. Insulin resistance is a cardinal feature of type 2 diabetes. Himwsorth and Kerr in 1936, may have been the first to appreciate this, having described grouped of patients who were either insulin-insensitive or insulin sensitive. (Hollenbeck, 1984).

Insulin resistance can be due to the general categories of causes:

a. An abnormal B cell secretory product;
b. Circulating insulin antagonists;
c. A target tissue defect in insulin action.

Insulin resistance is central to the pathogenesis of type 2 diabetes (DeFronzo, 1997). Increased obesity is associated with insulin resistance. However, there is a high degree of variation such that lean or obese individuals can be either relatively insulin sensitive or resistant. In fact, only 8% of individual differences in insulin sensitivity can be explained by differences in BMI. Thus, although obesity is an important factor (Bonora *et al,* 1992; Kahn 1993) individual variation in insulin sensitivity largely exists independent of the degree of generalized obesity (Bogardus and Lillioja, 1992; Ferrannini, 1997).

Other important factors contributing to insulin resistance include the accumulation of omental fat (*i.e.* upper body fat distribution) and fat in the intra-myocellular compartment, both of which can exist independent of the degree of general adiposity. Multiple authors have demonstrated that it is a relative redistribution of fat to the omental compartment that has the greatest adverse effects, although increased subcutaneous fat also contributes to insulin resistance. This observation is central to the hypothesis that increased omental adipose tissue helps promote the IRS via the secretion of free fatty acids (FAs) and adipocyte-derived proteins such as plasminogen activator inhibitor-1, TNFα, leptin, adiponectin, and others. Accumulating evidence suggests that these secreted factors may constitute a mechanistic link among increased omental fat, insulin resistance, and the expression of the IRS. Whether skeletal muscle insulin resistance or omental adipose tissue is primarily responsible for IRS is not known, but clearly both are integral to the pathogenesis of the IRS. (Cristina, 2004) An important study involving the Pima Indians of Arizona, USA, who has now the highest known rate of type 2 DM in the world, exemplifies the predictive value of increased insulin resistance. This study suggests that in this group the level of insulin resistance is relatively stronger predictor of the development of Type 2 DM than measures of insulin secretion. The final stage in the development of Type 2 DM is thought to be insulin secretory inadequacy. It is believed that the results from impairment of pancreatic B cell functions and

the inability of B cell to secret sufficient insulin to compensate for the increase in insulin resistance. The outcome is fasting hyperglycemia and the development of overt diabetes. (Kollerman *et al.* 1984). A continued increase in insulin resistance leads eventually to impaired glucose tolerance. World Health Organization criteria have been applied in a number of studies of IGT. These studies have determined the progression of type 2 DM at between 19% and 61%, 5-10 years after detection of IGT. IGT is currently the most reliable predictive marker of type 2 DM. Its presence usually indicates that the degrees of insulin resistance and compensatory insulin secretion rest in a precarious state of balance. Worsening of insulin resistance or diminishing insulin secretion will result in progressive hyper-glycemia and development of Type 2 DM (Holman *et al.* 1977). The progression though normal glucose tolerance to impaired glucose tolerance is marked by increase in both fasting and glucose stimulated plasma insulin levels and increasing insulin resistance.

It remains controversial whether insulin deficiency or insulin resistance is the earliest abnormalities in the natural history of the disease. It is, however, generally thought that both the defects are present in clinical cases of diabetes. The earliest detectable defect in beta-cell function is commonly thought to be a reduction in first-phase insulin release (Cerasi, 1967). The defects in insulin secretion include a markedly impaired and often absent first-phase insulin release, while second-phase insulin release is 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 young lean type 2 diabetic Bangladeshi subjects have substantially reduced insulin secretory capacity and the obese type 2 diabetic Bangladeshi subjects have both insulin secretory dysfunction and insulin resistance, but insulin deficiency seems to be predominant in the pathophysiology of type 2 diabetes in these patients (Al-Mahmood, 2000; Junaid, 2000).

Leptin, the obese (ob) gene product, originally identified, as an important regulator of body weight homeostasis and energy balance is multi-factorial hormone which may be involved in the pathogenesis of type 2 diabetes by influencing insulin production or sensitivity in humans. (Friedman, 1998; Segal, 1996). Leptin is a 16-kDa protein containing 146-amino acid with one disulfide bond located between cysteine residues 96 and 146 originating from the ob gene encoding a 4.5 kilo base adipose tissue mRNA with a highly conserved 167 amino acid open reading frame and is 84% identical of amino acid sequences between humans and mice. OB protein is homologous to the class I cytokine family (Ceddia *et al.* 1998).

Leptin plays its metabolic roles either indirectly (signaling to centers located in the central nervous system) or by acting directly on its targets cells. In endocrine pancreas, skeletal muscle and adipose tissue are three well-related sites where leptin seems to act directly. In adipose tissue Insulin-stimulated glucose uptake and lipogenesis are reduced and lipolysis is increased by leptin while in skeletal muscle leptin increases glucose uptake and glycogen synthesis under physiological conditions (normo-glycemia and normo-insulinemia), leptin shifts the flux of metabolites from adipocytes to skeletal muscle. This may function as a peripheral mechanism that controls body weight.

Two forms of leptin receptors (long and short forms) have been identified in various peripheral tissues that make them possible target sites for a direct action of leptin. The receptors are expressed in most tissues (brain, testes, ovary, kidney, spleen, intestines, brown and white adipose tissues, heart, lung, liver, skeletal muscle, and adrenals (Hoggard, 1998; Luoh *et al.* 1997), with the long form being prevalent in the choroids plexus, in addition to also being found in the medulla of the adrenal, the inner zone of the medulla of the kidney, pancreatic islets, and fat tissue (Wang, 1996; Emilsson, 1997; Luoh, 1997).

Recessive mutation in obr causes early onset of obesity though human obr mutation is rare. The amino acid sequences of mouse and humans are highly homologous though the length of the mouse protein, but human cDNA encodes much longer intracellular domain.

White adipocytes are the primary cells that synthesize and secrete leptin, although leptin has also been shown to be produced in muscle cells, gastric epithelium and placenta (Wang et al 1998). Leptin is expressed and secreted in proportion to adipocyte size and number and circulates in a concentration highly correlated with body fat mass in human and animal models (Meffei et al 1995). Its production is higher in subcutaneous than in visceral fat depots (Lonnqvist et al 1997), leptin gene expression may be a major factor causing regional difference in leptin secretion. Fat cell size and number is also appeared to be important. Subcutaneous fat cells are larger and constitute 80% of all fat tissue (Vanessa et al 1998). Despite these strong correlations, people with similar degrees of adiposity leptin concentration vary considerably. Several metabolic hormonal pathways affected by leptin including insulin sensitivity, lipogenesis, lipolysis in adipose tissue (Doehner & Anker, 2000). Phathophysiological factors include sex, race, puberty, fasting calorie loading, exercise and diurnal variation (Haffner et al 1996 and Mantzoros et al 1997). Furthermore leptin is under multihormonal control (such as, glucocorticoids and catecholamins).

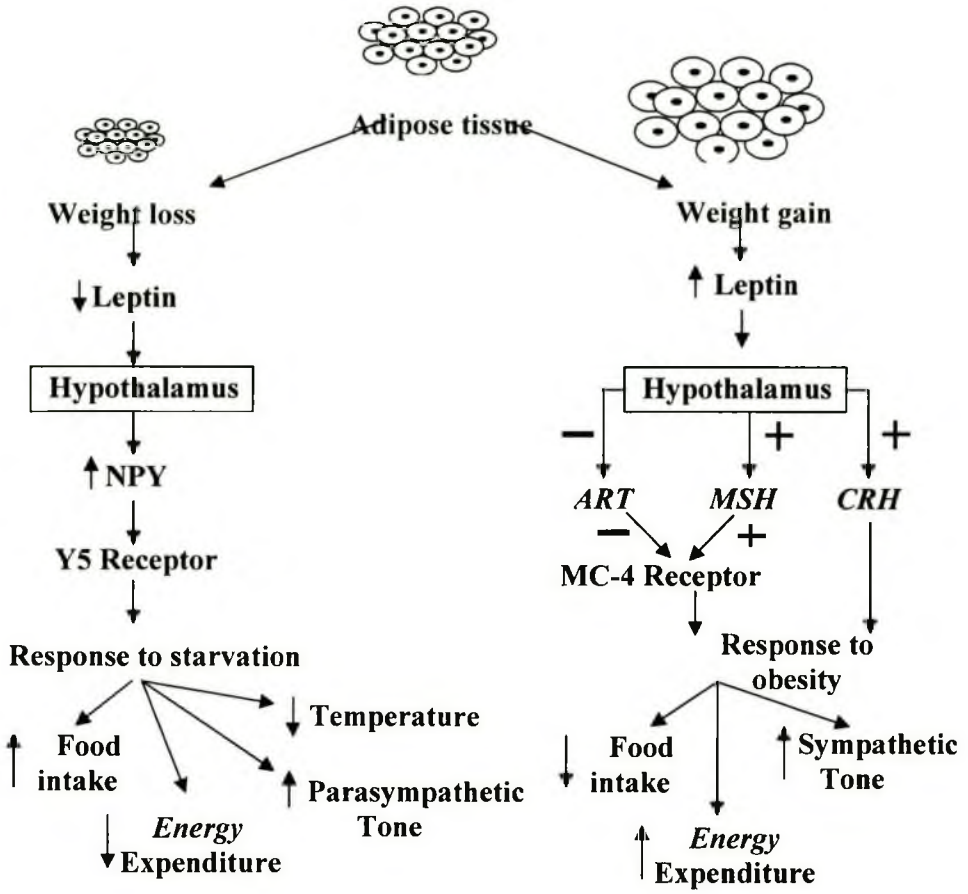

Fig 1: Leptin and energy homeostasis

Leptin is an important regulator in a feed back loop that maintains constant stores of adipose tissue. It acts in the arcuate nucleus to reduce food intake by decreasing NPY and increasing alpha-MSH. (Left) Loss of adipose tissue reduces circulating leptin concentration in the blood. A reduction in hypothalamic leptin binding increases NPY release, which induces central (increase in food intake, reduction in body temperature) as well as peripheral (reduction in energy expenditure), responses that counteract further weight loss. (Right) Gain of adipose tissue increases plasma leptin levels. Hypothalamic leptin stimulation increases levels of MSH, produced by cleavage of pro- opiomelanocortin (POMC), which binds to the melanocortin-4 (MC-4) receptor. MSH induces behavioral changes, such as a reduction in food intake and increase in sympathetic tone, that oppose further weight

gain. In addition, leptin stimulates the release of corticotrophin-releasing hormone (CRH), which also helps to mediate the reduction in food intake. However, the protein product of agouti-related transcript (ART) blocks the MC-4 receptor and promotes obesity. In this regulatory loop, two broad pathophysiological mechanisms lead to obesity:

(1) Deficiency of leptin, whether absolute (e.g. ob/ob mouse) or relative secondary to leptin resistance (e.g. db/db mouse) and
(2) Dysregulation of the hypothalamic neuropeptide system.

Leptin plays an important role in sexual dimorphism. It had been suggested that women have higher leptin level than men at any percent of body fat or fat mass. Adiposity, estrogen levels and gender are the major determinants of leptin concentration, which may be responsible to the sexual dimorphism. Subcutaneous adipose tissue is more abundant in females. leptin synthesis is inhibited by testosterone (Rosenbaum *et al.* 1996). The mechanism of the sexual dimorphism in leptin production is unclear. Low level of serum testosterone is predictive of the development of insulin resistance and type 2 diabetes androgen treatment improves insulin sensitivity and decrease plasma leptin level. Androgen is known to increase fat free mass and muscle size and to decrease visceral fat mass (Dominique *et al.* 2001) by inhibiting lipoprotein lipase activity. On the other hand, higher leptin levels in adolescents with hyperandrogenism (testosterone & dehydroepiandrosterone sulphate were significantly higher) than healthy girl (Zukauskaite *et al.* 2005).

The term " free fatty acid refers to fatty acids that are in un-esterified state also called an esterified (UFA) or nonesterified (NEFA) fatty acids. In plasma, longer-chain, FFA are combined with albumin, and in the cell they are attached to fatty acid binding protein or Z-protein, So that infact they are never really "free". Shorter-chair fatty acids are more water. Soluble and exist as the un-ionized acid over a fatty acid anion.

The free fatty acids (FFA, nonesterified fatty acids, and unspecified fatty acids) arise in the plasma from lipolysis of triacylglycerol in adipose tissue or as a result of the action of lipoprotein lipase during uptake of plasma triacylglycerol into the tissues. They are found in combination with albumin, a very effective solubilizer, in concentrations ranging between 0.1 and 2.0 μeq/ml of plasma. They comprise the long chain fatty acids found in adipose tissue, i.e., palmitic, stearic, oleic, palmitoleic, linoleic and other polyunsaturated acids and small quantities of other long chain fatty acids. Binding sites on albumin of varying affinity for the fatty acids have been described. Low levels of free fatty acids are recorded in the fat fed condition, rising to about 0.5 μeq/ml in the post absorptive and between 0.7 and 0.8 μeq/ml in the fat fasting state. In uncontrolled diabetes mellitus, the level may raise as much as 2 μeg/ml. The level fall just after eating and rises again prior to the next meal. Free fatty acids are removed from the blood extremely rapidly and oxidized (fulfilling 25-50% of energy requirements in starvation) or esterified to form triacyl glycerol in the tissues. In starvation, esterified lipids from the circulation or in the tissues are oxidized as well, particularly in heart and skeletal muscle cells, where considerable stores of lipid are to be found.

The free fatty acid uptake by tissues is related directly to the plasma free fatty acid concentration, which in turn is determined by the rate of lypolysis in adipose tissue. After dissociation of the fatty acid-albumin complex at the plasma membrane, fatty acids bind to a membrane fatty acid transport protein that acts as a trans-membrane co transporter with Na+. On entering the cytosol, free fatty acids are bound by intracellular fatty acid-binding proteins or Z protein. The role of this protein in intracellular transport is thought to be similar to that of serum albumin in extra cellular transport of long chain fatty acids (Murray, Herper 26th ed. & 22nd ed.).

In normal condition leptin protects nonadipocytes from steotosis. Intracellular FAs are derived from plasma TG, FFAs or from *de novo* synthesis and/or from hydrolysis of stored intracellular TG. FAs are activated to fatty acyl coenzyme A (CoA) by fatty acyl CoA synthetase. Although hepatocytes can export excess TG in the form of lipoproteins and skeletal muscle can increase fatty acid oxidation by exercising, most other non-adipocytes tissue lack the capacity to rid them of unwanted fat. Pancreatic β-cells falls in the later category and fatty acyl CoA that is not oxidized in mitochondria or peroxisomes may be re-esterified to TG. Moreover, the level of manganese super oxide dismutase, an enzyme that provides protection against free radicals (Rothstein, 1994), is relatively low in β-cells, making them vulnerable to proapoptotic factors generated by the excess TG.

Steatosis is an excess of triacylglycerol (TG) in a non-adipocytes but the term does not indicate whether or not adverse effects have occurred. Leptin is antisteatotic hormones that down regulate lipogenic enzymes and upregulate enzymes of FA oxidation in non-adipocytes, which increases, pari passu with the stockpiling of FA in adipocytes and decreases when adipocytes redistribute their FA during fast. When leptin action is lacking, fat accumulates in non-adipocytes. TG content is elevated in liver, heart and skeletal muscle in obese ZDF rats with defective leptin receptors (Koyama, 1997). The pancreatic TG content rises in parallel with that in other tissues. When one cultures normal non-adipocytes, such as islets, in 1 or 2 mM FA, TG content rises only slightly because FA-induced up-regulation of peroxisome proliferator-activated receptor α (PPARα) and the enzymes of oxidation, acyl CoA oxidase and carnitine palmitoyl transferase 1, direct the FA into oxidative rather than lipogenic pathways (Zhou *et al.,* 1998), thereby disposing of unwanted FA and preventing their accumulation as TG (Shimabukuro *et al.*, 1997). Conversely, islets from leptin-insensitive Zucker diabetic fatty (ZDF, *fa/fa*) rats with defective leptin receptors exhibit a marked increase in TG accumulation at the same concentrations of FA (Lee *et al.* 1997).

Longitudinal studies of islets of leptin-resistant ZDF rats indicate that the TG content of their non-adipocytes rises progressively in parallel with the increase in adipocyte TG (Lee, 1994; Unger, 1995), consistent with the prediction that without leptin action non-adipocytes are unprotected from over accumulation of fat. In addition to the functional abnormalities, there are extensive morphologic changes, including disorganization of the islet architecture, loss of the high K_m glucose transporter of β cells, GLUT-2 (Orci *et al.* 1990) and a high percentage of mitochondrial abnormalities, consistent with the view that leptin action on β cells (and perhaps other non-adipocyte tissues) serves to maintain a normal TG content and to guard against fat overload and lipotoxicity.

Leptin prevents steatosis is depicted in the fig described bellow.

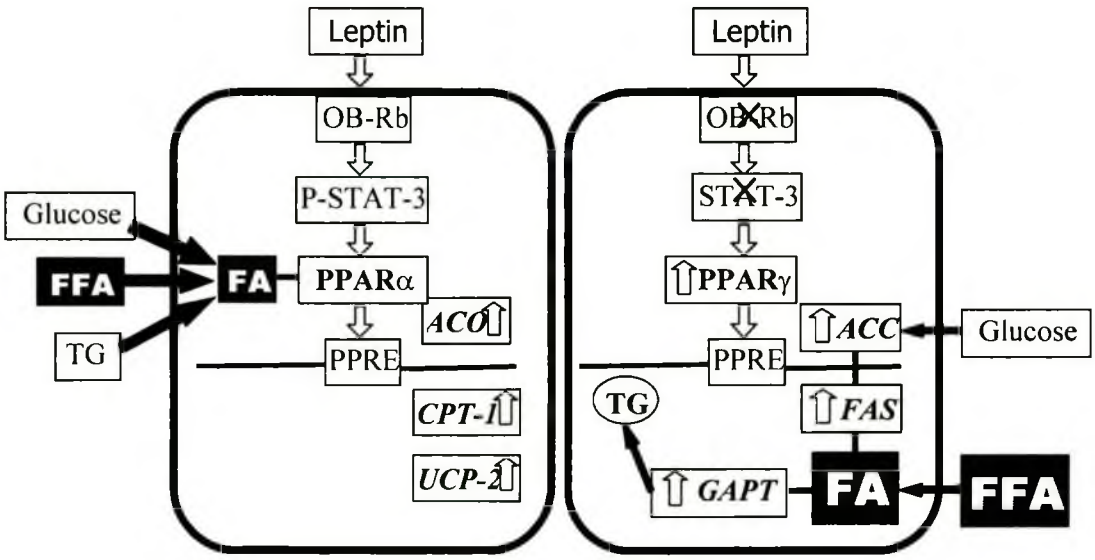

Fig 2: Fatty acid homeostasis in normal leptin-sensitive nonadipocytes (A) and leptin-resistant (B) nonadipocytes.

New evidence shows that steatosis causes defective insulin secretion and action (Le Roith *et al,* 2000). Obese patients with type 2 diabetes show altered insulin release with respect to prevailing blood glucose characterized

by elevated circulating insulin in the fasting state and relatively low insulin levels postprandially when glucose is high (Polonsky, 1994; Porte,1991). The biochemical basis of these alterations is not known, but the emerging evidence suggest that elevated free fatty acids (FFA) as well as long chain acyl- coA esters (Lc-coA) and triglyceride deposition in the β-cell may be causally implicated (McGarry 1992; Prentki, 1996). In skeletal muscle, the accumulation of triglycerides is believed to affect the sensitivity to insulin. The higher tissue fat, the greater the plasma insulin level required maintaining normoglycemia. The initial consequence of islets steatosis is to increase insulin secretion, thereby helping the β-cells to compensate for the increase in insulin requirements.

As fat content in islets increases, β cells at first become hyperplastic and insulin production rises (Minburn *et al.* 1995) but once the fat content exceeds ≈50 times normal, the hyperplastic β cells undergo lipoapoptosis (Shimabukuro *et al.* 1998) and diabetes appear. This finding implies that lack of leptin action in these non-adipocytes tissue caused the fat overload, which impaired the function and ultimately the viability of β cells.

Type 2 diabetes is often associated with obesity and increased levels of circulating FFA (Elks, 1990). In healthy individuals the levels of FFA usually range between 0.2-0.7 mM, whereas in diabetics the levels are higher and may reach concentrations of 1.0 mM (Chen, 1987; Coon, 1992; Swislocki, 1987). This has led to the proposal that high FFA levels might aggravate the diabetic condition by increasing the peripheral resistance to insulin and inducing hepatic gluconeogenesis (Elks, 1990). Moreover, increased levels of FFA seem to inhibit the function of the insulin-producing β cell. Indeed, reports have proposed that FFA directly impair the secretory capacity of pancreatic β-cells (Sako, 1990; Zhou, 1994). In a study it has been claimed that leptin is associated with *hyper-secretion* of beta cells when fat content is high.

The role of leptin in affecting *insulin secretion* was first demonstrated from *in vitro* studies. It has been shown that leptin interferes insulin secretion from pancreatic islets under normoglycemic and normoinsulinemic conditions to prevent obesity (Ceddia *et al.* 1998). Physiological concentrations of leptin also *reduced* basal insulin release from rat islets and β-cell pancreatic cell line but had no effect on glucose stimulated release (Emilsson V et al 1997), in their experiment islets were kept at 16.7 mM glucose in different leptin (1-100 nM) concentration. At 100 nM leptin concentrations the islets' insulin secretion response decreased to near 20% of the control group. At physiological leptin concentrations (1 nM) there was an apparent but non-significant reduction in insulin secretion. Supra physiological leptin concentration was necessary to elicit a significant reduction in insulin secretion from the islets. Other studies investigated the acute effect of supra physiological leptin (50 nM) on insulin secretion and on the fractional outflow rates of Ca^{2+} and $^{86}Rb^{+}$ from pancreatic islets isolated from male lean albino rats (Ceddia, William, Carpinelli and Curi, unpublished results). At a constant physiological glucose concentration (5.6 mM), the addition of leptin to the perifusion medium led to an increase of Ca^{2+} fractional outflow rate followed by a significant increase (26%) in insulin release from the islets. At low glucose concentrations (2.8 mM), leptin also elicited a significant (50-60%) increase in insulin secretion. At supra physiological (16.7 mM) glucose concentrations, they found that the rapid first phase insulin secretion response was blunted in the presence of leptin, but with continuation of perfusion it reached values close to those of the control group. These results led us to think of leptin as a molecule capable of modulating the insulin secretion process in beta cells, instead of just inhibiting or stimulating it (Ceddia, William, Carpinelli and Curi, unpublished results). William in 2003 suggests that leptin correlates better with glucose induced insulin release than sensitivity.

The initiating process of insulin secretion is the closure of ATP-sensitive K^+ (K^+-ATP) channels, which alters the electrical activity of the cell, causing an increase in cytoplasmic Ca^{2+} concentration.

The alteration of the Rb^+ efflux caused by leptin (Ceddia *et al.* 1998) suggests that its modulating effect might be associated with an alteration in the kinetics of K^+-ATP channel activity (gating) present in pancreatic beta cells.

Besides its effects on K^+-ATP channels, leptin has also been involved in metabolic alterations within the islets. Such metabolic alterations interfere with the islets' capability to secrete insulin in response to glucose and other fuels. Leptin alters the mRNA of the genes encoding the enzymes of free fatty acid (FFA) metabolism and uncoupling protein-2 (UCP-2) (Zhou YT et al 1997). Each normal islet contains approximately 24 ng of triglycerides (TG), and when it is depleted of fat by hyperleptinemia, the insulin response to glucose and other fuels is completely abolished, but is promptly restored by FFA. The size of the TG reservoir appears to be under the control of leptin. Islets of obese, leptin-resistant ZDF rats contain 990 ng TG per islet, while in rats with hypoleptinemia islet TG is zero.

It is also important to note that in islets of rats made hyperleptinemic by gene transfer, in which a recombinant adenovirus containing the rat leptin cDNA under the control of the cytomegalovirus (CMV) promoter (AdCMV-leptin) was administered, the mRNA of acetyl-CoA carboxylase was drastically reduced to 21% of the control group (free-feeding AdCMV-ß-gal infused) (Zhou YT 1997). This enzyme inhibits carnithine palmitoyltransferase (CPT-1) activity by generating malonyl-CoA (McGarry & Foster, 1980). In contrast, the mRNA of enzymes of FFA oxidation was significantly increased; acyl-CoA oxidase (ACO) mRNA was more than three times as high in islets of hyperleptinemic rats as in controls, and CPT-1 mRNA was almost 3 times as high. Furthermore, glycerol-3-phosphate acyltransferase mRNA, an enzyme

of FFA esterification, was reduced in the islets of hyperleptinemic rats to 16% of free-feeding AdCMV-ß-gal controls.

Insights from other *in vitro* and experimental studies have shown that some free fatty acids and lipoproteins are pro-apoptotic for the ß-cell while others are protective. Acute exposure of β-cells to fatty acids potentiates glucose-induced insulin secretion by mechanisms that apparently do not implicate K_{ATP} channels but may involve C-kinase activation by Lc-CoA and Ca^{2+} channels (Prentki *et al.* 1992; Warnotte *et al.* 1994). By contrast, over longer time ranges (>24 h) fatty acids such as palmitate and oleate cause both in vitro and in vivo high basal insulin secretion characterized by a lowered glucose set point and markedly reduced secretion in response to a further elevation of the sugar (Brun *et al.* 1994; Hosokawa *et al.* 1997; Zhou *et al.* 1995).

It has been postulated that ß-cell dysfunction is due to increased triacylglycerol (TAG) content in islets, which leads to increased production of nitric oxide and ceramide synthesis (Miriam *et al.* 2001) leading to expression of nitric oxide (NO) systhase and NO dependent β-cell apoptosis.

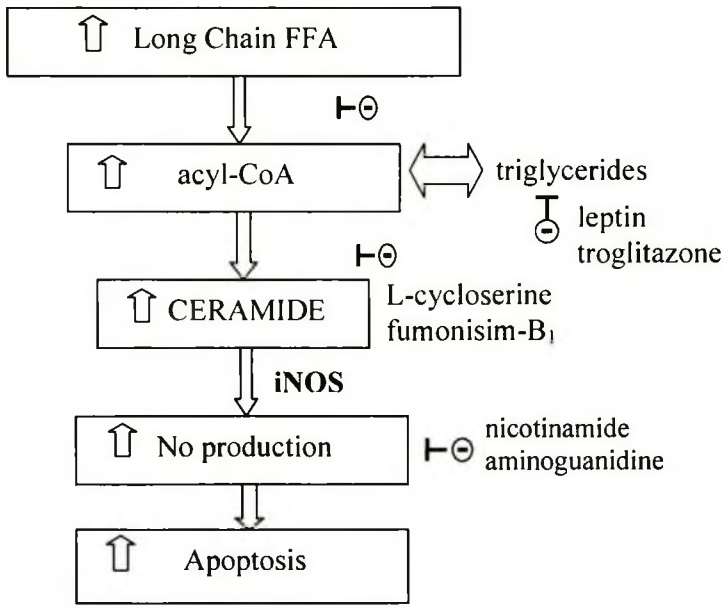

Fig 3: The proposed pathway of lipoapoptosis.

Several studies have suggested that the long-term action of fatty acids is caused by changes in lipid and glucose metabolism, possibly as a consequence of variations in the expression of genes encoding key enzymes implicated in fuel signaling. In this respect, a complex picture with conflicting results has emerged. Thus palmitate and oleate not only increase the total activity of hexokinase in rat islets (Hosokawa *et al.* 1997) and HC9 β-cell (Liang *et al.* 1997) but also decrease the expression of glucokinase and GLUT-2, possibly via reduced expression of the transcription factor IDX-1 (Gremlich *et al.* 1997). In addition, fatty acids induce the carnitine palmitoyltransferase I gene (CPT-I; Ref. AssimacopoulosJeannetI *et al.* 1997) and reduce acetyl-CoA carboxylase expression (Brun *et al.* 1997) in INS-1 β-cells, thus causing enhanced fat oxidation (Brun *et al.* 1997). An enhanced rate of oxidation of fatty acids might reduce glucose metabolism and consequently insulin secretion if a so-called Randle cycle (Randle *et al.* 1988) were operative in the β-cell after fat exposure. In this respect, FFA were shown to cause a modest (~20%) reduction of glucose oxidation in islets (Zhou *et al.* 1994) and HC9 β-cells (Liang *et al.* 1997) or to increase glucose oxidation in another islet study (Hosokawa *et al.* 1997). A reduced islet pyruvate dehydrogenase (PDH) activity predicted by the Randle cycle has been observed (Zhou, 1995), but the associated decreased glucose oxidation caused by fatty acid reported by the same group was noted only at very high (27 mM) glucose and not at intermediate (11 mM) or low glucose concentrations. Thus it is presently uncertain whether alterations in glucose metabolism provide an explanation for the long-term action of fatty acids on insulin secretion.

Recent studies suggest a complex interrelationship between leptin and *insulin sensitivity*. Leptin increases insulin sensitivity by reducing insulin secretion (Seufert, 1999; Emilsson, 1997). Some studies documented the association between serum leptin concentration and insulin sensitivity dependent on adiposity and serum leptin level was found significantly greater in the insulin resistant subjects than insulin sensitive subjects. Obesity and type 2

diabetes are closely related and insulin resistance characterizes both. In different studies on non-diabetic subjects it has been evidenced that leptin may affect the risk of type 2 diabetes separate from the risk due to obesity as increased serum leptin level were found to be associated directly with insulin resistance independent of adiposity (Zimmet, 1998; Donahue, 1999).

A number of studies it has been shown that obesity increases serum leptin level (Caro, 1996; Chu, 2000). A majority of study on obese IGT subjects' leptin level is increased in a proportion to fat mass (Turpeinen, 1997; Eriksson, 1999). Another study showed that leptin level is increased in type 2 diabetic subjects (Poitout, 2002). In contrast others showed leptin is reduced in obese type 2 diabetic subjects (Tatti, 2001; Abdelgadir, 2002). It was also evident that there is no difference of leptin level in between type 2 diabetes and non-diabetes subjects (Haffner, 1996; Bertin, 1998).

Roy *et al.* (2003) demonstrated that Bangladeshi diabetic subjects have highly significant β-cell dysfunction and also insulin resistance. The β-cell defect has been demonstrated in Impaired Fasting Glucose state (Rahman, 2006) suggesting it to be an important stage in the natural history of type 2 diabetes. Roy *et al.* (2003) also showed that Type 2 diabetic subjects had higher plasma leptin level suggesting its role in the pathogenesis of type 2 diabetes. Plasma leptin was associated directly with insulin resistance and inversely with β-cell function, the later being more pronounced. In a study on pregnancy induced hypertension it was also found that serum leptin was inversely correlated with β-cell function (Sultana, 2003). Leptin also possibly has a role in diabetic complications specifically diabetic nephropathy (Shahani, 2002).

Rationale of the present study

From the above discussion it seems that factors affecting leptin concentration in the pathogenesis of diabetes is still unclear particularly in the following respects:

- The evidence of combined and/or isolated effect of glucotoxicity and lipotoxicity on β-cell dysfunction has come from *in vitro* and animal studies but not in human studies.
- The human studies have suggested the possible role of leptin, but the exact nature of β-cell defect associated with altered leptin level in Type 2 diabetes has not been well characterized; and
- The possible mechanism altered leptin associated β-cell death/dysfunction has not been shown in previous *in vivo* human studies. Whether lipotoxicity alone or in combination with glucotoxicity produces the β-cell death/dysfunction that remains to conclusively answer.

Hypothesis

Insulin secretory dysfunction, insulin resistance and altered NEFA composition in Type 2 Diabetes Mellitus are associated with abnormalities in leptin levels.

OBJECTIVES

Objectives

General Objective

To determine the role of leptin in the pathogenesis of type 2 diabetes mellitus.

Specific Objectives

- To measure serum leptin levels in Bangladeshi Type 2 diabetic subjects and analyze the levels in relation to insulin secretory capacity and insulin sensitivity.
- To determine total and individual NEFAs in the serum of type 2 diabetic subjects.
- To explore whether serum leptin, insulin secretion and/or sensitivity are associated with total and individual NEFAs.

SUBJECTS AND METHODS

Subjects and Methods

Place of the study

The study was conducted in the Department of Biochemistry and Molecular Biology, University of Dhaka and Dept of Biochemistry & Cell Biology Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM), Dhaka, Bangladesh.

Study period

This study was done during the period of June 2004 to July 2006.

Study design

It was a cross-sectional analytical study.

Study Subjects

Fifty nine (59) type 2 diabetic and sixty two (62) age-sex matched control subjects were selected between the ages 30-45 years from the OPD of BIRDEM. Diabetes mellitus was confirmed according to the WHO criteria (WHO, 1999).

Inclusion criteria of type 2 diabetic subjects

Type 2 diabetic subjects were newly diagnosed. None of them had any history of using any antidiabetic agent or other medication including hormonal therapy known to affect on carbohydrate, lipid or insulin metabolism.

Inclusion criteria of the Control Subjects

Healthy subjects with normal glucose tolerance (fasting serum glucose <5.6 mmol/l and 2h serum glucose <7.8 mmol/l). They were non-

smokers, non-pregnant, sedentary and had stable body weight for at least 3 months before the study and were on their normal mixed diet.

Exclusion criteria

- Patients with serious comorbid diseases (infection, stroke, myocardial infarction, major surgery, malabsorption etc), hepatic, renal or edema.
- History of using drugs significantly affecting glucose and lipid metabolism (glucocorticoids, symvastatin, pravastatin, oral contraceptives containing levonorgestrel or high-dose estrogen, phenytoin, high-dose thiazide diuretics etc).

Collection of the subjects

After selection the purpose of the study was explained in details to each subject. They were advised to take unrestricted carbohydrate diet, to do normal physical activities and to avoid drugs that significantly interfere with blood glucose and lipid level (like glucocorticoids, symvastatin, pravastatin oral contraceptives containing levonorgestrel or high-dose estrogen, phenytoin, high-dose thiazide diuretics, etc.) for 3 days. Then they were requested to report to Biomedical Research Group Research Division, BIRDEM after 3 days at morning following an overnight (8-12 hours) fasting.

When the subjects reported, informed written consent (appendix IX) was taken. Then history was taken, clinical examination was done and printed questionnaire (appendix X) was filled up.

Anthropometric measurements

Height (m)

Standing height was measured using appropriate scales (Detect-Medic, Detect scales INC, USA) with minimal cloths. The patient was positioned fully erect, with the head in the Frankfurt plane (with the line connecting the outer canthus of the eyes and the external auditory meatus perpendicular to the long axis of the trunk); the back of the head, thoracic spine, buttocks, and heels touched the vertical axis of the anthrop meter, and the heels were together. Height was recorded to the nearest 5 millimeter. If the reading fell between two values, the lower reading was recorded.

Weight (kg)

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

BMI (kg/m^2)

Body mass index (BMI) of the subjects were calculated using standard formula.

$BMI = Weight\ (kg)/[Height\ (m)]^2$

Waist and Hip circumference (cm)

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

W/H ratio

Waist-to-hip ratio (W/H ratio) was calculated as the ratio of waist circumference divided by the hip circumference.

Skin fold thickness (mm)

Skin fold thickness was measured using Harpenden Skinfold Calipers. Triceps skin fold thickness was measured at the previously marked midpoint of the back of the upper right arm. The subject's stood with right arm hanging loosely by his side. It palpated the subjects measuring site to become familiar with soft tissues. Then it was grasp a vertical pinch of skin and subcutaneous fat between thumb and forefinger about 1 cm above the previously marked mid point. The skin fold was gently pulled away from the underlying muscle tissue and then the caliper jaws applied at right angels, exactly at the marked mid point. The caliper reading generally diminished for 2 to 3 second, and then the measurements were taken. Subscapular skin fold thickness was measured 1 cm bellow the inferior angle of right scapula with skin fold lying at 45 degree. Reading was taken rapidly and recorded at the nearest millimeter.

MUAC (mm)

Mid Upper Arm Circumference (MUAC) was measured at a point mid way between the acromial process of scapula and olecranon process of ulna of right arm hanging relaxed. A measuring tape was used to record the circumference following the above technique at the nearest millimeter.

Body fat mass (%)

Body fat mass was measured by Omron Body Fat Monitor. Height in cm, weight in kg, age in yrs and sex of patients were set to the monitor. Then the patient held the monitor by both hands with upper limbs horizontal in standing position. The machine was then put on and body fat mass (%) and body fat mass was recorded from the monitor.

Measurement of blood pressure

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

MAP= diastolic blood pressure + 1/3 pulse pressure (pulse pressure= systolic blood pressure – diastolic blood pressure)

Collection of blood samples

Fasting blood was collected between 8.00-9.00 am. Venous blood (10 ml) was taken in a heparinized (20μl into 2ml blood) tube for free fatty acid (FFA) and in a plain tube for insulin, leptin and other biochemical test. Then the patient was given 75g of glucose in 250-300 ml of water and advised to drink in 5 min. Patient was advised not to smoke, not to take any food and to take rest in a chair for 2 hours. After 30 min of glucose load blood sample was taken again to determine serum glucose as well as insulin and 2hr glucose only to the control subjects for the confirmation of diabetes. Plasma was separated immediately by centrifugation of heparinized blood samples. FFAs were estimated from the fresh plasma samples using Dole extraction method**. Serum sample was centrifuged at 3000 rpm for 10 min. All blood glucose in different time point and fasting biochemical test was measured in the same day. Rest of the serum samples was preserved separately in serum vials at -40^{0}C until the analysis of insulin and leptin.

Isolation of free fatty acid from plasma

For isolation of free fatty acids extraction mixture was prepared by mixing isopropanol, n-heptane and normal sulfuric acid (I N H_2SO_4) in the proportion of 40:10:1 in a volumetric flask according to Dole and Meinertz method (Dole et al, 1960). Extraction mixture (2 ml) was taken in a screw cap test tube (15 ml) containing plasma (300 μl). The mixture was vortexed and waited for 10 min. Then internal standard solution (heptadecanoic acid in heptane I mg/ml; 20 μl), heptane (1 ml) and water (I ml) were added to the mixture and was shacked or vortexed. The mixture was centrifuged at moderate speed of 2000 to 3000 rpm. Then the extracts were placed in a rack for half an hr to let the two phases separated. After half an hr the upper layer containing free fatty acids was separated through a pasteur pipette in a screw cap test tube containing anhydrous sodium sulfate ($NaSO_4$) in the bottom of the tube. The extract was filtered through a syringe filter and transferred to a reaction tube (10 ml with glass stopper). The solvent heptane was dried under nitrogen gas blow to retain the free fatty acids in the reaction tube. After isolation of free fatty acids from all the samples, following steps were under taken.

Preparation of fatty acid methyl ester (FAME)

Boron trifluoride-methanol complex (BF_3-CH_3OH) reagent (500 μl) and a small amount of anti-bumping powder (pumice stone powder) were added to the reaction tube containing free fatty acid (Metcalfe *et al.*, 1961). Then the tube was shielded with Teflon. The mixture was boiled on a boiling water bath for 3 to 5 min. The tube was removed from water bath. Hexane (1 ml) was added to it and was boiled further for 1 min. The tube was kept on the rack for a few minutes to cool the mixture. Then a saturated sodium chloride salt (1 ml) solution was added to it and vortexed. The mixture was kept standing for 30 min. Then the upper layer containing fatty acid methyl ester

(FAME) was transferred to a vial (3 ml) with anhydrous sodium sulfate at the bottom. Then the ester was filtered through syringe filter and transferred to a small vial (2 ml). If necessary, the solvent was concentrated by blowing nitrogen gas and stored in a refrigerator before analysis by GC.

Preparation of Standard fatty acid methyl ester (FAME)

Twenty-four of standard free fatty acids were individually weighed. About 10 mg of each was taken in a reaction tube (20 x 150 mm) and BF_3CH_3OH reagent (3 ml) was added (Metcalfe *et al*, .1961 and Murshed S *et al.*1993). The mixture was boiled on a boiling water bath for 5 min. Then the tube was removed from water bath. Hexane (3 ml) was added to it and boiled for further 1 min. After cooling the tube a solution of saturated salt (3 ml) was added and vortexed. Then the upper layer containing methyl esters was separated in the usual way as described before.

Gas chromatography (GC)

Gas chromatography was conducted with a gas chromatograph GC-14A series (Shimadzu Co, Tokyo, Japan) fitted with a flame ionization detector and an automated electronic recorder (CR-5A chromatopack, Shimadzu Co., Tokyo, Japan). For fatty acid analysis a capillary glass column (50 m X 0.25 mm ID) wall coated with 100% cyanopropyl polysiloxane open tubular (WCOT) fused silica (SP2340, Supelco, Bellefonte, Pennsylvania, USA) was used. Initial temperature of the column was 135°C with 14 minutes initial holding time and then programmed to increase at 3°C min^{-1} to a final temperature of 230°C in 6 min. The flow rate of helium gas was 3.5 ml per minute, split ratio 1:60. Temperature of the injection port was 235°C and the detector 240°C. Each FAME in extract was identified by comparing retention times with those of known standard FAME (LIPID STANDARD,

Sigma Chemical Co, St Louis, MO, USA). The area of fatty acids was measured with computer software (Crarity). The results were expressed as percentage composition method.

Analytical methods and lab analysis

- Serum glucose (fasting, 30 min and 2 hours after 75g glucose) was measured by Glucose Oxidase (GOD-PAP) method (Randox Laboratories Ltd., UK) (Appendix I).
- Serum total cholesterol by enzymatic colorimetric (Cholesterol Oxidase/ Peroxidase) method (Randox Laboratories Ltd., UK) (Appendix II).
- Serum HDL cholesterol by enzymatic colorimetric (Cholesterol CHOD-PAP) method (Randox Laboratories Ltd., UK) (Appendix III).
- The LDL-Cholesterol level was calculated by using Friedewald formula (Friedewald WT, 1972) (Appendix IV).
- Serum triglyceride by enzymatic colorimetric (GPO-PAP) method (Randox Laboratories Ltd., UK) (Appendix V).
- Serum creatinine was measured by alkaline-picrate method (Randox Laboratories, UK) (Appendix VI).
- SGPT was estimated by UV method using ALT (GPT) opt. kit (RANDOX) (Appendix VII).
- Serum total NEFA estimated by enzymatic colorimetric method (Randox Laboratories Ltd., UK) (Appendix VIII).
- Serum leptin by enzyme linked immunosorbent assay (ELISA) method (Linco Research Inc., USA) (Appendix IX).
- Serum insulin by enzyme linked immunosorbent assay (ELISA) method (Linco Research Inc., USA) (Appendix X).

Calculation of B cell function and insulin sensitivity

Homeostasis Model Assessment (HOMA) is a simple widely used method which derives separate indices of B cell secretion (HOMA B) and insulin sensitivity (HOMA S) from the serum glucose and insulin concentrations under basal conditions by using mathematical formula or software. Using HOMA, The HOMA index of insulin secretion (HOMA B-cell) is calculated as fasting insulin (mu/l) × 20/[fasting glucose (mmol/l)–3.5] (Levy, Mathews & Hermans 1998). Insulin sensitivity (HOMA-IR) is calculated as [fasting insulin (mu/l) × fasting glucose (mmol/l)/22.5]. The HOMA model has been incorporated in a simple MS-DOS-based computer program (HOMA-CIGMA software) that allows rapid determination of %B (B cell secretion) and %S (insulin sensitivity) from measured values using fasting plasma glucose in mmol/l and insulin in pmol/l. Although the simple equation gives a qualitatively useful approximation of the model prediction, most authors prefer the computer model. In this study HOMA-CIGMA software was used.

Estimation of 1st and 2nd Phases of insulin secretion

1st and 2nd phases of insulin secretion was calculated by the following equations:

$1^{st}\ phase_{est=}\ 1283+1.829.Ins30-138.7.Glu30+3.772.Ins0$

$2nd\ phase_{est=}\ 287+0.4164.Ins30-26.07.Glu30+0.9226.Ins0$

Statistical analysis

Statistical analysis was performed using SPSS (Statistical Package for Social Science) software for Windows version 11.5 (SPSS Inc. Chicago. IL. USA). All the data were expressed as mean±SD (standard deviation), median (range) and/or percentage (%) as appropriate. The statistical significance of differences between the values was assessed by ANOVA or Mann-Whitney U test (as appropriate). Correlation was also seen among the parameters. A two-tailed p value of <0.05 was considered statistically significant.

RESULTS AND OBSERVATIONS

Results and Observations

Total 121 subjects were included in this study in which 62 were healthy control and 59 were of diabetic subjects. Detailed results are given below. Values in Control and diabetic subjects are mentioned sequentially within parenthesis in the followings:

Anthropometric and clinical characteristics of the study subjects

The age (mean±SD, years) was 36±4 vs. 36±4 in diabetic vs. control subjects respectively. Both diabetic and control subjects BMI were matched [mean±SD (kg/m^2: 24.00±3.09 in diabetic vs. 23.62±2.01 in control] and there was no significant differences regarding anthropometrics measurements between both groups (p=ns). The diabetic subjects showed higher mean blood pressure [mean±SD (mmHg: 88±8.7 in diabetic vs. 82±7 in control, $p<0.001$)] when compared with control subjects.

Table I. Anthropometric and clinical characteristics of the study subjects

Variable	**Control** (n=62)	**Diabetic** (n=59)	**t/p value**
Age (years)	36±4	36±4	0.647/0.519
BMI (kg/m^2)	23.62±2.01	24.00±3.09	0.805/0.423
WHR	0.898±0.048	1.03±0.65	1.609/0.110
MUAC (mm)	289.66±20.87	283.56±25.26	1.451/0.149
STR	1.53±0.30	1.46±0.43	1.009/0.321
Body fat (%)	28.83±4.96	27.73±6.21	1.083/0.281
MBP (mmHg)	82±7	88±8	**4.171/0.001**

Results were expressed as Mean±SD. Means compared using Student's unpaired t-test. *n* = number of subjects; BMI, Body mass index; WHR, waist-to-hip ratio; MUAC, Mid upper arm circumference; STR, subscapular triceps ratio; MBP, Mean blood pressure.

Glycemic and Lipid profile status of the study subjects (Table II)

Regarding glycemic status, diabetic subjects had considerably higher serum fasting and after 30 min glucose level [mean±SD {mmol/l: (11.61±3.41); (17.64±3.63) in diabetic vs (5.01±0.434); (8.06±1.13) in control, $p<0.001$}] than the control subjects.

The serum cholesterol (mean±SD, mg/dl) were 214±44 in diabetic vs 193±34 in control subjects ($p<0.003$), TG median (range) were 176(52-681) in diabetic vs 102(49-370) in control; ($p<0.001$). Serum HDL-C level in diabetics was 33.45±6.77 on the other hand it was in control subjects 38.86±8.21 ($p<0.001$). There was no significant difference in LDL-C, between diabetic and control subjects (137±40 in diabetic vs 133±30 in control).

Insulinemic and leptinemic status of the study subjects (Table III)

This table represents that there was no significant difference in fasting but significant difference found in 30 min after glucose insulin and leptin level between diabetic and control subjects. Fasting serum insulin is [Median (range); 41.74 (14.73-99.04) vs 39.20 (12.41-169.02), $p=0.532$] and 30 min serum insulin is [Median (range); 79.56 (20.72-396.19) vs 380.84 (120.23-1389.0), $p=0.001$]. Serum leptin level in diabetic is [Median (range); 5.44 (0.65-34.70) in diabetic vs 8.35(1.36-55) in control subjects, $p=0.012$]. The ratio between diabetic and control subjects in fasting insulin was 1.06, in 30 min insulin was 0.209 and in leptin level was 0.651.

Table II. Glycemic and Lipid profile status of the study subjects

Variables	Control (n=62)	Diabetic (n=59)	p Value
F glucose (mmol/l)	5.01±0.434	11.61±3.41	**0.001**
30 min glucose (mmol/l)	8.06±1.13	17.64±3.63	**0.001**
Cholesterol (mg/dl)	193±34	214±44	**0.003**
HDL (mg/dl)	38.86±8.21	33.45±6.77	**0.001**
LDL (mg/dl)	133±30	137±40	0.516
TG (mg/dl)	102(49-370)	176(52-681)	**0.001**

Results were expressed as Mean±SD or Median (range). Difference between groups was calculated using Student's unpaired t-test and Mann-Whitney U test as appropriate. *n* = number of subjects; TG, Triacylglycerol; HDL-C, High density lipoprotein cholesterol; LDL-C Low density lipoprotein cholesterol; NEFA, Nonesterified fatty acid.

Table III: Insulinemic and leptinemic status of the study subjects

Variables	Control (n=62)	Diabetic (n=59)	DM: Control Ratio	U/p value
Insulin 0 min (pmol/l)	39 (12-169)	41 (14-99)	1.06	1708/0.532
Insulin 30min (pmol/l)	380 (120-1389)	79 (20-396)	0.209	**159/0.001**
S leptin (ng/ml)	8.35 (1.36-55)	5.44(0.65-34.7)	0.651	**1343/0.012**

Values were expressed as Median (range). Difference between groups was calculating using Man-Whitney U test. n=number of subjects.

Insulin secretory capacity and insulin resistance of the study subjects (Table IV)

In case of β- cell function, the diabetic subjects had significantly lower HOMA B % and HOMA S % value as compared to the control [HOMA B % Median (range); 20.20(4.20-89.60) vs. 78.35(35.50-365.7), p=0.001] [HOMA S % Median (range); 84.60 (39.1-226.4) vs. 118.8 (22.0-3573.7), p=0.004]. The 1st and 2nd phase of insulin secretion were significantly lower in diabetic subjects than the control subjects [Phase I: Median (range); 834.29 (235-2070) vs. 1037.02 (333-2929), p=0.001, Phase II: Median (range); 93.89 (35-204) vs. 274(124-704), p=0.001]. The ratio between diabetic and control subjects in HOMA B% was 0.26, in HOMA S% was 0.71, in phase I was 0.80 and phase II was 0.34.

Table IV: Insulin secretory capacity and insulin resistance of the study subjects

Variables	Control (n=62)	Diabetic (n=59)	DM: Control Ratio	U/p value
HOMA B %	78.35 (35.50-365.7)	20.20 (4.20-89.60)	0.26	**171/0.001**
HOMA S %	118.8 (22.0-3573.7)	84.6 (39.1-226.4)	0.71	**1271/0.004**
Phase I Insulin secretion	1037 (333-2929)	834 (235-2070)	0.80	**2.00/0.001**
Phase II Insulin secretion	274(124-704)	93(35-204)	0.34	**4.00/0.001**
Phase I: Phase II	3.7(2.6-4.1)	2.2 (16.2-36.5)	0.61	**866/0.001**

Values were expressed as Median (range). Difference between groups was calculating using Man-Whitney U test. n=number of subjects.

Plasma fatty acid composition (wt %) of the study subjects

Serum total NEFA level in diabetic was 0.652±0.196 vs 0.42±0.15 in control subjects. From this observation it was found that NEFA was significantly higher in diabetic compared to control subjects. Among saturated fatty acids; myristic, penta decanoic, palmitic and stearic acids were significantly higher in diabetic [Median (range): C 14:0; 1.49(0.86-7.28); (Mean ±SD): C15:0, 0.36 ±0.13; C16:0, 32.15±2.97; C18:0, 17.44±4.44 in diabetic vs. {Median (range)}:C14:0, 1.26(0.78-2.0); (Mean ±SD): C15:0, 0.26±0.11; C16:0, 27.73±2.69; C18:0, 22.04±4.99 in control]. Among monounsaturated fatty acids (MUFA) only oleic acid is significantly higher in diabetic compared to control subjects. [(Mean±SD): C18:1, 21.29±3.33 in diabetic vs. 16.23±3.5 in control. In case of polyunsaturated fatty acids (PUFA), linoleic and linolenic acids were significantly higher in control subjects. [(Mean±SD), C18:2, 14.72±3.38; C18:3, 0.74±0.31 in diabetic vs. C18:2, 20.79±4.92; C18:3, 1.01±0.43 in control].

Table V: Plasma fatty acid composition (wt%) in diabetic and control subjects

Fatty acids	**Control** (n=24)	**Diabetic** (n=23)	**p value**
Total NEFA (mmol/L)	0.42±0.15	0.652±0.196	**0.001**
NONEFA			
SFA			
C14:0	1.26(0.78-2.0)	1.49(0.86-7.28)	**0.02**
C15:0	0.26±0.11	0.36±0.13	**0.02**
C16:0	27.73±2.69	32.15±2.97	**0.001**
C18:0	22.04±4.99	17.44±4.44	**0.002**
C20:0	0.717(.10-1.45)	0.704(0.36-3.26)	0.949
C22:0	0.102(0.12-2.47)	0.479(0.24-2.4)	0.497
MUFA			
C16: 1	2.796±1.12	3.27±1.65	0.247
C18: 1	16.23±3.5	21.29±3.33	**0.001**
C20: 1	0.24(0.057-0.874)	0.35(0.15-1.27)	0.102
PUFA			
n-6 FA			
C18: 2	20.79±4.92	14.72±3.38	**0.001**
C18: 3	1.01±0.43	0.74±0.311	**0.02**
C20: 4	3.02±1.10	2.78±0.72	0.402
20:2	0.512±0.22	0.503(0.22-2.94)	0.88
20:3	0.53±0.33	0.73±0.28	0.298
n-3 FA			
C20: 5	0.582(0.256-2.89)	0.42(0.16-2.50)	0.296
C22: 6	1.00(0.33-5.87)	1.30(0.49-4.25)	0.194

Values were expressed as mean±SD or median (range). Difference between groups was calculated using Student's unpaired t-test and Mann-Whitney U test as appropriate. *n* = name of fatty acid; NONEFA, Non esterified fatty acid; SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid.

Multiple linear regression taking insulin secretory function (HOMA B%) as dependent variable

Table VI showed multivariate regression analysis using insulin secretory function (HOMA B%) as dependent variable and leptin, BMI, triceps, percent body fat, cholesterol, triglycerides, NEFA, HDL and LDL as independent variables. The independent variables, leptin ($\beta=0.437x10^{-3}$, p=0.008) and BMI ($\beta=0.001$, p=0.08) were found to be significant predictive value.

Multiple linear regression taking fat normalized serum leptin as dependent variable

Table VII showed multivariate regression analysis using serum fat normalized leptin level as dependent variable and BMI, triceps skin fold thickness, percent body fat, cholesterol, TG, NEFA, LDL, HDL, fglu, S glu 30 min, SBP, DBP, HOMA B% and HOMA S% as independent variables. The following independent variables were found to be significant predictive value: triceps skin fold thickness ($\beta=0.017$, p=0.001), percent body fat ($\beta=0.031$, p=0.001), TC ($\beta=0.007$, p=0.035), TG ($\beta=-0.001$, p=0.05), LDL ($\beta= -0.007$, p=0.047) and HOMA B% ($\beta=3.762$, p=0.019)

Table VI: Multiple linear regression taking insulin secretory function (HOMA B%) as dependent variable

Variable	β	P	95% Confidence Interval	
			Lower Bound	Upper Bound
leptin	$0.437x10^{-3}$	**0.008**	0.000	0.000
bmi	$1x10^{-3}$	**0.048**	0.000	0.003
triceps	$0.031x10^{-3}$	0.558	-0.001	0.000
P_b_fat	$0.004x10^{-3}$	0.456	-0.001	0.000
S_chol	$0.017x10^{-3}$	0.139	-0.001	0.000
S_tg	$0.049x10^{-3}$	0.215	0.000	0.000
nefa	-0.001	0.946	-0.017	0.016
S_ldl	$0.079x10^{-3}$	0.133	0.000	0.001
hdl_fac	0(a)	.	.	.

β for standard regression coefficient;

Table VII: Multiple linear regression taking fat normalized serum leptin as dependent variable

Parameter	β	Std. Error	P	95% Confidence Interval	
				Lower Bound	Upper Bound
BMI	-0.003	0.012	0.826	-0.026	0.021
Triceps skin fold thickness	0.017	0.005	**0.001**	0.007	0.027
Body Fat(%)	0.031	0.006	**0.000**	0.020	0.042
TC	0.007	0.003	**0.035**	0.000	0.013
TG	-0.001	0.001	**0.050**	-0.003	0.000
NEFA	-0.067	0.132	0.615	-0.329	0.196
S_ldl	-0.007	0.003	**0.047**	-0.013	0.000
hdl_fac	0(a)	.	.	.	.
fglu	-0.018	0.020	0.367	-0.057	0.021
fglu_30	-0.007	0.017	0.694	-0.041	0.028
SBP	-0.002	0.003	0.589	-0.008	0.005
DBP	0.000	0.004	0.958	-0.008	0.009
HOMA S	-0.423	0.409	0.303	-1.234	0.387
HOMA B	3.762	1.576	**0.019**	0.637	6.886

β for standard regression coefficient;

Correlation of serum leptin level with different variables

In bivariate analysis leptin level was found to be positively correlated with BMI, MUAC, % body fat, HDL, f insulin, insulin after 30 minutes, HOMA B%, phase I secretion, phase II secretion and C22:1 as well as negatively correlated with f glu, 30 min glu, TG, HOMA S% and C22:0.

Table VIII: Correlation of serum leptin level with different variables

Variables	r	p
BMI	0.457	**0.001**
MUAC	0.310	**0.001**
Percent body fat	0.796	**0.001**
f_ Glucose	-0.216	**0.017**
f_Glucose 30 min	-0.278	**0.002**
TG	-0.221	**0.015**
HDL	0.302	**0.001**
f_ Insulin (pmol/l)	0.623	**0.001**
s_Insulin 30 min (pmol/l)	0.500	**0.001**
HOMA B%	0.478	**0.001**
HOMA S%	-0.428	**0.001**
Phase I secretion	0.431	**0.001**
Phase II secretion	0.445	**0.001**
C22:0	-0.319	**0.039**
C22:1	0.561	**0.046**

The level of significance at $p<0.05$

Table IX: Correlation of serum leptin level, HOMA-B and HOMA-S with NEFA and different fatty acids*

	HOMA B%	HOMA S%	LEPTIN
NEFA	ns	**-.369/0.012**	ns
C14:0	ns	ns	ns
C15:0	ns	ns	ns
C16:0	**-0.577/0.01**	ns	ns
C16:1	ns	ns	ns
C18:0	ns	**0.093/0.037**	ns
C18:1	**-0.103/0.028**	ns	ns
C18:2	ns	ns	ns
C18:3	ns	ns	ns
C20:0	ns	ns	ns
C20:1	**-0.086/0.05**	ns	ns
C20:2	ns	ns	ns
C20:3	ns	ns	ns
C20:4	ns	ns	ns
C20:5		**0.354/0.001**	ns
C22:0	ns	ns	**-0.319/0.039**
C22:1	ns	ns	**0.561/0.046**
C22:6	ns	ns	ns
C16:0/C16:1	ns	ns	**-0.185/0.04**
C18:2/C18:3	ns	ns	**0.165/0.05**
C18:2/C20:4	**0.177/0.05**	ns	ns

*r/p values

Table X: Multiple linear regression analysis taking insulin sensitivity capacity (HOMA S%) as dependent variable

Variable	β	Std. Error	95% CI		P
C18:2/C20:4	.011	.008	-.006	.027	.189
C18:3/C20:5	-.040	.017	-.073	-.006	**.024**
C18:2/18:3	-.003	.002	-.007	.001	.116
C16:0/C16:1	-.005	.003	-.010	.001	.121
C18:0/C18:1	.053	.026	.001	.105	.047
Leptin	-.001	.001	-.002	.001	.350
BMI	-.006	.006	-.018	.006	.317
NEFA	-.246	.016	.0135	.568	**.003**

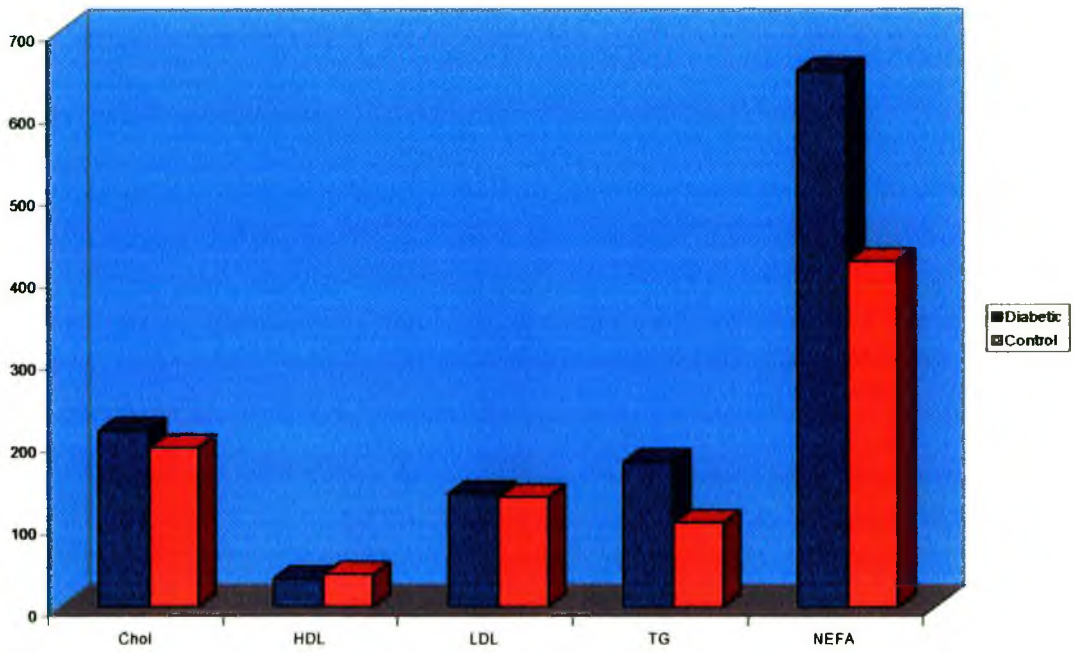

Fig IV: Lipidemic status of the study subjects

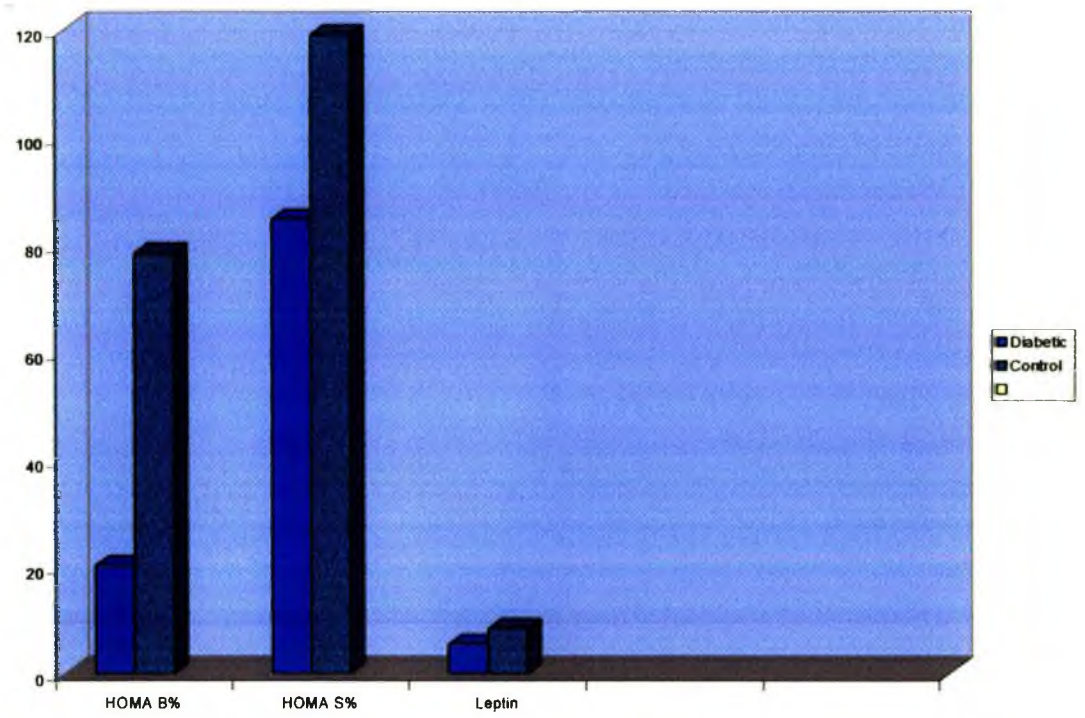

Fig V: Insulin secretion, sensitivity and leptin level of the study subjects

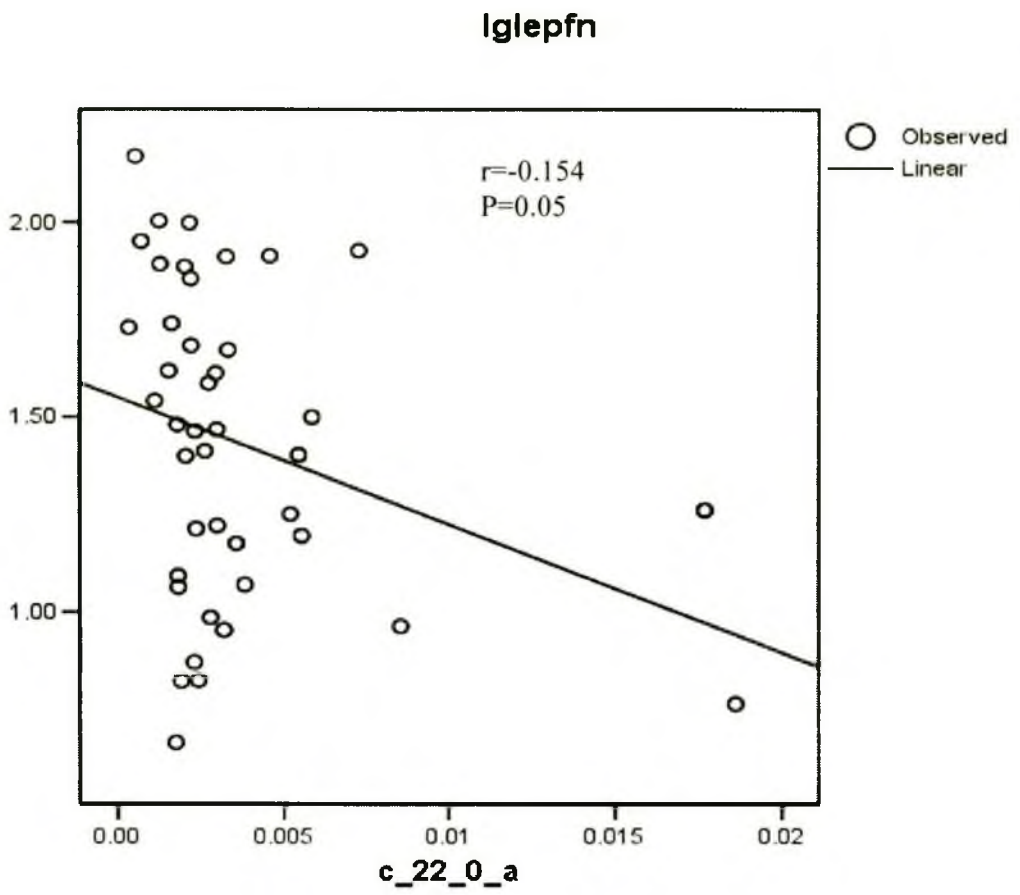

Fig VI: Correlation between serum leptin and C22:0

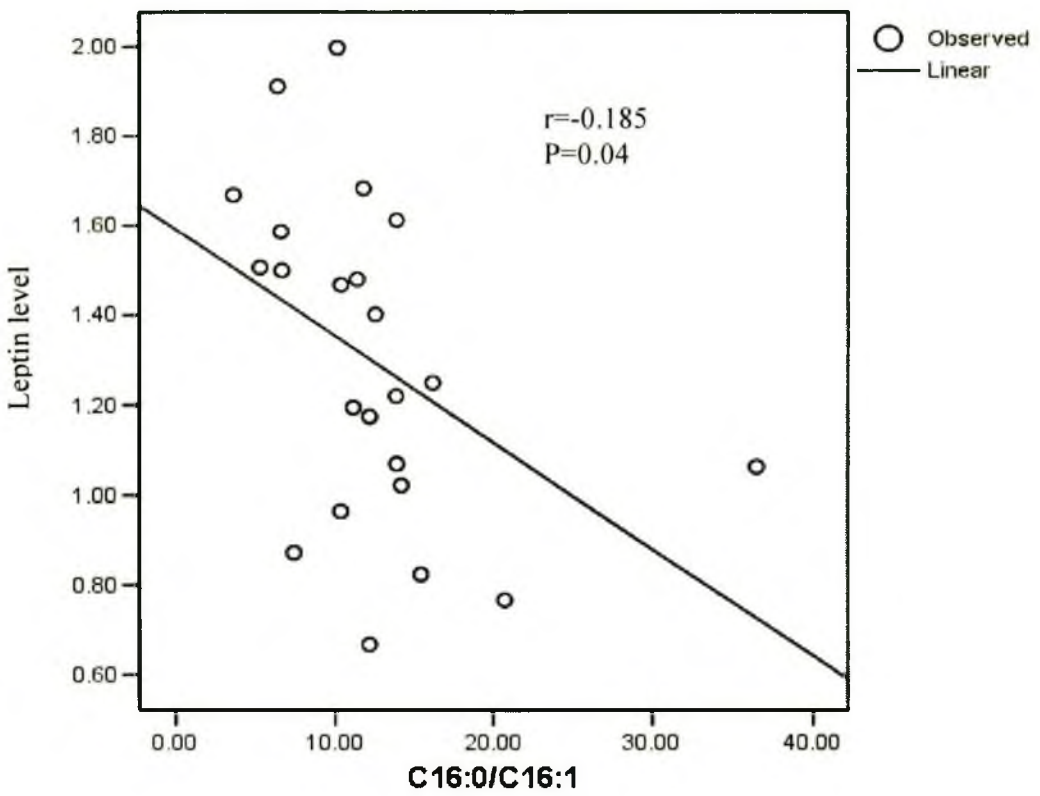

Fig VII: Correlation between serum leptin and C16:0/C16:1

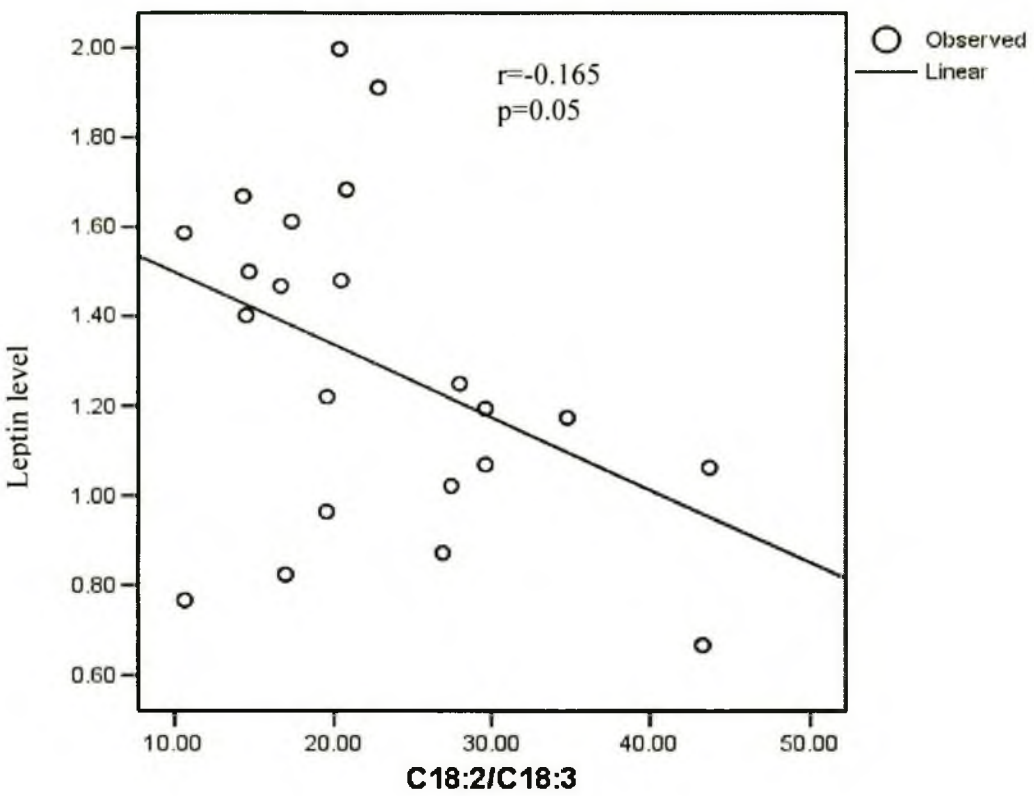

Fig VIII: Correlation between serum leptin and C18:2/C18:3

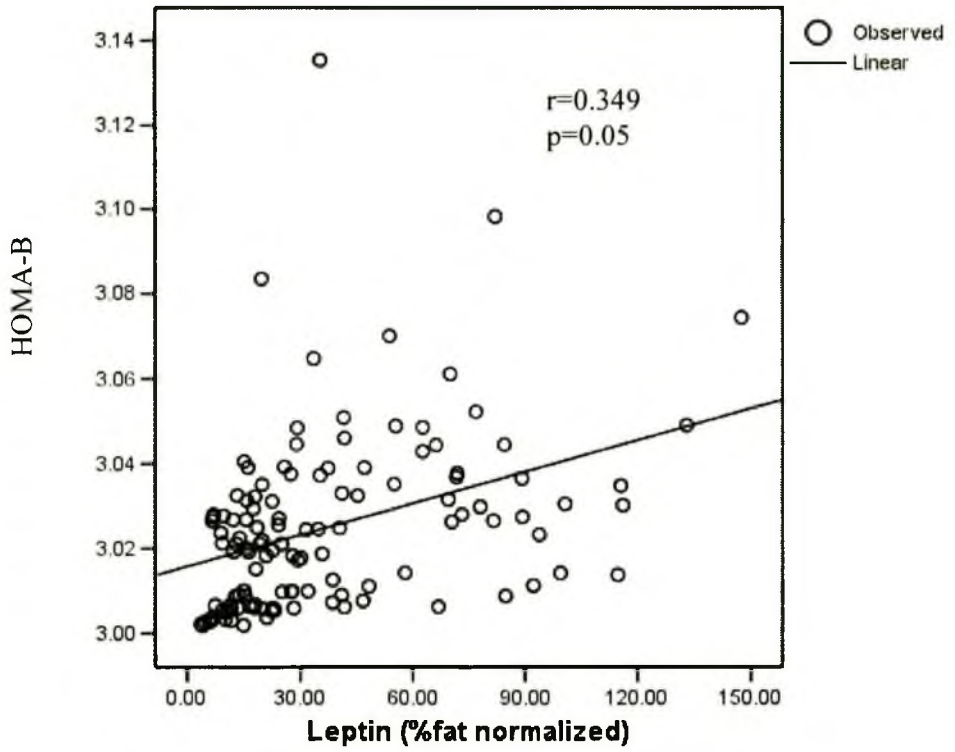

Fig IX: Correlation between insulin secretory function (HOMA B%) leptin level

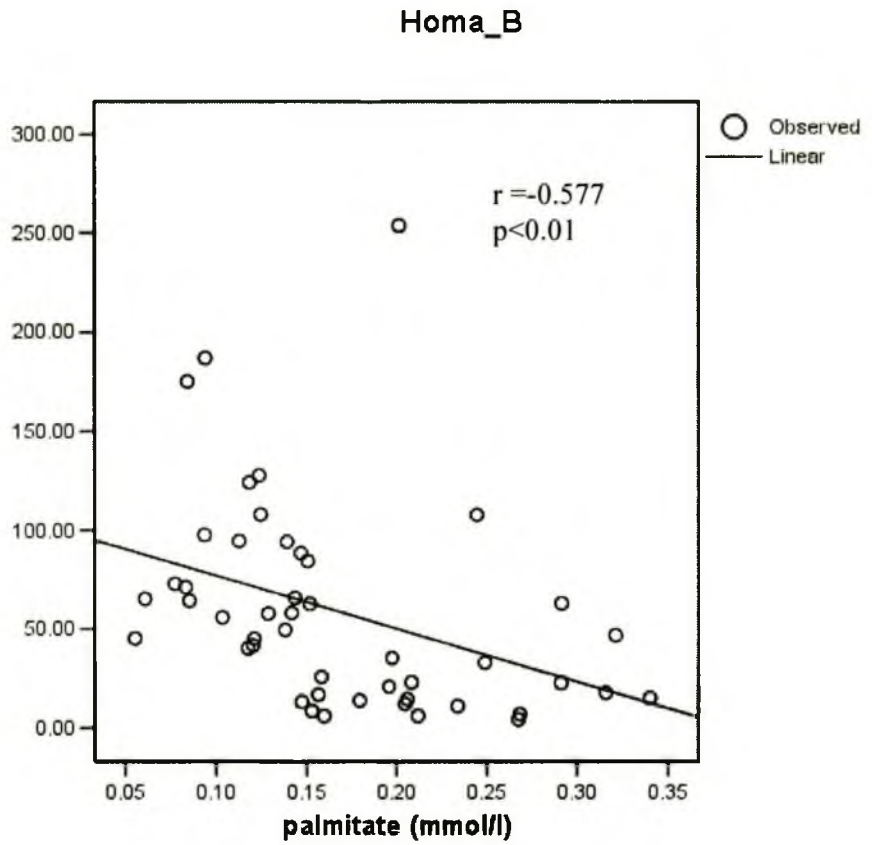

Fig X: Correlation between insulin secretory capacity (HOMA B %) and serum palmitate level

DISCUSSION

Discussion

Leptin is a multifunctional hormone that could impair insulin action as well as secretion and contribute to type 2 diabetes. A large number of studies showed conflicting result regarding the association of leptin with insulin secretion and sensitivity. In this study we explored the relationship of leptin and insulin secretary defect in type 2 diabetic subjects.

The diabetic subjects, in this study, had comparable anthropometric characteristics viz body mass index (BMI), waist to hip ratio (WHR), mid-arm upper circumference (MUAC), subscapular triceps ratio (STR) and body fat. As per study design newly diagnosed, homogeneous group of lean diabetics were selected to elucidate the relationship between leptin and insulin secretory defect. The diabetic subjects had higher mean blood pressure, as expected, than control subjects ($p<0.001$).

In this study, diabetic subjects were found to be dyslipidemic compared to control subjects. The diabetic subjects had higher serum total-cholesterol ($p<0.001$), TG ($p<0.001$) and lower HDL-C ($p<0.001$). These findings are consistent with findings in other studies (Stern and Haffner, 1997; Pickup and Williams, 1997; Mahtab *et al,* 1985).

In the present study both insulin secretory function (HOMA B%) and insulin sensitivity (HOMA S%) of diabetic subjects were significantly reduced. Reduction of HOMA B was more pronounced ($p<0.001$) than HOMA S ($p<0.004$). These findings are consistent with previous studies that found significant β-cell dysfunction and also insulin resistance (Al-Mahmood, 2000; Junaid, 2000). This observation in Bangladeshi subjects is in contrast to many studies on western population, where most type 2 diabetic subjects have impaired insulin action or sensitivity (Burtis and Ashwood, 1999; Sacks 1999).

Analysis of the different phases of insulin secretion in diabetic as well as in control and it was observed that both 1^{st} and 2^{nd} phases were markedly decreased in diabetic subjects ($p<0.001$). This finding is consistent with other studies. Weyer *et al.* (1999) reported a study where subjects were followed for several years from the NGT to IGT and diabetes; that longitudinal study allowed determination of the temporal sequence by which abnormalities in insulin action and insulin secretion progress during the development of the disease during the development of the disease and found both insulin secretion and insulin action significantly decrease early in the development of the disease. Individual with a first degree relative with type 2 diabetes already have reduction in first and second phase insulin release while having no change in insulin sensitivity. These findings suggest that impaired B-cell function precedes insulin resistance in those with a genetic predisposition to develop type 2 diabetes (Christian *et al* 1999).

In this study, serum body fat normalized leptin level was found to be lower in diabetic subjects compared to control ($p<0.001$). This finding is consistent with previous studies where they used fat normalized leptin level (Sivitz *et al.* 2003). This fat normalization is preferred as it removes anomalous interpretation from absolute leptin values due to confounders such as sex and degree of adiposity. Roy *et al.* (2003) reported higher absolute values in Bangladeshi type 2 diabetic subjects of variable degrees of adiposity (overweight to obese) without assaying total body fat mass/its relative proportion and thus such finding do not represent the actual alteration of leptin level in type 2 diabetes.

The serum leptin level inversely correlated with insulin secretory dysfunction. This observation is in consistent with previous reports (Abdelgadir *et al*, 2002; Sayeed *et al*, 2003; Abbas, 2003). The antisteatotic function of leptin is well documented (Unger, 1999). Leptin deficiency results in steotosis in ß-cells thereby impairing insulin secretory function (Unger, 1999; Zhou *et al*, 1998) with consequent development of diabetes. There is other possible explanation for reduced fat normalized leptin level may be due to

hyperglycemia. Hyperglycemic crises metabolically resemble the state of calorie deprivation; fasting and starvation may independently lower serum leptin. A period of 72-h fasting is known to inhibit serum leptin concentration, which could be reversed by feeding or glucose infusion (Abbas 2003; Guiller 2003). Moreover, leptin secretion may be dependent on adipocyte glucose utilization, which is clearly attenuated during hyperglycemic crises (Mueller *et al,* 1998).

The total NEFA level in the diabetic subjects was higher than control as expected (Le Roith *et al,* 2000; Groop *et al,* 1989). NEFA was positively correlated with fasting blood glucose level. As the diabetic subjects were untreated and newly diagnosed cases, raised NEFA level indicates increased lipolysis due to unabated action of lipoprotein lipase in absence or inaction of insulin.

Although leptin level had no correlation with total NEFA in this study, hypoleptinemia seems to be associated with altered fatty acid composition in type 2 diabetes (Unger, 1999;). Leptin level is positively correlated with fatty acid erucic acid (C22:1) and linoleic acid/linolenic acid (C18:2/C18:3); and inversely correlated with behenic acid (C22:0) and palmitic acid/palmitoleic acid (C16:0/C16:1).

There have been number of notable associations between insulin secretory function and different fatty acids. Palmitic acid (C16:0), (C18:0) and (C20:1) were individually inversely correlated with insulin secretory function, which may indicate their diabetogenic characteristic. It needs to be recalled that plasma palmitic acid/palmitoleic acid (C16:0/C16:1) ratio is also inversely related to leptin level suggesting a potential modulation by the hormone. However the change of (C18:0) and (C20:1) fatty acids in type 2 diabetes is independent of serum leptin level.

This study also revealed some associations of insulin sensitivity (ie insulin resistance^{-1}) with total NEFA and different fatty acids. Total NEFA, as

expected, was inversely correlated with insulin sensitivity. However stearic acid (C18:0) and eicosapentaenoic acid (C20:5) were found to be positively correlated with insulin sensitivity. This may indicate the antidiabetogenic /protective effect of the aforesaid fatty acids.

The relationship role of adipocytokines and different plasma fatty acids in the pathophysiology of type 2 diabetes is an area of complex relationships. Alteration in fatty acids may be the primary event or the sequelae which can only be properly deduced from prospective studies. Therefore interpretation of changes of a particular fatty acid level should very carefully drawn considering the background knowledge of its chemical and biological properties, and with after replication of findings in studies comprising large sample size.

SUMMARY AND CONCLUSIONS

Summary and Conclusions

Summary

Leptin, the adipose tissue-derived protein product of the obese (ob) gene, originally identified as an important regulator of body weight homeostasis and energy balance is a multifactorial hormone which may be involved in the pathogenesis of type 2 diabetes by influencing insulin production or sensitivity in humans. The present cross-sectional analytical study was designed to characterize insulin secretory defect in relation to leptin level in type 2 diabetic subjects and also to see whether blood fatty acid composition has any association with these abnormalities.

The study included 59 newly diagnosed type 2 diabetic subjects whose age range between 30 to 45 years. Sixty two age-matched healthy nondiabetic subjects were taken as control. Anthropometric measurement of all subjects were done following standard procedures with subsequent calculation for BMI, WHR and STR. Circulating fasting insulin and leptin were assayed by enzyme-linked immunoassay (ELISA). Fasting serum glucose, lipid profile and NEFA were measured by enzymatic-colorimetric method. Plasma fatty acid composition was characterized gas liquid chromatography using modified Dole Extraction method. Beta-cell function and insulin sensitivity were calculated from Fasting serum glucose and insulin values by the homeostasis model assessment (HOMA) using HOMA-CIGMA software.

The diabetic subjects, in this study, had comparable anthropometric characteristics viz body mass index (BMI), waist to hip ratio (WHR), mid-arm upper circumference (MUAC), subscapular triceps ratio (STR) and body fat. Mean blood pressure was significantly higher in diabetic as compared to control (88±8 vs. 82±7, $p<0.001$).

The diabetic subjects had higher serum total cholesterol (214±44 vs. 193±34, $p<0.001$), TG [176(52-681) vs. 102(49-370), $p<0.001$] than the control.

Serum HDL-C was significantly lower in diabetic as compared to control (33.45±6.77 vs. 38.86±8.21, $p<0.001$).

The diabetic subjects showed highly significant β-cell dysfunction and also insulin resistance as evident from HOMA B [20.20(4.20-89.60) vs. 78.35(35.50-365.7), $p<0.001$] and HOMA S [84.6(39.1-226.4) vs. 118.8(22.0-3573.7), $p<0.004$].

Analysis of the different phases of insulin secretion in diabetic as well as in control and it was observed that both 1^{st} and 2^{nd} phases were markedly decreased in diabetic subjects [Phase I 834 (235-2070) vs. 1037 (333-2929), $p<0.001$, Phase II 93 (35-204) vs. 274 (124-704), $p<0.001$].

Serum body fat normalized leptin level was found to be lower in diabetic subjects compared to control [(5.44 (0.65-34.70) vs. 8.35 (1.36-55), in control subjects, $p=0.012$]. The serum leptin level inversely correlated with insulin secretory dysfunction.

The total NEFA level in the diabetic subjects was higher than control as expected (0.652±0.196 vs. 0.42±0.15, $p<0.001$). NEFA was positively correlated with fasting blood glucose level.

Although leptin level had no correlation with NEFA in this study, hypoleptinemia seems to be associated with altered fatty acid composition in type 2 diabetes. Leptin level is positively correlated with palmitoleic (C16:1) acid, linolenic (C18:3) acid and inversely correlated with palmitic (C16:0) acid, linoleic (C18:2) acid, behenic acid as well as of linoleic/ linolenic (C18:2/C18:3) acid, palmitic/ palmitoleic (C16:0/C16:1) acid ratios.

Conclusions

The conclusions of the study are:

- Serum leptin level in normal to mildly overweight Bangladeshi type 2 diabetic subjects is lower compared to age and BMI-matched healthy population.
- Lower leptin in type 2 diabetes mellitus subjects is associated with insulin secretory dysfunction
- Modulation of serum leptin in type 2 diabetes mellitus is not related to total NEFA, but few individual fatty acids are associated with leptin both on higher and lower sides.
- Few individual NEFAs are associated with increasing or decreasing insulin secretion or sensitivity but no consistent feature are present among these two factors and leptin in their relation to total or individual NEFAs.

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APPENDICES

Appendix I

ESTIMATION OF SERUM GLUCOSE

Serum glucose was estimated by Glucose-Oxidase (GOD-PAP) method by the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of Randox Laboratories Ltd., UK.

Principle

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

Reaction Principle

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

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

Reagents composition

1. **Buffer:** Phosphate buffer (0.1 mol/l, pH 7.0) and phenol (11 mol/l)
2. **GOD-POD Reagent:** 4-aminophenazone (0.77 mmol/l), glucose oxidase (≥1.5 kU/l) and peroxidase (≥1.5 kU/l).
3. **Standard:** Glucose (5.55 mmol/l)

Procedure

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

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

Calculation of result for unknown sample is as follows:

Concentration of unknown sample = Standard concentration × optical density of unknown sample / optical density of standard

Appendix II

ESTIMATION OF SERUM TOTAL CHOLESTEROL

Serum total cholesterol was measured by enzymatic endpoint method (Cholesterol Oxidase/ Peroxidase) method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of Randox Laboratories Ltd., UK.

Principle

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

Reaction Principle

$$\text{Cholesterol-ester} + H_2 \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{fatty acids}$$

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

$$2\,H_2O_2 + \text{phenol} + \text{4–aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + H_2O$$

Reagent composition

1. **Enzyme Reagent:** Cholesterol oxides ($\geq$ 0.1 U/ml), cholesterol esterase (> 0.15 U/ml), peroxidase (> 0.5 U/ml), 4-aminoantipyrine (0.30 mmol/l), phenol (6 mmol/l) and Pipes buffer (80 mmol/l; pH 6.8).

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

Procedure

The equipment was calibrated before assay. Serum was taken in the sample cup and enzyme reagent was taken in the reagent container. The analyzer was programmed for the estimation of serum cholesterol and

allowed to run with the following steps: 5 μl sample and 500 μl reagent were taken to the reaction cell and mixed.The mixture was then incubated for 10 minutes at 37°C within the unit. Reading was taken at 500 nm.

Calculation of results for unknown sample is as follows:

Concentration of unknown sample = Standard concentration × optical density of unknown sample / optical density of standard

Appendix III

ESTIMATION OF SERUM HIGH DENSITY LIPOPROTEION (HDL) CHOLESTEROL

Serum high density lipoprotein (HDL) was measured by enzymatic colorimetric (cholesterol CHOD-PAP) method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of Randox Laboratories Ltd., UK.

Principle

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

Procedure

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

The supernatant was used as sample for determination of cholesterol content by the CHOD-PAP method. The sample and reagents were taken in specific cup. They were arranged serially then ID number for test was entered in the analyzer. Then 5 µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the cell. The reaction occurred in reaction cell. Reading was taken at 500 nm.

Calculation of result for unknown sample is as follows:

Concentration of unknown sample = Standard concentration × optical density of unknown sample / optical density of standard

Appendix IV

CALCULATION OF LDL- CHOLESTEROL

The LDL- cholesterol in serum was calculated by using the Friedewald formula.

The formula is as follows:

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

Appendix V

ESTIMATION OF SERUM TRIGLYCERIDE

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of Randox Laboratories Ltd., UK.

Principle

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

Reaction Principle

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

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

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

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

Reagents composition

1. **Buffer**: Pipes buffer (40 mmol/l, pH 7.6), 4-choloro-phenol (5.5 mmol/l), magnesium-ions (17.5 mmol/l).
2. **Enzyme Reagent**: 4-aminophenazone (0.5 mmol/l), glycerol-3-phosphate oxidase (GPO) (1.5 U/ml), lipases (>150 U/ml), ATP (1.0 mmol/l), peroxidase (POD) (0.5 U/ml).
3. **Standard**: Triglyceride = 200 mg/dl (2.29 mmol/)l.

Procedure

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

Calculation of result for unknown sample is as follows:

Concentration of unknown sample = Standard concentration × optical density of unknown sample / optical density of standard

Appendix VI

ESTIMATION OF FASTING SERUM CREATININE (RANDOX LABORATORIES, UK):

Estimation of serum creatinine was done by alkaline-picrate methods using reagents.

Principle

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

Sample

Serum

Reagents

Standard	177umol/1 (2mg/dl)
Picric acid surfactant	35 mmol/L
Sodium hydroxide	0.32 mol/L

Preparation of reagent

All reagents are supplied ready to use, stable to expiry date when stored at +15 to 25^0C.

Procedure

Wavelength		492 (490-510) mm
Cuvette		1 Cm Light Path
Temperature		25/30/37 C
Measurement		Against Air
Pipette into cuvette	Standard	Sample
Working reagent	1.0 ml	1.0 ml
Standard solutions	0.1 ml	---
Sample		

Mix and after 30 sec, read the absorbance A1 of the standard and sample

Exactly 2 min. later read the absorbance A2 of the standard and sample

Calculation

A2-A1 = ΔA sample or ΔA standard

Concentration of Creatinine in serum

$$\frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 100 = \text{mg/dl}$$

Appendix VII

ESTIMATION OF ALANINE AMINO TRANSFERASE (GPT)

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

Principle

$$\alpha\text{- oxogluterate + L - alanine} \xrightarrow{ALT} \text{L-glutamate + pyruvate}$$

$$\text{Pyruvate + NADH + } H^+ \xrightarrow{LD} \text{L-lactate + } NAD^+$$

Reagents

Contents	Concentration in the test
1. Buffer/Substrate	
Tris biffer	100 mmol/l, pH 7.5
L-alanine	0.6 mol/l
2. Enzyme/Coenzyme/α-oxoglutarate	
α-oxoglutarate	15 mmol/l
LD	≥1.2 U/ml
NADH	0.18 mmol/l

Preparation of Solutions

1. Buffer/Substrate: Buffer/Substrate supplied in the kit was used as it is.

2. Enzyme/Coenzyme/α-oxoglutarate : One vial of Enzyme/Coenzyme/α-oxoglu-tarate 2 was reconstituted with the appropriate volume of Buffer/Substrate 1:

2 ml	for the	20 x	2 ml	kit (AL 1200)
10 ml	for the	20 x	10 ml	kit (AL 1205)
20 ml	for the	5 x	20 ml	kit (AL 1268)

Cat No AL 2360 5 x 100 ml

One vial of Enzyme/Coenzyme/α-oxoglu-tarate 2 was reconstituted with a portion of Buffer/Substrate 1 and then the entire contents was transferred to bottle 1 rinsing bottle 2 several times.

Procedure

Wavelength:	340 nm (Hg 334 nm or Hg 365 nm)
Cuvette:	1 cm light path
Temperature:	25/30/37°C
Measurement:	against air

Pipetted into cuvette:

	Macro	Micro
Serum	0.2 ml	0.1 ml
Enzyme/Coenzyme/α-oxoglu-tarate 2	2.0 ml	1.0 ml

Mixed and initial absorbance was read after 1 minute. Again after 1, 2 and 3 minutes the absorbance was read. The absorbance change per minute was noted and if the value is between

0.11 and 0.16 at 340 nm/Hg 340 nm

0.06 and 0.08 at Hg 365 nm

Only then the values for the first 2 minutes were used for the calculation.

Calculation

To calculate the ALT activity the following formulae was used:

U/l	=	1746	x	ΔA 340 nm/min
U/l	=	1780	x	ΔA Hg 334 nm/min
U/l	=	3235	x	ΔA Hg 365 nm/min

Normal values in serum

	25°C	30°C	37°C
Men	Up to 22 U/l	Up to 29 U/l	Up to 40 U/l
Women	Up to 17 U/l	Up to 22 U/l	Up to 31 U/l

Linearity

If the absorbance change per minute exceeds

0.16 at 340 nm/Hg 334 nm

0.08 at Hg 365 nm

0.1 ml of sample was diluted with 0.9 ml pf 0.9% NaCl solution and reassessed. The result was multiplied by 10.

Appendix VIII

ESTIMATION OF NON ESTERIFIED FATTY ACID (NEFA)

Sample

Serum

Anticoagulant

EDTA

Serum NEFA was measured by colorimetric method using kits from Randox, UK, (Cat No FA 115). The measurement has been done using Microplate Reader, adopted in the Research Division, BIRDEM.

Principle

$$\text{NEFA} + \text{ATP} + \text{CoA} \xrightarrow{\text{Acyl CoA Synthetase}} \text{Acyl CoA} + \text{AMP} + \text{PPi}$$

$$\text{Acyl CoA} + O_2 \xrightarrow{\text{Acyl CoA Oxidase}} \text{2,3-trans-enoyl CoA} + H_2O_2$$

$$2H_2O_2 + \text{TOOS} + \text{4-AAP} \xrightarrow{\text{Peroxidase}} \text{purple adduct} + 4H_2O$$

4-AAP= 4-aminoantipyrine

TOOS= N-ethyl-N-(2hydroxy-3-sulphopropyl)m-toluidine

Reagents

Buffer	
Phosphate buffer	0.04mol/l, pH 6.9
$MgCl_2$	3mmol/l
Surfactant	
Enzyme/Coenzymes	
Acyl CoA Synthetase	0.3U/ml
Ascorbate Oxidase	1.5U/ml
Coenzyme A	0.9mmol/l
ATP	5.0 mmol/l
4-amino antipyrine	1.5mmol/Enzyme Diluents
Phenoxyethanol	0.3%(w/v)
Surfactant	
Maleimide	10.6mmol/l

Enzyme Reagent

Acyl Coenzyme A Oxidase	10U/ml
Peroxidase	7.5U/ml
TOOS	1.2mmol/l
Standard	1mmol/l

Preparation of reagent

Buffer

Contents were ready for use.

Enzyme/Coenzymes

The contents of one vial of Enzyme/Coenzyme were reconstitute with 10 ml of buffer which should remain stable for 5 days at +2 to $+8^0$C, should not freeze and protected from light.

Enzyme diluent

Contents supplied were ready for use at +2 to $+8^0$ C expiry date (concentration 1 mmol/l)

Maleimide

The contents of one bottle of maleimide were reconstituting with the entire contents of one bottle of enzyme diluent and dissolved completely.

Enzyme Reagent

The contents of one vial of enzyme reagent were reconstitute with one bottle of malemide solution which should remain stable for 5 days at +2 to $+8^0$ C, should not freeze and protected.

Standard

Contents supplied were ready for use (concentration 1mmol/l)

Procedure

Standard solution of 0.0,0.06,0.12,0.25,0.50,1.0 mmol/l was made using the given standard of 1mmol/l.

A microwell plate was taken and marked for reagent blank, standards and unknown samples.

Samples were taken out at the freezer and allowed to thaw

Deionized water (10µl) was used for reagent blank.

Standards samples (10µl) were pipetted into well in duplicate.

Working reagent 1(100µl) was added into each, mix and plate was incubated at 37^0C for 10 min.

Working reagent 2 (200µl) was added into each well.

Plate was incubated at 37^0C for 10 min.

Absorbance of sample (A_{sample}) and standard ($A_{standard}$) was taken against the reagent blank at 550 nm.

The standard curve was run on everyday experiment.

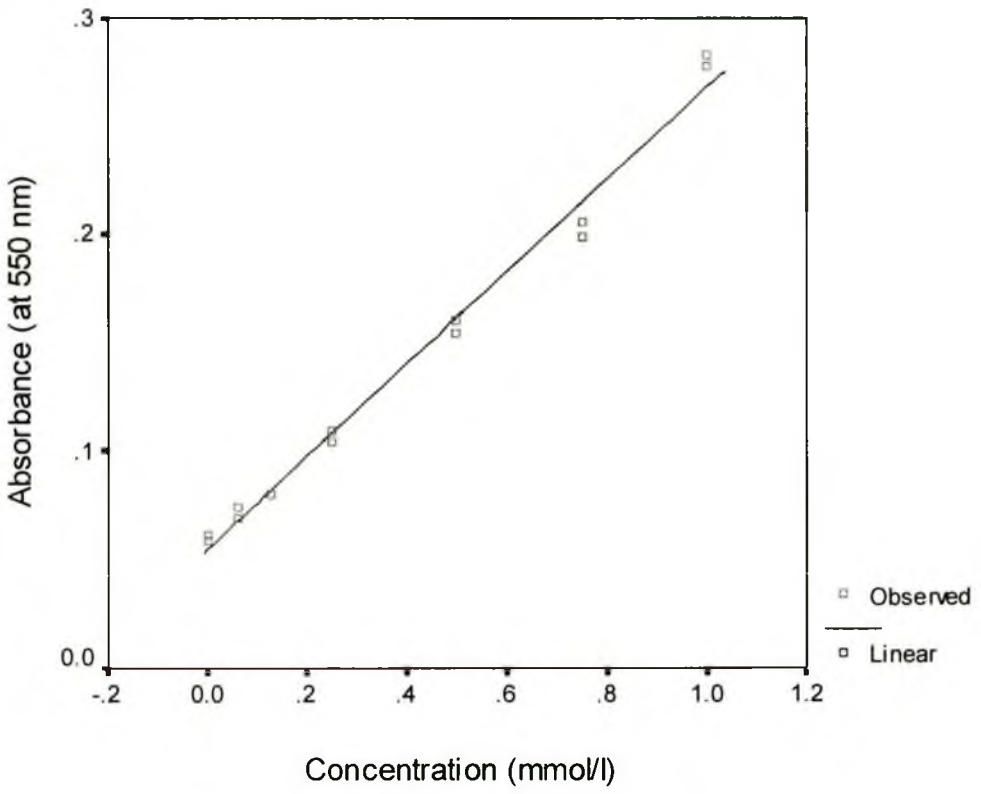

Figure 2: Standard curve of NEFA

Calculation

Optical densities obtained were used to calculate values of unknown samples using the software, Kinetical Calculation. Results were calculated by extrapolating the standard curve.

Table 6: Experiment plan

	Reagent blank (µl)	**Standard (µl)**	
Deionized water	10	-	-
Standards (1mmol/l)	-	10	-
Reagent solution (!)	100	100	100
Reagent solution (2)	200	200	200
Plasma	-	-	10

Appendix IX

ESTIMATION OF FASTING SERUM LEPTIN

Fasting serum leptin was measured by the Enzyme Linked Immunosorbentassay (ELISA) method with a research kit (LINCO Research, USA).

Principle

This assay is a direct quantitative ELISA technique. A polyclonal rabbit anti-human leptin antibody precoated onto a microtiterplate. Standard, control and samples were pipetted into the wells which react with the captured antibody. After incubation period washed away unbound materials. Binding of biotinylated monoclonal antibody forming sandwitch: coated antihuman leptin antibody, washed away the unbound free enzyme conjugates, a substrate solution was added and incubated. The reaction was stopped by the addition of stop solution. The amount of leptin was determined spectrophotometrically by measuring the absorbance which was proportional to the human leptin concentration. The result was determined by interpolation from a reference curve generated in the same assay with reference standard of known concentration of human leptin.

Reagents:

1. Microtiter plate with 96 Rabbit anti-Human Leptin Antibody coated wells.

2. Wash buffer 2×50 ml, should be diluted in 900 ml distilled water

3. Human leptin detection Antibody which is Biotinylated Mouse anti-Human Leptin Antibody, ready to use

4. Enzyme solution contain Streptavidin-Horseradish Peroxidase Conjugate in Buffer (12 ml), ready to use

5. Standards 0, 0.5, 2, 5,10,20,50 and 100 ng/ml leptin in assay buffer, ready to use

6. Control 1 and 2 in human plasma with preservatives ranges QC1: 2.7-5.7 ng/ml and QC2:18.0-37.4 ng/ml, ready to use

7. Assay buffer 1×10 ml, ready to use

8. Substrate solution: 3, 3'5, 5'-tetramethylbenzidine 1×12 ml, 1 ready to use

9. Stop solution 1×12 ml, ready to use

Material required

1. Deionized water

2. Precision micropipettes with disposable tips for 10μl-200μl

3. Buffer and reagent reservoirs

4. Vortex mixer

5. Absorbent paper

6. Microplate reader (450 nm)

7. Orbital microtiter plate shaker

8. Standard refrigerator

Reagent preparation:

1. Wash solution: The content of wash solution was diluted 10 fold by adding de-ionized water.

Assay procedure

All reagents and samples were brought to room temperature before use.

1. Removed required number of strips from the microtiter assay plate. Unused strips were resealed in the foil pouch containing the desiccant pack and then resealed.

2. Added 100 μl, 75 μl assay buffer into the blank as well as the rest of the wells respectively.

3. 25 μl of each standard, control and sample was dispensed into the appropriate wells. Standards and samples were run in dual determination. All additions were completed within one hour.

4. The plate was then covered with plate sealer and incubated at room temperature for 2 hours on an orbital microtiter plate shaker set at 400 rpm.

5. Removed plate sealer and decant solutions from the plate.

6. Washed wells 3 times with 300μl diluted wash buffer per wash. Decant and tap after each wash to remove residual buffer using manual procedure.

7. 100μl of detection antibody was added into each well and incubate 30 min as before.

8. Removed solution and added 100μl enzyme solution to each well. The plate was then incubate further 30 minutes at moderate speed (400 rpm)

9. After incubation, decant the residual solution and wash 5 times (300μl), decant and tap firmly after each wash to remove residual buffer by inverting on the paper towel.

10. 100μl of substrate solution was dispensed into each well as soon as possible.

11. The plate was then sealed and shake on the plate shaker for ~5 minutes.

12. The reaction was then stopped by adding 100μl of stop solution and shake plate by hand for complete mixing of solution in all wells.

13. Then the optical density of each well was determined within 10 minutes using a micro plate set at 450 nm (reference filter 630). Before taking reading ensured that no air bubbles was present in any well.

Calculation:

The duplicate readings for each standard and control were averaged and the average zero standard optical density was subtracted from it.

1. A standard curve was constricted by plotting absorbance (Y) of each reference standard against the concentration and the best sigmoid curve was drawn. The data were linearized by log/logit function.

2. The absorbance of each serum sample was used to determine the corresponding leptin value by simple interpolation.

Sensitivity: The lowest level of human leptin that detected by this assay was 0.5 ng/ml

Appendix X

ESTIMATION OF SERUM INSULIN

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

Principle

This human insulin is a sandwich ELISA based immunoassay in which a monoclonal antibodies had been precoated onto a microplate. Standard, controls and samples were bound with the coated monoclonal antibodies and the second antibody also boud to the captured insulin. A simple washing step removes unbound enzyme labelled antibody. The bound conjugate was detected by reaction with the substrate 3,3',5,5' tetra – methylbenzidine. Adding acid to give a colorimetric endpoint that is read spectrophotometrically stops the reaction.

REAGENTS SUPPLIED

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

Microtiter plate

Coated with mouse monoclonal anti-human insulin antibodies.

Adhesive Plate Sealer

10X Concentrate HRP Wash Buffer

Washing solution (buffer with preservatives) 2x50 ml, the vial content was diluted in1:10 with deionized water.

Standards

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

Quality Controls 1 and 2

Purified Recombinant Human insulin in Assay Buffer, ready to use.

Matrix Solution

Heat-treated charcoal stripped off the clot human insulin serum 2×1 ml, ready to use.

Assay Buffer

0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% Sodium Azide, 1% BSA, 2x9 ml, ready for use.

Human Insulin Detection Antibody

Pre-titered Biotinylated Monoclonal Mouse anti-Human Insulin antibody (2x12 ml), ready to use.

Enzyme Solution

Pre-titered Streptavidin-Horseradish Peroxidase

Conjugation Buffer (2x12 ml), ready to use.

Substrate

3,3',5,5'-tetramethylbenzidine in Buffer (12 ml), ready for use.

Stop Solution

0.3 M HCl (12 ml), ready for 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 590 nm

7. Orbital Microtiter Plate Shaker

8. Absorbant Paper

9. Standard Refrigerator

Reagent Preparation

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

Assay Procedure

1. The desired number of coated microtiter wells secured in holding frame.

2. Added 300 µl of dilute wash buffer into each well of monoclonal mouse anti-human insulin antibodies Coated microplate and incubated at room temperature for 5 minutes.

3. Removed wash buffer from all wells by inverting the plate. The plate was then sharply striked on absorbant paper to remove the residual droplets.

4. Added 20 µl of detection antibody to each well with a multichennel pipette

5. Added 20 µl matrix solution to the NSB, standard and quality control wells.

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

7. Added 20 µl of each Standard, control and sample was dispensed the appropriate wells. Run in duplicate determination for standard and control. Covered with plate sealer and incubated for 1 hrs at room temperature on orbital microtiter plate shaker set at 400 rpm.

8. The content of the wells was briskly shacked out.

9. The wells were rinsed 3 times by dispensing 300 µl diluted HRP wash buffer into each well and tap smartly onto the paper towel.

10. 100 µl of enzyme solution was dispensed into each well and incubated at room temperature for 30 minutes on an orbital microtiter plate shaker at 400 rpm.

11. The solution was then decanted from the plate and tapped as before to remove residual solutions in the wells.

12. The wells were washed five times as like as earlier.

13. Added 100 µl substrate solution to each solution, covered and kept for 8-10 minutes on the shaker.

16. Stopped the reaction by adding stop solution.

17. Absorbance was read at 450 nm.

Calculations : The insulin 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 (pmol) from the value of individual.
- The smooth curve was drown that fits 5 plotted points The results of unknown samples were calculated using logistic function.

Appendix XI

Consent Form

I Mr/Ms ..... hereby give my well informed and coercion free consent to participate in the study conducted by Salima Akter fully understand that my participation in the study will bring fruitful information to be useful for many others in future.

I am convinced that during participation in the study, I shall not be exposed to any physical, psychological, social or legal risks. My privacy and confidentiality will be safeguarded and any anonymity will be protected. I would like/ would not like to be monetarily compensated because of my loss of work. I also consent to use my blood for the study.

Signature of the Principal Investigator	Signature of the Subjects/ Thumb Impression
Date:	Date:

Appendix XII

CASE RECORD FORM

Sl No----------

Date:

Name---------- Phone:

Age----------

Sex: Male/female

Area: Rural / Urban / Semi-urban

Economic status: Lower/Middle/Upper

Present address----------

Permanent address----------

Subject: Type 2 Diabetic (Non-obese / Overweight / obese) / Control

Family history: Diabetes---------- Hypertension----------

Renal disease---------- Cardiac disease----------

Patient history: Hypertension---------- Renal disease----------

Hepatic disease---------- Cardiac disease----------

GDM in previous pregnancy.....

Smoking: Yes / No

Basis of Diagnosis:

Fasting plasma glucose----------mmol/l

Plasma glucose 30 minutes after glucose load----------mmol/l

A. Blood pressure (BP) in mm Hg: SBP DBP

B. **Antrhopometry:**

(i) Height (m): (ii) Weight (kg) (iii) BMI (kg/m^2)

(i) Skinfold thickness (mm)

Triceps: Subscapular:

(ii) MUAC (mm):

(iii) Waist circumference (cm): Hip circumference (cm):

(iv) Waist / Hip ratio:

(v) Percent body fat:

(vi) Free fat mass (FFM):

(vii) Changes in body weight in last 3 month

C. Results of Biochemical parameters:

Sl No	Biochemical parameter	Result	Result in SI unit	Ref range
1	S glucose (F)	mg/dl	**mmol/l**	**3.3-5.6**
2	S glucose 30 min after	mg/dl	**mmol/l**	
3	S glucose 2 hrs after	mg/dl	**mmol/l**	**<7.8**
4	S Insulin (F)	uIU/ml		6-27
5	β-cell function	%	%	
6	Insulin resistance			
7	S leptin (F)	**ng/ml**	pmol/l	
8	Blood HbA_{1c}	%	%	<6.0
9	S cholesterol	**mg/dl**	mmol/l	**150-200**
10	S HDL-C	**mg/dl**	mmol/l	M:>55,F:>65
11	S LDL-C	**mg/dl**	mmol/l	**<150**
12	S TG	**mg/dl**	mmol/l	**50-150**
13	S NEFA		mmol/l	
14	S creatinine	**mg/dl**	μmol/l	**0.67-1.2**
15	S GPT	***U/L***		Upto 40
16	S Uric Acid	**mg/dl**		**F:2.4-5.7, M:3.4-7.0**