



# **Incretin effect in prediabetic subjects**

**MPhil Thesis**

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*Dedicated*  
*To*  
*My Beloved Parents*

## **ACKNOWLEDGMENT**

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## ABSTRACT

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The global prevalence of diabetes mellitus (DM) is increasing and rising rate is higher in developing countries. The favorable influence of gut hormones GIP and GLP-1 (7 – 36) on metabolic disorders of DM has provided the mainstay of incretin based therapy of type 2 DM. Reduced incretin effect is a specific, important and early characteristic of type 2 DM and is associated with  $\beta$  cell failure. However, the interrelationship between incretins, insulin secretory dysfunction and insulin resistance in isolated IGT is still controversial and poorly understood. Increased insulin resistance in isolated IGT in Bangladeshi population has been reported. but its associated abnormalities in the secretion of gut hormones have not yet been investigated. In the present study, gut hormones (incretins) secretion in isolated IGT were evaluated by plasma concentrations of total GIP and GLP-1(7 – 36) to explore their relationship with insulin secretory capacity and insulin sensitivity.

Thirty four isolated IGT subjects and equal number of age-sex matched controls were recruited from the OPD of BIHS hospital. Blood specimens and anthropometric data were collected after obtaining written consent. Plasma glucose, lipid parameters, serum ALT and serum creatinine concentrations were measured by standard methods. Plasma concentrations of the gut hormones total GIP and GLP-1 (7 – 36), were determined by enzyme linked immunosorbent assay (ELISA). Results are expressed as median (range) analyzed by appropriated statistical tests.

According to inclusion criteria of the study subjects, acute and chronic glycemic status differed significantly ( $p < 0.001$ ) between control and isolated IGT groups. Fasting and postprandial TGs were found to be higher in isolated IGT compared to control. HDL cholesterol levels were significantly lower in isolated IGT subjects. Total cholesterol and LDL cholesterol levels were similar in control and isolated IGT subjects. Fasting and postprandial insulin levels were found to be 64.2 % [10.9 (2.8 – 25.6)  $\mu$ IU/ml vs 17.9 (7.7 – 44.8)  $\mu$ IU/ml and  $p < 0.001$ ] and 16.6% [69.4 (34 – 211)  $\mu$ IU/ml vs 80.9 (22 – 217)  $\mu$ IU/ml and  $p < 0.046$ ] higher in isolated IGT groups compared to control. Postprandial insulin levels compared to fasting levels were also found to be higher in both controls [10.9 vs 69.4  $\mu$ IU/ml] and IGT subjects (17.9 vs 80.9  $\mu$ IU/ml) and the changes in insulin levels were

remarkably lower in IGT compared to control (6.2 fold vs 4.5 fold). In subjects with IGT, insulin secretory capacity was found to be significantly higher compared to control (147.3 vs 164.3,  $p < 0.05$ ), insulin sensitivity was found to be significantly lower (63.9 vs 38.4) and insulin resistance was significantly higher (1.6 vs 2.6) compared to control.

Fasting GIP concentrations were 58% higher in isolated IGT compared to control (99.9 vs 158.1 pg/mL,  $p < 0.01$ ), but postprandial GIP concentrations were similar in both groups (only 2% lower in IGT,  $p = 0.864$ ). The changes in plasma GIP after mixed breakfast was 50% lower in isolated IGT compared to control (control vs IGT: 851% vs 424%). When GIP concentrations per unit of glucose were compared between control and IGT, fasting GIP per unit of glucose was 32% higher in IGT whereas postprandial GIP was 27% lower compared to control. Compared to control, increase in GIP concentration per unit of glucose was 50% lower in isolated IGT after mixed breakfast. Insulin concentrations per unit of GIP showed no significant difference between control and IGT in fasting state and postprandial state. On correlation analysis, fasting GIP showed no significant relationship with insulin secretory capacity, insulin sensitivity and insulin resistance. Fasting concentrations of GLP-1 (7 – 36) was found to be 58% lower in isolated IGT compared to control (3.3 vs 1.4 pmol/L,  $p < 0.05$ ). However, postprandial GLP-1 showed no significant differences between IGT and control subjects. The incretin effect in terms of plasma GLP-1 (7 – 36) showed no significant difference between fasting and postprandial state ( $p > 0.05$ ) in control. Whereas, GLP-1 (7 – 36) concentration in postprandial state in isolated IGT was found to be significantly higher compared to fasting state. GLP-1 (7 – 36) per unit of glucose was significantly lower in IGT compared to that of control; it was also significantly reduced in postprandial state compared to fasting state in both control and IGT. Insulin secretory capacity showed a significant positive relationship with fasting GLP-1 (7 – 36), postprandial GLP-1 (7 – 36) and BMI only in isolated IGT. Insulin sensitivity in isolated IGT was found to be inversely related to fasting GLP-1 (7 – 36), postprandial GLP-1 (7 – 36) and BMI. But in control insulin sensitivity showed no such relationship. GLP-1 (7 – 36) inversely related to insulin sensitivity and positively related to insulin resistance in IGT.

It may be concluded from this study that

- Isolated IGT is a hyperinsulinemic state and it is associated with insulin resistance.
- Hypersecretion of GIP and deficient secretion of GLP-1 in the fasting states are associated with isolated IGT.
- Insulin secretory dysfunction and insulin resistance can develop in the absence of any impairment of GIP secretions but they may be associated with defective GLP-1 secretion from the gut.

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## LIST OF ABBREVIATIONS

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ADA	American diabetic association
BMI	Body Mass Index
cAMP	Cyclic AMP
cDNA	Complementary DNA
CNS	Central Nervous System
CVD	Cardiovascular Disease
DPP-4	Dipeptidyl peptidase – 4
DM	Diabetes Mellitus
ELISA	Enzyme Linked Immunosorbent Assay
GLP-1	Glucagon Like Peptide – 1
GIP	Glucose dependent insulinotropic polypeptide
GIPR	GIP receptor
HOMA B	Homeostasis Model Assessment $\beta$ - cell function
HOMA S	Homeostasis Model Assessment Insulin Sensitivity
IDF	International Diabetic federation
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IDF	International Diabetes Federation
OPD	Out Patient Department
PKA	Protein Kinase A
SPSS	Statistical Package for Social Sciences
WHO	World Health Organization
WHR	West Hip Ratio

## **DECLARATION**

The Thesis titled as ‘Incretin effect in prediabetic subjects’ for the requirement for the Degree of Master of Philosophy (MPhil) in the Faculty of Biological Science, University of Dhaka. This study has been carried out in the Department of Biochemistry and Cell Biology, Bangladesh Institute of Health Sciences (BIHS) Dhaka, in collaboration with the Department of Biochemistry and Molecular Biology, University of Dhaka. To the best of our knowledge no part of the work has been submitted for another degree or qualification in any other institute at home or abroad.

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## Data collection Sheet

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MPhil (Thesis)

1) Identification NO:

2) Particulars of the patient:

Name-

Father/Husband name:

Age

Sex

Religion

Address-

Phone no:

Date of 1<sup>st</sup> examination:

3) Past medical history

4) Drug history

Anti-HTN-

Lipid lowering-

Anti obesity

Anti-ischemic

Others-

5) Family history:

Father- DM/HTN/CAD/CVD/Obesity

Mother- DM/HTN/CAD/CVD/Obesity

Children - DM/HTN/CAD/CVD/Obesity

Others - DM/HTN/CAD/CVD/Obesity

6) Physical Examination-

Height in meter-

Weight in kg-

BMI-

Waist Circumference-

Hip Circumference-

Waist-Hip ratio-

Mid Upper Arm Circumference (MUAC)-

Blood Pressure-

1<sup>st</sup> reading

2<sup>nd</sup> reading -

average-

Systolic-

Diastolic-

## CHAPTER -01

### INTRODUCTION

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The term “prediabetes” is used to describe the condition in which blood glucose levels are higher than normal but yet not diabetic. Patients with impaired fasting glucose (IFG) and /or impaired glucose tolerance (IGT) are referred to as prediabetes. It is a metabolic state between normal glucose homeostasis and diabetes. They are not interchangeable and represent different abnormalities of glucose regulation, one in fasting state and one postprandial (WHO, 1999). The categories of IFG and IGT are less commonly used in clinical practice but are routinely employed to define populations for clinical research. Both the American Diabetes Association (ADA) and the World Health Organization (WHO) recognize groups of patients with IFG and IGT. According to fasting and post load glucose concentration, at present, patients with prediabetes may be stratified into three subcategories – isolated IGT (i-IGT), isolated IFG (i-IFG) and combined IFG-IGT (IFG/IGT) (WHO and ADA, 2002). These categories are a part of the natural history of diabetes and not a type of diabetes.

IFG is defined as fasting plasma glucose between 6.1 and 6.9 mmol/l and 2h plasma glucose < 7.8 mmol/l (ADA, 2005). In 1997, the ADA published report mentioned IFG as a new category, which was also adopted in 1999 World Health Organization (WHO) report (Stern and Burke, 2002). Recently American Diabetic Association (ADA) has reduced the lower cut off value of fasting plasma glucose in IFG from 6.1 mmol/l to 5.5mmol/l (ADA 2005). IFG is subsequently more common among men. The prevalence of IFG tends to plateau in middle age (Unwin, 2002). The crude prevalence of IFG was 12.4% in rural population of Bangladesh; the age standardized prevalence of IFG was 13.0% (Sayeed et al 2003). In one study in Dutch population it was seen that the prevalence of IFG is 9.7% in men and 6.1% in women (Corpeleijn et al 2005). The age standard prevalence of IFG in European population was 2.8% (Boronat et al, 2002).

IGT is defined as fasting plasma glucose  $< 6.1$  mmol/l and 2h plasma glucose between 7.8 and 11.0 mmol/l (ADA, 2005) Historically the term IGT was first introduced by the National Diabetes Data Group in 1979 and later the same word was reestablished by WHO. IGT is more prevalent than IFG. IGT is slightly more common amongst women.

In abroad and in our country there are some prediabetic subjects who were diagnosed as having both the characteristics of impaired Fasting Glucose (IFG) and Impaired Glucose Tolerance (IGT) and they are frequently known as IFG – IGT subjects. IFG – IGT is defined as fasting plasma glucose between 6.1 mmol/l and 6.9 mmol/l and 2 hour plasma glucose between 7.8 mmol/l and 11.0 mmol/l (ADA, 2005). A study conducted in Denmark to observe the progression of IFG – IGT to diabetes has shown that the progression rate was 28 per 100 IFG – IGT per year ( Rasmussen et al, 2007).

The main features of IFG/IGT are: 1) a stage in the natural history of disordered glucose metabolism, 2) can lead to any type of diabetes, 3) increased risk of progression to diabetes, 4) increased risk of cardiovascular diseases 5) little or no risk of microvascular diseases, and 6) some patient may revert to normoglycemic condition (Balkau and Eschwege, 2003). IFG and IGT are asymptomatic and unassociated with manifested morbidity, but their sole significance lies in the fact that they predict future diabetes or cardiovascular diseases (Stern and Burke, 2002). Both IFG and IGT are similarly associated with an increased risk of diabetes mellitus. Risk is higher where IFG and IGT coexists (Unwin 2002). IGT is more prevalent than IFG, less than or equal to 50% of people with IFG has IGT and 20% - 30% of people with IGT also has IFG (Unwin, 2002).

### **Epidemiology of prediabetes**

Prediabetes is a common disorder in most populations. Epidemiological studies comparing the prevalence of IFG and IGT consistently have demonstrated that they define two distinct populations with only partial overlap (Unwin et al, 2002, Choi et al, 2002, Gabir et al, 2000, Dunstan et al, 2002, Shaw et al, 1999, Larsson et el, 1998, de vegt et al, 1998, Harris et al, 1997, The decode study group 1999, Sadikot et al, 2004, Botas et al, 2003). This observation was consistent in all ethnic groups studied. The prevalence of both IFG and IGT



varies considerably based on ethnicity, ranging from a low of 6.3% in Chinese (Ko GT et al, 1998) to a high of 20.3% in a Swedish population (Larsson et al, 1998). Both IFG and IGT increase in prevalence with age (Shaw et al, 1999). The prevalence of IFG is similar in men and women, but IGT is more frequent in women (Shaw et al, 1999). Although there is some overlap between IFG and IGT, most studies have shown that these criteria define different populations at risk for type 2 diabetes and other complications (e.g. CVD) ( Dunstan et al, 2004, Ko GT et al, 1998, Shaw et al, 1999).

The prevalence of IFG also varies among ethnic groups, but its prevalence consistently is lower than that of IGT in all populations. IGT and IFG also differ in their age and sex distribution (Decode Study Group 1998, Qiau et al, 2002, Qiau et al, 2000, Qiau et al, 2002). The prevalence of both categories increases with age, but under the age of 55, IGT is more frequent in women, while prevalence of IFG is more than twofold higher in men than women (Decode study group 1998, Qiau et al, 2002, Qiau et al, 2000, Qiau et al, 2002). The differences between IFG and IGT with respect to prevalence, age, and sex preference, as well as the lack of consistent overlap between both categories, suggest that even though IFG and IGT represent intermediate stages of glucose intolerance, they are likely to be distinct conditions with different pathophysiological etiology.

### **Pathophysiology of Prediabetes**

To clarify the pathophysiology of prediabetes, several studies have examined insulin resistance and insulin secretion in subjects with isolated IFG and isolated IGT (Weyer et al, 1999, Abdul Ghani et al, 2006). Both isolated IFG and isolated IGT are characterised by insulin resistance and impairments in insulin secretion. However, there are some differences in the nature of the defects between the two conditions. For example, individuals with isolated IFG manifest hepatic insulin resistance, but have relatively normal skeletal muscle insulin sensitivity. In contrast, those with isolated IGT are characterised by more severe muscle insulin resistance and less severe hepatic insulin resistance. Differences in insulin secretory abnormalities are also apparent between subjects with isolated IFG and isolated IGT. Whereas those with isolated IFG have defects in first-phase or early insulin secretion (in proportion to their fasting hyperglycemia), individuals with isolated IGT have more

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

### **Risk of developing diabetes**

The progression of prediabetes to type 2 diabetes has been examined in a number of populations with varying results. In general, epidemiological studies indicate that ~25% of subjects with IFG or IGT progress to type 2 diabetes in 5 years, whereas about ~50% remain pre-diabetic and ~25% revert to normal (Larson et al, 2004). The progression rate for IFG was 17.6 and for IGT 18.8 cases per 100 persons per year. When analyzing IGT as two separate categories, i.e. isolated IGT and combined IFG-IGT, the progression rates were 12.0 and 28.1 diabetes cases per 100 person per year respectively (Rasmussen et al 1997). Several recent prospective studies have suggested that the rate of progression to type 2 diabetes may be even higher, averaging ~10–12% per year (Pan et al, 1997, Toumlehto et al, 2001, Knowler et al, 2002). However, these higher rates are borne out by studies with longer observation periods suggesting that the risk of eventually developing type 2 diabetes may be as high as 70% among those with IFG or IGT (Pan et al, 1997). It is likely that the rate of progression varies between individuals due to genetic factors and differences in environmental factors. Diet, activity levels and obesity vary widely among populations, between individuals in a population and within individuals over time. Finally, it is clear that individuals with more severe defects in insulin resistance and insulin secretion who have higher glucose levels are at increased risk of developing type 2 diabetes. For example, those with the combination of IFG and IGT develop type 2 diabetes at approximately twice the rate as do individuals who manifest a single abnormality (Larson et al, 2004). Even within this group, those individuals with a higher FPG (i.e. 6.1–6.9 mmol/L [110–125 mg/dL]) are at much higher risk of progression to diabetes than those with lower (but abnormal) FPG levels (5.6–6.0 mmol/L [100–109 mg/dL]) (Knowler et al, 2002).

### **Risk of developing CVD**

Based on numerous longitudinal studies, prediabetes (IGT and IFG) has also been associated with an increased risk for cardiovascular events, with IGT being a slightly stronger risk predictor (The Expert committee Report, 1997, Coutinho et al, 1999, Selvin et al, 2010). IFG and IGT are frequently associated with other cardiovascular risk factors, such as obesity, in particular abdominal or visceral obesity; dyslipidemia with high triglyceride levels and/or low high-density lipoprotein cholesterol levels; and hypertension. However, there is substantial evidence that cardiovascular risk increases continually with increasing FPG levels alone and that the progressive relationship between glucose levels and cardiovascular risk also extends below the prediabetic threshold. Hoogwerf and colleagues (Hoogwerf et al, 2002) and others (Khaw et al, 2001, Barrett-Connor et al, 2001) showed that the relationship between glucose and coronary heart disease risk is also continuous and graded across the range of nondiabetic glucose values independent of traditional risk factors. Studies also suggest that most CVD in subjects with IFG and IGT occur among those who progress to type 2 diabetes (Levitan et al, 2004). Prediabetes is also associated with the development of microangiopathy as observed in particular in the Diabetes Prevention Program (Diabetes Prevention Program Research Group, 2007).

### **Economic Burden**

There is mounting evidence that prediabetes is associated with higher medical costs and increased risk for certain diabetic comorbidities. For example, even somewhat elevated blood glucose levels are associated with an increased risk of coronary heart disease (CHD), hypertension, retinopathy, and mortality (Zhang et al, 2009). The high prevalence of prediabetes suggests substantial implications for the health system with even a slight increase in per capita healthcare use and disease risk. A study conducted in 2009 by Zhang and colleagues (Zhang et al, 2009) used medical claims data to estimate per capita excess healthcare use associated with prediabetes, and found that patients with confirmed prediabetes had approximately 34% more ambulatory visits per year compared to the general population—ranging from 9% more visits for cardiovascular disease (CVD) and peripheral vascular disease to 92% more visits for hypertension.

Although the prevalence of both IFG and IGT varies considerably based on ethnicity (Dunstan et al, 2004, Tapp et al, 2004, KoGT et al, 1998, Cowie et al, 2006), the prevalence of IGT is consistently higher in various populations. Worldwide, the number of people with IGT is estimated to be 280 million ; by 2030, projections are that 398 million individuals will have IGT ( IDF, 2012)). The prevalence of both categories increases with age, but under the age of 55, IGT is more frequent in women (Decode study group 1998, Quiau et, al, 2002, Quiau et al, 2000). The prevalence of IGT rises in old age (Unwin 2002). It was found that the age standard prevalence of IFG in European population was 11.8% (Boronat et al, 2002) and in one study in Dutch population, it was seen that the prevalence of IGT is 13.8% in men and 14.6% in women (Corpeleijn et al, 2006). In a study of Asian ethnicity, it was found that IGT was more prevalent than IFG in all Asian populations studied for all age groups (DECODE Study Group, 2003). The rising prevalence rate of IGT may be mainly due to diabetogenic lifestyle factors that lead to obesity. The rising prevalence of IGT is assumed to increase from 8.2 to 9.0% worldwide and 7.1 to 7.8% in Bangladesh from 2003 to 2025 in adults in 20 – 79 yrs age groups ( IDF, 2003). It was found that approximately 20 -30% of people with IGT also has IFG (Sayeed et al, 2003).

We know that IFG and IGT are asymptomatic and unassociated with any manifested morbidity, but their sole significance lies in the fact that they predict future diabetes or cardiovascular diseases (Tony et al, 2003). The progression of prediabetes to type 2 diabetes has been examined in a number of populations with varying results. It is clear that individuals with more severe defects in insulin resistance and insulin secretion who have higher glucose levels are at increased risk of developing of type 2 diabetes. Diabetes develops sooner in patients with postprandial hyperglycemia than in those with IFG. The progression rate for IFG was 17.6 and for IGT 18.8 cases per 100 persons per year. When analyzing IGT as two separate categories, i.e. isolated IGT and combined IFG-IGT, the progression rates were 12.0 and 28.1 diabetes cases per 100 persons per year respectively (Rasmussen et al 1997). About 25% of people with IGT progress to type 2 diabetes within 5 years.

IGT is also associated with an increased risk for Cardiovascular Diseases (CVD) and all-cause mortality whereas the link between IFG and CV complications is not so strong.

Although other factors - such as family history of diabetes, waist to hip ratio, body mass index (BMI), and lipid levels may be independently associated with the development of diabetes, abnormal glucose levels are the best single predictor of those in whom diabetes will occur (Jaime et al, 2004). IGT is frequently associated with other cardiovascular risk factors, such as obesity, in particular, abdominal or visceral obesity; dyslipidemia with high triglyceride levels and / or low high – density lipoprotein cholesterol levels; and hypertension. In the Cardiovascular Health Study of elderly non-diabetic patients, IGT added information to the risk predicted by IFG, but IFG alone was not an independent predictor of cardiovascular risk (Smith et al, 2002). In France, a longitudinal cohort study showed that men with IGT had a relative risk for all – cause mortality of 1.6 times that of men with normal glucose tolerance (Balkau et al, 1993). More recently, the DECODE investigators analyzed data from 13 prospective studies and found an increased risk of cardiovascular death and all – cause mortality in patients with IGT compared with normal subjects ( DECODE Study Group, 2001). The DECODE investigators found that IGT status contributed to the predictive information of fasting glucose status with regard to cardiovascular death and all – cause mortality. However, IFG status did not add to the predictive information from glucose tolerance testing, suggesting IGT is a better predictor of clinical events (The DECODE investigators, 2003). IGT is also associated with the development of microangiopathy as observed in particular in the Diabetes Prevention Program, which demonstrated that 7.9% of participants with IGT had findings consistent with diabetic retinopathy ( Diabetes Prevention Program Research Group, 2007).

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. Insulin is the key regulatory hormone in glucose homeostasis. Besides insulin, some recently identified hormones play important role in glucose homeostasis, insulin sensitivity and secretion. It has recently been identified that some gut - derieved hormones, members of the glucagon superfamily, released in response to nutrient ingestion, exert a wide range of effects, including stimulation of pancreatic insulin secretion in a glucose dependent manner. The term incretin was subsequently used to denote these glucose – lowering gut derived hormones (La Barre et al, 1932). There is evidence that most of the incretin effect is due to

the gut-derived incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), secreted by L and K cells, respectively, in the jejunum, ileum, and colon, which enhance glucose-dependent insulin secretion by binding to specific receptors on the  $\beta$ -cell (Holst et al, 1994, Macdonald et al, 2002, MacDonald et al, 2002). These hormones are released during glucose or meal intake in proportion to nutrient transport across the intestinal epithelium (Ferrannini et al, 1982), their effects seem to be additive, and they stimulate insulin secretion both at fasting and postprandial plasma glucose levels (Vilsboll et al, 2003). Normally, the incretins GLP-1 and GIP are responsible for as much as half of the glucose-dependent insulin release after food ingestion. After secretion, incretins are rapidly degraded due to the action of dipeptidyl peptidase-4 (DPP-4), an ubiquitous enzyme found on the surface of epithelial and endothelial cells but also found in plasma (Hansen et al, 1999). The GLP-1 half life is <2 min. whereas that of GIP is ~ 5-7 minutes, and both are rapidly cleared by the kidneys (Baggio et al, 2007).

The physiological mechanisms by which incretins stimulate insulin release have not been investigated in much detail. GLP-1 secretion is reduced in patients with type 2 diabetes both after an oral glucose load (Vaag et al, 1996) and during a meal test compared with lean or obese nondiabetic subjects (Vilsboll et al, 2005). In first-degree relatives of type 2 diabetic patients, the incretin effect, calculated as the difference between oral glucose loading and isoglycemic intravenous glucose administration, on insulin secretion has been reported to be normal, and the plasma GLP-1 and GIP concentrations after the oral test have been found to be normal or even increased (Mari et al, 2005, Nauck et al, 2004, Nyholm et al, 1999, Quddusi et al, 2003). In subjects with impaired glucose tolerance (IGT), on the other hand, a slightly lower increment of plasma GLP-1 concentrations in the first 30 min of the oral glucose tolerance test (OGTT) has been described (Rask et al, 2004). Based on results obtained in the course of oral glucose tolerance testing and during meal testing, GIP secretion and fasting levels seem to be actually increased, in both the impaired and diabetic states. (Theodorakis et al, 2004, Vollmer et al, 2008), whereas the insulinotropic effect is almost totally lost in T2DM. Although different radioimmunoassays have been used over the years, most studies seem to agree that the secretion of GIP is normal or even higher in patients with T2DM compared with healthy control subjects. (Crockett et al, 1976, Ross et al, 1977, Ebert and Creutzfeldt, 1980, Jones et al, 1989, Vilsboll et al, 2001). For GLP -1

secretion in T2DM, the data has been confusing, especially those from earlier studies, although a consensus is finally emerging thanks to studies performed in newly diagnosed subjects and subjects with impaired glucose tolerance before receiving any treatment for glucose control.(Vollmer et al, 2008). Therefore, it is evident that T2DM develops in the setting of normal incretin secretion and reduced secretion cannot be evoked as causing the disease.It seems that abnormalities of incretin secretion are unlikely to be a primary pathogenic factor in the development of T2DM and are instead a consequence of the diabetic state. (Vaag et al, 1996, Meier et al, 2005, Knop et al, 2007).

Several lines of evidence support that the loss of incretin effect is secondary to the development of diabetes, therefore, it becomes of interest to many scientists to determine whether insulin resistance, defective  $\beta$ - cell function, or glucose intolerance are associated with impaired incretin function. It was observed that in the individuals developing insulin resistance only there was a significant reduction in incretin effect, whereas  $\beta$ - cell function was completely normal (Hansen et al, 2010). On the other hand, in the group developing impaired glucose tolerance, the incretin effect decreased, but it was accompanied by clearly impaired  $\beta$ - cell function (Jensen et al, 2010). These studies, therefore, indicate that impairment of the incretin effect is a very early sign of impaired glucose metabolism that may be observed before other signs of  $\beta$ - cell dysfunction are apparent, but that is aggravated further when  $\beta$ - cell function is impaired. It has recently been suggested (Meier et al, 2010) that the loss of incretin represents a diminished  $\beta$ - cell secretory capacity.

IGT subjects frequently are insulin resistant (Lillioja et al, 1988) and display similar defects in  $\beta$ - cell function as overtly diabetic patients, only of a lesser degree (Ferrannini et al, 1987, Ferrannini et al, 2003, Pimenta et al, 1996). Previous research suggested that the decrement of GLP-1 secretion might be related to insulin resistance (IR) or its risk factors too (Nauck et al, 2011, Kozawa et al, 2010). It is assumed that the earlier IR occurs, the more severe impaired GLP-1 secretion may appear. Meanwhile, the decreased GLP-1 release may induce the dysfunction of  $\beta$ - cell, exacerbation of IR and decrease in insulin sensitivity (Is). Different durations of pre-diabetes and diabetes may lead to the divergence in the  $\beta$ - cell function and insulin sensitivity (IS) and may further result in the divergence of the GLP-1 levels.

In one study it was found that the fasting GLP-1 level in patients with type 2 diabetes did not decrease significantly compared to that in normal glucose tolerance (NGT) group. And for the participants with impaired glucose tolerance (IGT), that level was in between the above mentioned two groups (the NGT and T2DM groups) (Toft Nielsen et al, 2001). Again a few scholars pointed out recently that the GLP-1 levels were not reduced in patients with IGT (Lee et al, 2010, Vollmer et al, 2008, Nauck et al, 2011, Kozawa et al, 2010). However, the interrelationship between incretins and insulin secretory capacity, insulin sensitivity and insulin resistance in isolated IGT is still controversial and poorly understood (Laakso et al 2008, Faerch et al 2008, Ahren et al 1997, Zhang et al 2012, Muscelli et al 2006, Vollmer et al 2008, Rask et al 2004). In Bangladeshi population, increased insulin resistance has been studied in isolated IGT (Rahman et al, 2010). But its association with the abnormalities in the secretion of gut hormones have not been studied in our population. Therefore the present study was undertaken with the aim in mind to investigate the role of incretin hormones in a Bangladeshi prediabetic population in terms of incretin response and their effects on  $\beta$ - cell function, insulin resistance (IR), and insulin sensitivity (IS).



## **Hypothesis**

Defects in insulin secretory capacity and insulin sensitivity are associated with altered levels of plasma incretins in isolated IGT subjects.

## **Objectives**

### **General objective**

To explore the association of incretin hormones with insulin secretory dysfunction and insulin resistance in subjects with isolated IGT.

### **Specific objectives**

- To estimate the plasma levels of fasting and postprandial total GIP and GLP-1 (7-36).
- To evaluate insulin secretory capacity and sensitivity by homeostasis model assessment (HOMA) in IGT.
- To determine the association of fasting and postprandial total GIP and GLP-1 with insulin secretory dysfunction and insulin resistance in isolated IGT subjects.

## Literature Review

### **The incretin system: a potential key to Understanding type 2 diabetes mellitus**

The concept that certain factors produced by the intestinal mucosa in response to nutrient ingestion are capable of stimulating the release of substances from the endocrine pancreas and thereby reducing blood glucose levels was first introduced in the early 1900s (Bayliss et al, 1902, Moore et al, 1906). The term incretin subsequently was used to denote these glucose-lowering, intestinal-derived factors (La Barre et al, 1932). With the development of the radioimmunoassay, this communication between the intestine and the endocrine pancreas was confirmed when it was shown that oral glucose administration is associated with a much greater increase in plasma insulin levels when compared with the same amount of glucose given intravenously. (McIntyre et al, 1964, Elrick et al, 1964). This phenomenon has been dubbed the incretin effect, and is estimated to account for approximately 50%–70% of the total insulin secreted after oral glucose administration. Thus, incretins are hormones that are secreted from the gastrointestinal tract into the circulation in response to nutrient ingestion that enhances glucose-stimulated insulin secretion. The first incretin hormone to be identified was isolated from crude extracts of porcine small intestine and initially were named gastric inhibitory polypeptide (GIP), based on its ability to inhibit gastric acid secretion in dogs (Brown et al, 1975). However, subsequent studies using more purified preparations of GIP revealed that GIP could also stimulate insulin secretion in animals and humans. Because the inhibitory effect of GIP on gastric acid secretion was seen only at pharmacologic doses, whereas its incretin action occurred at physiologic levels, GIP was renamed glucose dependent insulinotropic polypeptide, to reflect its physiologic action yet retain the acronym. In accordance with its role as an incretin hormone, GIP is released from K-cells of the small intestine, primarily in response to glucose or fat ingestion, and potentiates glucose-stimulated insulin secretion. It was recognized, however, that GIP alone could not fully account for the incretin effect in vivo. This was based on the observations that immunoneutralization of endogenous GIP activity attenuates but does not abolish the incretin effect in rodents and in humans surgical resection of the ileum is associated with diminished incretin activity, despite preservation of normal plasma GIP levels (Lauritsen et al, 1980). The discovery of a second incretin hormone, glucagon-like peptide-1 (GLP-1),

followed the cloning and sequencing of mammalian proglucagon genes and complementary DNAs (cDNAs). In addition to glucagon, the proglucagon gene also encoded 2 peptides that were approximately 50% homologous to glucagon and thus aptly were named glucagon-like peptide-1 and glucagon-like peptide-2. Based on their homology to glucagon, both peptides were tested for insulinotropic activity, but only GLP-1 was capable of stimulating insulin secretion. GLP-1 is a tissue-specific posttranslational proteolytic product of the proglucagon gene that is released from intestinal L-cells in response to nutrient ingestion and enhances glucose-stimulated insulin secretion (Mojsov et al, 1987, Kreymann et al, 1987). To date, only GIP and GLP-1 fulfill the definition of an incretin hormone in humans. Furthermore, studies have shown that these 2 peptides potentiate glucose-stimulated insulin secretion in an additive manner, likely contribute equally to the incretin effect, and together can fully account for the incretin effect in humans.

### **Incretin hormone candidates**

Many hormones have been suspected to be responsible for the incretin effect, but today there is ample evidence to suggest that the two most important incretins are GIP and GLP-1. Both have been established as important incretin hormones in mimicry experiments in humans, where the hormones were infused together with intravenous glucose to concentrations approximately corresponding to those observed during oral glucose tolerance tests. Both hormones powerfully enhanced insulin secretion, each of them actually to an extent that can fully explain the insulin response. Likewise, administration of GLP-1 and GIP receptor antagonists to rodents or immunoneutralization have clearly indicated that both hormones play an important role for the incretin effect (Lauritsen et al, 1980). However, there has been some uncertainty about the relative roles of the two hormones. GIP is circulating in 10-fold higher concentrations than GLP-1 [and this is true also with respect to the concentrations of the intact hormones], whereas GLP-1 appears more potent than GIP. Furthermore, it is often emphasized that both hormones require elevated plasma glucose concentrations for stimulation of insulin secretion, for GIP may be as much as 8 mM.

## **Glucose-dependent insulintropic polypeptide**

In 1971, a peptide hormone, named gastric inhibitory polypeptide (GIP) was isolated from porcine intestine (Brown et al, 1971), based on its ability to inhibit gastric acid secretion in dogs (Pederson et al, 1972). Subsequent studies, however, demonstrated a glucose-dependent stimulation of insulin secretion by GIP in animals and humans, suggesting an incretin role for the peptide (Dupre et al, 1973, Pederson et al, 1975). Because a physiological action of GIP to inhibit gastric acid secretion in man could not be demonstrated as opposed to its incretin action, GIP was renamed glucose-dependent insulintropic polypeptide, thus retaining the acronym.

### **Synthesis and secretion**

GIP is synthesized within and released from intestinal K-cells, the majority of which are located in the duodenum and proximal jejunum, with smaller numbers also occurring throughout the entire small intestine (Mortensen et al, 2003, Buchan et al, 1978). GIP is secreted in response to nutrient ingestion, especially glucose or fat. More specifically, it is the rate of nutrient absorption rather than the mere presence of nutrients in the intestine that stimulates GIP release. Thus, GIP secretion is reduced in individuals with intestinal malabsorption or after the administration of pharmacologic agents that reduce nutrient absorption (Besterman et al, 1979, Fushiki et al, 1992). There appear to be species-specific differences in the nutritional regulation of GIP release because fat is the most potent stimulator of GIP secretion in humans, whereas carbohydrates are the most effective in the rodent and pig. In humans, basal circulating GIP levels range between 0.06 and 0.1 nmol/L, depending on the assay used to measure total vs intact GIP, and increase to 0.2–0.5 nmol/L after a meal. GIP levels are normal or slightly increased in patients with T2DM (Vilsboll et al, 2001, Ross et al, 1977).

### **Degradation and elimination**

The half-life of intact biologically active GIP is less than 2 minutes in rodents, and approximately 7 and 5 minutes in healthy subjects and type 2 diabetic patients, respectively (Deacon et al, 2000). GIP has an alanine residue in position 2 and is also a target for DPP-4-mediated inactivation. A role for DPP-4 in the cleavage of GIP (1-42) and generation of the inactive metabolite GIP (3-42) has been established clearly and studies with rodents and both healthy and diabetic humans indicate that DPP-4 is the primary enzyme responsible for inactivating GIP in vivo (Kieffer et al, 1995, Deacon et al, 2000). Although pharmacologic doses of GIP (3-42) can function as weak antagonists of the GIP receptor invitro and in rodents, physiologic levels of GIP (3-42) do not antagonize the insulinotropic effects of GIP in vivo. Interestingly, a direct comparison of intact incretin hormones levels after exogenous intravenous infusion in humans found that 40% of GIP remains intact and bioactive versus 20% for GLP-1 (Kieffer et al, 1995, Deacon et al, 2000), indicating that GIP may be less susceptible to DPP-4 in vivo, and this is reflected in the slightly longer plasma half-life for GIP vs GLP-1. Administration of whey protein reduces DPP-4 activity in the proximal small intestine, but not in the distal gut or plasma, and is associated with increased intact GIP levels after glucose administration. The observations that GI levels are increased in uremic patients or individuals with chronic renal failure, together with impaired GIP clearance in nephrectomized rats, implicates the kidney as the major route of GIP clearance (Meier et al, 2004). Measurement of arteriovenous differences in GIP levels across various organ beds in the anesthetized pig also identifies the kidney as the major site of GIP metabolism, but the liver and extremities also contribute to GIP extraction (Deacon et al, 2001). The elimination rates for intact GIP and its metabolite are similar in obese type 2 diabetic patients and healthy individuals (Vilsboll et al, 2006).

### **The GIP Receptor**

The GIP receptor (GIPR) initially was cloned from a rat cerebral cortex cDNA library and was followed by the cloning of the hamster and human GIPRs. The GIPR gene is expressed in the pancreas, stomach, small intestine, adipose tissue, adrenal cortex, pituitary, heart,

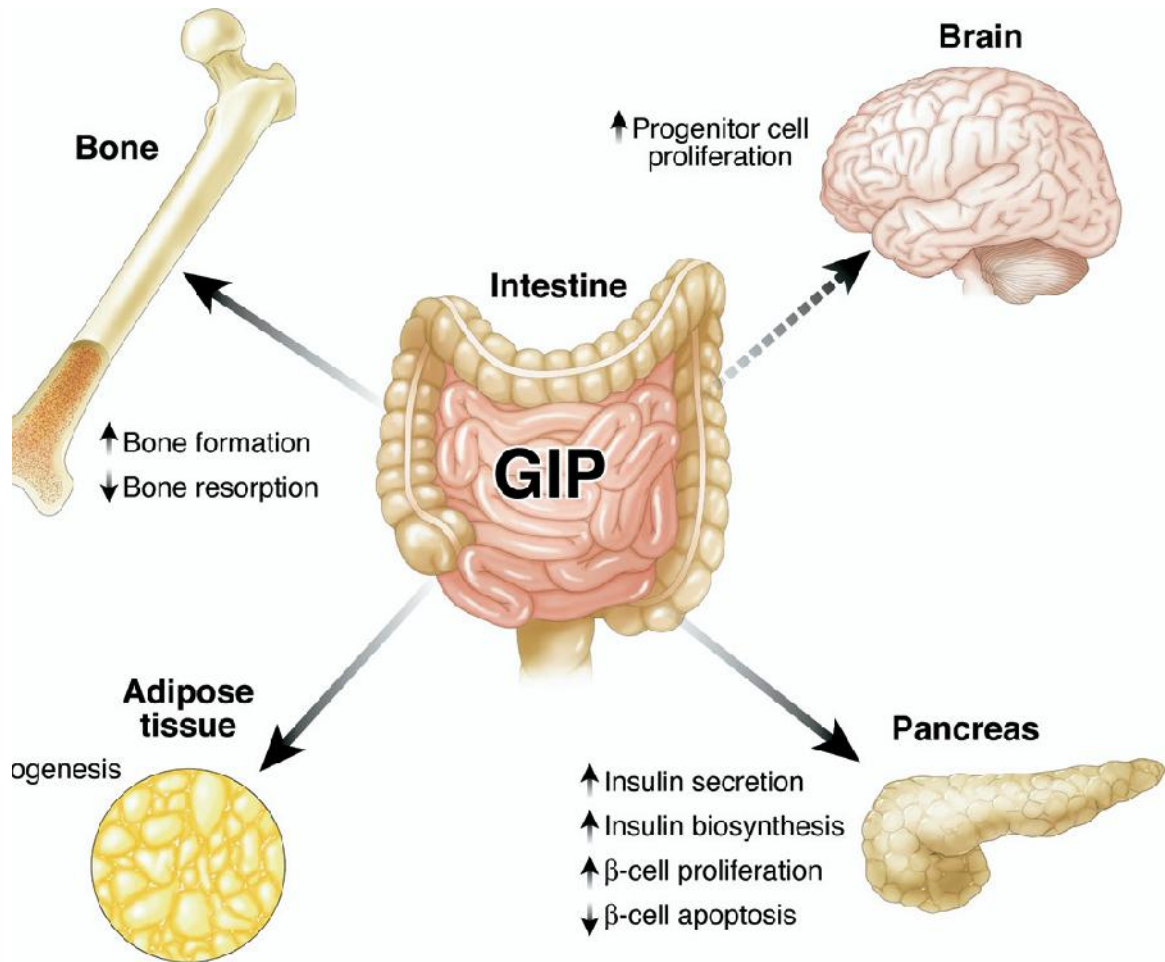
testis, endothelial cells, bone, trachea, spleen, thymus, lung, kidney, thyroid, and several regions in the CNS. Relatively little is known about the factors responsible for regulating GIPR expression. GIPR mRNA and protein levels are reduced in islets of diabetic rats, consistent with the observation of defective GIP action in diabetic animals and human beings (Lynn et al, 2001). Activation of GIPR signaling is coupled to increases in cAMP and intracellular  $Ca^{2++}$  levels. In vitro structure/function studies indicate that the N-terminal domain and the first extracellular loop of the GIPR are essential for high-affinity GIP binding, whereas portions of the N-terminal domain and the first transmembrane domain are important for receptor activation and cAMP coupling. Although the majority of the C-terminal tail of the GIPR appears to be dispensable for intracellular signaling, a minimum receptor length of approximately 405 amino acids is required for efficient transport and plasma membrane insertion. The GIPR undergoes very rapid and reversible homologous desensitization and site-directed mutagenesis, and C-terminal deletion analyses demonstrate the importance of particular serine residues in the C-terminal tail of the GIPR.

### **Biological Actions of GIP**

The actions of GIP on the pancreatic  $\beta$ -cell are analogous to those of GLP-1. However, GIP also exhibits unique physiologic actions in extrapancreatic tissues.

#### **Pancreas**

The primary physiologic role for GIP is that of an incretin hormone. GIP is released from intestinal K-cells in response to nutrient ingestion, binds to its specific receptor on pancreatic  $\beta$ -cells, and enhances glucose-dependent insulin secretion.



**Figure 1 : GIP actions in peripheral tissues.**

The molecular mechanisms where by GIP potentiates glucose-dependent insulin secretion overlap considerably with those of GLP-1. GIP also up-regulates  $\beta$ -cell insulin gene transcription and biosynthesis, as well as the expression of components of  $\beta$ -cell glucose sensors (Wang et al, 1996). The physiologic importance of GIP as an incretin hormone is illustrated by disruption of GIP action in vivo. Elimination of GIPR signaling using GIPR peptide antagonists, receptor-specific antisera, or by targeted inactivation of the murine GIPR gene (GIPR<sup>-/-</sup>) is associated with impaired oral glucose tolerance and defective glucose-stimulated insulin secretion in rodents (Tseng et al, 1996, Gelling et al, 1997, Baggio et al, 2000, Lewis et al, 2000, Miyawaki et al, 1999).

### **Central Nervous System**

In the CNS, GIP is expressed in the hippocampus and GIPR expression is detectable in several regions of the brain including the cerebral cortex, hippocampus, and olfactory bulb. Exogenous administration of GIP induces proliferation of hippocampal progenitor cells *in vivo* in rats as well as in adult-derived hippocampal progenitor cells cultured *in vitro*. Thus, GIP action in the CNS may play a role in neural progenitor cell proliferation and behavior modification (Nyberg et al, 2005).

### **Adipose Tissue**

Functional GIPRs are expressed on isolated rat adipocytes and 3T3-L1 cells (Yip et al, 1998) and GIP is implicated in the control of lipid metabolism and the development of obesity. Fat ingestion is a potent stimulator of GIP secretion in humans and GIP plasma levels are increased in some obese individuals (Creutzfeldt et al, 1978, Salera et al, 1982). The anabolic effects of GIP in fat include stimulation of fatty acid synthesis and re-esterification, enhancement of insulin stimulated incorporation of fatty acids into triglycerides, up-regulation of lipoprotein lipase synthesis, and reduction of glucagon-stimulated lipolysis. However, GIP also may have lipolytic effects. In addition, GIPR activation is associated with improvements in glucose tolerance and increased insulin secretion in animal models of diabetes. Hence, although type 2 diabetic patients are relatively resistant to the insulinotropic effects of exogenous GIP administration and there is no direct link between obesity and GIP in humans, the relative merits of inhibition versus activation of GIPR signaling need to be considered in any future therapeutic applications of GIP or its analogues.

### **Bone**

GIPR mRNA and protein are expressed in normal bone and osteoblast-like cell lines (Bollag et al, 2000). GIP stimulates increases in cAMP and intracellular  $Ca^{2++}$  levels in cultured osteoblasts and these effects are coupled to markers of new bone formation, including increases in alkaline phosphatase activity and collagen type 1 mRNA (Bollag et al, 2000). GIP also increases bone mineral density in ovariectomized rats, a rodent model of postmenopausal osteoporosis (Bollag et al, 2001).



## Other Tissues

GIP inhibits gastric acid secretion in the stomach, but only at supraphysiologic doses (Nauk et al, 1992), and GIP also has been shown to up-regulate intestinal hexose transport. In the liver, GIP attenuates glucagon-stimulated hepatic glucose production, likely through indirect mechanisms because GIPRs have not been detected in the liver. GIP can enhance insulin-dependent glucose disposal in animals, although this effect is not seen in humans (Deacon et al, 2001). GIP also stimulates glucocorticoid secretion in rats via a cAMP/PKA-dependent signaling pathway (Mazzocchi et al, 1999). Although GIP does not appear to regulate cortisol secretion in healthy humans, abnormal expression of the GIPR in adrenocortical adenomas is associated with the development of food-dependent Cushing's syndrome (Lacroix et al, 1992). The GIPR is present in the vascular endothelium and GIP stimulates increases in intracellular  $Ca^{2++}$  levels in endothelial cell cultures (Zhong et al, 2000). Although GIPR mRNA also is detected in the heart, testis, lung, and several other tissues, the physiologic actions of GIP in these tissues are not known.

## Glucagon like peptide – 1

The discovery of a second incretin hormone, glucagonlike peptide-1 (GLP-1), followed the cloning and sequencing of mammalian proglucagon genes and complementary DNAs (cDNAs). In addition to glucagon, the proglucagon gene also encoded 2 peptides that were approximately 50% homologous to glucagon and thus aptly were named *glucagon-like peptide-1* and *glucagon-like peptide-2*. Based on their homology to glucagon, both peptides were tested for insulinotropic activity, but only GLP-1 was capable of stimulating insulin secretion.

## Synthesis and secretion

GLP-1 is secreted from intestinal endocrine Lcells, which are located mainly in the distal ileum and colon. In contrast, GIP is released from intestinal K-cells that are localized to more proximal regions (duodenum and jejunum) of the small intestine. However, endocrine cells that produce GLP-1 or GIP, as well as cells that produce both peptides, can be found throughout all regions of the porcine, rat, and human small intestine (Mortensen et al, 2003,

Theodorakis et al, 2006). GLP-1 secretion from intestinal L-cells is stimulated by a variety of nutrient, neural, and endocrine factors. Meal ingestion, particularly one rich in fats and carbohydrates, is the primary physiologic stimulus for GLP-1 secretion (Brubaker et al, 2006). GLP-1 release can be stimulated by mixed meals or individual nutrients including glucose and other sugars, fatty acids, essential amino acids, and dietary fiber. Oral, but not intravenous, glucose administration stimulates GLP-1 secretion in humans (Unger et al, 1968, Hermann et al, 1995). The secretion profile of GLP-1 is sometimes biphasic starting with an early (within 10-15 min) phase that is followed by a longer (30-60 min) second phase, and it has been suggested that the stimulus for the early postprandial peak of GLP-1 is mediated indirectly through a neuroendocrine pathway. It was demonstrated that the sympathetic innervation to the gut is inhibitory for GLP-1 secretion, whereas the extrinsic vagal innervations had no effect. Intrinsic, cholinergic activity may play a minor role. As already pointed out, there are L-cells in the proximal jejunum, and these may very well be responsible for the early response. However, because L-cells seem to be present throughout the entire length of the small intestine, it is possible that early GLP-1 secretion also can occur by direct association of nutrients with L-cells located in more proximal regions of the small intestine (Mortensen et al, 2003, Theodorakis et al, 2006).

Multiple forms of GLP-1 are secreted *in vivo*, including GLP-1(1-37) and GLP-1(1-36)NH<sub>2</sub>, which are thought to be inactive, and GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub>, which are biologically active. The addition of an amide group to GLP-1(1-36) NH<sub>2</sub> and GLP-1(7-36) NH<sub>2</sub> likely is mediated by the enzyme peptidylglycine -amidating monooxygenase and may enhance the survival of GLP-1 in plasma (Wettergren et al, 1998,). In humans, the majority of GLP-1 in the circulation is GLP-1(7-36) NH<sub>2</sub> (Orskov et al, 1994).

### **Degradation and elimination**

The half-life of intact GLP-1 is less than 2 minutes in the circulation owing to rapid degradation. GLP-1 is extremely susceptible to the catalytic activity of the ubiquitous proteolytic enzyme dipeptidyl peptidase -4 ( DPP-4), which cleaves off the two NH<sub>2</sub> terminal amino acids ( Deacon et al, 1995). A large part of the GLP-1 that leaves the gut has already been degraded to the metabolite, such that less than 25% of newly secreted GLP-1 enters the portal vein in intact, insulinotropic form (Hansen et al, 1999). A similar

degradation amounting to ~40-50% takes place in the liver, and it can be calculated that only ~10-15% of newly secreted GLP-1 reaches the systemic circulation in the intact form (Pridal et al, 1996). In agreement with this, the concentrations of intact GLP-1 in plasma are very low (Vilsboll et al, 2003).

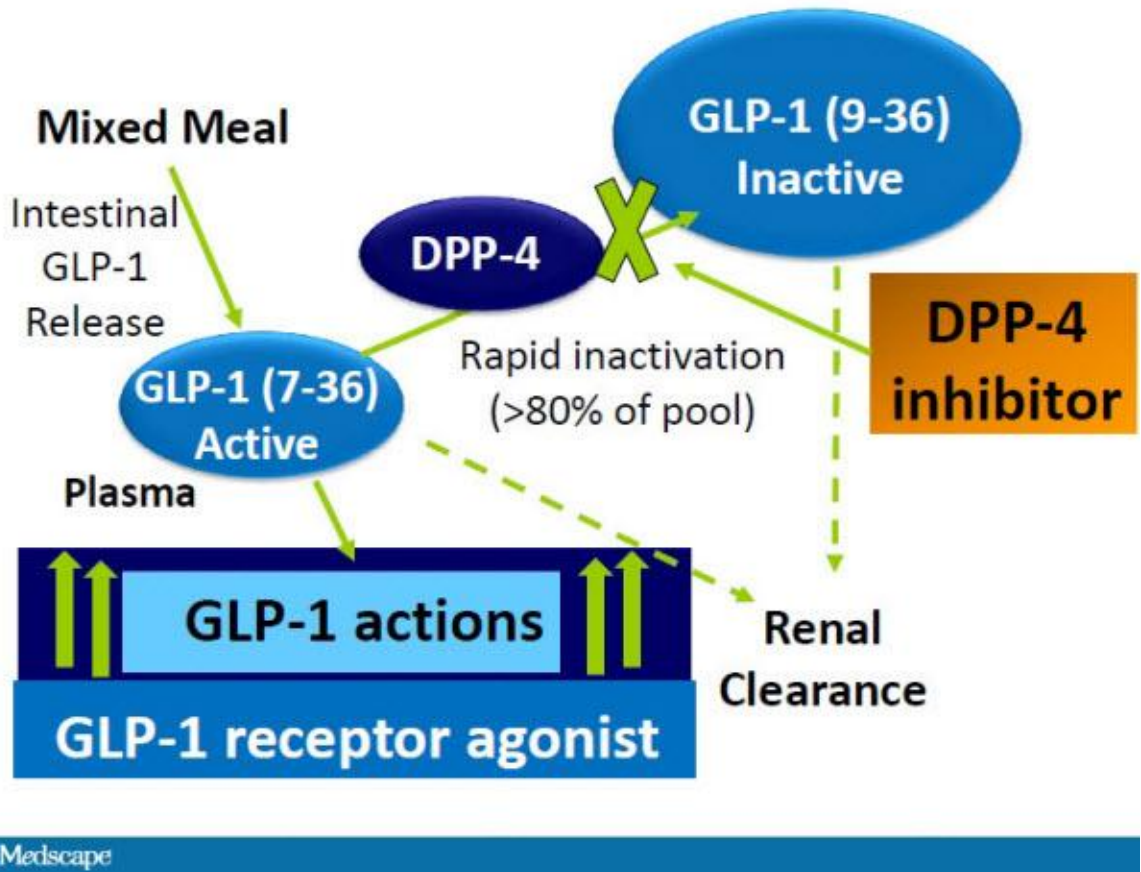


Figure 2: Secretion and metabolism of glucagon-like peptide-1 (GLP-1). Following meal ingestion, GLP-1 is released by intestinal L cells in its active form in plasma, which is rapidly degraded to the inactive form by dipeptidyl peptidase-4 (DPP-4).

The plasma half-life of intact GLP-1 is approximately 2 minutes, whereas that of its metabolite has been estimated to be approximately 5 minutes as a result of renal clearance. The major route of GLP-1 elimination is through the kidney and involves mechanisms that include glomerular filtration and tubular uptake and catabolism (Ruiz-Grande et al, 1993).

### **The GLP-1 Receptor**

The GLP-1 receptor is a class 2, G protein-coupled receptor (Mayo et al, 2003) and was first cloned by expression cloning from a rat pancreatic islet library. The highly homologous human receptor was also cloned (Thorens et al, 1992, Thorens et al, 1993) and it was also confirmed that the 53% homologous lizard peptide exendin 4 is a full agonist and the truncated peptide exendin (9–39) is a potent antagonist of the receptor (Goke et al, 1993). GLP-1 receptors have been found in various organs including pancreatic islets, lung, heart, kidney, stomach, intestine, pituitary, skin, nodose ganglion neurons of the vagus nerve, and several regions of the CNS, including the hypothalamus and brainstem. The GLP-1 receptor belongs to the same family as the GIP and the glucagon receptors (Mayo et al, 2003). The receptor typically couples via a stimulatory G protein to adenylate cyclase (Thorens et al, 1996, Wheeler et al, 1995). Numerous attempts have been made to identify alternative GLP-1 receptors or subtypes, but at present only a single GLP-1 receptor has been identified, whether expressed in the brain, the stomach, or the pancreas (Wei et al, 1995). As mentioned earlier, exendin (9–39) acts as a potent and specific antagonist at the GLP-1 receptor (Thorens et al, 1993), and all effects of GLP-1 transmitted via this receptor would be expected to be blocked by the antagonist.

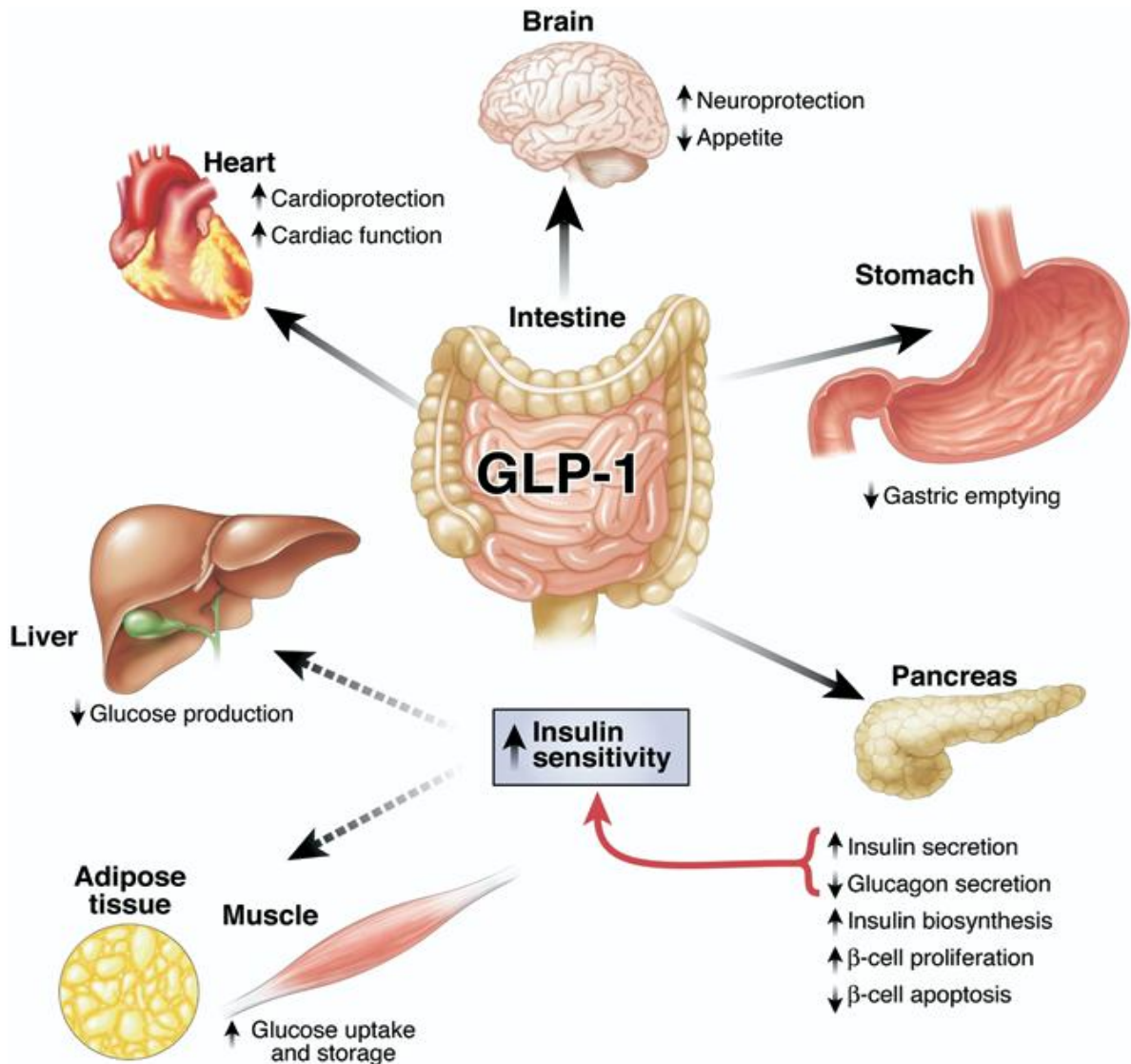


Figure 3: GLP-1 actions in peripheral tissues. The majority of the effects of GLP-1 are mediated by direct interaction with GLP-1Rs on specific tissues. However, the actions of GLP-1 in liver, fat, and muscle most likely occur through indirect mechanisms

## Biological Actions of GLP-1

### Effects on the Beta-Cells

GLP-1's insulinotropic activity, which is strictly glucose dependent, is, at least partly, exerted via interaction with the GLP-1 receptor located on the cell membrane of the beta

cells (Holst et al, 2004). Binding of GLP-1 to the receptor causes activation, via a stimulatory G- protein, of adenylate cyclase resulting in the formation of cAMP. Most of the actions of GLP-1 are secondary to the formation of cAMP (see figure below). Subsequent activation of protein kinase A and the cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII, also known as Epac2) leads to a plethora of events including altered ion channel activity, elevation of intracellular calcium concentrations, and enhanced exocytosis of insulin-containing granules (Holz et al, 2004). GLP-1 also stimulates coordinated oscillations in both intracellular calcium and cAMP, and these are potentiated by glucose (Dyachok et al, 2006). Furthermore, sustained elevations of cAMP concentrations induce nuclear translocation of the catalytic subunit of the cAMP-dependent protein kinase, presumably leading to CREB activation and likely cell proliferation and survival. The effects of glucose and GLP-1 may converge at the level of the KATP channels of the beta cells. These channels are sensitive to the intracellular ATP levels and, thereby, to glucose metabolism of the beta cells, but may also be affected by protein kinase A (PKA) activated by GLP-1 (Gromada et al, 1998, Holz et al, 1993, Light et al, 2002). There is also evidence that GLP-1 acts as a glucose sensitizer. Thus GLP-1 has been found to facilitate glucose-dependent mitochondrial ATP production (Tsuboi et al, 2003). At any rate, it is of potential clinical importance that sulfonylurea drugs, which bind to and close the KATP channels and thereby cause membrane depolarization and calcium influx, may uncouple the glucose dependency of GLP-1 (De Heer et al, in press). Indeed, 30–40% of patients treated with both sulfonyl urea compounds and a GLP-1 agonist (exendin) experience, usually mild, hypoglycemia. cAMP generated by activation of the GLP-1 receptor may also influence the exocytotic process directly, and this process has been estimated to account for up to 70% of the entire secretory response (Holst et al, 2004).

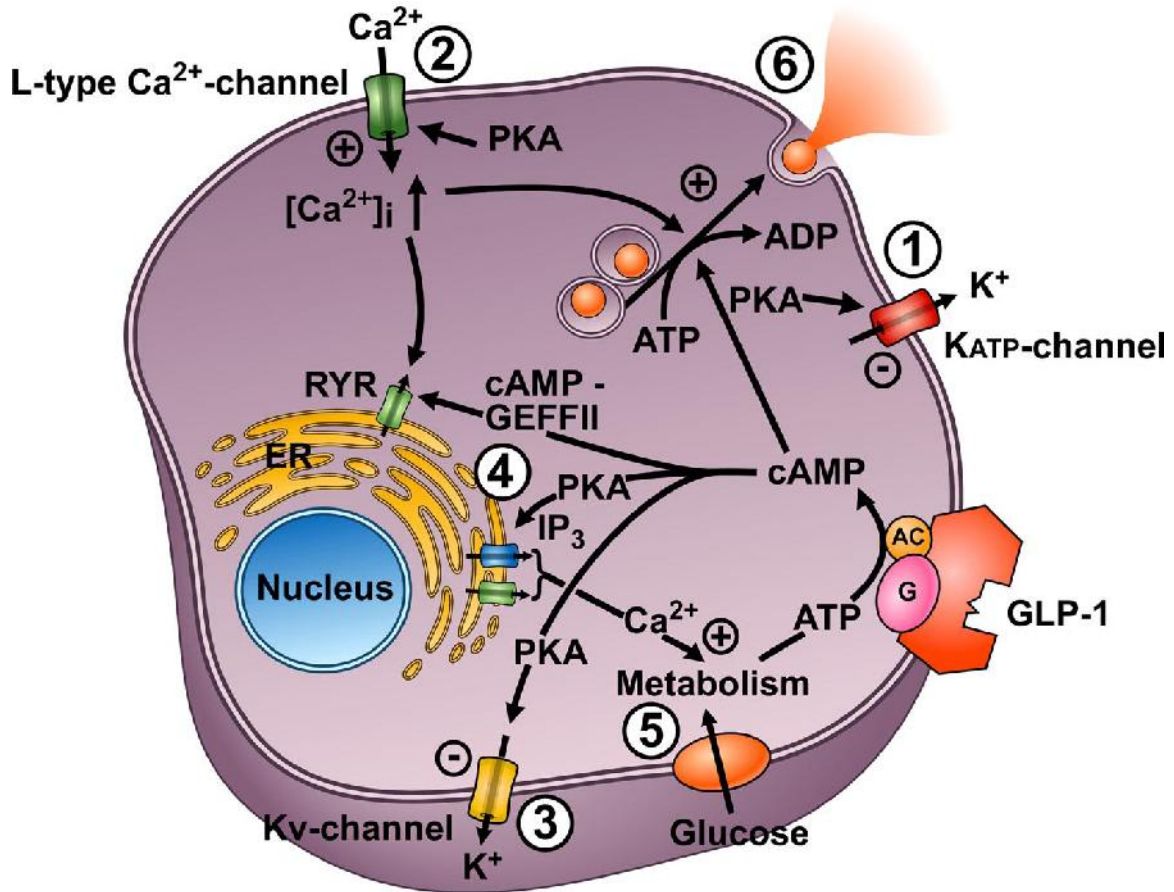


Figure 4. Summary of the cellular actions of GLP-1 that lead to stimulation of insulin secretion. Binding of GLP-1 to its receptors couple to activation of adenylate cyclase; intracellular cAMP levels are elevated leading to activation of protein kinase A (PKA) and cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII, also known as Epac2). These two proteins are likely to mediate the plethora of molecular mechanisms (summarized below in points 1–6). 1) GLP-1 acts synergistically with glucose to close ATP-sensitive  $K^+$  (KATP) channels and thus facilitates membrane depolarization and the induction of electrical activity. 2) Once electrical activity is initiated, the slower time course of inactivation of the  $Ca^{2+}$  channels results in prolonged bursts of action potentials. In addition, each action potential will be associated with a slightly greater  $Ca^{2+}$  influx because the amplitude of the  $Ca^{2+}$  current is moderately increased. 3) Antagonism by GLP-1 of the delayed rectifying  $K^+$  ( $K_v$ ) channels will increase excitability and results in prolongation of the duration of action potentials. 4) In the presence of stimulatory levels of glucose and GLP-1,  $Ca^{2+}$  influx through the  $Ca^{2+}$  channels feeds forward into mobilization of  $Ca^{2+}$  from intracellular stores by  $Ca^{2+}$ -induced  $Ca^{2+}$  release through PKA and cAMP-GEFII-dependent mechanisms. 5)  $Ca^{2+}$  mobilization from intracellular stores will stimulate mitochondrial ATP synthesis, which will promote further membrane depolarization via closure of KATP channels. ATP is also required for stimulation of exocytosis of the insulin-containing granules. 6) The elevation in the cytoplasmic free  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) triggers the exocytotic response that is further potentiated by increased cAMP levels. This effect is principally attributable to the ability of cAMP to accelerate granule

mobilization resulting in an increased size of the pools of granules that are immediately available for release. These effects depend both on cAMP binding to PKA and cAMP-GEFII. Quantitatively the last mechanism is by far the most important one and may account for 70% or more of the total insulinotropic activity of GLP-1 and GIP (Gromada J et al, 1998). [Modified from Holst and Gromada (Holst et al, 2004)].

Other changes that occur in the beta cells appear to be PKA independent. Thus the actions of GLP-1 on the insulin gene promoter appear to be mediated by both PKA-dependent and – independent mechanisms, the latter possibly involving the mitogen-activated protein kinase pathway (Kemp et al, 2001). The effect of GLP-1 on the insulin promoter appears to be mediated by two distinct *cis*-acting sequences, both in a PKA-dependent and PKA-independent manner (Skoglund et al, 2000). Much attention was aroused by the finding that GLP-1 appeared to be essential for conveying “glucose competence” to the beta cells, i.e., without GLP-1 signaling, beta cells would not be responsive to glucose (Gromada J et al, 1998, Holz et al, 1992). As already alluded to, GLP-1 also has trophic effects on beta cells (Egan et al, 2003). Not only does it stimulate beta-cell proliferation (Edvell et al, 1999, Farilla et al, 2002, Stoffers et al, 2000, Xu G et al, 1999), it also enhances the differentiation of new beta cells from progenitor cells in the pancreatic duct epithelium (Zhou et al, 1999). Most recently, GLP-1 has been shown to be capable of inhibiting apoptosis of beta cells including human beta cells (Buteau et al, 2004, Farilla et al, 2003). Since the normal number of beta cells is maintained in a balance between apoptosis and proliferation (Bonner-Weir et al, 2001), this observation raises the possibility that GLP-1 could be useful as a therapeutic agent in conditions with increased beta-cell apoptosis, which would include type 1 as well type 2 diabetes in humans (Butler et al, 2003). Thus treatment of mice with the GLP-1 agonist exendin 4 reduced beta-cell apoptosis induced by streptozotocin (while GLP-1 receptor knockout mice were abnormally susceptible to streptozotocin-induced beta-cell apoptosis) (Li et al, 2003). It is important to note that the trophic effects of GLP-1 agonists in rodents, like their insulinotropic properties, are coupled to the presence of hyperglycemia, and also to note that as GLP-1 alleviates hyperglycemia, which is in itself a very strong stimulus to beta-cell growth in rodents; this growth stimulus is reduced (Sturis et al, 2003). A most striking demonstration of the beta-cell protective/ proliferative effects of GLP-1 receptor activation was provided by Stoffers et al. (Stoffers et al, 2003), who studied the



diabetes developing in rats subjected to intrauterine growth retardation. Treatment with exendin 4 in the neonatal period completely prevented development of diabetes and restored beta-cell mass, which otherwise is strongly reduced in these animals. The complicated and incompletely elucidated mechanisms that could be involved in the GLP-1-induced trophic effects on the beta cells were reviewed recently (Brubaker et al, 2004, Drucker et al, 2006, Synclair et al, 2005).

### **Effects on Glucagon Secretion**

GLP-1 strongly inhibits glucagon secretion (Orskov et al, 1988). Since in patients with type 2 diabetes there is fasting hyperglucagonemia as well as exaggerated glucagon responses to meal ingestion (Toft-Nielson et al, 2001), and since it is likely that the hyperglucagonemia contributes to the hyperglycemia of the patients (Shah et al, 2000), this effect may be as important clinically as the insulinotropic effects. Indeed, in patients with type 1 diabetes and complete lack of beta cell activity (C-peptide negative), GLP-1 is still capable of lowering fasting plasma glucose concentrations, presumably as a consequence of a powerful lowering of the plasma glucagon concentrations (Creutzfeldt et al, 1996). The mechanism of GLP-1-induced inhibition of glucagon secretion is not completely elucidated. Insulin is generally thought to inhibit glucagon secretion, and local elevations of insulin levels around the alpha cells might inhibit their secretion in a paracrine manner, but the preserved and pronounced inhibitory effect of GLP-1 in type 1 diabetic patients without residual beta-cell function (Creutzfeldt et al, 1996) would suggest that other mechanisms must also be involved.. GLP-1 stimulates pancreatic somatostatin secretion (Orskov et al, 1998), which in turn might inhibit glucagon secretion by paracrine interaction (Fehmann et al, 1995). The inhibitory effect of GLP-1 on glucagon secretion in vivo is only observed at glucose levels at or above fasting levels. In studies involving graded hypoglycemic clamping in humans, the inhibitory effect of GLP-1 was lost at glucose levels just below normal fasting levels, and the normal stimulation of glucagon secretion at hypoglycemic levels was unimpeded by GLP-1 (Nauk et al, 2002). This is important because it implies that treatment with GLP-1 does not weaken the counterregulatory responses to hypoglycemia and, therefore, does not lead to an increased risk of hypoglycemia.

## **Effects on the Gastrointestinal Tract**

Further important effects of GLP-1 include inhibition of gastrointestinal secretion and motility (Nauk et al, 1997, Wettergren et al, 1993). It was first noted that GLP-1 inhibits gastrin-induced acid secretion in humans (Schjoldager et al, 1989), and subsequently demonstrated that GLP-1 also inhibits meal-induced secretion as well as gastric emptying and pancreatic secretion (Wettergren et al, 1993). The effect on pancreatic exocrine secretion was first suspected to be secondary to the inhibition of gastric emptying, but in subsequent studies, GLP-1 was demonstrated to also inhibit pancreatic secretion in response to intraduodenal stimulation (Groger et al, 1997). The inhibitory effect of GLP-1 on acid secretion could be elicited by physiological elevations of the GLP-1 concentrations in plasma and was, remarkably, additive to the inhibitory effects of PYY, which is released from the L-cell in parallel with GLP-1 (Wettergren et al, 1997). Together the two peptides almost abolished gastrin-stimulated secretion, indicating that these two peptides are the likely mediators of the “ileal brake effect,” i.e., the endocrine inhibition of upper gastrointestinal functions elicited by the presence of unabsorbed nutrients in the ileum (Holst et al, 1997, Layer et al, 1992, Read et al, 1994). These effects of GLP-1, also designated enterogastrone effects, are likely to be physiological, since stimulation of endogenous GLP-1 secretion by intraileal instillation of nutrients corresponding to the “physiological malabsorption,” resulted in concomitant inhibition of gastric and pancreatic secretions (Layer et al, 1995). All of the actions of GLP-1 on gastric functions are mediated via vagal pathways (Wettergren et al, 1997). In recent studies Schirra et al. (Schirra et al, 2006) were able to demonstrate the importance of endogenous GLP-1 for regulation of antroduodenal motility (and pancreatic endocrine secretion) by administration of the GLP-1 receptor antagonist exendin (9–39). The physiological relevance of the ileal-brake effects of GLP-1 in humans thus seems established.

### **Effects on Food intake and appetite**

Several studies have shown that infusions of GLP-1 significantly and dose dependently enhance satiety and reduce food intake in normal subjects (Flint et al, 1998, Verdich et al, 2001). Flint et al reported a 21% reduction in food intake as well as an increase in satiety and fullness during GLP-1 administration in healthy human subjects (Flint et al, 1998), and similar results were obtained in patients with type 2 diabetes (Gutzwiller et al, 1999). The mean reduction in energy intake observed during GLP-1 infusion in different studies was 12% (Verdich et al, 2001). In a long-term study with the subcutaneous infusion for over 6 weeks in patients with type 2 diabetes, GLP-1 treatment caused a progressive and sustained weight reduction of 1.9 kg (Zander et al, 2002). However, the mechanisms by which GLP-1 inhibits food intake and appetite remain unclear. Substantial evidence suggests that brain GLP-1 and GLP-1R play a role in the control of feeding, as do peripheral GLP-1 and GLP-1R. Different studies support the view that peripheral GLP-1 reduces food intake through an effect on peripheral GLP-1R. The albuminbound GLP-1 does not cross the Blood brain barrier (bbb), as mentioned, but still reduces feeding when administered systemically (Baggio et al, 2004). Although GLP-1 can cross bbb, several lines of evidence suggest that peripheral GLP acts to reduce food intake primarily via vagal afferent activation. GLP-1R mRNA is expressed in the nodose ganglion and GLP-1R has been observed on vagal terminals innervating the hepatic portal vein (HPV). It has been demonstrated that either total subdiaphragmatic vagotomy (Abbot et al, 2005) or selective vagal deafferentiation (Ruttimann et al, 2009) prevents intraperitoneal (ip) injected GLP-1-induced anorexia. Recent pharmacological studies using exendin(9-39) provide further support for this view. Peripherally administered exendin(9-39) increases food intake when delivered to satiated rats and also blocks the satiety induced by nutrient preloads (Williams et al, 2009). These effects appear to be based on ip administered exendin(9-39)'s ability to block peripheral GLP-1R, because the same dose of ip exendin(9-39) that blocked the anorexic effect of systemic GLP-1 failed to blunt the anorexic effect of CNS GLP-1 administration. Conversely, intracerebroventricular (icv) exendin(9-39) blocked the feeding-inhibitory effect of icv GLP-1 but failed to attenuate peripheral GLP-1-induced anorexia (Williams et al, 2009). Taken together, these data support the idea that GLP-1 released by the intestine promotes satiety by activating peripheral GLP-1R, whereas neuronal GLP-1 affects feeding

through GLP-1R in the brain. It is clear that brain GLP-1 is involved in food intake and appetite. Thus, early studies demonstrated that the icv administration of GLP-1 inhibited appetite and food intake (Tang-Christensen et al, 1996, Turton et al, 1996), while gastric distension has been shown to increase the activity of neurons (expression of c-Fos) in the brainstem that produce GLP-1 (Vrang et al, 2003). Recently, Shick and colleagues identified the lateral, dorsomedial and ventromedial hypothalamus to be involved in the mediation of satiety effects (Schick et al, 2003).

### **Nutrient regulation of GLP -1 Secretion**

Enteral nutrient ingestion is the primary stimulus for GLP -1 secretion. Following a mixed meal, GLP secretion is induced in both human subjects (Elliott et al, 1993, Xiao et al, 1999) and animal models. Interestingly, GLP-1 secretion depends on the specific nutrient composition of the meal, and there are clear differences in the response to carbohydrates, lipids and proteins. Furthermore, several observations have led to the concept that there is a caloric threshold that must be attained in any meal for GLP-1 secretion to occur. In healthy male volunteers, Schirra et al. found that a duodenal glucose delivery rate above 1.4 kcal/min was required to induce GLP-1 secretion. Therefore, a minimum nutrient intake threshold must be reached to induce GLP-1 secretion, and that the extent of enteral nutrition and rate of nutrient delivery to the intestine are critical factors in this response.

### **Carbohydrates**

Ingested carbohydrates are potent GLP -1 secretagogues in vivo, and circulating GLP-1 are increased ( from fasting levels of 5-10 pmol/l) by two-to three fold in humans following glucose ingestion. (Elliott et al, 1993, Xiao et al, 1999, Kreymann et al, 1987, Balks et al, 1997). Interestingly, GLP-1 secretion in humans occurs in a pulsatile manner at a frequency of five to seven per hour, and oral glucose ingestion acts to increase the amplitude, but not the frequency of these pulses (Balks et al, 1997). Despite the well-established stimulatory role of glucose in human GLP-1 secretion in vivo, the effects of other carbohydrates are less well-defined. Kong et al. observed a significant increase in GLP-1 secretion upon fructose ingestion in humans; however, this response was significantly less than response to glucose.

(Kong et al, 1999). Complex carbohydrates appear unable to stimulate GLP-1 secretion, at least when ingested as either brown rice or barley in isolation. (Elliott et al, 1993).

### **Lipids**

Lipids are arguably the most potent stimulus for GLP secretion, and ingestion of fats in humans leads to sustained secretion of GLP-1 (Elliott et al, 1993). Administration of orlistat, a gastrointestinal lipase inhibitor, to diabetic humans results in increased transit of fat to the ileum and a corresponding increase in postprandial GLP-1 secretion. (Damci et al, 2004), emphasizing the direct impact of fat on the ileal L-cell. Given the role of GLP-1 in reducing gastric emptying, it is interesting to speculate that ileal fat-induced GLP-1 secretion may play a role in the “ileal brake” response. Although the L-cell is unresponsive to plasma glucose levels, GLP-1 secretion appears to be inhibited by circulating non esterified fatty acids (Ranganath et al, 1999); this, in part, may play a role in altered GLP-1 secretion in obese humans.

### **Protein**

Although mixed meals containing protein are able to stimulate GLP-1 secretion, proteins themselves do not appear to be a major stimulus for the L-cell. In vivo studies on humans and animal models have failed to demonstrate a consistent stimulatory effect of proteins or amino acids on GLP-1 secretion (Elliott et al, 1993, Xiao et al, 1999).

### **Incretin secretion in Type 2 Diabetes**

T2DM is characterized by a severely impaired or absent GIP insulinotropic effect (Nauck et al, 1986) that most likely results in worsening insulin secretion. However, T2DM seems unlikely to result from deficient incretin secretion. One of the reasons frequently given for using exenatide or DPP4 inhibitors is that lead to “normalization” of incretin levels that are supposedly reduced compared with non diabetic subjects (Drucker and Nauck, 2006). However, on closure analysis of all the data in print, it is far from certain that incretin secretion is reduced in this condition. For GIP, the data seem to actually favor the opposite conclusion. Based on results obtained in the course of oral glucose tolerance testing and during meal testing, GIP secretion and fasting levels seem to be actually increased, in both

the impaired and diabetic state. (Theodorakis et al, 2004, Vollmer et al, 2008), whereas the insulinotropic effect is almost totally lost in T2DM. Although different radioimmunoassays have been used over the years, most studies seem to agree that the secretion of GIP is normal or even higher in patients with T2DM compared with healthy control subjects. (Crocket et al, 1976, Ross et al, 1977, Ebert and Creutzfeldt 1980, Jones et al, 1989, vilssboll et al, 2001). For GLP -1 secretion in T2DM, the data has been confusing, especially those from earlier studies, although a consensus is finally emerging thanks to studies performed in newly diagnosed subjects and subjects with impaired glucose tolerance before receiving any treatment for glucose control.(Vollmer et al, 2008). In general, GLP -1 levels reach maximum secretion 17 to 20 min after oral glucose administration, followed by a slow decline toward fasting levels; In contrast, peak secretion occurs 60 to 90 min after a mixed meal. Data from the Baltimore Longitudinal Study of Aging shows that GLP-1 secretion is not deficient in either the fasting state or after oral glucose in glucose-impaired or diabetic subjects not taking any drugs affecting glucose homeostasis. Vollmer et al. (2008) actually found a trend toward higher plasma GLP-1 levels in 17 well controlled subjects with T2DM after a mixed meal. (Vollmer et al, 2008).Therefore, it is evident that T2DM develops in the setting of normal incretin secretion and reduced secretion cannot be evoked as causing the disease.It seems fair therefore to conclude that abnormalities of incretin secretion are unlikely to be a primary pathogenic factor in the development of T2DM and are instead a consequence of the diabetic state. (Vaag et al, 1996, Meier et al, 2005, Knop et al, 2007).

## CHAPTER -02: SUBJECTS & METHODS

### **Place of study**

The study was conducted in the department of Biochemistry and Cell Biology, Bangladesh Institute of Health Sciences (BIHS), Dhaka, Bangladesh.

### **Study period**

This study was done during the period of August 2012 to March 2013.

### **Study design**

It was a case-control study. The study subjects were recruited purposively.

### **Study subjects**

According to inclusion-exclusion criteria thirty four (34) cases and age-sex matched thirty four (34) apparently healthy controls were included in this study. There was no specific predilection for race, religion and socioeconomic status.

### **Inclusion criteria**

Isolated Impaired Glucose Tolerance (IGT) subjects according to WHO definition.

### **Exclusion criteria**

- Patients with serious comorbid diseases (infection, stroke, myocardial infarction, major surgery, mal absorption etc)
- History of using drugs significantly affecting glucose metabolism (anti-hyperglycemic agents, glucocorticoids, thiazide diuretics etc.).

### **Collection of study subjects**

From the out-patient department of BIHS attending for the diagnosis of diabetes mellitus, subjects with isolated Impaired Glucose Tolerance (according to WHO criteria) were recruited. Written consent was taken before data collection, clinical examination and specimen collection.

### **Anthropometric measurements**

#### **Height (m)**

Standing height was measured using appropriate scales without shoes. The subjects 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 heels were together. Height was recorded to the nearest 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<sup>2</sup>)**

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

$$\text{BMI} = \text{Weight (Kg)} / [\text{Height (m)}]^2$$

### **Waist circumference (cm)**

Waist 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 12<sup>th</sup> rib and the highest point of the iliac crest on the mid-axillary line at the end of normal expiration.

### **Hip circumference (cm)**

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.

### **Supplied breakfast**

Between 8:30 and 9:00 am, a mixed meal containing 524 k cal was served and the whole meal was ingested within 10-12 minutes. The meal consisted of 3 pieces (153 gm) of handmade ruti (made of wheat), 133 gm of lentil and 1 piece of egg (53 gm). The carbohydrate, protein and fat content were 82.5 kcal, 24.9 kcal and 10.5 kcal respectively (Ali et al, 1992) which correspond to 63%, 19% and 18% of the total energy content (Lindgren et al, 2009). The study included two separate test days. On the first day venous blood was taken for Oral Glucose Tolerance Test (OGTT). The patients were advised to



avoid rich diet, to do normal physical activities. After one day the patients were advised to come in fasting condition (overnight fast) and fasting blood sample was taken. They ingested the test meal (mixed meal) within 10-12 minutes and venous blood was taken after one hour of taking meal. Blood was distributed into different vacutainers for different biochemical analyses both with fasting blood sample and one hour after mixed test meal sample.

### **Collection of blood specimens**

Fasting blood was collected between 8.00-9.00 am. Venous blood (10 ml) was taken by venepuncture with the subject sitting comfortably in a chair in a quiet room. Then the patient was given 75 g of glucose in 250-300 ml of water and advised to drink in 5 minutes. Patient was advised not to smoke, not to take any food and to take rest in a chair for 2 hours. Then blood specimen was taken 2 hour after glucose load. A portion of blood specimen was poured into a test tube containing sodium fluoride. Another portion of blood specimen was poured into another test tubes without anticoagulant and antiglycolytic agent. After 15 minutes blood samples were centrifuged for 10 minutes at 3000 rpm to obtain plasma and serum. Fasting and 2h plasma glucose were measured in the same day.

Subjects were finally selected from fasting and 2h plasma glucose values who fulfilled the inclusion criteria of the study. Fasting and postprandial serum of the selected subjects were aliquoted into 7 vacutainers ( one for blood sugar, one for insulin, lipid profile and other biochemical parameters, one for GLP-1 and total GIP in and another for hemoglobin A1c measurement. BD P700 Blood collection and preservation system containing a DPP-4 protease inhibitor cocktail was used for collection and preservation of active GLP-1(7-36) and total GIP and kept frozen at  $-80^{\circ}\text{C}$  until analysis.

### **Analytical methods**

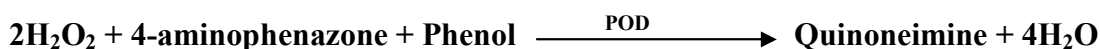
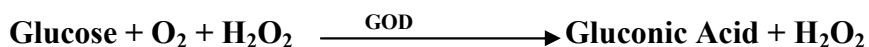
#### **Estimation of plasma glucose**

Plasma glucose was measured by Glucose-Oxidase (GOD-PAP) method by automated chemistry analyzer, Hitachi 912 (Japan) using reagents of Randox Laboratories Ltd. UK (Cat. No. GL 366).

## 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



## Reagent composition

1. Buffer: Phosphate buffer (0.1 mol/L, pH 7.0) and phenol (11 mol/L)
2. GOD-PAP reagent: 4-aminophenazone (0.77 mmol/L), glucose oxidase ( $\geq 1.5$  kU/L) and peroxidase ( $\geq 1.5$  kU/L).
3. Standard: 5.55 mmol/L

## Procedure

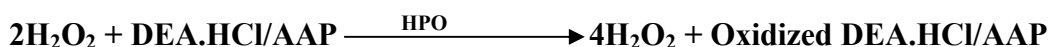
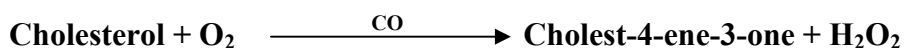
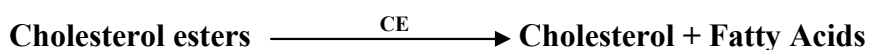
The analyzer was programmed for blood glucose estimation in an open channel according to the instructions of the analyzer manufacturer with the aid of expert (usually used for blood glucose estimation in routine analysis). In this system, 3  $\mu\text{L}$  of plasma and 250  $\mu\text{L}$  of reagent are mixed automatically and final reading was taken after 10.34 minutes of incubation (at 37°C) at 505 nm as primary and 720 as secondary wave length. The analyzer was calibrated before the assay. Plasma was taken in the sample cup and working reagents were placed in the reagent holder in the reagent chamber. Plasma samples were placed in the sample tray sequentially and the analyzer was programmed for glucose estimation in a batch mode and allowed to run. The Hitachi 912 system automatically calculates and prints the results sequentially. To assure quality, Bio-rad quality control materials (level 1 and 2) were used.

### Estimation of serum total cholesterol

Serum total cholesterol concentration was measured by Dimension® clinical chemistry system (Siemens Healthcare Diagnostics Inc. USA) using reagents (Cat. No. DF27, Siemens Healthcare Diagnostics Inc. USA).

#### Principle

Cholesterol esterase (CE) catalyzes the hydrolysis of cholesterol esters to produce free cholesterol which, along with preexisting free cholesterol, is oxidized in a reaction catalyzed by cholesterol oxidase (CO) to form cholest-4-ene-3-one and hydrogen peroxide. In the presence of horseradish peroxidase (HPO), the hydrogen peroxide thus formed is used to oxidize N,N diethylaniline-HCl/4-aminoantipyrine (DEA-HCl/AAP) to produce a chromophore that absorbs at 540 nm. The absorbance due to oxidized DEA-HCl/AAP is directly proportional to the total cholesterol concentration and is measured using a polychromatic (452, 540, 700 nm) end point technique.



#### Reagent composition

1. Reagent 1 (wells 1-3): CE (0.7 U/ml), CO (0.1 U/ml), HPO (2.4 U/ml) as tablet. AAP (4.5 μmol), buffer, Cholate as tablet.
2. Reagent 2 (wells 4-6): DEA (5.8 μmol) and surfactant as liquid.

#### Procedure

Dimension® clinical chemistry system automatically recognizes the flex reagent when inserted and prepare reagent as required. Before assay, the method was calibrated using a 3 point calibration procedure according to the recommendation of the manufacturer using calibrator cat. No. DC16 (Siemens Healthcare Diagnostics Inc. USA). After calibration, serum samples were placed in the sample tray sequentially and programmed for total

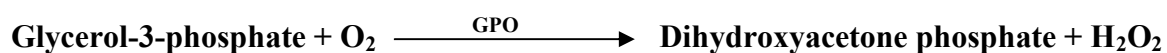
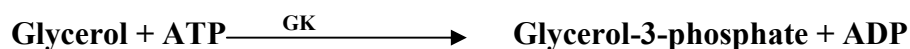
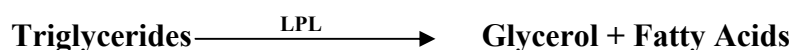
cholesterol, triglyceride, HDL cholesterol in a batch mode. The Dimension system automatically added 3  $\mu\text{L}$  of serum into a freshly automatically prepared reaction cell in which 88  $\mu\text{L}$  of reagent 1 and 26  $\mu\text{L}$  of reagent 2 were added. After incubation ( $37^\circ\text{C}$ ), optical density was measured and the system automatically calculated and printed the results. For quality control, Bio-rad quality control material level 1 and level 2 were used.

### **Estimation of serum triglycerides**

Serum triglycerides concentration was measured by Dimension® clinical chemistry system (Siemens Healthcare Diagnostics Inc. USA) using reagents (Cat. No. DF69A, Siemens Healthcare Diagnostics Inc. USA).

### **Principle**

The triglycerides method is based on an enzymatic procedure in which a combination of enzymes are employed for the measurement of serum or plasma triglycerides. The sample is incubated with lipoprotein lipase (LPL) enzyme reagent that converts triglycerides into free glycerol and fatty acids. Glycerol kinase (GK) catalyzes the phosphorylation of glycerol by adenosine-5-triphosphate (ATP) to glycerol-3-phosphate. Glycerol-3-phosphate-oxidase oxidizes glycerol-3-phosphate to dihydroxyacetone phosphate. The catalytic action of peroxidase (POD) forms quinoneimine from  $\text{H}_2\text{O}_2$ , aminoantipyrine and 4-chlorophenol. The change in absorbance due to the formation of quinoneimine is directly proportional to the total amount of glycerol and its precursors in the sample and is measured using a bichromatic (510, 700 nm) end point technique.



### **Reagent composition**

Reagent (wells 1-6): Lipoprotein lipase (7.5 KU/L), ATP (3 mmol/L), Glycerol Kinase (0.5 KU/L), Glycerol-3-phosphate-oxidase (2.2 KU/L), 4-Aminoantipyrine (0.75 mmol/L), 4-Chlorophenol (6 mmol/L), Peroxidase (5 KU/L), Mg<sup>++</sup> (22.5 mmol/L), Buffer pH 7.2 (50 mmol/L).

### **Procedure**

Dimension® clinical chemistry system automatically recognizes the TGL flex reagent (ready to use) when inserted. Before assay, the method was calibrated using a 3 point calibration procedure according to the recommendation of the manufacturer using CHEM II calibrator cat. No. DC20 (Siemens Healthcare Diagnostics Inc. USA). After calibration, serum samples were placed in the sample tray sequentially and programmed for triglyceride in a batch mode. The Dimension system automatically added 4 µL of serum into a freshly automatically prepared reaction cell in which 133 µL of reagent was added. After incubation (37°C), optical density was measured and the system automatically calculated and printed the results. For quality control, Bio-rad quality control material level 1 and level 2 were used.

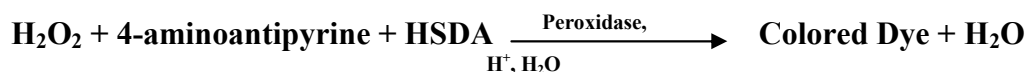
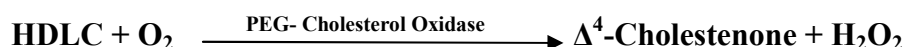
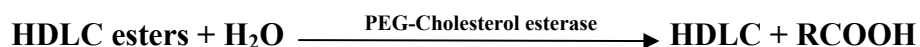
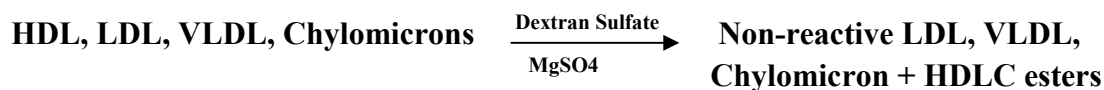
### **Estimation of serum high density lipoprotein cholesterol**

Serum high density lipoprotein cholesterol (HDL) concentration was measured by Dimension® clinical chemistry system (Siemens Healthcare Diagnostics Inc. USA) using HDL Flex® reagents (Cat. No. DF69A, Siemens Healthcare Diagnostics Inc. USA).

### **Principle**

This method measures HDL levels directly without the need for sample pretreatment or specialized centrifugation steps, using a two reagent format. In the first reaction, chylomicrons, very-low-density lipoprotein cholesterol (VLDL) and low-density lipoprotein cholesterol (LDL) form water soluble complexes with dextran sulfate in the presence of magnesium sulfate. These are resistant to the polyethylene glycol (PEG)-modified cholesterol esterase and cholesterol oxidase that react with HDL. In the presence of oxygen, the HDL is oxidized to  $\Delta^4$ -cholestenone and hydrogen peroxide. The generated hydrogen peroxide then reacts with 4-aminoantipyrine and sodium N-(2-hydroxy-3-

sulfopropyl)-3,5-dimethoxyaniline (HSDA) in the presence of peroxidase to form a colored dye that is measured using a bichromatic (600/700 nm) endpoint technique. The color intensity of the dye is directly proportional to the serum HDLC concentration.



### Reagent composition

Reagent 1 (wells 1-3): HEPES buffer pH 7.4 (10.07 mmol/L), 2-(N-cyclohexylamino)-ethanesulfonic acid (96.95 mmol/L), Dextran sulfate (1.5 g/L), Magnesium nirtate hexahydrate ( $\geq 11.7$  mmol/L), N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (0.96 mmol/L), Ascorbate oxidase ( $\geq 50$   $\mu\text{Kat/L}$ ), Peroxidase ( $\geq 16.7$   $\mu\text{Kat/L}$ ), Preservative.

Reagent 2 (Well 4): HEPES buffer pH 7.0 (10.07 mmol/L), PEG-Cholesterol esterase ( $\geq 3.33$   $\mu\text{Kat/L}$ ), PEG-Cholesterol oxidase ( $\geq 127$   $\mu\text{Kat/L}$ ), Peroxidase ( $\geq 333$   $\mu\text{Kat/L}$ ), 4-amino-antipyrine (2.46 mmol/L), Preservative.

### Procedure

Dimension® clinical chemistry system automatically recognizes the AHDL flex® reagent (ready to use) when inserted. Before assay, the method was calibrated using a 3 point calibration procedure according to the recommendation of the manufacturer using AHDL calibrator cat. No. DC48B (Siemens Healthcare Diagnostics Inc. USA). After calibration, serum samples were placed in the sample tray sequentially and programmed for HDL cholesterol in a batch mode. The Dimension system automatically added 4  $\mu\text{L}$  of serum into a freshly automatically prepared reaction cell in which 300  $\mu\text{L}$  of reagent 1 and 100  $\mu\text{L}$  of reagent 2 were added successively. After incubation for 8.6 minutes at 37°C, optical density

was measured and the system automatically calculated and printed the results. For quality control, Bio-rad quality control materials level 1 and level 2 were used.

### **Calculation of serum low-density lipoprotein cholesterol (LDLC)**

The LDLC concentrations in serum were calculated by Friedewald's formula (Friedewald et al, 1972)

$$\text{LDLC} = \text{Total Cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDLC}$$

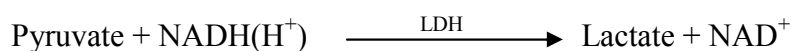
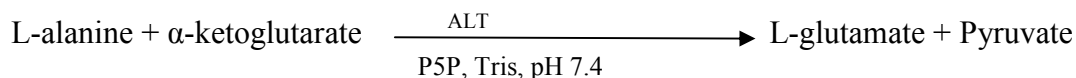
(When all concentrations were expressed in mg/dl and serum triglyceride concentration  $\leq$  400 mg/dl).

### **Estimation of serum alanine aminotransferase (ALT)**

Serum ALT activity was measured by IFCC recommended method by Dimension® clinical chemistry system (Siemens Healthcare Diagnostics Inc. USA) using ALT Flex® reagents (Cat. No. DF43A, Siemens Healthcare Diagnostics Inc. USA).

### **Principle**

Alanine aminotransferase (ALT) catalyzes the transamination of L-alanine to  $\alpha$ -ketoglutarate, forming L-glutamate and pyruvate. The pyruvate formed is reduced to lactate by lactate dehydrogenase (LDH) with simultaneous oxidation of reduced nicotinamide-adenine dinucleotide (NADH). The change in absorbance is directly proportional to the ALT activity and is measured using a bichromatic (340, 700 nm) kinetic technique.



### Reagent composition

Reagent 1 (wells 1-3): LDH (3000 U/L), NADH (0.22 mmol/L), P5P (0.15 mmol/L)

Reagent 2 (wells 4-6):  $\alpha$ -ketoglutarate (20 mmol/L)

Reagent 3 (well 7): Alanine (260 mmol/L)

Reagent 4 (well 8): Tris buffer (100 mmol/L)

### Procedure

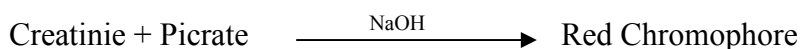
Dimension® clinical chemistry system automatically recognizes the ALT flex® reagent when inserted. Before assay, reagents were prepared (hydrated) by automation and the method was calibrated using a 3 point calibration procedure according to the recommendation of the manufacturer using enzyme verifier cat. No. DC19 (Siemens Healthcare Diagnostics Inc. USA). After calibration, serum samples were placed in the sample tray sequentially and programmed for ALT in a batch mode. The Dimension system automatically added 35  $\mu$ L of serum into a freshly automatically prepared reaction cell in which 30  $\mu$ L of reagent 1, 80  $\mu$ L of reagent 2 and 215  $\mu$ L diluent were added successively. After addition of all reagents at 37°C, optical density was measured for kinetic technique and the system automatically calculated and printed the results. For quality control, Bio-rad quality control materials level 1 and level 2 were used.

### Estimation of serum creatinine

Serum creatinine concentration was measured by a modified kinetic Jaffe reaction (Larsen 1972) by Dimension® clinical chemistry system (Siemens Healthcare Diagnostics Inc. USA) using CREA Flex® reagents (Cat. No. DF33A, Siemens Healthcare Diagnostics Inc. USA).

### Principle

In the presence of string base such as NaOH, picrate reacts with creatinine to form a red chromophore. The rate of increasing absorbance at 510 nm due to the formation of this chromophore is directly proportional to the concentration of cratinine in the sample and is measured using a bichromatic (510, 600 nm) rate technique.





### **Reagent composition**

Reagent 1 (wells 1-3): Lithium picrate (25 mmol/L)

Reagent 2 (wells 4-6): NaOH (100 mmol/L),  $K_3Fe(CN)_6$

### **Procedure**

Dimension® clinical chemistry system automatically recognizes the CREA flex® reagent when inserted. Before assay, reagents were prepared (hydrated) by automation and the method was calibrated using a 3 point calibration procedure according to the recommendation of the manufacturer using CHEM I calibrator cat. No. DC18A (Siemens Healthcare Diagnostics Inc. USA). After calibration, serum samples were placed in the sample tray sequentially and programmed for total cholesterol, triglyceride, HDL cholesterol, ALT and creatinine in a random access batch mode. The Dimension system automatically added 20  $\mu$ L of serum into a freshly automatically prepared reaction cell in which 74  $\mu$ L of reagent 1, 18  $\mu$ L of reagent 2 and 258  $\mu$ L diluent were added successively. After addition of all reagents at 37°C, optical density was measured for kinetic technique and the system automatically calculated and printed the results. For quality control, Bio-rad quality control materials level 1 and level 2 were used.

### **Estimation of serum insulin**

Serum insulin was measured by a solid phase enzyme-linked immunosorbent assay (ELISA) using DRG Insulin ELISA Kit (Cat no. EIA-2935).

### **Principle**

The ELISA method is based on the sandwich principle. The microtitre wells are coated with a monoclonal antibody directed towards a unique antigenic site on the insulin molecule. An aliquot of patient sample containing endogenous insulin is incubated in the coated well with enzyme conjugate, which is an anti-insulin antibody conjugated with Biotin. After incubation, the unbound conjugate is washed off. During the second incubation step Streptavidin Peroxidase enzyme complex binds to the biotin-anti-insulin antibody. The amount of bound HRP complex is proportional to the concentration of insulin present in the

sample. Having added the substrate solution, the intensity of color developed is proportional to the concentration of insulin in the patient sample.

### **Reagents**

1. Microtiter wells (Wells coated with anti-insulin antibody, monoclonal).
2. Zero standard (0  $\mu$ IU/ml, 3 ml)
3. Standard (1-5): Concentrations: 6.25, 12.5, 25, 50 and 100  $\mu$ IU/ml (Standardized against WHO approved reference material NIBSC 66/304).
4. Enzyme conjugate (mouse monoclonal anti-insulin conjugated to biotin).
5. Enzyme complex (Streptavidin-HRP complex).
6. Substrate solution (Tetramethylbenzidine).
7. Stop solution (0.5 M  $H_2SO_4$ ).
8. Wash solution.

### **Reagent Preparation**

All reagents and required number of strips were kept at room temperature prior to use.

**Wash Solution:** Deionized water was added to the 40X concentrated Wash Solution. 30 ml of concentrated Wash Solution was diluted with 1170 ml deionized water to a final volume of 1200 ml.

**Specimen preparation:** If in an initial assay, a specimen is found to contain more than the highest standard, the specimens can be diluted with Zero Standard and re-assayed as described in Assay Procedure. For the calculation of the concentrations this dilution factor has to be taken into account.

### **Procedure**

1. The desired number of Microtiter wells was placed in the frame holder.
2. 25  $\mu$ l of each Standard, control and samples were dispensed with new disposable tips into appropriate wells.
3. 25  $\mu$ l Enzyme Conjugate was dispensed into each well.
4. Sample and enzyme conjugate were mixed thoroughly for 10 seconds. It is important to have a complete mixing in this step.

5. Incubated for 30 minutes at room temperature.
6. The wells were briskly shaken out.
7. The wells were rinsed 3 times with diluted Wash Solution (400  $\mu$ l per well) and the wells were struck sharply on absorbent paper to remove residual droplets.
8. 50  $\mu$ l of Enzyme Complex was added to each well.
9. Then incubated for 30 minutes at room temperature.
10. The wells were briskly shaken out.
11. The wells were rinsed 3 times with diluted Wash Solution (400  $\mu$ l per well) and the wells were struck sharply on absorbent paper to remove residual droplets.
12. 50  $\mu$ l of Substrate Solution was added to each well.
13. Then incubated for 15 minutes at room temperature.
14. 50  $\mu$ l of Stop Solution was added to each well.
15. The absorbance (OD) of each well was taken at  $450 \pm 10$  nm with a microtiter plate reader after 10 minutes of adding the stop solution.

### **Calculation of concentration**

Eight point calibration curve was drawn using computer software and concentration of insulin in each sample was obtained using non-linear 4 point theoretical curve (GraphPad Prism version 5.04 for Windows).

### **Measurement of active glucagon-like peptide -1 (GLP-1 active):**

#### **Assay principle**

The high sensitive enzyme-linked immunosorbent (ELISA) kit [EIA-5096, DRG International, Inc., USA; according to protocol revised 13 Aug, 2012 rm (Ver. 4.0)] was used for the quantitative determination of bioactive GLP-1 (7 – 36) level in human plasma samples. The assay utilizes the two-site “sandwich” technique with two selected GLP-1 (7 – 36) specific antibodies. The microplate wells are coated with streptavidin. Assay standards, controls and test samples are directly added to the microplate wells. When a mixture of biotinylated GLP-1 (7 – 36) specific antibody and a horseradish peroxidase (HRP)

conjugated GLP-1 (7 – 36) specific antibody is added to each well and incubated, a “sandwich” immunocomplex of “Streptavidin – Biotin-Antibody – GLP-1 (7 -36)-HRP conjugated antibody” is formed and attached to the wall of the well. The unbound HRP conjugated antibody is removed in a subsequent washing step. For the detection of this immunocomplex, each well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to GLP-1 (7-36) on the wall of the microtitre well is directly proportional to the amount of GLP-1 (7-36) in the sample.

### **Reagents**

1. Streptavidin Coated Microplate – One well-breakable microplate with 12×8 strips (96 wells total) coated with streptavidin. The plate is framed and sealed in a foil zipper bag with desiccant.
2. GLP-1 Tracer Antibody (TA) – One vial contains 0.6 mL HRP labeled Anti-GLP-1 specific antibody in a stabilized protein matrix. This reagent was mixed with GLP-1 (7 – 36) Capture Antibody (CA) and TA diluent before use.
3. GLP-1 (7 – 36) Capture Antibody (CA) – One vial contains 0.6 mL of biotinylated GLP-1 (7 – 36) specific antibody. This reagent was mixed with GLP-1 TA and TA diluent before use.
4. ELISA wash concentrate – One bottle contains 20 mL of 30 fold concentrate. Before use the contents were diluted with 580 mL of distilled water and mixed well and stored at room temperature.
5. ELISA HRP Substrate – One bottle contains 24 mL of tetramethylethylbenzidine (TMB) with stabilized hydrogen peroxide.
6. ELISA Stop Solution – One bottle contains 12 mL sulfuric acid.
7. GLP-1 Standard – Six vials contain different levels of lyophilized GLP-1 (7 – 36) in a liquid protein matrix with a non-azide, non-mercury based preservative.
8. GLP-1 Controls – Two vials contain different levels of lyophilized GLP-1 (7 – 36) in a liquid protein matrix with a non-azide, non-mercury based preservative.
9. Tracer Antibody Diluent – One vial contains 12 mL ready to use buffer.

### **Reagent preparation**

- a. All reagents were allowed to come to room temperature.
- b. ELISA wash concentrate was diluted 30 times using distilled water.
- c. All standards and control materials were reconstituted by adding 1.0 mL of demineralized water to each vial and allowed to sit undisturbed for 10 minutes, and mixed well by gentle vortexing. These reconstituted standards and controls were stored at  $-20^{\circ}\text{C}$  or below and used within three consecutive days with one freeze-thaw cycles.

### **Sample preparation**

- a. Specimens were allowed to come to room temperature.
- b. Specimens were collected for direct measurement of active GLP-1 (7 – 36) in BDTM P-700 Blood Collection and Preservative System (BD) and thus no sample preparation was required.

### **Assay procedure**

1. Sufficient number of streptavidin coated microwell strips/wells were placed in a holder to run GLP-1 (7 – 36) standards, controls and unknown samples according to the instructions revised in 13 Aug 2012 rm (Var. 4.0).
2. GLP-1 (7 – 36) antibody mixture was prepared by mixing GLP-1 tracer antibody and capture antibody by 1:21 fold dilution of the tracer antibody (code#30229) and by 1:21 fold dilution of the biotinylated capture antibody (code#30230) with the TA diluent (code#30017).
3. 100  $\mu\text{L}$  of standards, controls and test samples were added into the designated microwell.
4. 100  $\mu\text{L}$  of GLP-1 (7 -36) antibody mixture was then added to each well.
5. The plate was then covered with one plate sealer and incubated for 20 – 24 hours at 2 to 8  $^{\circ}\text{C}$  in a static condition.
6. After incubation, plate sealer was removed. The contents of each well were then aspirated and washed 5 times using 350  $\mu\text{L}$  of working wash buffer.
7. 200  $\mu\text{L}$  of ELISA HRP substrate was then added into each of the wells.

8. The plate was then covered with plate sealer and also with aluminum foil to avoid light exposure and incubated at room temperature, static for 20 minutes.
9. Aluminum foil and plate sealer were removed and 50  $\mu$ L of ELISA stop solution was then added into each well. After gentle mixing, absorbances were read at 450nm/620nm within 10 minutes using a microplate reader.

### **Interpretation of results**

The optical density was corrected for the 0 concentration. A standard curve was generated by plotting corrected absorbances of all standard levels against the standard concentrations on the abscissa. Quadratic curve fit using statistical software GraphPad Prism version 5.04 for windows was used for the calculation of results.

### **Estimation of GIP**

#### **Principles of procedure**

The Human GIP (Total) enzyme-linked immunosorbent (ELISA) kit (EIA-4527, DRG International, Inc., USA; according to protocol revised 20 Aug, 2012 cc (Vers. 5.0) was used for the non-reactive quantification of Human GIP in human serum samples. This kit had 100% cross reactivity to human GIP(1-42) and GIP(3-42).

This assay was a Sandwich ELISA based, sequentially, on: 1) capture of human GIP molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of anti-GIP monoclonal antibodies, 2) wash away of unbound materials from samples, 3) binding of a second biotinylated anti-GIP polyclonal antibody to the captured molecules, 4) wash away of unbound materials from samples, 5) incubation of streptavidin-Horseradish peroxidase conjugate to bind to the immobilized biotinylated antibodies, 6) wash away of free enzyme conjugates, and 7) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine. The enzyme activity was measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to

the amount of captured human GIP in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human GIP.

### **Reagents**

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

- A. GIP ELISA Plate: Coated with anti-GIP Monoclonal Antibodies.
- B. 10X HRP Wash Buffer Concentrate: 10× concentrate of 50 mM Tris Buffered Saline containing Tween-20 which was diluted 1:10 with deionized water.
- C. Human GIP Standard: Human GIP (1-42), 0.5 ml/vial, lyophilized which was reconstituted with 0.5 ml deionized water.
- D. Human GIP Quality Controls 1 and 2: Human GIP (1-42), 0.5 ml/vial, lyophilized which was reconstituted with 0.5 ml deionized water.
- E. Assay Buffer: Buffer containing BSA and 0.08% Sodium Azide
- F. Human GIP Detection Antibody: Pre-titered Biotinylated Rabbit anti-Human GIP Polyclonal Antibody
- G. Enzyme Solution: Pre-titered Streptavidin-Horseradish Peroxidase Conjugate in Buffer
- H. Substrate (Light sensitive): 3, 3', 5, 5'-tetramethylbenzidine in buffer
- I. Stop Solution: 0.3 M HCl
- J. Matrix Solution

### **Sample preparation**

No dilution or preparation was needed for normal serum or plasma samples. In the event that any sample was above 2000 pg/mL range, dilutions were performed using the Matrix Solution provided.

### **Assay procedure**

All reagents were allowed to come to room temperature prior to setting up the assay.

1. The 10X concentrated Wash Buffer was diluted 10 fold by mixing the entire contents of both buffer bottles with 900 ml deionized water.

2. The required number of strips was removed from the Microtiter Assay Plate. Strips were assembled in an empty plate holder and each well was filled with 300  $\mu\text{L}$  of diluted Wash Buffer, incubated at room temperature for 5 minutes, wash buffer was then decanted and the residual amount was removed from all wells by inverting the plate and tapping it onto absorbent towels several times.
3. 80  $\mu\text{L}$  Assay Buffer was added to the blank wells and sample wells.
4. 60  $\mu\text{L}$  Assay Buffer was added to Standard wells, QC1 and QC2 wells.
5. 20  $\mu\text{L}$  Matrix Solution was added to the Blank wells, Standard wells, and QC1 and QC2 wells.
6. 20  $\mu\text{L}$  Human GIP Standards was added in the order of ascending concentration to the appropriate wells. Then 20  $\mu\text{L}$  QC1 and 20  $\mu\text{L}$  QC2 were added to the appropriate wells. 20  $\mu\text{L}$  of the unknown samples were added sequentially to the remaining wells. For best result all additions were completed within 30 minutes.
7. The plate was then covered with plate sealer and incubated at room temperature for 1.5 hours on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400 to 500 rpm.
8. Plate sealer was removed and solutions were decanted from the plate.
9. Wells were washed 3 times with diluted Wash Buffer, 300  $\mu\text{L}$  per well per wash, Decanted and tapped firmly after each wash to remove residual buffer.
10. 100  $\mu\text{L}$  Detection Antibody was added to all wells. The plate was covered with plate sealer and incubated at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400 to 500 rpm.
11. Plate sealer was removed and solutions were decanted from the plate and tapped as before to remove residual solutions in the wells.
12. Wells were washed 3 times with diluted Wash Buffer, 300  $\mu\text{L}$  per well per wash, Decanted and tapped firmly after each wash to remove residual buffer.
13. 100  $\mu\text{L}$  Enzyme Solution was added to each well and incubated with moderate shaking at room temperature for 30 minutes on the microtiter plate shaker.
14. After removing sealer, solutions were decanted from the plate, and plate was tapped to remove the residual fluid.



15. Wells were washed 3 times with diluted Wash Buffer, 300  $\mu\text{L}$  per well per wash, decanted and tapped firmly after each wash to remove residual buffer.
16. 100  $\mu\text{L}$  of Substrate Solution was added to each well, plate was covered with sealer and shaken on the plate shaker for approximately 8 to 20 minutes. Blue color was formed in wells of GIP standards with intensity proportional to increasing concentrations of GIP.
17. 100  $\mu\text{L}$  Stop Solution was added and plate was shaken by hand to ensure complete mixing of solution in all wells. The blue color turned to yellow after acidification. Absorbance was read at 450 nm and 590nm in a plate reader within 5 minutes and ensured that there are no air bubbles in any well.
18. The difference of absorbance units was recorded.

### **Interpretation of results**

The optical density was corrected for the 0 concentration. A standard curve was generated by plotting corrected absorbances of all standard levels against the standard concentrations on the abscissa. Quadratic curve fit using statistical software GraphPad Prism version 5.04 for windows was used for the calculation of results.

### **Estimation of glycated hemoglobin (HbA1c)**

Glycosylated hemoglobin levels (HbA1c) were measured by D-10™ Hemoglobin A1c Program (Bio–Rad Laboratories, Inc., USA)

### **Principle of the procedure**

The D-10 Hemoglobin A1c Program utilizes principles of ion-exchange high-performance liquid chromatography (HPLC). The samples are automatically diluted on the D-10 and injected into the analytical cartridge. The D-10 delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobins are separated based on their ionic interactions with the cartridge material. The separated hemoglobins then pass through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are measured. The D-10 software performs reduction of raw data collected from each analysis. Two-level calibration is used for quantitation of the HbA1c values. A sample

report and a chromatogram are generated for each sample. The A1c area is calculated using an exponentially modified gaussian (EMG) algorithm that excludes the labile A1c and carbamylated peak areas from the A1c peak area.

### **Calculation of B cell function and insulin secretion**

Homeostasis Model Assessment (HOMA) is a simple and widely used method which derives separate indices of  $\beta$  cell secretion (HOMA B) and insulin sensitivity (HOMA S) from plasma glucose and insulin concentration under basal conditions by using mathematical formula or software.

### **Statistical analysis**

Statistical analysis was performed using IBM SPSS (Statistical Package for Social Science) Statistics version 19 for Windows (SPSS Inc., Chicago, Illinois, USA), GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA). All data were expressed as median (range) and/or percentage (%) as appropriate. The statistical significance of differences between the values were assessed by independent sample *t* test or *Mann-Whitney U* test (as appropriate). Correlation was also seen among the parameters (*Spearman's correlation* as appropriate). A two-tailed *p* value of  $<0.05$  was considered statistically significant. Graphical presentations were done by GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA).

### CHAPTER -03: RESULTS AND OBSERVATIONS

In this study, 68 subjects were recruited according to inclusion-exclusion criteria among them 34 were cases (isolated IGT) and rest were age and sex matched controls. The study was planned to determine levels of incretins and to evaluate the association of insulin sensitivity and insulin resistance with incretins [GLP-1 (7 -36) (active) and GIP (total)] in subjects with isolated IGT.

#### **Characteristics of the study subjects**

The median (range) age of the total study subjects was 46 (29 – 67) years. Body mass index (BMI), mid upper arm circumference (MUAC), waist circumference (WC), hip circumference (HC), Waist to Hip ratio (WHR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were also expressed as median (range) and the values were 24.9 (18.2 – 34.25) kg/m<sup>2</sup>, 29.2 (23.3 – 38.1) cm, 91.4 (72.5 – 116.8) cm, 96.8 (83.8 – 113.0) cm, 0.95 (0.81 – 0.98), 120 (80 – 180) mm(Hg), 80 (60 – 100) mm(Hg) respectively. Table 1 shows the clinical characteristics of controls and subjects with isolated IGT. Two groups were matched for age and sex ( $p>0.05$ , Table 1) but not matched for BMI, MUAC, WC, HC, acute/chronic glycemc status and diastolic blood pressure ( $p<0.05$ , Table 1). Serum creatinine, WHR and systolic blood pressure were also similar in control and IGT groups. Among the lipid parameters studied, fasting TG, postprandial TG, fasting HDLC and postprandial HDLC differed significantly between control and IGT group. Fasting cholesterol, postprandial cholesterol and fasting LDLC showed no significant difference between control and IGT group. Serum TG, Cholesterol and HDLC differed significantly between fasting and postprandial conditions in both groups.

**Table 1: Clinical and biochemical characteristics of the study subjects**

Parameters	Control (n=34)	IGT (n=34)	p value
Age (yrs)	45.5 (30 – 67)	45.5 (29 – 67)	0.985
Sex (male/female)	18/16	18/16	1.000 <sup>†</sup>
<b>BMI (kg/m<sup>2</sup>)</b>	24.0 (20.5 – 28.9)	27.0 (18.2 – 34.3)	<b>0.023</b>
<b>MUAC (cm)</b>	29.0 (23 – 34)	30.5 (25 – 38)	<b>0.009</b>
<b>Waist circumference (cm)</b>	88.3 (72.5 – 100.3)	92.9 (81.3 – 116.8)	<b>0.001</b>
<b>Hip circumference (cm)</b>	93.9 (86 – 109)	99.9 (84 – 113)	<b>0.001</b>
<b>WHR</b>	0.94(0.81 – 1.04)	0.95 (0.83 – 1.06)	0.241
<b>SBP (mm-Hg)</b>	110 (80 – 180)	120 (90 – 140)	0.069
<b>DBP (mm-Hg)</b>	70 (60 – 100)	80 (60 – 90)	<b>0.023</b>
<b>FPG (mmol/L)</b>	4.6 (3.6 – 5.4)	5.1 (4.2 – 5.6)	<b>&lt;0.001</b>
<b>PPG<sup>††</sup> (mmol/L)</b>	5.6 (3.0 – 7.3)	9.5 (8.1 – 10.9)	<b>&lt;0.001</b>
<b>HbA<sub>1c</sub> (%)</b>	5.6 (4.4 – 6.3)	6.1 (5.2 – 7.3)	<b>&lt;0.001</b>
<b>Fasting TG (mg/dl)</b>	104 (51 – 351)	158 (44 – 859)	<b>0.011</b>
<b>Postprandial TG (mg/dl)</b>	113 (61 – 349)	167 (52 – 758)	<b>0.007</b>
<b>Fasting Chol (mg/dl)</b>	166 (60 – 369)	171 (95 – 268)	0.790
<b>Postprandial Chol (mg/dl)</b>	161 (79 – 386)	169 (90 – 264)	0.564
<b>Fasting HDLC (mg/dl)</b>	41 (25 – 58)	36 (22 – 48)	<b>0.007</b>
<b>Postprandial HDLC (mg/dl)</b>	39 (23 – 60)	34 (20 – 46)	<b>0.018</b>
<b>Fasting LDLC (mg/dl)</b>	110 (64 – 295)	98 (48 – 205)	0.329
<b>S Creatinine (mg/dl)</b>	0.8 (0.5 – 1.1)	0.8 (0.6 – 1.2)	0.461
<b>ALT (U/L)</b>	23 (12 – 42)	31 (14 – 48)	<b>0.008</b>

Results are expressed as median (range) and compared by Mann-Whitney test, <sup>†</sup>Compared by Fisher's exact test, BMI = Body Mass Index, MUAC = Mid Upper Arm Circumference, WHR =Waist-Hip ratio, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, FPG =Fasting plasma glucose, PPG<sup>††</sup> =Postprandial Plasma Glucose 2 hours after 75g oral glucose load, TG = Serum Triglycerides, Chol = Serum Total Cholesterol, HDLC = High- Density Lipoprotein Cholesterol, LDLC = Low-Density Lipoprotein Cholesterol, ALT = Serum alanine amiono transferase.

## Comparison of biochemical parameters

Comparison of various biochemical parameters and indices studied in control and IGT group, and in fasting and postprandial conditions is presented in Table 2. The median value of fasting plasma glucose and postprandial plasma glucose (one hour after mixed breakfast) differed significantly between control and IGT ( $p < 0.001$ ), and between fasting and postprandial conditions ( $p < 0.001$ ). Compared to control, fasting and postprandial insulin levels were found to be significantly higher in IGT group. Postprandial insulin was also significantly higher than fasting insulin in both groups. Compared to control, HOMA %B was significantly higher ( $p = 0.032$ ), HOMA %S was significantly lower ( $p < 0.001$ ) and HOMA IR was significantly higher in IGT group ( $p < 0.001$ ).

Fasting total GIP concentrations were significantly higher (58%,  $p = 0.003$ ) in IGT group compared to control (Fig. 5A) but postprandial GIP concentrations were similar in both groups (only 2% lower in IGT,  $p = 0.864$ , Fig. 5B). Both fasting and postprandial GIP concentrations were increased one hour after mixed breakfast (Fig. 6). The changes in plasma GIP after mixed breakfast was 50% lower in isolated IGT compared to control (control vs IGT: 851% vs 424%; Fig. 6). Compared to control, fasting GIP to Glucose ratio was 32% higher ( $p = 0.024$ ) but postprandial GIP to glucose ratio was 27% lower ( $p < 0.001$ ) in isolated IGT (Table 2). In the control, GIP to glucose ratio was also significantly higher (573%,  $p < 0.001$ ) and in the isolated IGT it was significantly higher (274%,  $p < 0.001$ ) in postprandial state compared to fasting state. The changes of GIP per unit of glucose one hour after mixed breakfast was 50% lower in isolated IGT compared to that of control (573% vs 274%). Insulin per unit of GIP showed no significant difference between control and isolated IGT. Compared to fasting, postprandial insulin to GIP ratio was significantly lower only in control ( $p = 0.016$ ) (Table 2).

Both fasting and postprandial GLP-1 (7 – 36) (active) concentrations were lower in IGT group compared to that in control and median value significantly ( $p = 0.020$ ) differed only for fasting state (Fig. 7). Compared to the GLP-1 (7 – 36) concentrations in fasting state,

postprandial concentrations of GLP-1 (7 – 36) were found to be higher but the median value significantly ( $p = 0.003$ ) differed only in isolated IGT (Fig. 8). When compared GLP-1 (7 – 36) concentrations per unit of glucose in blood between control and isolated IGT, median values of GLP-1 per unit of glucose (GLP-1:Glucose) were found to be significantly ( $p < 0.01$ , Table 2) lower in isolated IGT for both fasting and postprandial state. The ratios of GLP-1 and glucose (GLP-1:Glucose) also showed significant ( $p < 0.05$ ) reduction in the postprandial state in both control and isolated IGT. Insulin concentrations per unit of GLP-1 (Insulin:GLP-1) were significantly higher in isolated IGT compared to that in control in both fasting and postprandial state. In fasting state it was ~4 fold higher and in postprandial state it was ~2 fold higher in isolated IGT compared to that in control. The Insulin:GLP-1 ratios were also significantly higher in postprandial state (one hour after having mixed breakfast) compared to that in fasting state but the increase in insulin concentrations per unit of GLP-1 was lower in isolated IGT compared to that in control (~3 fold vs ~7 fold).

**Table 2: Comparison of plasma glucose, insulin, insulin secretory capacity, insulin sensitivity, insulin resistance, GLP-1 and GIP between control and IGT, and between fasting and postprandial conditions**

Parameters	Control (n=34)	IGT (n=34)	p value
FPG (mmol/L)	4.7 (3.9 – 5.5)	5.2 (4.4 – 6.1)	<b>&lt;0.001</b>
PPG <sup>†</sup> (mmol/L)	6.0 (3.2 – 8.2)	8.8 (6.2 – 11.8)	<b>&lt;0.001</b>
<i>p value</i>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	
F Insulin (μIU/ml)	10.9 (2.8 – 25.6)	17.9 (7.7 – 44.8)	<b>&lt;0.001</b>
PP Insulin (μIU/ml)	69.4 (34 – 211)	80.9 (22 – 217)	<b>0.046</b>
<i>p value</i>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	
HOMA %B	147.3 (68.7 – 272.7)	164.3 (96.4 – 324.0)	<b>0.032</b>
HIOMA %S	63.9 (27.2 – 255.4)	38.4 (15.7 – 89.0)	<b>&lt;0.001</b>
HOMA IR	1.6 (0.4 – 3.7)	2.6 (1.1 – 6.4)	<b>&lt;0.001</b>
Fasting Insulin:Glucose	2.29 (0.68 – 4.92)	3.40 (1.60 – 8.11)	<b>&lt;0.001</b>
Postprandial Insulin:Glucose	11.43 (4.86 – 29.13)	9.42 (3.53 – 24.99)	<b>0.089</b>
<i>p value</i>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	
F GIP (pg/mL)	99.9 (35.9 – 351.9)	158.1 (66.4 – 565.5)	<b>0.003</b>
PP GIP (pg/mL)	949.8 (413 – 1514)	928.7 (490 – 2188)	<b>0.864</b>
<i>p value</i>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	
F GIP:Glucose	22.0 (8.2 – 80.0)	29.1 (12.1 – 115.4)	<b>0.024</b>
PP GIP:Glucose	148.1 (59.3 – 337.8)	108.7 (62.9 – 248.6)	<b>&lt;0.001</b>
<i>p value</i>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	
Fasting Insulin:GIP	0.10 (0.02 – 0.42)	0.12 (0.02 – 0.43)	<b>0.436</b>
Postprandial Insulin:GIP	0.07 (0.03 – 0.28)	0.08 (0.03 – 0.35)	<b>0.175</b>
<i>p value</i>	<b>0.016</b>	<b>0.120</b>	
F GLP-1 (pmol/L)	3.3 (0.6 – 27.1)	1.4 (0.2 – 33.5)	<b>0.020</b>
PP GLP-1 (pmol/L)	3.5 (0.9 – 16.0)	2.6 (0.3 – 31.9)	<b>0.088</b>
<i>p value</i>	<b>0.138</b>	<b>0.003</b>	
F GLP-1:Glucose	0.65 (0.14 – 5.26)	0.30 (0.03 – 5.77)	<b>0.006</b>
PP GLP-1:Glucose	0.51 (0.13 – 3.35)	0.28 (0.04 – 3.66)	<b>0.004</b>
<i>p value</i>	<b>0.033</b>	<b>0.015</b>	
Fasting Insulin:GLP-1	3.32 (0.19 – 17.50)	12.04 (0.63 – 105.3)	<b>&lt;0.001</b>
Postprandial Insulin:GLP-1	21.90 (3.29 – 179.0)	41.92 (2.49 – 188.7)	<b>0.037</b>
<i>p value</i>	<b>&lt;0.001</b>	<b>&lt;0.001</b>	

Mann Whitney test between case and control; Wilcoxon matched-pairs signed rank test between pairs of each group. <sup>s</sup> Postprandial plasma glucose level 1 hour after defined food, <sup>ss</sup> Post prandial plasma glucose 2 hours after 75g oral glucose load; FPG, Fasting plasma glucose; PPG, Postprandial plasma glucose.

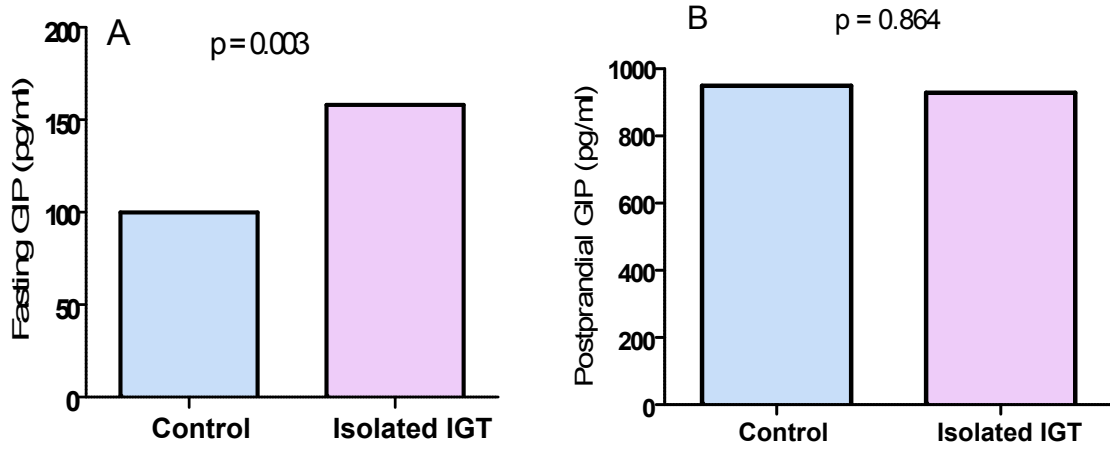


Figure 5. Comparison of fasting GIP (A) and postprandial GIP (B) between control and IGT.

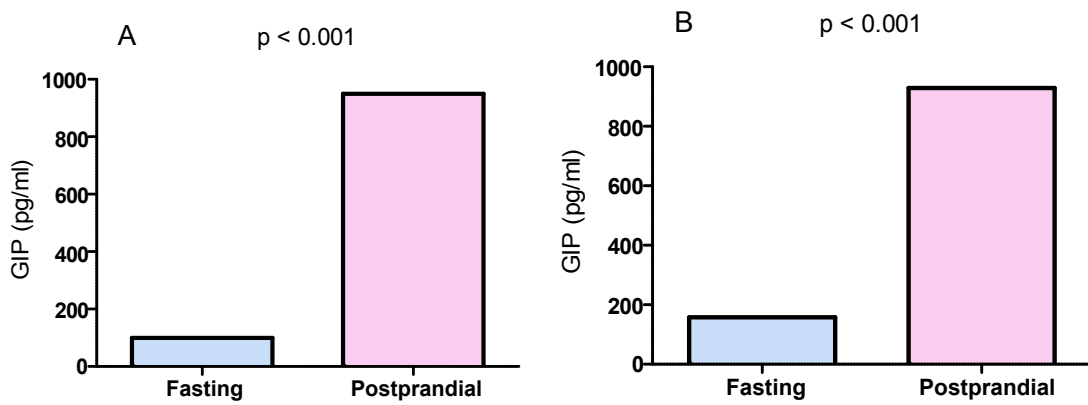


Figure 6. Comparison of GIP between fasting and postprandial states in control (A) and in IGT (B).



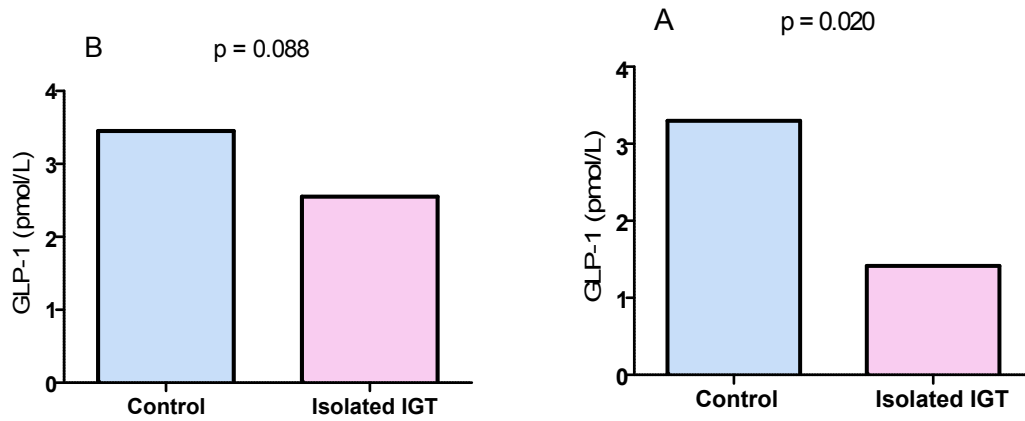


Figure 7. Comparison of fasting GLP-1 (A) and postprandial GLP-1 (B) between control and IGT.

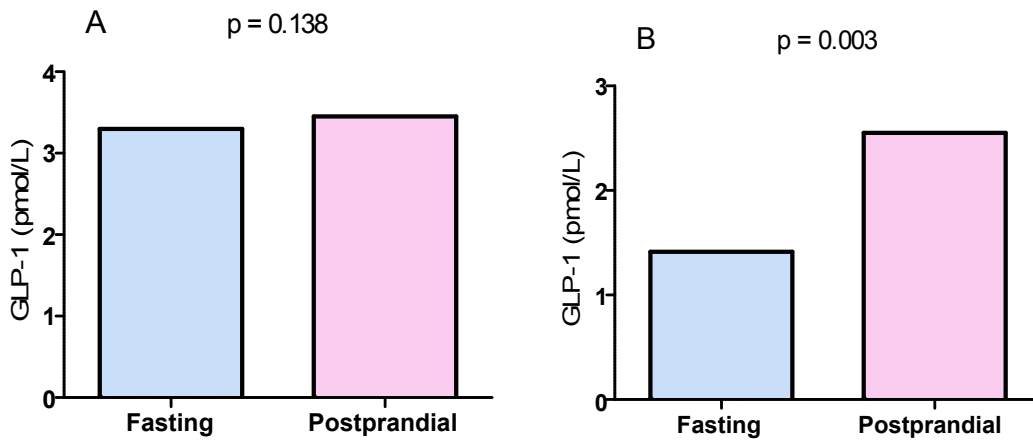


Figure 8. Comparison of GLP-1 between fasting and postprandial states in control (A) and in IGT (B).

### Relationship of HOMA %B, HOMA %S and HOMA IR with total GIP, GLP-1 and other variables

Table 3 shows the correlation coefficient of HOMA %B with incretins in control and isolated IGT. In the control, no incretin showed statistically significant relationship with HOMA %B but in isolated IGT, fasting GLP-1 showed significant positive relationship with HOMA %B ( $r = 0.421, p = 0.013$ ). The correlation coefficient of HOMA %B with age was not significant in both groups ( $p > 0.05$ ). HOMA %B showed significant positive relationship with BMI ( $r = 0.433, p = 0.010$ ) in isolated IGT.

The correlation coefficient of HOMA %S for incretins is presented in table 4. Fasting GIP showed no significant relationship with HOMA %S in control and isolated IGT but fasting GLP-1 showed a significant negative relationship with HOMA %S in isolated IGT. Among the anthropometric variables, age showed no significant relationship with HOMA %S in both groups but BMI showed a significant inverse relationship with HOMA %S ( $r = -0.440, p < 0.05$ ) in isolated IGT.

The correlation coefficient of HOMA IR for incretins is presented in table 5. Fasting GIP showed no significant relationship with HOMA IR but fasting GLP-1 was found to be positively related to HOMA IR in isolated IGT. BMI was also positively related to HOMA IR in isolated IGT ( $r = 0.439, p = 0.009$ ).

**Table 3. Correlation of HOMA %B with fasting incretins**

Parameters	Control (n=34)	IGT (n=34)
	r (p)	r (p)
Fasting GIP	0.295 (0.090)	0.119 (0.501)
Fasting GLP-1	-0.316 (0.069)	<b>0.421 (0.013)</b>

**Table 4. Correlation of HOMA %S with fasting incretins**

Parameters	Control (n=34)	IGT (n=34)
	r/p	r/p
Fasting GIP	-0.257 (0.142)	-0.177 (0.316)
Fasting GLP-1	0.144 (0.418)	<b>-0.416 (0.014)</b>

**Table 5. Correlation of HOMA IR with fasting incretins**

Parameters	Control (n=34)	IGT (n=34)
	r/p	r/p
Fasting GIP	0.243 (0.166)	0.186 (0.293)
Fasting GLP-1	-0.138 (0.438)	<b>0.401 (0.019)</b>

## CHAPTER -04: DISCUSSION

Global health burden is increasing due to upward trend in noncommunicable diseases (NCDs) and diabetes mellitus (DM) is considered as an important component of NCDs. The incidence of DM is increasing worldwide (Shaw et al 2009, Boyle et al 2010) and rising rate is higher in developing countries (Shaw et al 2009). The favorable influence of GLP-1 (7 – 36) on metabolic disorders of DM includes stimulation of insulin secretion, (Holst et al 1987, Nauck et al 1993), stimulation of  $\beta$  cell proliferation and differentiation (Neumiller 2009), inhibition of  $\beta$  cell apoptosis (Neumiller 2009), inhibition of glucagon secretion (Nauck et al 1993, Orskov et al 1988), hepatic glucose production (Hvidberg et al 1994, Larsson et al 1997), gastric emptying (Wattergren et al 1993, Willms et al 1996) and appetite (Gutzwiller et al 1999, Flint et al 1998) has provided the mainstay of GLP-1(7 – 36) targeted therapy of type 2 DM. It is now considered as a safer and effective treatment of type 2 DM. Reduced incretin effect is a specific, important and early characteristic of type 2 DM (Knop et al 2007, Holst et al 2011) and is associated with  $\beta$  cell failure (Holst et al 2011). However, the interrelationship between incretins and insulin secretory capacity, insulin sensitivity and insulin resistance in isolated IGT is still controversial and poorly understood (Laakso et al 2008, Faerch et al 2008, Ahren et al 1997, Zhang et al 2012, Muscelli et al 2006, Vollmer et al 2008, Rask et al 2004). Increased insulin resistance in isolated IGT in Bangladeshi population has been studied (Rahman et al 2010). But its associated abnormalities in the secretion of gut hormones have not yet been studied. In this study, gut hormone (incretin) secretion in isolated IGT were evaluated by plasma concentrations of GIP and GLP-1 to explore their relationship with insulin secretory capacity, insulin sensitivity and insulin resistance.

Thirty four (34) isolated IGT subjects and equal number of age-sex matched controls were recruited from the OPD of BIHS hospital. According to inclusion criteria of the study subjects, acute and chronic glycemic status differed significantly ( $p < 0.001$ ) between control and isolated IGT groups. Fasting and postprandial TGs were found to be higher in isolated IGT compared to control. HDL cholesterol levels were significantly lower in isolated IGT subjects. Total cholesterol and LDL cholesterol levels were similar in control and isolated IGT subjects. Fasting and postprandial insulin levels were found to be 64.2 % [10.9 (2.8 –

25.6)  $\mu\text{IU/ml}$  vs 17.9 (7.7 – 44.8)  $\mu\text{IU/ml}$  and  $p < 0.001$ ] and 16.6% [69.4 (34 – 211)  $\mu\text{IU/ml}$  vs 80.9 (22 – 217)  $\mu\text{IU/ml}$  and  $p < 0.046$ ] higher in isolated IGT groups compared to control. Postprandial insulin levels compared to fasting levels were also found to be higher in both controls [10.9 vs 69.4  $\mu\text{IU/ml}$ ] and IGT subjects (17.9 vs 80.9  $\mu\text{IU/ml}$ ) and the changes in insulin levels were remarkably lower in IGT compared to control (6.2 fold vs 4.5 fold). This is consistent with the general characteristic of hyperinsulinemia in prediabetic subjects. In subjects with IGT, insulin secretory capacity was found to be significantly higher compared to control (147.3 vs 164.3,  $p < 0.05$ ), insulin sensitivity was found to be significantly lower (63.9 vs 38.4) and insulin resistance was significantly higher (1.6 vs 2.6) compared to control. These findings are partly consistent with the findings of Rahman et al (2010) who observed significantly lower insulin sensitivity in isolated IGT compared to control in Bangladeshi population. Unlike the results of Rahman et al (2010) we observed significant increase in insulin secretory capacity in isolated IGT compared to control. Increased insulin resistance, as we observed, occurs frequently in IGT subjects (Laakso, 2007). The difference between the two studies in this population may be linked to the duration of IGT state. So increased insulin resistance may be responsible for the increased blood glucose level in isolated IGT. Further, decreased insulin sensitivity and increased blood glucose level may be linked to enhanced insulin secretion.

Fasting GIP concentrations were 58% higher in isolated IGT compared to control (99.9 vs 158.1  $\text{pg/mL}$ ,  $p < 0.01$ ) but postprandial GIP concentrations were similar in both the groups (only 2% lower in IGT,  $p = 0.864$ ). The changes in plasma GIP after mixed breakfast was 50% lower in isolated IGT compared to control (control vs IGT: 851% vs 424%). When GIP concentrations per unit of glucose were compared between control and IGT, fasting GIP per unit of glucose was 32% higher in IGT whereas postprandial GIP was 27% lower compared to control. Compared to control, increase in GIP concentration per unit of glucose was 50% lower in isolated IGT after mixed breakfast. Insulin concentrations per unit of GIP showed no significant difference between control and IGT in fasting and postprandial states. On correlation analysis, fasting GIP showed no significant relationship with insulin secretory capacity, insulin sensitivity and insulin resistance. These results indicate that higher GIP concentration in fasting state in IGT may be secondary to insulin resistance to compensate

increased fasting levels of plasma glucose or it may be stimulated by increased blood glucose level to promote insulin secretion from  $\beta$  cell as evidenced by hyperinsulinemia in isolated IGT.

Fasting concentrations of GLP-1 (7 – 36) was found to be 58% lower in isolated IGT compared to control (3.3 vs 1.4 pmol/L,  $p < 0.05$ ). However, postprandial GLP-1 showed no significant difference between IGT and control subjects. The incretin effect in terms of plasma GLP-1 (7 – 36) showed no significant difference between fasting and postprandial state ( $p > 0.05$ ) in control and this finding is consistent with the study of Vollmer et al (2007). Unlike the results of Vollmer et al (2007) we found significant increase in plasma GLP-1 (7 – 36) concentration in postprandial state in isolated IGT and this is consistent with the findings of Rask et al (2004) who observed a significant increase in GLP-1 (7 – 36) in isolated IGT after 30 minutes of oral glucose load. On the other hand Zhang et al (2012), Nauck et al (1986) and Toft-Nielsen et al (2001) observed a significant reduction of GLP-1 concentration in isolated IGT on oral glucose challenge or mixed meal. GLP-1 (7 – 36) per unit of glucose was significantly lower in IGT compared to that of control; it was also significantly reduced in postprandial state compared to fasting state in both control and IGT. This result indicates that hyperglycemia is related to a reduced GLP-1 (7 – 36) secretion. On the other hand higher insulin per unit of GLP-1 (7 – 36) in isolated IGT reflects the compensatory effect of insulin resistance or to overcome the reduced insulin sensitivity by increasing insulin secretion from beta cell. These results also support the results of correlation analysis. Insulin secretory capacity showed a significant positive relationship with fasting GLP-1 (7 – 36), postprandial GLP-1 (7 – 36) and BMI only in isolated IGT. Insulin sensitivity in isolated IGT was found to be inversely related to fasting GLP-1 (7 – 36), postprandial GLP-1 (7 – 36) and BMI. But in control insulin sensitivity showed no such relationship. The inverse relationship of GLP-1 (7 – 36) with insulin sensitivity and the positive relationship with insulin resistance in IGT is inconsistent. It may be apparent as evidenced by decreased GLP-1 per unit of plasma glucose in postprandial state or due to compensatory insulin resistance and insulin sensitivity in IGT. Thus impairment of insulin secretory capacity, insulin sensitivity and insulin resistance in isolated IGT is associated with impaired secretion of gut hormone GLP-1 or reduced bioavailability of incretins in IGT

in the population studied. This finding is consistent with the findings of Zhang et al (2012) who reported a remarkable relationship between impaired GLP-1 secretion and insulin secretory capacity, insulin sensitivity and insulin resistance. On the other hand Muscelli et al (2006) observed no significant difference in incretin effect between IGT and control.

It may be concluded from this study that

- Isolated IGT is a hyperinsulinemic state and it is associated with insulin resistance.
- Hypersecretion of GIP and deficient secretion of GLP-1 in the fasting states are associated with isolated IGT.
- Insulin secretory dysfunction and insulin resistance can develop in the absence of any impairment of GIP secretions but they may be associated with defective GLP-1 secretion from the gut.

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